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Cellular mechanisms that regulate the endogenous mono-ADP-ribosylation of the G protein $\beta\gamma$ subunit

Nadia Dani

Discipline: Life sciences
Sponsoring establishment: Consorzio Mario Negri Sud

Thesis submitted in accordance with the requirements of the Open University for the degree of Doctor of Philosophy
January 2006
What I have done
is only the first step
of a long way.
(P.Picasso)

In my uncle's memory
and to my family.
Abstract

Mono-ADP-ribosylation is a reversible, post-translational modification of cellular proteins that has been implicated in regulation of signal transduction, muscle cell differentiation, and protein trafficking and secretion. The reaction is catalysed by mono-ADP-ribosyltransferases that transfer a single ADP-ribose moiety from β-NAD⁺ to a specific amino-acid of acceptor proteins. An ADP-ribosylation reaction occurs in intact cells on the β subunit of heterotrimeric G proteins that is carried out by an arginine-specific, plasma-membrane-associated, mono-ADP-ribosyltransferase. This modification is reversed by a cytosolic ADP-ribosylhydrolase that regenerates native βγ dimer by releasing the bound ADP-ribose. Once ADP-ribosylated, the βγ dimer is inactive towards its effector enzymes, such as adenylyl cyclase, phosphoinositide 3-kinase and phospholipase C. It thus appears that endogenous β subunit mono-ADP-ribosylation might represent a novel cellular mechanism for the modulation of the G-protein-mediated signal transduction machinery through a direct regulation of the βγ dimer. In this study, the mechanisms that regulate endogenous mono-ADP-ribosylation of the β subunit have been investigated. The reaction appears to be under hormonal control both in vitro and in vivo, since the levels of ADP-ribosylated β are increased upon activation of certain G-protein-coupled receptors (GPCRs), such as thrombin, serotonin and cholecystokinin receptors. Conversely, hormonal stimulation by additional GPCRs, such as the GnRH receptor, can lead to a decrease in β subunit mono-ADP-ribosylation. Thus, ADP-ribosylation of the βγ dimer can be differentially regulated by different GPCRs in a receptor-type-dependent manner. In addition, the involvement of the ADP-ribosylating factor ARF6 in GnRH-mediated regulation of β subunit mono-ADP-ribosylation is demonstrated. Indeed, removal of ARF6 from plasma membranes results in loss of GnRH-mediated inhibition of β subunit mono-ADP-ribosylation, which can be fully restored by re-addition of purified ARF6. In
conclusion, the results reported in this thesis allow the definition of the mechanisms that regulated endogenous ADP-ribosylation of the β subunit, and demonstrate a novel role for ARF6 in hormonal regulation of β subunit mono-ADP-ribosylation.
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Chapter 1

Introduction

1.1 Heterotrimeric G-protein-mediated signalling

Heterotrimeric guanine-nucleotide-binding proteins (G proteins) mediate the ability of eukaryotic cells to respond to a wide variety of extracellular signals and sensory stimuli. The G-protein-mediated signalling pathway is composed of three major molecular entities (Morris and Malbon, 1999; Figure 1.1):

1) the G-protein-coupled receptor (GPCR): a seven-transmembrane-spanning protein that exposes a binding site for specific ligands and an interaction site for G proteins on its extracellular and intracellular faces, respectively.

2) the transducer: one or more frequently, two or three different families of G proteins that are capable of translating the GPCR activation into the regulation of specific effectors, through its $\alpha$ and $\beta\gamma$ subunits.

3) the effector: enzymes and ion channels that control the intracellular concentrations of the second messengers, which in turn amplify and propagate the stimulus within the cell, thus leading to physiological responses, including contraction, relaxation, cell division, cell differentiation and secretion.

Due to the central role of the heterotrimeric G proteins in the modulation of almost all cell functions, both the $G\alpha$ and $G\beta\gamma$ subunits are strictly regulated by different mechanisms. In this study, the endogenous mono-ADP-ribosylation reaction of the $G\beta$ subunit is discussed as a new mechanism controlling the $G\beta\gamma$-dimer-mediated signalling (Section 1.2). In particular, the experimental work presented here is focused on an understanding of the molecular mechanisms that regulate this reaction.
Figure 1.1. General characteristics of the GPCRs. Model of G protein mediated signalling pathways, showing: 1) the G-protein-coupled receptor, 2) the trimeric G protein composed of an α subunit and a βγ dimer, 3) effectors, which can be enzymes such as adenylyl cyclase and phosphodiesterases or ion channels.
1.1.1 G-protein-coupled receptors

The GPCRs constitute the largest family of membrane receptors involved in signal transduction. Members of this family are able to recognize and bind molecules as different as neurotransmitters, hormones, chemokines, lipids, odorants and even photons of light. Despite the remarkable structural diversity of their activating ligands, all of the GPCRs share a common molecular architecture, predicted principally from the crystal structure of the mammalian visual pigment rhodopsin, the GPCR stimulated by light (Palczewski et al., 2000). The typical GPCR protein sequence includes seven transmembrane helices (TM I-VII) joined by three intracellular (i₁, i₂, i₃) and three extracellular (e₁, e₂, e₃) loops (Baldwin, 1993). Two cysteine residues (one in e₁ and one in e₂) that are conserved in most GPCRs form a disulfide bridge, which is probably important for the packing and stabilization the TMs. The extracellular surface of the receptor, including the N-terminal domain, is critical for ligand binding, while the intracellular surface is involved in G protein recognition and activation (Schwartz, 1994; Strader et al., 1994). This region also comprises canonical sites for protein phosphorylation by protein kinase A (PKA), protein kinase C (PKC), G-protein receptor kinases (GRKs), receptor tyrosine kinases (RTK; e.g., insulin and IGF-I) and non-receptor tyrosine kinases (e.g., Src family kinases) (Lefkowitz, 1998).

Both structural and physiological features were used by Kolakowski to classify GPCRs into several families, most of which in turn contain subfamilies (Kolakowski, 1994). According to this classification, family A comprises rhodopsin and a huge number of rhodopsin-related receptors, like the receptors for catecholamines, peptides, glycoproteins, and olfactory molecules; family B consists of secretin-like receptors, activated by large peptides, like calcitonin, parathyroid hormone, and glucagon; family C contains metabotropic glutamate receptors and related subfamilies. Frizzled and
smoothened receptors and a subgroup of pheromone receptors form two additional classes of GPCRs.

The entire known GPCRs shuttle between inactive and active conformations. While increasing evidence is emerging that some GPCRs exhibit constitutive activity (Leurs et al., 1998; Mitchell et al., 1998), it is generally assumed that in the absence of activating ligands, the inactive conformation of the receptor is favoured. This implies that the receptor is functionally uncoupled from the G proteins. Upon activation by ligands, the GPCR shifts to the active conformation through a 30° clockwise rotation of TM VI (viewed from the cytoplasmic face), which moves apart from TM III. This conformational change in the core domain affects the conformation of the i₂ and i₃ loops that constitute key sites for G protein coupling and activation (Wong and Ross, 1994). In this state, the activated receptor is able to complex with and activate heterotrimeric G proteins, which in turn transmit the signal into the intracellular lumen of the cells, initiating the signalling cascade.

Several mechanisms exist to regulate the length and strength of GPCR signals. In many cases, a time-dependent decrease in the cellular response to the external signal occurs despite the continued presence of the signalling ligand. This attenuation of signalling is known as desensitization, and consists in the uncoupling of a GPCR from its associated G proteins. Studies of rhodopsin (Kuhn et al., 1984; Wilden et al., 1986) and the β₂-adrenergic receptor (β₂-AR) (Ferguson, 2001; Lefkowitz, 1998) have revealed that desensitization initiates with the recruitment of a GRK that phosphorylates the receptor. The “uncoupling” protein arrestin is then recruited from the cytosol to bind the phosphorylated receptor, causing the uncoupling of the receptor and the G protein (Lefkowitz and Shenoy, 2005). The next event is the internalization (endocytosis) of the receptor from the plasma membrane into the intracellular compartment (Figure 1.2).

Multiple pathways of receptor internalization have been described, which are mediated by clathrin-coated pits, caveolae or other uncoated vesicles (Claing et al., 2002;
Le Roy and Wrana, 2005). Once internalized, the receptors can be dephosphorylated, resensitized and recycled to the cell surface, or targeted to lysosomes for degradation, or engaged in additional intracellular signalling.

Monomeric GPCRs are believed to be the signalling unit; however, more recently, this view has been challenged, and largely supplanted by results consistent with the existence of GPCR receptors as dimers or higher-order oligomers (Filizola and Weinstein, 2005; Maggio et al., 2005; Park et al., 2004). As an example, the functional GABA\textsubscript{B} is an obligatory heterodimer of GABA\textsubscript{B}R1 and GABA\textsubscript{B}R2: the heterodimerization is crucial for its correct intracellular transport to the cell surface, for the binding of the ligand and for the activation of the G proteins (Jones et al., 1998; Kuner et al., 1999). Importantly, heterodimerization among different GPCRs may explain some aspects of the cross-talk regulation that occurs between distinct signalling systems. The angiotensin AT\textsubscript{1} and the bradykinin B\textsubscript{2} receptors form stable heterodimers that enhance the activation of the Go\textsubscript{q} and Go\textsubscript{i} proteins, the two major transducer proteins triggered by AT\textsubscript{1} (AbdAlla et al., 2000). Such integration and communication between receptors can presumably tune the strength and duration of the signals, thereby leading to the fine regulation of the ultimate physiological responses.

1.1.2 Heterotrimeric G proteins

The heterotrimeric G proteins are the signal transducers that switch the biological signal from the cell surface to the intracellular compartment (Preininger and Hamm, 2004). These proteins are composed of three different polypeptides: the $\alpha$ subunit that binds and hydrolyses GTP, the $\beta$ and the $\gamma$ subunits, which form the tightly associated $\beta\gamma$ dimer that dissociates only under denaturing conditions, and is, therefore, considered a functional monomer. Both the Go$\alpha$ subunit and the G$\beta\gamma$ dimer are involved in signal transduction pathways.
Figure 1.2. General model of GPCR desensitization and internalization via clathrin-coated vesicles. The stimulation of a GPCR by its agonist leads to activation of the G protein. The active Gβγ dimer recruits G-protein-receptor kinases (such as GRK2 and GRK3) to the receptor, where they specifically phosphorylate the agonist-occupied receptor. The uncoupling protein arrestin is then recruited from the cytosol to bind the phosphorylated receptor, sterically uncoupling the receptor and the G protein. Arrestin, in turn, targets the receptor to internalization via clathrin-coated pits. Once internalized, the receptor can be de-phosphorylated and recycled to the cell surface, or targeted to lysosomes for degradation.
Based on the amino acid similarities among the α subunits, G proteins are divided into four major families, namely Gs, Gi/0, Gq/11 and G12/13 (Table 1.1). The Gs family is mainly involved in the activation of adenylyl cyclase (AC), whereas the Gi family inhibits AC, but also regulates ion channels, phospholipases and phosphodiesterases. Gq/11 activates phospholipase C (PLC), and G12/13 is able to regulate the monomeric GTP-binding protein Rho, through the interaction with its exchange factor Rho-GEF (Freissmuth et al., 1999).

1.1.2.1 G protein subunits: classification and structure

According to current knowledge, there are 16 Ga genes in the human genome that encode for 23 known Ga proteins (McCudden et al., 2005), which range in size from 39 to 45 kDa. The solution of crystal structures for Ga1 and Ga4 in both the active and inactive states has revealed two distinct domains: a nucleotide binding domain with high structural homology to the Ras-superfamily GTPases, and an α-helical domain that in combination with the Ras-like domain, helps to form a deep pocket that is involved in guanine nucleotide binding (Sprang, 1997). Three flexible regions, designated switch I, II and III, change conformation in response to GTP binding and hydrolysis.

The Gβ subunit exists in five isoforms, known as β1, β2, β3, β4 and β5, with molecular weights ranging from 35 kDa and 39 kDa; and 12 Gγ subunits have been cloned, with molecular weights ranging from 5 and 10 kDa (Clapham and Neer, 1997). The 12 Gγ subunits are considerably different in their primary sequences (27-76% amino acid identity). In contrast, the Gβ subunits are highly homologous (Gβ1 to Gβ4 share 78-88% amino acid identity), with the most divergent member of the family being Gβ5, which shares only 51-53% amino acid identity with the other Gβ subunits. Moreover, while β1, β2, β3 and β4 are ubiquitously expressed, β5 appears to be tissue-specific, and has been detected only in the retina, neuronal tissue and platelets. Also, the Gγ subunits show tissue expression profiles that indicate unique roles in specific signal transduction pathways.
<table>
<thead>
<tr>
<th>Class</th>
<th>Gα subunit</th>
<th>Effectors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gs</td>
<td>Gα5</td>
<td>Adenylyl cyclase (+)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Calcium channel (+)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sodium channel (+)</td>
</tr>
<tr>
<td>Gi</td>
<td>Gαolf</td>
<td>Adenylyl cyclase (+)</td>
</tr>
<tr>
<td></td>
<td>Gα1</td>
<td>Adenylyl cyclase (-)</td>
</tr>
<tr>
<td></td>
<td>Gα2</td>
<td>Potassium channel (+)</td>
</tr>
<tr>
<td></td>
<td>Gα3</td>
<td>Calcium channel (-)</td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Phospholipase A2 (?) (+)</td>
</tr>
<tr>
<td></td>
<td>Gα0,A,B</td>
<td>Potassium channel (+)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Calcium channel (-)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phospholipase C (?) (+)</td>
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<td></td>
<td>Gα1,2</td>
<td>cGMP-phosphodiesterase (+)</td>
</tr>
<tr>
<td></td>
<td>Gαg</td>
<td>cGMP-phosphodiesterase (+)</td>
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<tr>
<td></td>
<td></td>
<td>Phospholipase C-β (?)</td>
</tr>
<tr>
<td></td>
<td>Gαz</td>
<td>Adenylyl cyclase (?)</td>
</tr>
<tr>
<td>Gq/11</td>
<td>Gαq</td>
<td>Phospholipase C-β (+)</td>
</tr>
<tr>
<td></td>
<td>Gα11</td>
<td></td>
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<td></td>
<td>Gα16</td>
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<td></td>
<td>Gα14</td>
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<td></td>
<td>Gα15</td>
<td></td>
</tr>
<tr>
<td>G12</td>
<td>Gα12</td>
<td>GEFs, GDIs, GAPs (?)</td>
</tr>
<tr>
<td></td>
<td>Gα13</td>
<td>GEFs, GDIs, GAPs (?)</td>
</tr>
</tbody>
</table>

Table 1.1. Heterotrimeric G proteins: classification and effectors.
Indeed, the expression of $\mathrm{G}\gamma_1$ is restricted to retinal rod cells, and the $\mathrm{G}\beta\gamma_1$ dimer interacts better with rhodopsin than with other receptors (Kisselev and Gautam, 1993).

The $\mathrm{G}\beta$ subunit is a WD-40-containing protein, and is thus characterised by the presence of tandem-repeating regions of amino acids that contain two conserved dipeptide sequences, Gly-His (GH) and Trp-Asp (WD) (Garcia-Higuera et al., 1998). The WD repeats have been proposed to act as building modules for multimeric complexes. The crystal structure of the $G$-protein heterotrimer reveals that the core WD-repeat portion of $\mathrm{G}\beta$ is made up of seven four-twisted $\beta$ sheets arranged in a ring, forming a propeller-like structure (Clapham and Neer, 1997; Neer and Smith, 1996). The circular structure is held closed by a molecular “velcro snap” in the seventh blade of the propeller. Conserved residues establish a hydrophilic environment between the $\beta$-sheets, and the stabilisation of the propeller structure is achieved by inter- and intra-blade hydrogen bond triads (Neer and Smith, 1996). In addition to its WD-40 repeat regions, $\mathrm{G}\beta$ has a unique N-terminal domain that adopts an $\alpha$-helical structure. This region forms a coiled-coil interaction with the $\gamma$ subunit, which is critical for proper folding and function of the proteins, and which cannot be interrupted without strong denaturing conditions (Figure 1.3) (Clapham and Neer, 1997).

The major interaction between $\mathrm{G}\alpha$ and $\mathrm{G}\beta\gamma$ involves the $\mathrm{G}\alpha$ N-terminus (Lambright et al., 1996; Wall et al., 1995). The $\mathrm{G}\alpha$ subunit binds asymmetrically over the narrow end of the $\mathrm{G}\beta\gamma$ dimer, making contacts principally with residues in blades 1, 2 and 3 (Figure 1.4). The $\mathrm{G}\alpha$ switch-II region that changes its conformation in the GDP- and GTP-bound states is positioned over the central tunnel of $\mathrm{G}\beta$ (Sondek et al., 1996). The association with the $\mathrm{G}\beta\gamma$ dimer produces a significant change in conformation of the $\mathrm{G}\alpha$ switch I and II regions. By contrast, the structure of $\mathrm{G}\beta\gamma$ is not appreciably modified following its association with the $\mathrm{G}\alpha$ (Cabrera-Vera et al., 2003). This interaction of $\mathrm{G}\beta\gamma$ with $\mathrm{G}\alpha$ increases the affinity of $\mathrm{G}\alpha$ for GDP, both by increasing the rate of association.
Figure 1.3. G protein βγ dimer. A: Model of the βγ dimer. The WD repeats are represented by circles connected to the variable regions. Each WD repeat is predicted to be a structure made up of a β-strand-turn-β-strand. The putative α-helical region at the N-terminus is shown as a rectangle. The area of γ that determines the specificity of interaction with β is represented by violet box. The C-terminal prenyl group is indicated by the zigzag line. B: Structure of the WD40 propeller domain of the Gβ subunit.
**Figure 1.4. G protein structure.** The Gα subunit (green) interacts through its N-terminal region principally with residues in blades 1, 2 and 3 of the β subunit (orange). The Gα region that changes its conformation in the GDP- and GTP-bound states is positioned over the central tunnel of Gβ (indicated by the blue arrow). See text for more details.
and by decreasing the rate of dissociation, probably due to the stabilisation of switch I and switch II. The overall effect is that G\(\beta\gamma\) facilitates the inactivation of the stimulated G\(\alpha\) subunit. Apparently in contrast with this view of G\(\beta\gamma\) as a negative regulator, G\(\beta\gamma\) needs to be present for efficient binding of G\(\alpha\) to the receptor. A direct interaction of G\(\beta\gamma\) with the receptor has been shown in the case of the purified rhodopsin receptor (Phillips and Cerione, 1992): although the G\(\alpha\) of transducin (the G protein involved in visual transduction, G\(\alpha_{\text{t}}\)) can bind to rhodopsin without G\(\beta\gamma\), G\(\beta\gamma\) appears to increase the affinity of G\(\alpha_{\text{t}}\) for rhodopsin. This effect is independent of the ability of G\(\beta\gamma\) to enhance binding of G\(\alpha\) to phospholipid vesicles, hence it appears to be independent of an increased binding of G\(\alpha\) to the membrane.

### 1.1.2.2 The heterotrimeric G-protein cycle

The G-protein activity is regulated by an activation/deactivation cycle that allows transmission of signals from receptor to effector (Gilman, 1987). In the inactive state, the G protein is an \(\alpha\beta\gamma\)-heterotrimer with GDP bound to the G\(\alpha\) subunit. In this state, the rate of GDP release (\(K_{\text{off}} \leq 0.1 \text{ min}^{-1}\)) is much lower than the rate of GTP hydrolysis (\(K_{\text{cat}} \geq 3 \text{ min}^{-1}\)); this kinetic feature clamps the system in the “off” position (Freissmuth et al., 1999). When a ligand activates a GPCR, the receptor becomes activated and changes its conformation, and now acts as a guanine nucleotide exchange factor for the G\(\alpha\). GDP is released from the G\(\alpha\), which then binds GTP present at a significant molar excess over GDP in cells. The binding of GTP results in conformational changes within the three flexible switch regions of the G\(\alpha\) subunit (Morris and Malbon, 1999), leading to the dissociation of the G\(\beta\gamma\) dimer. The cycle terminates when the intrinsic guanosine triphosphatase (GTPase) activity of the G\(\alpha\) subunit catalyses the hydrolysis of GTP to GDP. The inactive G\(\alpha\)-GDP then re-associates with G\(\beta\gamma\), turning off the signalling (Figure 1.5). Interestingly, with the notable exception of transducin, there is only weak evidence
Figure 1.5. **Receptor-mediated G-protein activation.** In the inactive state (1) the G protein is a heterotrimer with GDP bound to the Gα subunit. Upon activation by an appropriate signal (2), the receptor interacts with the G protein and catalyses the exchange of GDP for GTP on Gα. The GTP-bound Gα and Gβγ dimer dissociate from the receptor, as well as from each other, and initiate cellular responses through their interactions with effector molecules (3). The duration of the signal is determined by the intrinsic GTP hydrolysis rate of Gα (4) and the subsequent re-association of Gα-GDP with the Gβγ dimer (5).
for the physical separation of the Gα subunit from the Gβγ dimer (Fung, 1983). Recent data indicate that Gα and Gβγ dissociation may not be required for downstream signalling: i.e. fusion of Gα to its associated βγ dimer does not impair G protein function (Levitzki and Klein, 2002). In addition, using a fluorescence resonance energy transfer (FRET)-based assay, it has been demonstrated in intact cells that fluorescent Gα i and αz subunits and Gβ1γ2 dimers do not dissociate, although conformational changes take place upon activation (Bunemann et al., 2003). According to this “clamshell” model of G protein activation, receptor-induced changes in G protein conformation lead to the exposure of previously buried interfaces between the Gα and Gβγ subunits that can thus interact with their respective effectors. However, a different study, using the same FRET-based approach to investigate G protein activation in Dictyostelium demonstrated the receptor-mediated dissociation of Gα (Janetopoulos et al., 2001). The most likely explanation for these contradictory results might reside in the difference between the two G protein families taken in consideration. Thus, G i and G s, which are quite different in their primary structure, can differentially undergo either subunit dissociation (G s) or an intersubunit rearrangement (G i) upon activation.

1.1.2.3 The accessory proteins that regulate the G protein signalling

The GTPase activity of the Gα subunit is regulated by accessory proteins, among which the RGS (Regulators of G protein Signalling) proteins have attracted attention due to their ability to act as GTPase-activating proteins (GAPs) for the Gα subunit (Siderovski et al., 1996). Each RGS protein contains a hallmark ~120 amino-acid “RGS-box” domain, which contacts the Gα switch region, increasing their intrinsic GTPase activity (Berman et al., 1996; Watson et al., 1996). This provides an explanation for the paradox between the rapid physiological timing described for GPCR-mediated signal transduction in vivo and the slow rate of GTP hydrolysis (0.1 to 0.3 Pi/mol Gα/min for most Gα; Gilman, 1987) exhibited by purified Gα subunits in vitro.
A recent addition to the group of regulators of the G-protein cycle is a family of Ga-interacting proteins characterised by one or more Go Loco motifs (otherwise known as GPR or "G-Protein Regulatory" motifs; Siderovski et al., 1999). These generally bind to GDP-bound Ga subunits of the Gi class and act as guanine nucleotide dissociation inhibitors (GDIs), slowing the spontaneous exchange of GDP for GTP (Willard et al., 2004). In this way, Gβγ-effector signalling can continue in the absence of receptor-catalysed Ga-GTP formation.

A completely different family of regulators of the G-protein cycle is constituted by the AGS (Activators of G-protein Signalling) proteins, which have been characterised as putative GEFs for the Ga subunit. To date, four AGS have been identified (AGS1-4, Lanier, 2004) that interact with different subunits and/or conformations of the heterotrimeric G proteins, and that can selectively activate different G proteins, providing an as yet to be defined mechanism for receptor-independent regulation of the G-protein cycle.

1.1.2.4 The G-protein signalling and diseases

Once activated, the Ga and Gβγ subunits can interact with a variety of target effectors. Because of this, abnormal G-protein signalling can lead to serious biological consequence. Gene mutations that alter the GTPase activities of Ga subunits correlate with several human diseases, such as some human thyroid and pituitary tumours (reviewed in Dhanasekaran et al., 1995), and sporadic McCune-Albright syndrome, a disease characterised by the hyperactivity of the endocrine tissues and hyper-proliferation (Weinstein et al., 1991). Recently, a critical role in pathogenesis has also been demonstrated for Gβγ (Bookout et al., 2003). In vitro, the expression of GRK2ct, a peptide derived from the C-terminus of GRK2 that inhibits Gβγ signalling, attenuates serum-induced prostate cancer cell growth. In vivo, the inhibition of Gβγ signalling induces a significant decrease in rate the of prostate tumour growth in mice. In parallel, altered levels
of Gγ subunit expression have been noted in different tumour tissues. Indeed levels of Gγ are reduced in pancreatic and gastrointestinal tract cancers (Shibata, 1998; Shibata et al., 1999).

Thus, G proteins are crucial elements that define a correct cellular response. The study of the mechanisms that regulate signal transduction is an essential step towards being able to connect inappropriate responses from external signals with an abnormal cell function. Many current lines of research focus on how the G proteins transmit and integrate signals coming into the cell, and data that has accumulated over the last few years indicates that the Gβγ dimer is a crucial modulator of a broad range of cellular activities, as is the Gα subunit. The role of Gβγ dimers in signal transduction pathways is extensively discussed in the next Section.

1.1.3 The G-protein βγ dimer: role in signal transduction

For a long time, the prevalent hypothesis for the mechanism of G-protein-mediated signal transduction gave merit for the activation of effector proteins to the Gα-GTP subunit exclusively. The Gβγ dimer was regarded only as a negative regulator of Gα subunit activity. Release of free Gβγ from an abundant G protein, such as Gi, was thought to deactivate other Gα subunits by forming inactive heterotrimers (Gilman, 1987). The first clear evidence that the Gβγ dimer can itself regulate effectors emerged when Logothetis (Logothetis et al., 1987) showed that the Gβγ dimer can activate a K+ -selective ion-conducting channel in cardiac atrial cells. Now, we know that Gβγ dimers regulate Ca2+ homeostasis through the modulation of ACs and PLCβs, and can activate mitogen-activated protein kinase (MAPK) pathways through phosphoinositide 3-kinase (PI3K) and Ras activation, and are therefore involved in the regulation of cell growth and differentiation (Table 1.2; Cabrera-Vera et al., 2003).
<table>
<thead>
<tr>
<th>EFFECTOR</th>
<th>REGULATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLC-β</td>
<td>Stimulation</td>
</tr>
<tr>
<td>ACI</td>
<td>Inhibition</td>
</tr>
<tr>
<td>ACII, IV, and VII</td>
<td>Stimulation</td>
</tr>
<tr>
<td>K+ channels (GIRK 1, 2, 4)</td>
<td>Stimulation</td>
</tr>
<tr>
<td>Ca2+ channels</td>
<td>Inhibition</td>
</tr>
<tr>
<td>G protein receptor kinase</td>
<td>Recruitment to membrane</td>
</tr>
<tr>
<td>PI3K</td>
<td>Stimulation</td>
</tr>
<tr>
<td>Bruton’s tyrosine kinase</td>
<td>Stimulation</td>
</tr>
<tr>
<td>Tsk tyrosine kinase</td>
<td>Stimulation</td>
</tr>
<tr>
<td>Protein kinase D</td>
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</tr>
<tr>
<td>Calmodulin</td>
<td>Inhibition of calmodulin kinase</td>
</tr>
<tr>
<td>Tubulin</td>
<td>Increased GTPase activity</td>
</tr>
<tr>
<td>Dynamin I</td>
<td>Increased GTPase activity</td>
</tr>
<tr>
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<td>Indirect activation of MAPK</td>
</tr>
<tr>
<td>Raf-1 protein kinase</td>
<td>Sequestration of Gβγ</td>
</tr>
<tr>
<td>Ras exchange factor</td>
<td>Indirect activation of MAPK</td>
</tr>
<tr>
<td>KSR-1</td>
<td>Sequestration of Gβγ</td>
</tr>
</tbody>
</table>

Table 1.2. **Effectors regulated by Gβγ dimers.** GIRK, G protein-activated inwardly rectifying potassium channel. Modified from Cabrera-Vera, 2003.
1.1.3.1 The Gβγ dimer composition directs effector coupling

Current research indicates that the Gβγ dimer composition determines the quality, the efficiency and the specificity of effector activation. Since structural constraints preclude the formation of certain Gβγ dimers, (most notably those involving the β2, β3 and γ1 subtypes; Yan et al., 1996) not all of the potential Gβγ pairs exist in nature. As an example, Gβ5γ2 can activate in vitro PLCβ1 or β2, but not PLCβ3, whereas Gβ1γ2 is effective with all of these PLC isoforms (Maier et al., 2000). This observation is supported by the demonstration of a greater level of Gβγ specificity in vivo: while in neurons recombinant Gβ1γ2, β3γ2, and β5γ2, but not Gβ2-containing dimers, activate PLCβ, in vitro both Gβ1γ2 and Gβ2γ2 are equally effective at activating PLCβ, although at a 30-fold higher concentration than in vivo (Diverse-Pierluissi et al., 2000). Moreover, Bayewitch and co-workers (Bayewitch et al., 1998) have shown that different configurations of the Gβγ subunit can modulate the ACs: Gβ5γ2 inhibits ACII, whereas Gβ1γ2 and a pool of βγ subunits purified from tissues stimulate ACII. More recently, the specificity of coupling between Gβ1.5γ2 dimers and ACI and ACII has been analysed in Sf9 insect cells. All of these dimer combinations activate ACII and inhibit ACI (with β3γ2 having the weakest effect), with the sole exception of Gβ5γ2 (McIntire et al., 2001). Finally, the extent of inhibition of voltage-dependent N-type Ca2+ channels is related to the Gβ subtype (Gβ1 = Gβ2 > Gβ5 >> Gβ3 = Gβ4; Ruiz-Velasco and Ikeda, 2000), and it depends on the abilities of the different β subunits to interact physically with the Ca2+ channels.

Together, these data demonstrate that the primary sequence of the Gβ subunit is a major determinant for effector coupling efficiency and specificity.

1.1.3.2 The βγ binding sites for effectors

The Gβγ proteins have so far not been crystallised in complexes with their effector molecules. However, using the two-hybrid system, it has been demonstrated that the N-
terminus of the Gβ subunit (including the N-terminal coiled-coil, the outer β strand of blade 7, and blade 1) interacts with three different effectors: ACII, the K+ channel and PLCβ2 (Yan and Gautam, 1996). In parallel, data from molecular modelling and the use of peptides that are able to inhibit the Gβγ-mediated activation of the ACs have implicated residues 85-143 of the Gβ subunit in effector interactions (Chen et al., 1997; Weng et al., 1996). This region overlaps the domain that was identified with the two-hybrid system (Yan and Gautam, 1997). Moreover, mutational analysis of the Gβ subunit N-terminal 100 residues demonstrates that the N-terminal helix within these 100 residues takes part in γ-subunit binding, but not in effector interactions, whereas different domains that are downstream of this helix interact with AC and with the K+ channel and PLCβ (Yan and Gautam, 1997). These domains form the first and the second β sheets of the Gβ subunit. The Goα subunit N-terminus lies close to this region of the Gβ subunit, providing an explanation for the ability of the Goα subunit to turn off Gβγ signalling. (Lambright et al., 1996; Wall et al., 1995). However, although the well-defined interacting domains of the Gβ subunit are covered by the Goα subunit in the resting heterotrimeric conformation, it appears that different domains may be used by the Gβγ dimer for activation of different effectors. This conclusion was reached through testing various Gβ mutated in the switch interface between the Goα and Gβ subunits for their ability to interact with several Gβγ-effectors, such as β-adrenergic receptor kinase (βARK), PLCβ, ACII and ion channels (Ford et al., 1998). In line with this, by mutating multiple amino acids in each of seven blades of the β propeller, Panchenko and colleagues (Panchenko et al., 1998) showed that mutations in blades 2, 6 and 7 greatly inhibit the ability of Gβγ to activate PLCβ2, while being completely unaffected for ACI and ACII. Moreover, Buck and co-workers (Buck et al., 1999) have shown that a peptide mimicking the Gβ amino acidic sequence 115-135 blocks Gβγ-dimer-dependent activation of PLCβ2. This sequence is located on the outer surface of the Gβ blade 2, and overlaps one of the domains identified by Panchenko.
Panchenko et al., 1998) as being involved in PLCβ2 activation. Another important region for interactions with effectors is the C-terminal region of the Gβ subunit: the sequence between Gly 306 and Gly 319 that is located at the top surface of the β propeller of β1 is involved in the activation of PLCβ2 and ACII (Myung and Garrison, 2000). Overall, these results indicate that the amino acids at the top surface and at the edges of the β torus are important for PLCβ activation, whereas the activation of ACII depends more on its interaction with the top surface of Gβ, thus demonstrating that various regions of Gβγ are involved in its interaction with its targets.

1.1.3.3 Gβγ dimers modulate the activity of ion channels

1.1.3.3.1 Potassium channels

The inward rectifier G-protein-gated K+ channel (GIRK) was the first identified effector of Gβγ. Early studies demonstrated that the channel is acutely gated by Gβγ, with channel openings increasing up to 1,000-fold upon activation of the muscarinic receptor or application of Gβγ to the intracellular face of an excised membrane patch (Logothetis et al., 1987). These results were confirmed upon cloning of the GIRK channel, with the demonstration of a functional interaction between the Gβγ dimer and the full-length GIRK protein (Krapivinsky et al., 1995) and with segments of the channel subunits (Huang et al., 1995; Inanobe et al., 1995).

Mammalian GIRKs are homo- or heteromultimers of GIRK monomers (Corey et al., 1998) that are found in brain, where they mediate the postsynaptic inhibitory effects of various transmitters (dopamine, somatostatin, opioids and others), and in heart, where they underlie a large part of the negative chronotropic parasympathetic regulation (reviewed by Dascal, 1997). Gβγ interacts directly with the N- and C-terminal intracellular domains of each GIRK subunit. The identification of amino acid residues within the pore that if mutated, render the channel constitutively active and Gβγ insensitive has contributed to the
definition of the gating mechanism mediated by Gβγ (Sadja et al., 2001; Yi et al., 2001). According to this model, the binding of Gβγ results in a conformational rearrangement of the channel structure that allosterically regulates the gating apparatus in the pore. More recently, studies with chimeras between Gy2 and yeast Gy have revealed that the C-terminal half of the Gy2 subunit is required for channel activation by the Gβγ complex (Peng et al., 2003). In addition, point mutations of Gy2 have identified several amino acids that significantly reduce the ability of Gβγ to stimulate channel activity, an effect that is not due to improper association with Gβ. Most of the critical Gy residues that have been identified cluster together, forming an intricate network of interactions with the Gβ subunit, and defining an interaction surface of the Gβγ complex with GIRK channels. These results reveal a functional role for Gy in Gβγ-mediated effector modulation.

Further studies indicated an additional level of complexity in the regulation of the GIRKs. Activation of the channel by Gβγ requires permissive levels of membrane phosphatidylinositol4,5-bisphosphate (PI4,5P2). In turn, binding of PI4,5P2 to GIRK channels is stabilized by Gβγ (Huang et al., 1998; Zhang et al., 1999). Probably the gating of the channel by Gβγ proceeds through the modulation of the channel-PI4,5P2 interactions (Logothetis and Zhang, 1999).

1.1.3.3.2 Calcium channels

The Gβγ subunit also has an important modulatory role in inhibition of some presynaptic Ca2+ channels (Ikeda, 1996). Voltage-gated Ca2+ channels are transducers that couple cellular excitability to several cellular processes, including contraction, secretion and gene regulation. These channels are multimers that are composed of a common α1 subunit that forms the conducting pore, which is associated with the regulatory subunits (α2, β, γ, and δ). Inhibition of Ca2+ channels by G proteins was first reported in the chick dorsal root ganglion (Marchetti and Robello, 1989), and subsequent studies using rat GH3
pituitary cells have provided evidence for an involvement of Gβγ subunits in the receptor-dependent inhibition of Ca^{2+} channel activity (Hescheler and Schultz, 1994). The over-expression of Gβγ in rat sympathetic neurons and in cell lines expressing the reconstituted whole Ca^{2+} channel complex have contributed to the confirmation of these initial observations (Ikeda, 1996).

Gβγ inhibits the Ca^{2+} current by forming contact with two regions on the channel α1 subunits: the intracellular I-II loop (De Waard et al., 1997; Page et al., 1997) and the C-terminus (Zhang et al., 1996), forcing the channel into a state of low open probability. Although direct binding studies between full-length α1 subunits and Gβγ have not been performed, indirect kinetic evidence suggests the presence of a single Gβγ binding site per channel (Zamponi and Snutch, 1998). The extent of inhibition is dependent on the Gβ subunit isoform and it is antagonized by PKC-dependent phosphorylation of the channel (Zamponi et al., 1997). Finally, additional subunits and proteins can affect the binding of Gβγ and its effects on Ca^{2+} gating. First, the β subunit of the channel appears to be indispensable for the Gβγ activity (Meir et al., 2000) although the mechanism of this interaction is still unclear. Secondly, syntaxin.1A (a protein that is crucial for transmitter release) modulates the channels by binding to the α1 subunit and Gβγ, and it supports the Gβγ-induced inhibition (Jarvis et al., 2000). Finally, Gαo and Gαs subunits form contacts with the Ca^{2+} channel and probably serve as donors of Gβγ (Furukawa et al., 1998; Kinoshita et al., 2001). These results suggest that an integrated crosstalk between G protein signalling and other cellular pathways takes place at the level of the Ca^{2+} channel.

1.1.3.3 Other ion channels

Direct interactions of Gβγ with other ion channels have also been reported. Kir2.3, an inwardly rectifying K^{+} channel that does not belong to the GIRK family interacts
directly with Gβγ and is inhibited by Gβγ co-expression in Xenopus oocytes (Cohen et al., 1996). The physiological role of this Gβγ modulation is unclear.

Gβγ subunits also regulate ATP-sensitive K⁺ (K_{ATP}) channels. These are normally inhibited by resting levels of cytosolic ATP and they have a protective role during metabolic stress and cardiac excitability (Ashcroft, 1988). In COS cells, Gβγ interacts directly with the accessory proteins of the K_{ATP} channel leading to its activation by reducing the inhibition by ATP (Wada et al., 2000). Notably, the affinity of Gβγ revealed by experiments in excised patches, is very high (5-10 pM caused substantial activation), suggesting a sensitivity to Gβγ at least two orders of magnitude higher than that of the GIRKs.

1.1.3.4 Gβγ dimers and signal generating enzymes

1.1.3.4.1 PLCs

The PLC enzymes have key roles in signal transduction through their hydrolyzing of PI4,5P₂ with the release of the second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP₃) (James and Downes, 1997). This leads to activation of protein kinase C (PKC) in the case of DAG and to the release of Ca⁺ from intracellular stores in the case of IP₃. A number of PLC isoforms have been purified and cloned. PLCβ, γ and δ were identified over a decade ago and two additional members of the family, PLCɛ and PLCξ have been cloned more recently.

The four PLCβ isoenzymes (PLCβ₁₋₄) that have been identified to date can be activated by both Gα_q₁₁ and Gβγ subunits, and these pathways are important in the regulation of cardiovascular function and neuronal plasticity (James and Downes, 1997). In reconstitution assays, it has been demonstrated that the Gβγ-dependent activation of the PLCβs is due to a direct interaction, while by expression of single PLCβ isoforms in cells or by using purified PLCβ, it has been shown that Gβγ can activate all of the PLCβ
isoenzymes. PLCβ2 and PLCβ3 appear more sensitive to stimulation by Gβγ than PLCβ1 and PLCβ4 (Park et al., 1993; Rhee, 2001). In the case of PLCβ3, Gαq and βγ are about equally effective as activators (on a molar basis). For other members of the PLCβ family, the Gαq subunits are considerably more potent (50- to 100-fold) than the Gβγ dimer.

The activation of the PLCβ isoforms by Gαq and the βγ subunit is independent, and not conditional upon the priming by either subunit (Smrcka and Stemweis, 1993). This reflects the different sites on the PLCβ molecule to which they bind. Blank and colleagues (Park et al., 1993) isolated a shortened form of PLCβ3 that is activated by βγ but not by Gαq, and truncation of a sequence of amino acids at the C-terminus eliminated activation by Gαq, but not by Gβγ (Lee et al., 1993). From these observations, it has been postulated that Gβγ binds to the N-terminus while Gα binds to the C-terminal region (Wu et al., 1993). The PLCβ enzymes contain a pleckstrin homology (PH) domain at the N-terminus, but there are no direct investigations of the role for this domain in the binding of the Gβγ dimer.

Expression of Gβ1γ2, Gβ1γ3 or Gβ2γ2 in COS7 cells markedly stimulates the enzymatic activity of PLCε that is involved in the regulation of cell morphology, development and proliferation (Wing et al., 2003). Whether this activation of PLCε by Gβγ occurs by a direct interaction is not known. Since PLCε is also modulated by the monomeric G proteins Ras and Rho, it can be considered as a nexus protein that senses and mediates crosstalk between heterotrimeric GTPase and small GTPase signalling pathways.

1.1.3.4.2 ACs

The Gβγ subunit also regulates some form of the ACs (Patel et al., 2001; Sunahara et al., 1996). These catalyse the formation of cyclic AMP (cAMP), that in turn activates cAMP-dependent kinases (cAPK), such as PKA. Molecular cloning techniques have
identified nine mammalian AC isoforms, types I-IX, that encode membrane-bound ACs, and one gene encoding a soluble isoforms.

The Gβγ subunit is a strong modulator of AC activity that can be either stimulated synergistically with Gαs (type II, IV and VII), or inhibited (ACI and ACVIII) (Tang et al., 1991). The relationship between Gβγ and the other cyclase isoforms, such as type V and type VI, is however less understood. Transfection experiments suggest that Gβγ can inhibit ACV and ACVI activities, perhaps in an indirect manner (Bayewitch et al., 1998). A failure to detect inhibition of AC activity by isolated Gα subunits has led to the proposal that sequestration of Gαs by Gβγ is the mechanism underpinning this inhibitory mechanism.

Interestingly, the Gβγ-inhibited and some Gβγ-independent isoforms of ACs (ACI, ACIII and ACVIII) are stimulated by Ca²⁺/calmodulin (Cali et al., 1994; Tang et al., 1991), while ACV and ACVI are inhibited in vitro by micromolar calcium concentrations (Cooper and Brooker, 1993; Cooper et al., 1994; Yoshimura and Cooper, 1992). Ca²⁺ levels are regulated by the phosphoinositide pathway through PLCβ, and thus the Gβγ-mediated activation of PLCβ that regulates calcium levels indirectly modulates the ACs, serving as a cross-link between these two pathways.

1.1.3.5 Gβγ dimers and the MAP kinase cascade

In eukaryotic cells, the participation of the Gβγ subunit in the cell cycle was suggested when it emerged that Gβγ dimers directly elicit signalling pathways leading to MAPK activation in response to Gαo/Gαt-linked receptor activation (Crespo et al., 1994). Then, under a variety of experimental conditions, it was demonstrated that MAPK activation by Gβγ is blocked by a dominant interfering mutant of the monomeric G protein Ras, and that Gβγ can induce accumulation of the activated form of Ras (Crespo et al., 1994; Koch et al., 1994). Taken together, these findings indicate that signalling from
GPCRs to MAPK involves a Gβγ-mediated Ras-dependent pathway and provides strong evidence that the GPCRs and RTK signalling use the same pathway to activate MAPK. First, the Gβγ- and RTK-dependent pathways converge on the activation of Ras (Gutkind, 2000); secondly, the pathway by which Gβγ stimulates MAPK may include a non-receptor tyrosine kinase, such as Lck, Fyn or cSrc. Finally, in certain cells the activation of MAPK by Gβγ may also require trans-activation of RTKs for the assembly of the Ras-activating complex (Della Rocca et al., 1999).

Several intracellular effectors have been proposed as the first step towards activation of MAPK by Gβγ dimers (Figure 1.6). Since Gβγ can activate PLC, it has been hypothesised that the IP₃ derived from this PLC pathway can increase intracellular Ca²⁺, thus leading to Ca²⁺/calmodulin activation that, in turn, activates Pyk2 kinases. Pyk2 phosphorylates Shc, recruiting Grb2 and Sos, and the protein complex Shc/Grb2/Sos initiates the sequential activation of Ras, Raf and MEK. In confirmation of this proposal, the disruption of this complex blocks Gβγ-mediated MAPK activation (Della Rocca et al., 1997). Gβγ-mediated activation of PLC also leads to an increase in DAG and activation of PKC. Therefore, at least in some cell types, Gβγ should activate the MAPK pathway through a PKC-dependent, but Ras-independent, pathway, similar to the MAPK pathway activated by Gαq.

In addition, PI3K type IB is considered to be an early intermediate in Gβγ-mediated MAPK activation (Hawes et al., 1996) since it is directly activated by Gβγ dimers. The PI3Ks are a large family of enzymes that phosphorylate the phosphoinositides (PIs) in the 3-phosphate position of the inositol rings (Fruman et al., 1998; Krugmann and Welch, 1998), forming lipids (PI3P, PI3,4P₂ and PI3,4,5P₃) that are crucial in various signalling pathways involved in cell proliferation, differentiation, apoptosis, cytoskeleton organization and membrane trafficking. The PI3Ks are subdivided into three main classes (I, II, III) based on sequence similarities, substrate specificities and physiological roles.
Figure 1.6. Gβγ dimer and MAP kinase cascade. Biochemical routes initiated by Gβγ subunit activation can stimulate Ras by activation of non-receptor tyrosine kinases, such as Lck, Fyn and/or cSrc, or by activation of PLC and PI3K. IP₃ derived from the PLC pathway can increase intracellular Ca²⁺, which in turn activates Pyk2. Pyk2 phosphorylates Shc, recruiting Grb2 and Sos, and the Shc/Grb2/Sos protein complex initiates sequential activation of Ras, Raf and MEK. Gβγ-mediated activation of PLC also leads to an increase in DAG and activation of PKC, causing activation of the MAPK pathway through a PKC-dependent, but Ras-independent, pathway. PI3Kβ mediates MAPK stimulation through activation of the Gab1/SHP2 complex, while PI3Kγ mediates MAPK stimulation through activation of the Shc/Grb2/Sos complex.
(reviewed in Fruman et al., 1998). The class I PI3Ks are the best characterized, and they are composed of a 110-kDa catalytic subunit (four isoforms, p110α, β, γ and δ), that is associated with a regulatory subunit (p85 or p101) that defines two subclasses (A and B, respectively). Gβγ dimers directly activate the sub-type IB PI3Kγ that is formed by p110γ and p101 (Stephens et al., 1997), both in vivo and in vitro (Krugmann et al., 1999). When expressed in Sf9 cells or COS7 cells, p110γ has a significant basal activity that is only slightly stimulated by the Gβγ dimer, but when p110γ is co-expressed with its associated subunit, the p110γ/p101 heterodimer is stimulated to a much greater extent (Stephens et al., 1997). Thus, one of the functions of p101 is to increase the sensitivity to Gβγ-stimulation. Leopoldt and colleagues (Leopoldt et al., 1998) using co-purification of Gβγ with a p110γ-GST fusion protein provided evidence of the direct binding of Gβγ to multiple regions of the p110γ subunit. In addition, since the Gβγ-stimulated activity of PI3Kγ requires the N-terminus of p101, the p101 subunit is also involved in binding the Gβγ dimer (Krugmann et al., 1999). Thus, the mechanism by which Gβγ activates PI3K involves direct binding of Gβγ to both components of the p110γ/p101 heterodimer. Finally, the p110γ catalytic subunit in turn mediates MAPK stimulation through the activation of the Shc/Grb2/Sos complex (Bondeva et al., 1998). However, the molecular events underlying these observations remain to be clarified.

Also the activation of the IB PI3Kβ by Gβγ dimers appears to be involved in MAPK activation. Early studies showed that PI3Kβ is greatly stimulated by Gβγ and by peptides containing phosphotyrosyl residues (pY) (Kurosu et al., 1997), although the molecular determinants of this synergism have not been defined. In addition, a dominant-negative mutant of p110β inhibits MAPK stimulation (Yart et al., 2002b), and activation of p110β is essential for cell-cycle progression of NIH-3T3 cells stimulated with lysophosphatidic acid (Yart et al., 2002a). The Gβγ/PI3Kβ-mediated MAPK activation probably involves the pY-motif-containing adapter protein Gab1, and the tyrosine phosphatase SHP2 that is
recruited through PI3K and Gab1. These findings indicate that the p110β/Gab1/SHP2 pathway provides a novel link between Gβγ and Ras in the activation of the MAPKs.

Gβγ also regulates the UV-induced activation of p38 and JNK (Seo et al., 2002), suggesting that G proteins mediate the activation of MAPK stimulated by stress, although this signalling cascade downstream of Gβγ is not well understood. The over-expression of Gβγ in COS-1 and human keratinocytes increases UV-induced activation of the small G protein Cdc42, and over-expression of a constitutively active Cdc42 increases UV-induced p38 activation (Seo et al., 2004). This activation of p38 enhances the resistance of normal human keratinocytes to apoptosis (Chouinard et al., 2002). In addition, Gβγ increases UV-induced phosphorylation of the epidermal growth factor receptor (EGFR), and UV-induced p38 activation is blocked by an EGFR kinase inhibitor. These results indicate that Gβγ mediates UV-induced activation of p38 in a Cdc42-dependent way and that activation of EGFR is required (Seo et al., 2004).

The recruitment of different signalling proteins, such as Src, Pyk2, monomeric G proteins and PI3Ks, by Gβγ subunits and the cross-talk between the G-protein and receptor tyrosine kinase signalling pathways allows cells to integrate the information from many different sources and this facilitates their delicate control over multiple regulatory systems.

1.1.3.6 Other Gβγ interactors

The Gβγ dimer has also been shown to interact directly with a variety of regulatory proteins, mainly through a region that contains sequences homologous to PH domains (Touhara et al., 1994). An important group of Gβγ interactors is represented by the ARF family of monomeric G proteins. In its inactive GDP-bound form, recombinant ARF1 was shown to bind the Gβγ subunit of transducin on phospholipid vesicles, rather than the entire Gαβγ protein heterotrimer. The physiological significance of this interaction remains uncertain due to its low affinity, although it may be involved in the dynamics of Golgi membranes (Franco et al., 1995). Concomitantly, using a different in vitro approach, it was
demonstrated that both Gβγ and Gαs interact with ARF1 and ARF4 (Colombo et al., 1995). In particular, Gβγ interacts directly with ARF-GDP, suggesting that the complex Gβγ/ARF-GDP allows the interaction of ARF proteins with nucleotide exchange factors, thus facilitating ARFs activation. Further demonstrations that ARFs and Gβγ can interact in vivo come from the work of Galas and colleagues (Galas et al., 1997), who demonstrated that ARF6 is specifically associated with the membranes of secretory chromaffin granules through its interaction with the Gβγ subunit and, they proposed that the Gβγ dimer represents the membrane receptor that stabilises the interaction of ARF6 with secretory granules. In line with these observations, Le Stunff (Le Stunff et al., 2000) using an in vitro assay, provided evidence that in myometrial membranes, inactive ARF6 is bound to the Gβγ subunit and that ammonium sulphate disrupts this association, enabling ARF6 to mediate PLD activation.

The interaction site between the ARFs and Gβγ has not been defined. Although ARFs lack a PH domain, the ARF accessory proteins, such as GAPs or GEFs, do contain such a PH domain, and this may regulate ARF activation in conjunction with Gβγ.

Recently, Dell et al. (Dell et al., 2002) used a yeast two-hybrid screen to demonstrate that Gβγ, through its own WD-40 domain can interact with several WD-40 repeat proteins. These include RACK1, a partner of an activated PKC isozyme (PKCβII), and dynein intermediate chain (DIC), a retrograde microtubule motor protein involved in cell division and intracellular transport. The possible functional roles of the interactions between Gβγ and RACK1 and DIC remain to be determined. One possibility is that it regulates the binding of RACK1 to the membrane. RACK1 lacks any post-translational modification that might promote its membrane association so its interaction with Gβγ may constitute the membrane receptor similar to that proposed for ARF6.

Interactions between other WD-40 repeat proteins have also been reported in the literature (Tcherkasowa et al., 2002). These encompass diverse cellular processes, ranging
from signal transduction to cell growth and vesicular trafficking. In general, interactions between Gβγ and other WD-40-containing proteins might represent a new mechanism for the integration of signals across different pathways.

1.1.4 Regulation of βγ dimer activities

The broad spectrum of action and the ability of free Gβγ dimers to activate so many effectors implies that its activity has to be tightly controlled. Based on present knowledge, the activation of Gβγ is mainly regulated by the same mechanisms that regulate the Ga subunits: it depends on GPCR activation, as well as its deactivation which depends on the Ga subunit GTPase activity. In addition, the activation state of Gβγ can be modulated by regulators of the Ga guanine nucleotide cycle, like the RGS, GoLoco and AGS proteins (as described in Section 1.1.2.3). Nevertheless, Gβγ functionality is also controlled by interactions with proteins other than the Ga subunits, making the regulation of this subunit much more complex than would have been thought. In the following section, an overview of the regulatory mechanisms active on the Gβγ dimer in a Ga-subunit-independent manner is reported.

1.1.4.1 Proteins that directly regulate signalling of the βγ dimer

1.1.4.1.1 G-Protein coupled receptor kinase

Important proteins involved in the regulation of Gβγ dimers are the G-protein-coupled Receptor Kinases GRK2 and GRK3 (originally known as β-adrenergic receptor kinase, βARK). Interactions between Gβγ and GRK2 mediate the membrane association of the kinase, and enhance GRK2-mediated receptor phosphorylation. The phosphorylation of the receptor does not interfere with G protein activation itself; instead, it begins the receptor-desensitization processes (Penela et al., 2003).
Free Gβγ subunits bind GRK2 through basic residues on two distant sites of the C-terminal PH domain of GRK2. One of these sites (R587) is in the N-terminal half of the PH domain, and the second is in the extended α-helix and involves three lysine residues: 663, 665 and 667 (Carman et al., 2000). Modulation of GRK2 by Gβγ could be more complex, since the occurrence of a regulatory Gβγ-binding site in its N-terminus (amino acids 1-53) has recently been suggested (Eichmann et al., 2003). In addition, the PH domains of GRK2 and GRK3 can interact directly with PI4,5P₂, the production of which is regulated by Gβγ (DebBurman et al., 1996). This suggests that Gβγ contributes to GRK2 localization and activation with multiple pathways.

1.1.4.1.1 Phosducin and phosducin-like protein

Another family of Gβγ-binding proteins is the phosducins, which have been shown to block the functions of the Gβγ dimers. This protein family consists of phosducin, phosducin-like protein (PhLP), and several isoforms of each.

Phosducin is a 33-kDa, soluble phosphoprotein that is expressed at very high levels in retinal photoreceptor cells and in pinealocytes. In the retina, phosducin binds the photoreceptor-specific Gβγ with high affinity and inhibits its interaction with the Gα subunit (Lee et al., 1992; Yoshida et al., 1994). When phosducin is phosphorylated at Ser73 by cAMP-dependent PKA, it no longer blocks the association with Gα. The phosphorylation state of phosducin is determined by light, being phosphorylated in the dark and de-phosphorylated upon illumination (Lee et al., 1990). These data have led to the hypothesis that phosducin participates in light adaptation. In the light, the de-phosphorylated phosducin binds Gβγ and this blocks the re-association with Gα₃-GDP, thereby inhibiting further activation of the heterotrimeric G_i by light-activated rhodopsin.

PhLP also binds Gβγ with high affinity (Schroder and Lohse, 1996; Thibault et al., 1997) and prevents Gβγ from binding Gα. However, PhLP is widely expressed in many
tissues beyond the retina and pinealocytes. Because of this, it has been postulated that PhLP is a general regulator of Gβγ signalling in non-retinal tissues.

Both phosducin and PhLP possess two distinct domains that interact with Gβγ subunits. The N-terminal domains bind loops on the top of the Gβ surface, overlapping the binding surface for Gα and other effectors, explaining why phosducin and PhLP block the functionality of Gβγ (Ford et al., 1998; Hawes et al., 1994). Interactions with the N-terminal domain contribute to a conformational change in Gβγ that tucks its farnesyl group into a protein pocket, thereby decreasing the affinity of Gβγ for the membrane (Savage et al., 2000). The C-terminal domains of phosducin and PhLP appear to have no effects on the association of Gβγ with its effectors but bind the blades 1 and 7 at the putative membrane-binding site of Gβγ (Savage et al., 2000; Sondek et al., 1996).

Besides the ability to scavenge Gβγ subunits, another previously unrecognized mechanism of inhibition of Gβγ-mediated signalling was recently suggested. Humrich and colleagues (Humrich et al., 2005) reported that transfection of PhLPs (a splice variant of PhLP) is associated with down-regulation of transfected and endogenously expressed Gβγ, and that this involves a proteasome-dependent pathway. Since, PhLP also interacts with and inhibits the function of the cytosolic chaperonin complex involved in the folding of several proteins, it was suggested that this interaction results in the misfolding of the G-protein subunits and might thereby provide an indirect mechanism of inhibition.

1.1.4.2 Post-translational modifications

1.1.4.2.1 Lipid modifications

Heterotrimeric G protein functions are also regulated by lipid modifications: depending on their identities, Gα subunit are myristoylated or palmitoylated at the N-terminus, while Gγ subunits are farnesylated or geranylgeranylated at the C-terminus. Gα-myristoylation is an irreversible, co-translational modification occurring only on the Gαi
family and this anchors these G proteins to the plasma membrane (Casey, 1994) and is of importance for the interactions of Go with Gβγ and effectors (Linder et al., 1991; Taussig et al., 1993). The reversible, post-translational palmitoylation is also important for G-protein anchoring to the plasma membrane, and in addition, it increases the affinity of Go for Gβγ. It has the effect of inhibiting the interaction of GzGAP (an RGS protein) with Go (Tu et al., 1997).

The post-translational prenylation of Gy is not necessary for Gβγ dimer formation; indeed, Gy subunits in which the cysteine (that is the target of prenylation) is mutated to serine can form Gβγ dimers as efficiently as wild-type Gy (Casey et al., 1994). However, lipid modifications of Gy are necessary for membrane attachment of the Gβγ dimer (Chen and Manning, 2001) and for the productive interactions of Gβγ with other proteins, including the Go subunit and effectors as well as the receptor.

1.1.4.2.2 Phosphorylation

In addition to lipid modifications, βγ subunits can be modified by phosphorylation. The first indication that Gβ is phosphorylated was provided by Wieland (Wieland et al., 1993; Wieland et al., 1991). Interestingly, the target of the phosphotransfer reaction is a histidine residue, which is phosphorylated by GTP. The phosphate can be re-transferred onto GDP so generating GTP, which can activate Gi and Gs heterotrimer. Recent evidence suggests that this unusual phosphotransfer reaction is catalysed by the ubiquitous membranous isoforms of nucleoside diphosphate kinase (NDPK-B) (Cuello et al., 2003). Gβγ and NDPK-B are able to interact directly, forming a complex that allows the phosphorylation of Gβ and then the transfer of the phosphate on the GDP derived from the Go subunit. A physiological role of this reaction is suggested by the increase in cAMP formation that is dependent on the presence of NDPK-B complexes with Gβγ that occurs in rat heart myocytes (Hippe et al., 2003). These observations fit with a model where the
phosphorylated NDPK-B/Gβγ complex contributes to the receptor-independent activation of G proteins. Very recently, it has been reported that the phosphorylated Gβ subunit is a substrate of a widely expressed protein histidine phosphatase (PHP), both in vitro and in vivo (Maurer et al., 2005). This finding delineates a cycle in which Gβ is phosphorylated by NDPK and dephosphorylated by PHP. As Gβ, NDPK and PHP are ubiquitously expressed, these data hint at a novel but widespread mechanism in eukaryotes for the regulation of the basal, receptor-independent activation of heterotrimeric G proteins.

Both the Ga and Gβγ subunits are subject to phosphorylation by Ser/Thr-protein kinases, such as PKC, and this results in altered functional properties. Indeed, phosphorylation of Ga by PKC inhibits signal transduction through the Ga5, Ga1z and Ga12 family members. Interaction of phosphorylated Ga subunit with free βγ is greatly impaired: it is likely that this prolongs Gβγ-effector activation, while reducing Ga effector stimulation (Fields and Casey, 1995).

Similar to the Ga subunit, Gβγ containing γ12 are substrates of PKC phosphorylation (Asano et al., 1998). The free Gβγ12 is phosphorylated on an N-terminal Ser residue of the Gy subunit. Compared to the non-phosphorylated form, the phosphorylated protein is more resistant to digestion by calpain and appears to interact with the Ga subunit more tightly. Interestingly, phosphorylation of βγ12 affects the interaction of Gβγ only with certain effectors. Indeed, phosphorylation inhibits Gβγ-mediated AC activation while having no effect on activation of PLCβγ (Yasuda et al., 1998). This demonstrates that several different mechanisms must cooperate to regulate signalling mediated by Gβγ subunit.

1.1.4.2.3 ADP-ribosylation

G proteins can also be regulated by the mono-ADP-ribosylation reaction. There are a number of bacterial toxins that alter the action of specific heterotrimeric G proteins as
part of their mechanisms of toxicity. Our laboratory provided the first demonstration that the G proteins can also be mono-ADP-ribosylated by an endogenous enzyme. Indeed, we showed that the Gβ subunit is a substrate of a cycle of ADP-ribosylation/de-ribosylation that modulates its activity (Lupi et al., 2000; Lupi et al., 2002). This cycle represents the means to regulate the Gβγ dimers independently from the α subunits. A deeper description of the endogenous mono-ADP-ribosylation reaction involving the Gβ subunit is reported in Section 1.2.3.3.2.

1.1.4.2.4 Ubiquitylation

The ubiquitin proteasome pathway (UPP) provides yet another level of regulation (Obin et al., 1994). This is a conserved pathway of selective protein modification and degradation that controls levels and activities of highly regulated eukaryotic proteins (reviewed in Ciechanover et al., 2000). Substrates of the UPP are covalently ligated to one or more monomers of the 8.5-kDa protein ubiquitin by the sequential activities of three families of enzymes: ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (Ubc/E2) and ubiquitin-ligase (E3). Interestingly, the Gβγ subunit of transducin is one of the targets of UPP (Obin et al., 1994). Ubiquitylation of Gβγ on Gγ is catalysed by two different ubiquitin-conjugating enzymes (E2) and this coincides with the degradation of the entire Gβγ dimer. The association of Gβγ with phosducin blocks Gβγ ubiquitylation and its subsequent degradation (Obin et al., 2002), but phosphorylation of phosducin, which inhibits the phosducin-Gβγ complex formation, completely restores ubiquitylation and degradation. Thus, ubiquitylation of Gβγ can be considered as a selective proteolytic mechanism that regulates transducin activities with phosducin probably serving as a protective factor for Gβγ following the light-dependent dissociation of Gαβγ.
1.2 The enzymatic ADP-ribosylation reaction

Cells adapt rapidly to changes in their environment by regulating the functional status of signalling protein. This is achieved by various mechanisms, among which there are several post-translational modifications that are crucial for protein activation and/or inactivation. Whereas the best-described modifications involve phosphorylation of specific amino acids, evidence is also accumulating that endogenous ADP-ribosylation can have a similar role.

ADP-ribosylation consists of the binding of an ADP-ribose group, and it can occur by non-enzymatic or an enzymatic reaction. In the first case, ADP-ribose that is produced from NAD$^+$ by glycohydrolase activity is attached non-enzymatically to reactive nucleophilic amino acids residues, primarily lysine and cysteine (Ziegler, 2000).

Alternatively, the ADP-ribose moiety is enzymatically transferred from NAD$^+$ to specific amino acids of the acceptor proteins. ADP-ribosyltransferases, the enzymes that catalyse the enzymatic reactions, are capable of cleaving β-NAD$^+$ at its β-N-glycosidic bond and covalently attaching either a single (mono-ADP-ribosylation) or multiple (poly-ADP-ribosylation) ADP-ribose moieties to the target proteins (Ame et al., 2004; Corda and Di Girolamo, 2003). These two classes of enzyme-catalysed ADP-ribosylation reaction differ not solely in the length of the ADP-ribose chain, but also in the chemical nature of the ADP-ribosyl-bond (N-glycoside versus O-glycoside) and the site of reaction (cytoplasm and cell membrane versus nucleus) (Shall, 1995).

1.2.1 The poly-ADP-ribosylation reaction

Poly-ADP-ribosylation is a modification that occurs widely in nature and that has important roles in processes aimed at preserving the functional integrity of the genome. It thus influences processes such as recombination, transcription, differentiation and apoptosis.
The PARPs (poly-ADP-ribosylpolymerases) that catalyse this reaction are present in almost all eukaryotic cells, although they are apparently absent in yeast (Hassa and Hottiger, 2002). PARP1, the prototypical and most widely expressed member of the PARP family, is a nuclear protein with highly conserved sequence, especially at amino acids comprising structural motifs and functional domains. These include: (1) an N-terminal double zinc-finger domain (DBD) with a high affinity for DNA single strand breaks; (2) a C-terminal NAD\(^+\)-binding catalytic domain, with a structure similar to that of diphtheria toxin, indicating a phylogenetic relationship among the ADP-ribosyltransferases; and (3) a central auto-modification domain with several conserved glutamate residues (Ame et al., 2004; Rolli et al., 1997). Auto-modification can inhibit PARP1-DNA binding, protein-protein interactions and ADP-ribosyltransferase activity, which ultimately inactivates the protein (D'Amours et al., 1999).

The product of the reaction catalysed by PARP1 is represented schematically in Figure 1.7. Initially, PARP1 catalyses NAD\(^+\) hydrolysis with concomitant release of free ADP-ribose that is attached to the acceptor amino acid (initiation phase). Then, this ADP-ribose serves as an acceptor for the successive attachment of ADP-ribose to the 2' position (elongation phase). Branching occurs at approximately every 20-25\(^{th}\) ADP-ribose units (branching phase) (Alvarez-Gonzalez et al., 1999). The resulting tree-like structure comprises many negative charges and profoundly alters the function of the modified protein. The main target of poly-ADP-ribosylpolymerase is PARP-1 itself, but the polymer is also attached to other proteins, including histones, topoisomerases, and high mobility group (HMG) proteins.

An important feature of PARP1 is the requirement for DNA strand breaks to activate its catalytic functions in DNA repair processes. In addition, to direct binding to DNA, PARP1 activity can also be stimulated by interactions with protein binding partners. In particular, PARP1 recruits repairing enzymes at sites of DNA damage and participates in the ligation step (Creissen and Shall, 1982). Indeed, 3-aminobenzamide, a specific
Figure 1.7. Poly-ADP-ribosylation. Poly-ADP-ribose polymers (PARs) are synthesised by the distinct enzymatic activities of PARP1, including (A) initiation, (B) elongation, and (C) branching. PARs are attached to glutamate residues in target proteins via covalent linkages at the position labelled 1 in the expanded view of an ADP-ribose unit. The ADP-ribose units in the PAR polymers are linked via glycosidic ribose-ribose bonds at the positions labelled 1 and 2 in the expanded view. The blue arrows indicate the sites of action of poly-ADP-ribose glycohydrolase (PARG) and ADP-ribosyl protein lyase.
PARP inhibitor, prevents the ligation of 10kb-DNA-replication-intermediates to the high-molecular-weight DNA-replication forks in a human melanoma cell line (Lonn and Lonn, 1985). In striking contrast to this cytoprotective function, in case of serious DNA damage, PARP1 becomes over-activated and cells undergo apoptosis due to the depletion of the NAD$^+$ pool. Excessive NAD$^+$ consumption is prevented by the cleavage of PARP1 by caspases that generate two inactive fragments of 24 and 89 kDa (reviewed in Soldani and Scovassi, 2002). The N-terminal fragment (p24) remains in the nucleus, retaining its DNA-binding activity, and inhibits the catalytic activity of uncleaved PARP1 and impairs DNA repair. The p89 fragment migrates from the nucleus to the cytoplasm in late apoptotic cells with advanced nuclear fragmentation and becomes a potential target of autoimmunity (Rodenburg et al., 2000; Figure 1.8).

In the absence of DNA damage, PARP1 regulates normal cellular functions, such as transcription and maintenance of chromatin structure. The available data suggest that PARP1 regulates transcription by modifying histones, the structural proteins that constitute chromatin, with severe effects on both the packaging of nucleosomes into high-ordered structures and on the stability of single nucleosomes (D'Amours et al., 1999). Indeed, poly-ADP-ribosylation of H1 and H2 histones in vitro by purified PARP1 leads to nucleosome decondensation (Poirier et al., 1982).

The negatively charged ADP-ribose polymers can be recognised by histones, that dissociate from DNA, destabilizing the nucleosome structure (Realini and Althaus, 1992). These effects might allow access of the enzymes involved in transcription to regions normally occluded. In addition, PARP1 regulates transcription by functional interactions with various non-histone proteins, many of which are DNA-binding transcription factors (D'Amours et al., 1999).

Telomeres represent another important cellular target for poly-ADP-ribosylation. Tankyrases (TNKS1 and TNKS2, also termed PARP5), PARP1 and PARP2 ADP-ribosylate the telomere-binding protein TRF1, a negative regulator of telomere-length
Figure 1.8. The involvement of PARP1 in DNA repair and apoptosis. PARP1, a 113-kDa protein, has three functional domains: the N-terminal DNA-binding domain (DBD); the central auto-modification domain (AD); and the C-terminal catalytic domain (CD). PARP1 is a DNA nick-sensor that is recruited to DNA damage sites where it poly-ADP-ribosylates itself. During DNA repair, auto-ADP-ribosylated PARP1 is detached from the DNA, thus allowing DNA damage to be accessible to DNA repair factors. During apoptosis, to avoid excessive NAD consumption, auto-ADP-ribosylated PARP1 is cleaved by caspases into its p24 and p89 inactive fragments.
maintenance (Kaminker et al., 2001; Smith et al., 1998). In vitro, poly-ADP-ribosylated TRF1 moves from telomeric DNA, suggesting that poly-ADP-ribosylation is a positive regulator of telomere length in telomerase-expressing cells.

Like other covalent protein modifications, poly-ADP-ribosylation is reversible. A poly-ADP-ribose glycohydrolase (PARG) catalyses the hydrolysis of ADP-ribose units (Ame et al., 1999), and in vivo the steady-state cellular levels of poly-ADP-ribosylation are determined by the opposing actions of PARP and PARG.

1.2.2 The mono-ADP-ribosylation reaction

The mono-ADP-ribosylation reaction consists of the transfer of a single ADP-ribose moiety from NAD⁺ to target proteins (Figure 1.9). The physiological and most efficient acceptor is usually a specific amino acid residue, although some of the mono-ADP-ribosyltransferase can use water as an acceptor, leading to the glycohydrolysis of NAD⁺ (NADase activity).

The mono-ADP-ribosylation was identified originally as the pathogenic mechanism of action of diphtheria, cholera, and other bacterial toxins (Ueda and Hayaishi, 1985). Only later were the enzymes related to the toxins identified in cells, and their potential physiological roles explored (Corda and Di Girolamo, 2003). In mammalian cells, enzymes that reverse the reaction and regulate the mono-ADP-ribosylation include the cytosolic ADP-ribosylhydrolases and the cytosolic and extracellular pyrophosphatases (Moss et al., 1992; Zolkiewska and Moss, 1995). The former hydrolyse the protein-ADP-ribose linkage with the release of the ADP-ribose moiety, while the pyrophosphatases hydrolyse the pyrophosphate linkage releasing AMP and a ribosylphosphoryl-protein. The presence of these activities in the cell strongly suggests that the mono-ADP-ribosylation reaction represents a normal regulatory mechanism.
Figure 1.9. The mono-ADP-ribosylation reaction. A schematic representation of the reversible mono-ADP-ribosylation reaction that is catalysed by an arginine-specific mono-ADP-ribosyltransferase and an ADP-ribosylhydrolase (see text for details). The other amino acid residues that can be modified by this reaction are cysteine, diphthamide and asparagine (From Corda and Di Girolamo, 2003).
1.2.2.1 Mono-ADP-Ribosylation in viruses and prokaryotes as a mechanism of pathogenesis

Mono-ADP-ribosyltransferase enzymes were historically characterised as virulence factors of several bacterial pathogens that pose significant human health threats (Domenighini et al., 1994; Ueda and Hayaishi, 1985). Indeed, upon translocation into mammalian cells, bacterial toxins ADP-ribosylate crucial host cell proteins, such as the Gα subunits of the heterotrimeric G proteins, the small G proteins Rho and Ras, monomeric actin and elongation factor 2 (EF-2), resulting in permanent activation or inactivation of the cellular functions modulated by these proteins (reviewed by Krueger and Barbieri, 1995; Table 1.3).

Diphtheria toxin (DTX) produced by Corynebacterium diphteriae was the first bacterial toxin to be identified as an ADP-ribosyltransferase. Using radiolabeled preparations of NAD+, Honjo et al. (Honjo et al., 1968) demonstrated that DTX catalyzes the transfer of ADP-ribose to EF2, a 100-kDa protein that functions in polypeptide chain elongation on ribosomes. Subsequent studies demonstrated that DTX specifically ADP-ribosylates diphthamide, a modified histidine present uniquely in eEF2, with drastic consequences for the host cells due to the inhibition of protein synthesis (Collier and Cole, 1969).

Since then, a wide variety of other toxins have been shown to use the mono-ADP-ribosylation reaction to disrupt metabolic processes within host cells.

Cholera toxin (CTX) and the closely related heat-labile enterotoxins LT-1 and LT-2 from Escherichia coli are the etiological agents of cholera and traveller’s diarrhoea, respectively. They are arginine-specific ADP-ribosyltransferases that are able to modify the α subunit of the heterotrimeric Gα proteins. The modified arginines are localized close to the domain responsible for the interaction with the γ-phosphate of GTP, locking the G protein in its active conformation through the reduction of its intrinsic GTPase activity.
<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Source</th>
<th>Substrate/ amino acid</th>
<th>Effect of the reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Viruses:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALT</td>
<td>Bacteriophage T4</td>
<td>RNA polymerase/ Arg265</td>
<td>enhances viral transcription</td>
</tr>
<tr>
<td>Mod A</td>
<td>Bacteriophage T4</td>
<td>RNA polymerase/ Arg265</td>
<td>enhances viral transcription</td>
</tr>
<tr>
<td>Mod B</td>
<td>Bacteriophage T4</td>
<td>RNA polymerase/ Arg265; S1 ribosomal protein/ Arg</td>
<td>enhances viral transcription</td>
</tr>
<tr>
<td><strong>Prokaryotes:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Toxins:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diphtheria</td>
<td><em>C. diptheriae</em></td>
<td>EF2/diphtamide715</td>
<td>inhibits protein synthesis</td>
</tr>
<tr>
<td>Exotoxin A</td>
<td><em>P. aeruginosae</em></td>
<td>EF2/diphtamide715</td>
<td>inhibits protein synthesis</td>
</tr>
<tr>
<td>Exotoxin S</td>
<td><em>P. aeruginosae</em></td>
<td>Ras family/Arg41</td>
<td>disrupts actin microfilaments</td>
</tr>
<tr>
<td>Cholera</td>
<td><em>V. cholerae</em></td>
<td>Gox, Gat/Arg187</td>
<td>inhibits GTPase activity</td>
</tr>
<tr>
<td>LT1, LT2</td>
<td><em>E. coli</em></td>
<td>Gox, Gat/Arg187</td>
<td>inhibits GTPase activity</td>
</tr>
<tr>
<td>Pertussis</td>
<td><em>B. pertussis</em></td>
<td>Gox/0, Gat/Cys351</td>
<td>uncouples receptor and G proteins</td>
</tr>
<tr>
<td>C3</td>
<td><em>C. botulinum</em></td>
<td>Rho, Rac/Asn41</td>
<td>disrupts actin cytoskeleton</td>
</tr>
<tr>
<td>EDIN</td>
<td><em>S. auras</em></td>
<td>Rho/Asn41</td>
<td>disrupts Golgi apparatus</td>
</tr>
<tr>
<td>SpvB</td>
<td><em>S. enterica</em></td>
<td>actin</td>
<td>prevents actin polymerisation</td>
</tr>
<tr>
<td><strong>Intracellular:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DRAT</td>
<td><em>R. rubrum</em></td>
<td>dinitrogenase reductase/ Arg101</td>
<td>inhibits dinitrogenase reductase</td>
</tr>
</tbody>
</table>

Table 1.3. Mono-ADP-ribosylation in viruses and prokaryotes. Modified from Corda and Di Girolamo, 2003.
The prolonged activation and the consequent increase in cAMP in the intestinal epithelium causes net intestinal salt and water secretion, resulting in massive diarrhoea, and changes in cell morphology presumably due to activation of cAMP-dependent PKA (Barbieri et al., 2002; Donta et al., 1976; Peterson and Ochoa, 1989; Scott et al., 2003; Spangler, 1992). Structurally, CTX and the LTs are oligomeric proteins, consisting of a monomeric A subunit, with ADP-ribosyltransferase activity (CTA), coupled to a homopentamer B subunit that binds to the extracellular surface of mammalian cells via specific interactions with the GM1-ganglioside receptors (Zhang et al., 1995). To cause disease, both CTX and LT co-opt molecular machineries of the host cell: the toxin enters by endocytosis of the toxin-receptor complexes, moves to the Golgi cisternae or to the endoplasmic reticulum (ER) via retrograde transport, and finally translocates to its site of action on the inner surface of the plasma membrane (Lencer et al., 1999). The A subunit then requires proteolytic cleavage that allows the release of the 22-kDa catalytically active fragment (CTA1). The enzymatic activity of CTA1 is stimulated by the host protein family of ADP-ribosylation factors (ARFs) (Moss and Vaughan, 1995; see Section 1.3). Another well-characterised bacterial ADP-ribosyltransferase is pertussis toxin (PTX), which is produced by Bordetella pertussis as the etiologic agent of whooping cough. It catalyses the ADP-ribosylation of the Gαi, Go and Gαt subunits on a cysteine residue. Unlike CTX, PTX-catalyzed mono-ADP-ribosylation occurs when Gα is associated with the βγ dimer, thus generating a modified G protein that is unable to transduce signals (Figure 1.11; Hepler and Gilman, 1992). PTX is a heterohexameric protein that is divided functionally into an A-B architecture similar to CTX (Tamura et al., 1982). The A protomer consists of a single subunit (S1) with ADP-ribosyltrasferase activity. The B oligomer is a pentameric complex of five polypeptides, which confers binding specificity to the target cell membrane, thus initiating toxin internalization by receptor-mediated endocytosis (Xu and Barbieri, 1995; Xu and Barbieri, 1996).
Figure 1.10. Effects of cholera toxin on the cycling of G$_s$$\alpha$ between its active and inactive forms. Normally, GTP in active G$_s$$\alpha$-GTP is rapidly hydrolyzed (blue arrow), so that the activation of adenylyl cyclase and rise in cAMP persist only as long as does hormone stimulation. In the presence of cholera toxin (CTX), G$_s$$\alpha$ is irreversibly modified by mono-ADP-ribosylation; the modified G$_s$$\alpha$ can bind GTP but cannot hydrolyze it (green arrows). As a result, there is an excessive, non-regulated rise in the intracellular cAMP levels.
Figure 1.11. Effects of pertussis toxin on the cycling of G<sub>i</sub>α between its active and inactive forms. Normally, G<sub>i</sub> inhibits eukaryotic adenylyl cyclase. In the presence of pertussis toxin (PTX), G<sub>i</sub>α is irreversibly modified by mono-ADP-ribosylation. The modified G<sub>i</sub>α cannot bind GTP (green arrows), and is unable to perform its normal function of inhibition of adenylyl cyclase. The conversion of ATP to cyclic AMP cannot be stopped and the intracellular levels of cAMP increase.
Unlike cholera toxin, there is no evidence that the ADP-ribosyltransferase activity of the S1 subunit requires cofactors, such as the ARFs.

In recent years, the bacterial-mediated ADP-ribosylation of the monomeric G proteins Rho and Ras has been studied in detail due to their pivotal role in the regulation of the actin cytoskeleton. The Rho proteins are modified by a variety of toxins that have been collected into the C3-like family. This group includes *Clostridium botulinum* exoenzyme C3, and the *C. limosum* exotoxin and transferases produced by *Bacillus cereus* and *Staphylococcus aureus* (Aktories and Just, 2005). All of these transferases are single-chained 23-kDa exoenzymes that enter the host cells mostly via fluid phase endocytosis and pinocytosis. C3 toxin causes disorganization of actin filaments due to ADP-ribosylation of RhoA, B and C (Sekine et al., 1989). ADP-ribosylation of Rho does not impair the binding of GTP or effectors; rather, the recruitment of Rho to the membrane is affected. In line with this concept, there is the finding that activation of ADP-ribosylated Rho by GEFs is inhibited (Sehr et al., 1998), and downstream Rho signalling is blocked (Fujihara et al., 1997). The ADP-ribosylation of Rho catalysed by the *S. aureus* toxin EDIN (Epidermal cell Differentiation INhibitor) also disrupts the Golgi complex by a process that is similar to that used by brefeldin A (Sugai et al., 1992). The other Rho subfamily members, Rac and Cdc42, are poor substrates for ADP-ribosylation under artificial conditions and do not seem to be the relevant *in vivo* targets.

Diverse members of the Ras family are targets of arginine-specific ADP-ribosylation by exotoxin S (ExoS) from *Pseudomonas aeruginosa* (Fraylick et al., 2002; McGuffie et al., 1998). Once modified, Ras and its related protein Rap, are unable to interact with their respective guanine nucleotide exchange factors; this results in their inactivation. This causes changes in cytoskeleton architecture induced by the consequent actin rearrangements (Riese et al., 2001). In parallel, ExoS can affect the cellular cytoskeleton structure with a mechanism that is unrelated to ADP-ribosylation of the Ras family but involving the protein moesin (Maresso et al., 2004). Ezrin and radixin, two
moesin homologues, are also ADP-ribosylated, indicating that the ezrin/radixin/moesin family (ERMs) collectively represent specific substrates of ExoS. Since the ERM protein complex contributes to cytoskeleton dynamics, the ability of ExoS to modify ERM links ADP-ribosylation to the actin cytoskeleton changes associated with ExoS intoxication.

The actin network is also affected by the activity of bacterial toxins that directly ADP-ribosylate actin monomers. This is the case for *C. botulinum* type C and type D, which produce the C2 toxin that causes ADP-ribosylation of monomeric actin on arg177, thereby inhibiting polymerization (Figure 1.12; Aktories et al., 1986) and actin ATPase activity (Geipel et al., 1989). In addition, actin polymerisation is affected by the activity of the *Salmonella enterica* virulence-associated protein SpvB. The spvB gene codes for a transferase that modifies actin monomers to prevent polymerization; thus, infected cells lose all of their F-actin filaments (Lesnick et al., 2001). SpvB is expressed by *Salmonella* strains after invasion of epithelial cells and after phagocytosis by macrophages, and its activity causes local and global actin depolymerisation that affects either vesicular trafficking or cellular physiology, or both, to promote *Salmonella* proliferation.

There is an alternative means by which bacterial toxins interfere with signal transduction (Quan et al., 1999). In this case, a mycobacterial transferase causes ADP-ribosylation and inactivation of the antibiotic rifampicin. This constitutes a new mechanism used by pathogens to induce sickness in a host organism through resistance to antibiotic treatment.

Interestingly, some bacterial toxins are encoded by lysogenic phages that incorporate into the DNA of their host bacteria (Uchida et al., 1971). So far, three different ADP-ribosyltransferases have been discovered that are encoded by the bacteriophage T4: Alt, ModA and ModB. Alt, a structural component of the phage head, ADP-ribosylates Arg265 of the host RNA polymerase (Goff, 1984), with the consequent enhancement of viral transcription (Sommer et al., 2000). ModA and ModB both ADP-ribosylate the α-subunit of the bacterial RNA polymerase and the S1 ribosomal protein of *E. coli*
Figure 1.12. Mechanism of F-actin disruption by bacterial toxins. In the host, there is a dynamic equilibrium between G-actin monomers and polymerized F-actin filaments. The SpvB protein and other actin-modifying toxins catalyse the covalent attachment of ADP-ribose to G-actin monomers, preventing their incorporation into polymers. Since F-actin is continuously depolymerised by cytoskeletal regulatory proteins, the monomers are eventually trapped in an inactive form, and no more G-actin is available for polymerisation. The cell thus becomes completely depleted of F-actin filaments.
(Tiemann et al., 1999), both of which support the programme for the phage to gain control over the infected host cell. Computer analysis reveals that both the Mod and Alt protein sequences exhibit structural similarities to the catalytic domains of prokaryotic ADP-mono-ribosyltransferases.

1.2.2.2 Endogenous mono-ADP-ribosylation in the prokaryotic world: the DRAT/DRAG cycle

Bacterial mono-ADP-ribosylation reactions occur not only through the secreted toxins, but also within the bacteria themselves, to control their metabolic enzymes. The best studied endogenous ADP-ribosylation reaction in bacteria is the modification of dinitrogenase reductase in *Rhodospirillum rubrum* (Figure 1.13; Ludden, 1994; Ludden et al., 1989). This modification regulates nitrogen fixation only in certain nitrogen-fixing bacteria (Halbleib et al., 2000), and it was the first clear demonstration of the physiological role of this reaction. The dinitrogenase reductase (an enzyme that reduces nitrogen to ammonium) is inactivated rapidly and reversibly by mono-ADP-ribosylation on arginine, thus preventing non-productive nitrogen fixation during energy-limiting or nitrogen-sufficient conditions. This reaction is catalysed by dinitrogenase reductase/mono-ADP-ribosyltransferase (DRAT), a 30-kDa monomer having high specificity for Arg101 of oxidised dinitrogenase reductase (Lowery and Ludden, 1988). DRAT is activated by environmental stimuli such as darkness or a source of fixed nitrogen. The reaction catalysed by DRAT is very similar to that of CT although there is no obvious amino acid sequence homology between these enzymes, even though some residues are conserved between the two families (Bazan and Koch-Nolte, 1997).

Dinitrogenase reductase is fully reactivated by a specific ADP-ribosylarginine-hydrolase, called dinitrogenase reductase-activating glycohydrolase (DRAG), a 32-kDa monomeric, manganese-dependent enzyme that hydrolys the N-glycosidic bond of the ADP-ribosylated protein (Fitzmaurice et al., 1989). DRAG is activated after exposure of
Figure 1.13. A model of *in vivo* nitrogenase regulation in *R. rubrum* by reversible ADP-ribosylation. Nitrogenase activity is regulated by reversible ADP-ribosylation of dinitrogenase reductase. Dinitrogenase reductase ADP-ribosyltransferase (DRAT) catalyzes the transfer of the ADP-ribose moiety from NAD to an arginine, rendering the dinitrogenase reductase inactive. The enzyme is reactivated upon the removal of the ADP-ribose by dinitrogenase reductase-activating glycohydrolase (DRAG). The redox-dependent conformational change in the protein is shown by a shift in protein subunit position.
the bacteria to light or depletion of the nitrogen source and acts to regenerate free arginine and activating dinitrogenase reductase, thus completing the ADP-ribosylation cycle (Ludden, 1994). The DRAT/DRAG regulatory approach has also been found in other nitrogen-fixing bacteria such as the phototroph *Rhodobacter capsulatus* (Masepohl et al., 1993; Willison et al., 1993) and the non-photosynthetic *Azospirillum brasiliense* and *Azospirillum lipoferum* (Fu et al., 1989; Zhang et al., 1992). This DRAT/DRAG-induced modification represents the best-characterised endogenous ADP-ribosylation cycle and demonstrates that ADP-ribosylation represents a physiological mechanism to regulate the activities of cellular proteins.

**1.2.3 Mono-ADP-Ribosylation in eukaryotes: enzymes and substrates**

After the discovery of the bacterial ADP-ribosyltransferases, similar enzymatic activities were sought in eukaryotic cells. However, the enzyme responsible eluded molecular cloning until the groups of Moss and Shimoyama succeeded in purifying and sequencing proteins with ADP-ribosylating activities from rabbit skeletal muscle and chicken bone marrow cells (Tsuchiya and Shimoyama, 1994; Zolkiewska et al., 1992). Then, using oligonucleotides derived from known mono-ADP-ribosyltransferases (mADPRTs), additional family members were cloned from chicken, mouse, human and other species (Davis and Shall, 1995; Koch-Nolte and Haag, 1997; Koch-Nolte et al., 1996). Cloning of the mADPRTs gave new impetus to the study of the ADP-ribosylation reaction; indeed, mADPRTs represent an exciting possibility for the characterisation of the physiological role of this modification. The growing number of mADPRTs identified has prompted the need to introduce a systematic nomenclature for the corresponding genes and proteins (Haag and Koch-Nolte, 1997). The mammalian mADPRTs are thus designated as ARTs and numbered from 1 to 5 (ART1-5). In Table 1.4, the main features of the cloned ARTs are summarised.
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Species</th>
<th>Preferred site of expression</th>
<th>Predicted cellular localisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ART1</td>
<td>human, mouse, rat, rabbit</td>
<td>skeletal and cardiac muscle</td>
<td>cell surface</td>
</tr>
<tr>
<td>ART2A, ART2B</td>
<td>human (pseudogene), mouse, rat</td>
<td>peripheral T cells, intraepithelial lymphocytes NK cells testis</td>
<td>cell surface</td>
</tr>
<tr>
<td>ART3</td>
<td>human, mouse, rat, rabbit</td>
<td>testis</td>
<td>cell surface</td>
</tr>
<tr>
<td>ART4</td>
<td>human, mouse, rat, rabbit</td>
<td>lymphatic tissue</td>
<td>cell surface</td>
</tr>
<tr>
<td>ART5</td>
<td>human, mouse, rat, rabbit</td>
<td>testis</td>
<td>secreted</td>
</tr>
<tr>
<td>ART6A, ART6B</td>
<td>chicken</td>
<td>bone marrow, heterophils</td>
<td>secreted (granules)</td>
</tr>
<tr>
<td>ART7</td>
<td>chicken</td>
<td>erythroblasts</td>
<td>secreted</td>
</tr>
</tbody>
</table>

Table 1.4. The ART family. Cloned vertebrate members of the mono-ADP-ribosyltransferase gene family (modified from Haag and Koch-Nolte, 1998).
1.2.3.1 The ART family

Ecto-ADP-ribosyltransferases (ARTs) constitute a relatively small group of structurally related proteins that modify extracellular substrates. There are six mammalian ARTs: ART1 (the rabbit skeletal muscle ART), ART2.1 and ART2.2 (identified in rat and mouse), ART3, ART4 and ART5. Three further forms have been described in birds: ART6A, ART6B (Tsuchiya et al., 1991) and ART7 (Davis and Shall, 1995). There are only four functional ART genes in the human genome (ART1, ART3, ART4 and ART5; ART2 is present as a pseudogene; Glowacki et al., 2002). All the ARTs have a hydrophobic N-terminal signal sequence, which indicates that they are extracellular proteins; ART1-ART4 also have hydrophobic carboxyl-terminal signal sequences characteristic of glycosylphosphatidylinositol (GPI)-anchored membrane proteins, with an extracellular catalytic domain (Koch-Nolte and Haag, 1997; Koch et al., 1990; Zolkiewska et al., 1992). Mammalian ART5 and avian ART6 and ART7 possess hydrophobic N-terminal signal sequences and they are secreted proteins (reviewed in Bazan and Koch-Nolte, 1997; Moss et al., 1999; Okazaki and Moss, 1999). Ecto-ARTs ADP-ribosylate proteins on the cell surface of the same cells (cis-ADP-ribosylation), and/or of neighbouring cells in vivo during cell-cell contact (trans-ADP-ribosylation).

Members of this enzyme family share very limited amino acid sequence identity, with 20-30% identity among the ART parologue members within any species; the exception is mouse ART2.1 and ART2.2, with 85% sequence identity indicating a recent evolutionary duplication of the mouse art2 gene (Braren et al., 1998). As the human and mouse genome sequences have been completely determined, all of the recognizable members of these toxin-related ARTs have now been identified for these two species, with the identity among orthologues ranging between 75% and 85%.

Despite the limited primary sequence homology among the different ADP-ribosyltransferases, a central cleft bearing the NAD-binding pocket has been remarkably
conserved between bacterial toxins and eukaryotic mono- and poly-ADP-ribosyltransferases. This core comprises six β-strands, two α-helices and a single helical turn. The six β-strands fold in the form of two β sheets (in complex strand order 6-3-1 and 2-5-4) that are perpendicular to each other, and the two α-helices in turn principally form the “walls” of a bi-lobed crevice formed by the curled sheets (Figure 1.14). Moreover, there are common structural features that characterize this family of mammalian ARTs. Rappuoli and colleagues (Domenighini and Rappuoli, 1996) have proposed a structural model that applies to prokaryotic and eukaryotic ARTs. According to this, three common domains have been identified in the ART catalytic core. Region 1, near the N-terminus of the protein, is characterised either by a conserved arginine or by a conserved histidine (DTX, Pseudomonas exotoxin A, and eukaryotic PARP). Region 2 is characterized either by hydrophobic amino acids that are involved in NAD⁺ binding or by the serine-x-serine motif (where x represents threonine, serine or alanine), and region 3 is highly acidic and characterised by a conserved glutamate residue that is crucial for catalysis (Domenighini et al., 1994). In ART1 from rabbit, the conservative glutamate 240 to aspartate (E240D) substitution abolishes the transfer of ADP-ribose to arginine; the neighbouring glutamate 238 has also been shown to be important for ADP-ribose transfer (Hara et al., 1996; Takada et al., 1995). In several of the ARTs, the replacement of this second glutamate abolishes the ability of these transferases to use arginine as acceptors, thus further supporting the hypothesis that this region is involved in substrate recognition (Han and Tainer, 2002). Indeed, through a comparative analysis of crystallographic structures, Han and Tainer have extended the significance of region 3 sequences by identifying an ARTT motif (ADP-ribosylating turn-turn motif) that they have implicated in substrate recognition (Han and Tainer, 2002). In line with the relevance of the ARTT motif, it has been shown that the auto-ADP-ribosylation of ART2.b is abolished by mutation of its arginine 204, which is part of the ARTT motif. Similarly, if tyrosine 204 of ART2.a is mutated to an arginine (Y204R), it is possible to promote ART2.a auto-ADP-ribosylation
Figure 1.14. The ART family. A, Core features of the ADP-ribosyltransferase fold as a schematic topology diagram. The location of the catalytic Glu is marked by a pink circle. B, Gene structures of the ART1, ART2 and ART5 proteins. Exons (boxes) encode domains common to the ARTs. The transferase catalytic core contains the conserved amino acids that are critical for the formation of the active site. In ART1 and ART5, an arginine- and lysine-rich region is encoded by separate exons, whereas in ART2, this region is included in the exon that contains the catalytic core. The lines between the exon boxes represent the introns of the gene.
(Stevens et al., 2003). Finally, four cysteines that are conserved among all of the ART isoforms form disulfide bridges at the C-terminus of the molecule, which are important for protein stabilization (Mueller-Dieckmann et al., 2002).

The possibility that the N- and C-terminal domains of the ARTs are involved in the regulation of ART activity has been investigated recently by measuring both the transferase and NAD glycohydrolase (NADase) activities of truncated mutants of ART1 (Bourgeois et al., 2003). In mouse ART1, the amino acids at 24-38 (an ART1-specific extension) modulate both the transferase and NADase activities, and amino acids 39-45 (a common ART coil) are essential for both activities. The removal of the C-terminal basic domain decreases the transferase, but enhances the NADase activity. The N- and C-terminal regions of ART1 are therefore required for its transferase activity, while the enhanced NADase activities of the shorter mutants indicate that there are sequences outside of the catalytic site that exert structural constraints, and that modulate the substrate specificity and catalytic activity (Bourgeois et al., 2003).

1.2.3.2 The ecto-ARTs: expression, distribution and biological significance in mammals

Although substantial progress has been made with regard to the molecular characterisation of ARTs, identification of their physiological target proteins has been difficult. This may be related to their high turnover or their low net modification in vivo, and to the low abundance of acceptor proteins. It is also difficult to recognise a common denominator for the target proteins described so far. They are highly diverse with respect to structure, function and cellular localization. In contrast, a common feature of the ecto-ART-encoding genes is that their expression is restricted to one or few tissues (Seman et al., 2004; see Table 1.4). Northern blotting and RT-PCR with human and animal tissues have revealed a relatively broad distribution of ART3 and ART4, and a rather specific expression of ART1 in heart and skeletal muscle in both species. ART5 is mainly expressed in testis, while ART2, which is absent in man, has been detected in mouse
peripheral and rat lymphoid tissues (Adriouch et al., 2001). The pattern of ART expression in some cases is related to cell differentiation and/or activation state. In human polymorphonuclear neutrophils, translocation of ART1 to the cell surface depends on the presence of activating ligands such as formyl-Met-Leu-Phe (fMLP), IL-8 or platelet activating factor (PAF) (Kefalas et al., 1999). Similarly, the rate of ART3-transcription is increased upon exposure of human monocytes to lipopolysaccharide, which also induces expression of ART4 mRNA (Grahnert et al., 2002). Another typical example is ART2 expression on murine T cells. ART2 is expressed only on a small subpopulation of mature thymocytes, and thus appears to be developmentally regulated (Koch-Nolte et al., 1999). Moreover, whereas ecto-ART expression increases with human PMN or monocyte activation, ART2 is shed from the membrane upon T-cell activation (Kahl et al., 2000; Nemoto et al., 1996).

Expression of ecto-ARTs on the cell membrane also appears to depend on genetic factors. Analysis of ART2.2 levels on T lymphocytes has revealed a considerable variation from one mouse strain to another (Koch-Nolte et al., 1999), which might result from cis-acting regulatory elements in the promoters and their distribution in alternative splice variants (Sardinha et al., 1999).

1.2.3.2.1 ART1

ART1 is expressed predominantly in leukocytes and in skeletal muscle, where its substrates include integrin α7. Although the functional consequences of integrin α7 ADP-ribosylation remains to be determined, it has been postulated that this modification affects both cell adhesion and outside-inside cell signalling. In murine T-cell lymphoma EL-4 cells, over-expressed ART1 ADP-ribosylates distinct cell surface molecules, including lymphocyte-function-associated antigen-1 (LFA-1), CD45, CD43 and CD44, causing inhibition of T-cell receptor signalling (Liu et al., 1999).
Another important and recently identified ART1 substrate is the human defensin HNP-1 (Paone et al., 2002). Defensin belongs to a large family of cationic arginine-rich peptides of 2 to 6 kDa that are released from the granules of activated neutrophils. These peptides have a broad spectrum of antimicrobial and cytotoxic activities, and play an important role in the innate immune response. Moreover, it has recently been shown that defensins might also be a component of adaptive immunity: human natural killer cells and T and B lymphocytes produce HNP1-3 when stimulated with cytokines. The mechanisms of defensin action are not completely understood, but they are generally believed to involve disruption of microbial membranes (Yang et al., 2002). Paone and colleagues (Paone et al., 2002) have demonstrated in vitro that Arg14 of synthetic HNP-1 is a specific substrate for ART1. The ADP-ribosylation of the peptide reduces both its antimicrobial and cytotoxic activities, but enhances its ability to release IL-8 from epithelial cells. Conversely, modified and unmodified HNP-1 show similar chemotactic activities when evaluated for their ability to recruit T lymphocytes. These data are in line with the concept that once modified, HNP-1 acquires properties that can result in the recruitment of neutrophils (by the release of IL-8) and in the modulation of its own antimicrobial and cytotoxic activities. Evidence that ADP-ribosylation of HNP-1 occurs in vivo is obtained in broncho-alveolar wash-fluid from habitual smokers (and not from non-smokers). Cigarette smoking results in pulmonary inflammation with increased numbers of neutrophils in the lungs. Here, HNP-1 reaches concentrations that are cytotoxic for airway epithelial cells in vitro. By inhibiting its antimicrobial and cytotoxic properties while promoting neutrophil recruitment, the ADP-ribosylation of HNP-1 may thus have an important role in the regulation of the inflammatory response. This observation has two interesting implications: it indicates that ADP-ribosylated HNP-1 participates in the inflammatory response, and it demonstrates that endogenous ADP-ribosylation occurs in humans.

Additional substrates of ART1 including growth factors and membrane receptors have been identified in various cell lines over-expressing this enzyme. ART1-transfected
rat adenocarcinoma cells have been used to demonstrate ADP-ribosylation of basic fibroblast growth factor (FGF-2), which had been initially detected on the surface of adult bovine aortic endothelial and human hepatoma cells (Boulle et al., 1995; Jones and Baird, 1997). FGF-2 has a specific extracellular receptor (FGFR), but also a relatively high affinity for heparin. It can thus be sequestered by the heparan sulphates on the cell surface and in the extracellular matrix. Heparin inhibits the ADP-ribosylation reaction, which would imply that the heparin binding of FGF-2 and its ADP-ribosylation are mutually exclusive reactions. Furthermore, the ADP-ribosylated site of FGF-2 is in its receptor-binding domain and so it is possible that ADP-ribosylation modulates the binding of FGF-2 to its receptor and to heparin, thus regulating its availability to the cell (Boulle et al., 1995; Jones and Baird, 1997).

In ART1-transfected V79 Chinese hamster lung fibroblasts, platelet-derived growth factor-BB (PDGF-BB) is a substrate for ART1 whereas its structural homologue PDGF-AA is not (Saxty et al., 2001). The ADP-ribosylated PDGF-BB loses its ability to stimulate mitogenic and chemotactic responses in human pulmonary smooth muscle cells, and it shows a reduced capacity for binding to PDGF receptors in competition-binding assay, as compared to unmodified PDGF-BB (Saxty et al., 2001). This indicates that PDGF-BB-dependent signalling can be regulated by ART1 activity at the cell surface.

1.2.3.2.2 ART2

ART2 is expressed in resting T cells and in natural killer cells, and it appears to be specific to the immune system. The presence of ART2 on the surface of immune cells thus suggests an immunomodulatory activity. Indeed, in different animal models, autoimmune disorders such as juvenile diabetes and systemic lupus have been found to coincide with defects in the structure and function of ART2 (Greiner et al., 1986; Koch-Nolte et al., 1995). In the rat, susceptibility and resistance to diabetes correlate with the absence and presence of ART2-expressing T cells, respectively (Fowell and Mason, 1993). Moreover,
treatment of the animals with ART2 specific antibodies can induce an autoimmune reaction, up to diabetes (Greiner et al., 1987).

Nolte and co-workers (Seman et al., 2003) recently uncovered an intriguing function of ART2: ADP-ribosylation activates the P2X7 purinoceptor. This is a member of the P2X family of ATP-gated ion channels, and it is widely expressed in several type of blood cells (Di Virgilio et al., 2001). The interest in this purinoceptor rose because of its peculiar ability to induce the formation of large membrane pores (Di Virgilio et al., 2001). Thus, the activation of P2X7 with millimolar concentrations of ATP triggers calcium fluxes, phosphatidylserine exposure and apoptosis (Di Virgilio et al., 2001). These same effects are triggered by NAD+ at micromolar concentrations via the ADP-ribosylation of P2X7. However, these effects are not seen in ART2-deficient T-cells, demonstrating that the activation of P2X7 by NAD+ is ART2 dependent (Seman et al., 2003). These data provide an explanation for the rapid apoptosis induced by extracellular NAD (NAD-induced cell death or NICD) in naive T cells (Scheuplein et al., 2003). How does ADP-ribosylation activate P2X7? Covalently immobilized ADP-ribose could function as a ligand for the adenosine-binding site on P2X7. Alternatively, ADP-ribosylation could activate P2X7 by an allosteric conformational change independent of the ligand-binding site. Identification of the ADP-ribosylated arginine residues will help to solve this question. Altogether, these data show that not only is ART2 expressed in the immune system, akin to ART1, but also that both of these two arginine-specific ARTs have clear roles in the regulation of the immune response.

1.2.3.2.3 ART3, ART4 and ART5

The biological functions of ART3, ART4 and ART5 remain poorly defined. ADP-ribosyltransferase activity on the surface of human monocytes correlates with the presence of ART3 in unstimulated cells, while ART4 is expressed only in response to lipopolysaccharide stimulation (as, for example, in bacterial infection) in lymphatic tissue
(Grahnert et al., 2002). Cell-surface ADP-ribosylated proteins on human monocytes are modified on cysteine residues, suggesting that ART3 and ART4 may be cysteine-specific enzymes (Grahnert et al., 2002). This is in line with the observation that when these two enzymes were assayed \textit{in vitro}, neither displayed an arginine-specific enzymatic activity (Glowacki et al., 2002).

1.2.3.2.4 Extracellular $\beta$-NAD$^+$ availability

A point that remains to be clarified is the occurrence of extracellular NAD$^+$ that is required to sustain the extracellular ADP-ribosylation reaction. The steady-state concentration of NAD$^+$ in the serum of healthy individuals is around 0.1 $\mu$M, and it can be kept low by the extracellular NAD-glycohydrolase CD38 (Berthelier et al., 1998). However, to be used by ecto-ARTs, extracellular NAD$^+$ should reach the concentrations of 1-10 $\mu$M that are required for ADP-ribosylation of P$_2$X$_7$ (Seman et al., 2003), or higher if the K$_m$ of the ARTs (from \textit{in vitro} assay) is considered (Okazaki et al., 1996). The probable mechanism is that NAD$^+$ is released from the cells, where its concentration is in the range of 0.5-1.0 mM, as a consequence either of cell lysis during inflammatory immune reactions and apoptosis, or of non-lytic release, for example through the connexin 43 channels (Bruzzone et al., 2001).

1.2.3.3 Intracellular ADP-ribosyltransferases and endogenous substrates

All of the human and mouse ecto-mono-ADP-ribosyltransferases have now been identified (Glowacki et al., 2002) and classified as either GPI-anchored or secretory proteins, implying that the targets of these enzymes are extracellular proteins. However, mono-ADP-ribosylation has also been demonstrated for intracellular proteins involved in cell signalling and metabolism (Table 1.5). The enzymatic activities involved here have been shown to be both cytosolic and membrane associated, although there is very little further information available concerning their identities. Indeed, the intracellular ADP-ribosyltransferases probably constitute a different family of proteins having no obvious
sequence similarities or structural relationships to the well-known ARTs described above. An example in line with this hypothesis is given by the sirtuin family. Thus, it has recently been shown that yeast Sir2p (silent information regulator 2 protein) has ADP-ribosyltransferase activity. Frye and colleagues (Frye, 1999) showed that a human Sir2p homologue (SIRT2) can transfer the label from NAD$^+$ to BSA, providing evidence in support of the idea that human sirtuins might also act as mono-ADP-ribosyltransferases.

More recently, the in vitro characterization of mouse SIRT6 confirms that this enzyme is a nuclear protein with a robust auto-ADP-ribosylation activity (Liszt et al., 2005). All of the seven human sirtuins so far cloned possess a conserved histidine that is important for the ADP-ribosyltransferase activity, although they lack any obvious sequence homology with the ARTs (Frye, 2000).

Sirtuins are not the only examples of unusual ADP-ribosyltransferases; indeed, the intracellular enzymes pierisin-1 and pierisin-2 were discovered recently in the cabbage butterfly, *Pieris rapae*, as mono-ADP-ribosyltransferases (Matsushima-Hibiya et al., 2000; Watanabe et al., 1999). Pierisin-1 is a 98-kDa protein having a role in the development of the insect, and it induces apoptosis in a variety of human cancer cell lines (Kono et al., 1999). Only after the characterisation of its enzymatic activity did a very remote similarity to the ADP-ribosylating subunit of CT emerge (Watanabe et al., 1999). The use of known inhibitors for ADP-ribosylating enzymes greatly reduces pierisin-induced apoptosis, thus linking pierisin ADP-ribosylating activity to apoptosis (Watanabe et al., 1999). Once inside the target cell, intracellular proteases cleave pierisin, releasing a fragment that contains the ADP-ribosyltransferase activity (Kanazawa et al., 2001). The targets of pierisin-1 were recently described as the 2'-deoxyguanosine residues in DNA so that the reaction yields N(2)-ADP-ribosyl-2'-deoxyguanosine, as determined by NMR and mass spectrometry (Takamura-Enya et al., 2001). Pierisin-1 induces apoptosis of HeLa cells by activating a mitochondrial pathway involving Bcl2 and caspase (Kanazawa et al., 2002). Whether this process is due to the ADP-ribosylation of deoxyguanosine in a specific DNA region or to a
<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Source</th>
<th>Substrate/ amino acid</th>
<th>Effect of the reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sir2p</td>
<td>Yeast</td>
<td>Histone/Sir2p/ acetyl-lysine</td>
<td>involved in histone deacetylation</td>
</tr>
<tr>
<td>Sirtuin2</td>
<td>Human</td>
<td>Albumin/ acetyl-lysine</td>
<td>involved in histone deacetylation</td>
</tr>
<tr>
<td>Piersin1, 2</td>
<td>Cabbage butterfly</td>
<td>DNA</td>
<td>Cytotoxic</td>
</tr>
<tr>
<td>Arginine-specific</td>
<td>Hamster, human</td>
<td>Gβ/Arg129</td>
<td>inhibits substrate activity</td>
</tr>
<tr>
<td>Cysteine-specific</td>
<td>Human</td>
<td>GDH/Cys</td>
<td>inhibits substrate activity</td>
</tr>
</tbody>
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Table 1.5. Intracellular mono-ADP-ribosylation in eukaryotes. Modified from Corda and Di Girolamo, 2003.
more extensive modification of these residues remains to be clarified. The function of pierisin-1 in the butterfly as well as the mechanism underlying pierisin-induced apoptosis in cancer cells clearly require further analysis.

As an alternative to unidentified ADP-ribosyltransferases that are unrelated to the ARTs, secretory ecto-ARTs, such as ART5 or ART2, shed from activated T cells might conceivably translocate to the cytoplasm. This could be achieved in a way similar to that of the bacterial toxins, which have their own specific receptors on the plasma membrane (Lencer et al., 1999). However, the sorting mechanism reported for GPI-anchored proteins, which involves endocytic vesicles, would result in a lumenal localization of the catalytic domain, separating it from cellular substrates, including the G proteins (Muniz and Riezman, 2000). Our laboratory is now actively working to identify and define potential new intracellular ART isoforms.

The intracellular mono-ADP-ribosylation reaction has been associated with cell metabolism in intact cells, and thus far, it has been demonstrated for three substrate proteins: the ER-resident chaperone protein GRP78/BiP (Laitusis et al., 1999; Leno and Ledford, 1989), the heterotrimeric G protein \( \beta\gamma \) subunit (Lupi et al., 2000; Lupi et al., 2002), and mitochondrial glutamate dehydrogenase (GDH; Herrero-Yraola et al., 2001).

1.2.3.3.1 GRP78/BiP

GRP78/BiP is a molecular chaperone that resides in the lumen of the ER and that functions in the folding and assembly of proteins entering the secretory pathway. Specific mono-ADP-ribosylation of GRP78/BiP leads to its inactivation. The modified protein has been detected in response to nutritional stress, a condition that depletes the ER of processible proteins. Laitusis and co-workers (Laitusis et al., 1999) have hypothesized that in intact Swiss 3T3 and GH3 pituitary cells this ADP-ribosylation is related to the rate of protein synthesis and processing. According to this idea, in cells with high rates of protein synthesis, unmodified GRP78 is complexed with protein-folding intermediates. A slowing
of protein synthesis would result in the accumulation of the free, active form of GRP78, which is subject to subsequent inactivation by ADP-ribosylation. The ADP-ribosylated form of the chaperone thus provides a buffering system that allows the rates of protein processing to be balanced with those of protein synthesis. It should be noted that while this mono-ADP-ribosylation occurs intracellularly, from a topological point of view, the enzyme involved (that has not yet been characterized) should be an ectoenzyme, due to the luminal localization of GRP78.

1.2.3.3.2 G protein βγ dimer

An ADP-ribosylation reaction may also occur on the β subunit of heterotrimeric G proteins. Indeed our group has provided direct evidence of a functional, enzymatic modification of the β subunit both in isolated plasma membranes from different cell lines (including Swiss 3T3, CHO and HL60 cells) and in intact cells (Lupi et al., 2000). Modification of the βγ subunit occurs on Arg129, a residue critically located in the β common-effector-binding surface (Chen et al., 1997; Weng et al., 1996; Yan and Gautam, 1997; Yan et al., 1996). It is catalyzed by a plasma membrane-associated, non-GPI-anchored intracellular mono-ADP-ribosyltransferase that has yet to be characterised. Interestingly, the modified β subunit can be de-ADP-ribosylated by a cytosolic ADP-ribosylhydrolase, which regenerates native βγ by releasing the bound ADP-ribose (Figure 1.15). In intact cells under resting condition, ca. 0.2% of the total βγ heterodimer is modified, an amount that could correspond to a cellular pool of free βγ dimer heterodimer that remains inactive. This hypothesis is supported by the demonstration that ADP-ribosylation occurs only on the activated βγ heterodimer, and that this inactivates the β subunit by impairing its interactions with its effectors enzymes. This has been shown directly in the case of type 1 AC and PLCβ and PI3K (Lupi et al., 2000; Lupi et al., 2002). Thus, the ADP-ribosylation/de-ribosylation cycle might correspond to a functional activation/inactivation cycle of the βγ dimer for the modulation of its functions. The
Figure 1.15. The ADP-ribosylation/de-ribosylation cycle of the heterotrimeric G protein β subunit. Modification of the Gβγ subunit occurs on Arg129 and is catalyzed by a plasma-membrane-associated, intracellular mono-ADP-ribosyltransferase. The modified Gβγ subunit then serves as a substrate for a cytosolic ADP-ribosylhydrolase, which regenerates the native Gβγ by releasing the bound ADP-ribose.
demonstration that the β subunit is subject to covalent modification is of particular interest because of the central role that the βγ dimer plays in the regulation of signal transduction pathways downstream of GPCRs. This was the first in vivo demonstration of a post-translational modification for the G protein β subunit (Lupi et al., 2000; Lupi et al., 2002).

1.2.3.3.3 Mitochondrial GDH

An ADP-ribosylation/de-ADP-ribosylation cycle has also been proposed to occur in mitochondria, which involves the cysteine-specific ADP-ribosylation of liver mitochondrial GDH. ADP-ribosylation of GDH causes substantial inhibition of its enzymatic activity in intact Hep-G2 cells (Herrero-Yraola et al., 2001). The ADP-ribosylation cycle could then be completed by an ADP-ribosylcysteine hydrolase present in mitochondria, which restores the active form of GDH. Given the central position of GDH in metabolism where it lies at the crossroads of several important pathways, a tight control of its catalytic activity is essential. In line with this suggestion, it has been demonstrated that enhanced ADP-ribosylation of GDH occurs if glutamate is added to the cell culture medium. Inhibition of excessive glutamate use by GDH would prevent the concomitant accumulation of ammonia that is toxic for the cells. This enzyme is also central to cellular nitrogen metabolism, and thus ADP-ribosylation of GDH may be part of the complex regulatory system controlling the cellular nitrogen balance. As for GRP78/BiP and for the G protein β subunit, the nature of this ADP-ribosyltransferase activity remains uncharacterised.

Further substrates of mono-ADP-ribosylation have also been identified, including the membrane-fissioning proteins CtBP1-s/BARS (De Matteis et al., 1994; Di Girolamo et al., 2005; Di Girolamo et al., 1995) and the cytoskeletal proteins actin, tubulin and desmin (Huang et al., 1993; Zhou et al., 1996). However, to date there has been no direct evidence for their in vivo modification.
Besides mADPRTs, the other class of enzymes involved in the ADP-ribosylation reaction are the ADP-ribosylhydrolases. These are soluble proteins that release ADP-ribose from proteins that are modified on an arginine or on other amino acid residues (Herrero-Yraola et al., 2001; Moss et al., 1992). Specific hydrolase activities have been detected in mammalian, avian and bacterial systems. However, the functional interplay of the constituents of these postulated ADP-ribosylation cycles remain to be demonstrated. The ADP-ribose moiety is critical for substrate recognition; indeed, the ADP-ribosylhydrolase enzyme hydrolyzes ADP-ribosylarginine and (2-phospho-ADP-ribosyl)arginine, but is inactive with phosphoribosylarginine or ribosylarginine.

ADP-ribosylation of integrin catalysed by the extracellular GPI-anchored ART is reversed by a phosphodiesterase. As a result, 5'-AMP is released from the protein while phosphoribose remains attached to the modified arginine residue (Zolkiewska and Moss, 1995). The ADP-ribosylhydrolases have been cloned from rat (Takada et al., 1994), mouse and human brain (Takada et al., 1993). Hydrolase activities of rat and mouse brain are sensitive to Mg$^{2+}$ and dithiothreitol (DTT), whereas the human hydrolase appears to be DTT independent (Moss et al., 1992). The nucleotide and deduced amino acid sequences of the mouse hydrolase shows 92% and 94% identity with rat; the human and rat hydrolases show 82% and 83% identity in their nucleotide and deduced amino-acid sequences.

### 1.3 The ADP-ribosylating factors (ARFs)

During the 1980s, several cellular factors that enhance CTX-catalysed ADP-ribosylation of $\mathrm{G}\alpha_s$ were described. Kahn and Gilman (Kahn and Gilman, 1984) reported the purification of a ~20-kDa membrane-associated protein that is able to stimulate CTX
activity in vitro, which they called ADP-Ribosylation Factor (ARF). Later, the purified soluble ARF proteins were found to stimulate both toxin-catalysed ADP-ribosylation of Gα4 as well as toxin auto-ADP-ribosylation (Tsai et al., 1988). These reactions require the presence of GTP, which suggests that the ARFs belong to the monomeric small G protein family. Kinetic studies revealed that only ARF-GTP acts as an allosteric activator of the toxin, interacting with the catalytic subunit to lower the apparent K_m for both NAD^+ and acceptor substrates (Noda et al., 1990). Later, the ARF proteins were documented as being involved in several other cellular functions, including PLD activation, membrane traffic regulation, and actin cytoskeleton remodelling (Randazzo et al., 2000).

We now know that the ARFs constitute a ubiquitous family of small G proteins that shares structural elements with both the monomeric G proteins of the Ras family and the α subunit of the heterotrimeric G proteins in the region involved in guanine-nucleotide binding and hydrolysis (Bobak et al., 1989; Sewell and Kahn, 1988). The ARFs are present in all eukaryotic cells, from Giardia lamblia to human, with high levels of conservation across phylogenetic lines. To date, six ARFs (ARF1-6) have been identified in mammals, and through sequence homology, additional members have been identified, including the ARF-domain proteins (ARDs) (Mishima et al., 1993; Vitale et al., 1996), ARF-like proteins (ARLs) (Clark et al., 1993; Moss and Vaughan, 1998) and ARF-related proteins (ARPs). The functions of these extended family members remain to be clearly defined. All members of the ARF family are modified post-translationally by myristoylation at the N-terminus, which is necessary for membrane association, and is clearly important for ARF functions.

The ARF proteins are divided into three classes based on their amino acid sequences, genetic structure and phylogeny. ARF1, 2 and 3 belong to class I, ARF4 and 5 are in class II, and ARF6 is the only member of class III (Moss and Vaughan, 1998; Tsuchiya et al., 1991). In Table 1.6, a comparison between the coding region nucleotides and the amino acid sequences of all of the mammalian ARFs is given, and it emerges that
ARFs differ in their sequences mainly at the N- and C-terminal. Indeed, ARF6 has a gap corresponding to amino acids 8-11 in the other ARFs. The members within each group have high levels of protein sequence identity (96% amongst the class I ARFs, and 90% among class II ARFs). The single class III ARF is the least conserved among the ARF family members being only 65-70% homologous to the others members of the family (Moss et al., 1993; Tsuchiya et al., 1991). The low level of similarity between the class III and class I-II proteins correlates to differences in localization and function. When bound to GDP, mammalian ARF1-ARF5 appear to be predominantly cytosolic but upon exchange of GDP for GTP, they become bound to a variety of intracellular membranes. ARF1, ARF3 and ARF5 are recruited to isolated Golgi membranes and endosomes both in *in vitro* and in *in vivo* assays (Honda et al., 1999; Hosaka et al., 1996; Takatsu et al., 2002; Tsai et al., 1992; Whitney et al., 1995). Conversely, activated ARF4 is recruited to the plasma membrane following binding of epidermal growth factor (EGF) to its receptor (Kim et al., 2003).

ARF6, on the other hand, and again depending on its nucleotide status, cycles between intracellular vesicles such as the endosomes and secretory vesicles and the plasma membrane (Cavenagh et al., 1996; Galas et al., 1997), where it regulates endocytosis, actin cytoskeleton remodelling, cell shape and cell migration (reviewed by Donaldson, 2003). In addition to these well-described activities, a new and previously unknown function of ARF6 has arisen from the present study in which I found that ARF6 acts as a modulator of the endogenous mono-ADP-ribosylation reaction that occurs on the G protein βγ subunit. For this reason, the second part of this section focuses mainly on ARF6-mediated cellular functions.
Table 1.6. Comparison of nucleotide and deduced amino-acid sequences of the ARFs.

Percent identity of the indicate Arf deduced amino acid sequences is indicated above the diagonal and percent identity of nucleotide sequences (coding regions) is below the diagonal. Abbreviations: h, human; b; bovine. Modified from Moss and Vaughan 1993; Tsuchiya 1991.
1.3.1 The GDP-GTP cycle of the ARF proteins

The ARF proteins cycle between their GDP-bound inactive, and GTP-bound active forms. The spontaneous release of GDP from ARF is slow and it needs to be catalysed by a guanine-nucleotide exchange factor (GEF). In parallel, the hydrolysis of bound GTP to yield ARF-GDP is also very slow in the absence of specific GTPase-activating proteins (GAPs) (reviewed in Donaldson and Jackson, 2000).

For most ARFs, the GDP-bound form is primarily cytosolic, whereas ARF-GTP is membrane bound. Early thinking proposed that once GTP substitutes for GDP, ARF undergoes a conformational change that allows its myristoylated N-terminal helix to interact with phospholipid bilayers (Amor et al., 1994). However, further studies have indicated that ARF can expose its myristoylated tail even in its GDP-bound form allowing weak, but measurable, membrane association. This is stabilised by the loading of GTP onto ARF, whereas it is completely abolished when the myristate is removed (Antonny et al., 1997a). It is likely that membrane association has an essential role in ARF activation, by allowing its interaction with GEF proteins. Thus the ARFs first undergo a lipid-mediated conformational switch, and then they form a productive complex with a membrane-associated GEF (Franco et al., 1996). Hence, ARF activation takes place after both the GEF and its target, ARF, have been localised to membranes.

1.3.1.1 The ARF GEFs

Many GEFs for ARF have been identified in both yeast and mammals, and all possess a conserved region of approximately 200 amino acids that is known as the Sec7 domain, with a strong similarity with the yeast sec7 secretory protein (Jackson and Casanova, 2000). The guanine-nucleotide-exchange activity resides within the Sec7 domain that is sufficient by itself for the exchange activity (Chardin et al., 1996). The crystal structure of the Sec7 domain of the exchange factor ARNO (ARF nucleotide binding site opener) has been determined at ~2 Å resolution (Cherfils et al., 1998). This
consists of ten α-helices (A-J) arranged as an elongated cylinder having a deep hydrophobic groove in the central region (comprising helices F, G and H) that forms the binding site for nucleotide-free ARF (Figure 1.16). A second highly conserved motif comprises most of helix H and contains a large number of solvent-exposed hydrophobic residues. Point mutations within these conserved motifs reduce exchange factor activity, demonstrating that this region constitutes the active site of the Sec7 domain. In addition, there is an invariant glutamate residue at the C-terminus of the F-G loop that forms a "glutamic finger" that inserts into the nucleotide-binding site of ARF, displacing the coordinating Mg^{2+} ion and possibly the β-phosphate of bound GDP (Beraud-Dufour et al., 1999). Further analysis of the crystal structure of the Sec7 domain of Gea2p, another ARF exchange factor, in complex with nucleotide-free ARF1 support this scheme (Goldberg, 1998) indicating that the glutamate side chain comes within 3 Å of the β-phosphate site, and it is therefore likely to exert both steric and electrostatic repulsive forces on the bound nucleotide. The structure of nucleotide-free ARF1 in a complex with the Sec7 domain is very similar to that of the GTP-bound form (Goldberg, 1998).

The ARF GEF family can be subdivided into two groups based on sequence similarities and functional differences (Figure 1.17). The first class comprises the large GEFs (>100 kDa) that have been found in all eukaryotes examined to date, while the second contains the small GEFs (<100 kDa) that are peculiar to the higher eukaryotes (Jackson and Casanova, 2000). The high molecular weight ARF-GEF subfamily includes yeast Sec7, Gea1 and Gea2, and mammalian BIG1/p200, BIG2 and GBF1. A feature of this family of ARF GEFs (with the exception of GBF1) is their sensitivity to the fungal metabolite brefeldin A, which inhibits their ARF-GEF activity thus stabilising the ARF-GDP-Sec7 domain complex (Peyroche et al., 1999). The low-molecular-weight GEFs include mammalian ARNO, cytohesin-1, GRP1 and cytohesin 4, all of which are insensitive to brefeldin A. EFA6, a GEF specific for ARF6, can also be included in this subfamily because it is brefeldin A-insensitive and shows the structural Sec7 domain
Figure 1.16. Structure of the ARNO-Sec7 domain. A, Overall view. The Sec7 domain consists of ten α helices, from A to J, arranged to form an elongated cylinder. The hydrophobic groove in the central region that forms the binding site for nucleotide-free ARF is indicated by the blue arrow. B, Alignment of the ARNO-Sec7 sequence with sequences of homologous Sec7 domains. The helices are shown by the red lines. Identical amino acids are boxed in black. The two conserved motifs that form the putative ARF-binding site are boxed in blue. The invariable glutamate residue, which is involved in nucleotide exchange on ARF, is indicated with a black arrowhead. Taken from Cherfils, 1998.
Figure 1.17. The ARF-GEF family. Representative members of different subfamilies of the Sec7-domain-exchange factors for ARFs. The grey boxes represent the Sec7 domain, which catalyses the GDP/GTP exchange on the ARFs. For the high-molecular-weight proteins, the coloured boxes represent regions showing significant levels of sequence similarity; the functions of these domains have not yet been determined. The PH domain (PH, green boxes) and coiled-coiled regions (CC, red boxes) of the lower molecular weight GEFs are indicated. The proline-rich regions of EFA6 are designed by “pr” (yellow boxes).
despite having a slightly larger size. As shown in Figure 1.17, the ARF family members are outnumbered by the ARF-GEFs. Thus, an individual ARF protein may be activated by more than one GEF. A key question is the physiological specificity of the different GEFs for the different ARFs. For example, in several \textit{in vitro} systems, ARNO, cytohesin-1 and GRP1 catalyse GDP/GTP exchange more efficiently on the class I ARFs than on the members of the other classes (Jackson and Casanova, 2000). However, \textit{in vivo}, ARNO, and GRP1 partially co-localise with ARF6 and have key roles in ARF6 activation (Frank et al., 1998). Moreover, the overexpression of cytohesin-1 and GRP1 lead to a greater increase in ARF6-GTP in cells than in ARF1-GTP, thus supporting the concept that cytohesin-1 and GRP1 can act as GEFs for ARF6 \textit{in vivo} (Langille et al., 1999). Cytohesin-4 efficiently catalyses exchange on ARF1, and to a lesser extend on ARF5 \textit{in vitro}, but it is inactive towards ARF6 (Ogasawara et al., 2000). Overall, these observations suggest that ARNO/cytohesins are able to localise at, and probably to act on, both Golgi and plasma membranes, although their ARF substrates remain to be clearly assigned. The situation is much clearer for the GEF EFA6, which activates ARF6 more efficiently than ARF1 both \textit{in vitro} (Macia et al., 2001) and \textit{in vivo}. Studies on the over-expression of the wild-type or a dominant-negative form of EFA6 have revealed effects on membrane traffic and cytoskeletal organization that are consistent with its function as an ARF6-specific GEF \textit{in vivo} (Luton et al., 2004).

\textbf{1.3.1.2 The ARF GAPs}

The hydrolysis of GTP is equally critical to its binding in the regulation of ARF functions. GTP hydrolysis is stimulated by ARF GAPs, the functions of which are regulated by ARF itself and by phospholipids and DAG (Antonny et al., 1997b; Ding et al., 1996). Over the past few years, 16 ARF GAPs have been identified (Figure 1.18); however, their substrate specificities have not yet been systematically characterised. All these proteins share a common GAP domain of 70 amino acids that includes a zinc finger
motif of CXXCX(16-17)CXXC (where C is cysteine and X is any amino acid) that is essential for their GAP activity. In addition to the zinc finger, all of the ARF GAPs have a conserved arginine within the GAP domain. In the GAPs ASAP1 and PAPβ, mutation of this arginine results in a strong decrease in GTPase activity (Mandiyan et al., 1999; Randazzo et al., 2000), suggesting an arginine finger mechanism for GTP hydrolysis. The crystal structure of the GAP reveals that this arginine is positioned on the surface of the molecule, near to several hydrophobic residues. Mutation of these adjacent hydrophobic residues also impairs GAP activity, suggesting that this region may represent the ARF interaction site (Mandiyan et al., 1999). The first GAP for the ARFs that was cloned was ARF GAP1. This protein is cytosolic and is recruited to the Golgi complex in mammalian cells where it acts on ARF1 (Makler et al., 1995). This is mediated by the seven-pass transmembrane KDEL receptor Erd2 (Aoe et al., 1997), which recognises a Lys-Asp-Glu-Leu carboxy-terminal motif on certain proteins destined for Golgi-to-ER transport. This suggests a role for ARF GAPs in retrograde transport to the ER (Lanoix et al., 1999). A remarkable parallel to the observation that Erd2 recruits ARF GAP1 to the Golgi is that a peripheral ARF GAP, Git1, is recruited to the GPCRs at the plasma membrane level. Git1 is a PIP3-stimulated GAP for all ARFs. It was isolated as a binding partner of the GRKs, which are implicated in the down-regulation of receptor signalling by mediating receptor internalisation via clathrin-dependent endocytosis (Vitale et al., 2000). In other studies, dioleylglycerol increased the activity of recombinant GAP. Because monosaturated diacylglycerols are produced chiefly by the action of PLD, it has been suggested that PLD activity could be the major regulator of ARF-GAP (Antonny et al., 1997b).

Finally, Arf GAPs also appear to function independently of the ARFs. For example, they are incorporated into transport intermediates that lack ARFs, leading to speculation of a structural role in vesicle coat formation (Yang et al., 2002). Moreover, the multidomain nature of the GAPs suggests that they can interact with signalling proteins and adaptor proteins. Consequently, Arf GAPs could form a scaffold for large protein complexes.
Figure 1.18. The ARF-GAP family. Gcs 1 and Glo 3 are from *S. cerevisiae*; the others are all mammalian proteins. All of the proteins share homology in their GAP domains (orange), which contain a critical zinc finger motif. A number of the GAPs shown also have a PH domain (green); these GAPs are known as the centaurins. The ankyrin repeats (AR, blue) and Src homology 3 (SH3) domains (pink) are indicated.
1.3.2 The structures of ARF1 and ARF6

Recently, it has been noted that the ARFs have a unique structural feature that allows propagation of the structural modification occurring at the nucleotide-binding site to the N-terminus of the proteins. Indeed, as previously described, the ARFs require both a lipid-mediated conformational switch and productive complex with a membrane to be activated. The interaction with the membrane is mediated by a myristoylated N-terminal helix, which flips open upon binding GTP and inserts into the lipid bilayer. Communication between the N-terminal helix and the nucleotide-binding site, which are located on opposite sides of the protein, is mediated by two β-strands and a β-hairpin loop that connect the switch I and II regions (called the interswitch region). Switch I, and to a lesser extent switch II, form the major sites for the interactions of ARF proteins with their cellular partners. Surprisingly, these sequences are almost identical among the ARFs, suggesting that they may have the same three-dimensional structure. Upon nucleotide exchange, the interswitch region passes from a retracted conformation in which it hosts the N-terminal α helix, to an open conformation in which the N-terminal hasp protrudes out of the protein and is free to insert into the lipid bilayer (Pasqualato et al., 2002). This conformational shift does not take place in other small GTP-binding proteins.

The finding that ARF6 localises primarily to membranes even when bound to GDP, unlike ARF1 which is mostly cytosolic in this state, and that the ARF6 N-terminus is shorter than that of ARF1, raises the possibility that its membrane/nucleotide cycle may differ from that of the other ARFs (Yang et al., 1998). Biochemical and crystallographic studies on both ARF6 and ARF1 bound with GDP or GTP support this idea (Goldberg, 1998; Goldberg, 1999; Menetrey et al., 2000: Pasqualato et al, 2001). The crystal structure of the full-length, non-myristoylated ARF6-GDP differs in conformation with respect to ARF1-GDP (Figure 1.19). The N-terminus is folded into an α-helix that is positioned in the hydrophobic pocket opposite the nucleotide binding site, similar to that of ARF1-GDP.
The missing residues do not shorten the helix as expected, but instead shorten the linker that connects the helix to the protein core. As in ARF1-GDP, the interswitch region is buried in the protein core and interacts with the switch I region, which forms an ordered β-strand. Thus, it is likely that the structural principles for the activation of ARF6 closely mimic those for the activation of ARF1, even including the rearrangements of the N-terminus and the interswitch region. Therefore, the localization of ARF6-GDP to membranes in cells cannot be due to the inability of its N-terminal helix to bind in a pocket in the protein core. Moreover, the structure of ARF6-GDP reveals that the switch components are also the regions where ARF1 and ARF6 diverge most, while other regions have essentially the same main-chain conformations. In particular, switch II prominently differs between ARF6 and ARF1. This region is flexible in ARF1-GDP and undergoes a disorder-to-order transition upon the binding of GTP (Goldberg, 1998). In contrast, it is well organised in ARF6-GDP, where it rotates as a rigid body to adopt the GTP-bound conformation (Pasqualato et al., 2001; Figure 1.20). These disparities result in different conformations, and thus in distinctive nucleotide-binding properties that are large enough to affect protein-protein interactions. This could explain how ARF-GEF discriminates between ARF1 and ARF6, and suggests a model for the peculiar GDP/GTP cycle of ARF6. The ARF proteins are recognized by their specific ARF-GEFs at the initial GDP-containing complex stage, rather than at the nucleotide-free stage, due to the different conformations and flexibility of their switch regions. Unlike their GDP-bound forms, the active forms (with bound GTPγS) of ARF6 and ARF1 are very similar (Pasqualato et al., 2001). Thus, the switch regions are discriminatory elements between ARF isoforms in their inactive, and not in their active forms. This suggests that the GTP-bound ARFs may establish specific interactions outside the switch regions and/or might be recognized in their cellular context, possibly in complexes with other proteins, rather than as isolated proteins.
Figure 1.19. Superposition of ARF1–GDP and ARF6–GDP. Superposition is not seen for the N-terminal helix and linker, the switch regions and the interswitch loop. Superimposable regions and the GDP nucleotide are in grey, and are shown only for ARF6 for clarity. GDP is shown as a ball-and-stick model. Diverging regions are shown for both ARF1 and ARF6, with ARF6 in bright colours and a continuous outline, and ARF1 in pastel shades and a dotted outline. The N-terminal helix is yellow, the linker is green, switch I is purple, switch II is blue and the interswitch loop is red. Taken from Menetrey, 2000.
Figure 1.20. The interswitch toggle of human ARF6. ARF6-GDP (Menetrey et al., 2000) (left) and ARF6-GTP\(\gamma\)S (Pasqualato et al., 2001) (right) are shown in the same orientation. Residues with disordered electron density are indicated by a dashed line and are expected to interact with membranes in activated ARF. Invariant residues of the signature are shown.
1.3.3 The sub-cellular localisation of ARF6 and its physiological functions

The sub-cellular localisation of ARF6 is quite different from that of the other ARF proteins. The class I and class II ARFs reversibly associate with Golgi complex membranes during their GTP cycle with ARF1-GDP being cytosolic and ARF1-GTP bound to the membranes. ARF6, on the other hand does not localise to the Golgi complex (Peters 1995) although it is present at the plasma membrane and to some extent on endosomal membranes (Donaldson, 2003). Two mutants of ARF6, Q67L and T27N, that are considered to mimic the GTP- and the GDP-bound forms respectively, have been used to reveal the localisation and the function of the protein. Both immunoelectron microscopy and immunofluorescence analysis have revealed that the ARF6(Q67L) mutant is restricted to the plasma membrane, while the ARF6(T27N) mutant is on internal structures resembling endosomes (D'Souza-Schorey et al., 1998; Radhakrishna et al., 1996). Thus, based on the localisation of these two mutants, it has been proposed that ARF6 cycles between the plasma membrane (ARF6-GTP) and a recycling endosomal compartment (ARF6-GDP). In this way, ARF6-GDP would encounter its exchange factor at the surface of an internal compartment to promote the formation of vesicles destined for the plasma membrane. However this hypothesis has been challenged by other observations. Firstly, EFA6, a specific ARF6-GEF, is present only at the plasma membrane, implying that ARF6-GDP should also be located here (Franco et al., 1999). Secondly, by using free-flow electrophoresis, ARF6 was found to be a stable component of the plasma membrane in CHO cells (Cavenagh et al., 1996). It now appear that the ARF6(T27N) mutant may not exactly reflect the phenotype of ARF6-GDP, as it has a tendency to lose its nucleotide in vitro, and to denature or to aggregate in the cytosol in vivo (Macia et al., 2004). A resolution of this question has come from the use of a mutant T44N that has a reduced affinity for GTP and thus remains in the GDP-state (Menetry et al., 2000; Pasqualato et al., 2001). In transfected cells, ARF6(T44N) is present only at the plasma membrane.
where it co-localises with the exchange factor EFA6, thus demonstrating that the GTP-GDP cycle of ARF6 takes place at the plasma membrane. This peculiarity implies that ARF6 is essentially involved in the regulation of plasma membrane-related processes, such as membrane ruffling, endocytosis, exocytosis and membrane recycling, as well as the reorganization of the cortical actin cytoskeleton, activation of phospholipase D, and phagocytosis. In addition, several recent studies have linked ARF6 either to the regulation of GPCR signalling, desensitization and internalization, or to the regulation of cell adhesion, migration and cancer cell invasion (Claing, 2004; Hunzicker-Dunn et al., 2002; Sabe, 2003).

These functions possibly take place through: (1) the recruitment of cytosolic coat proteins onto membranes to facilitate sorting and vesicle formation; (2) the activation of lipid modifying enzymes; and (3) the reorganization of the actin structure.

1.3.3.1 Recruitment of cytosolic coat proteins and vesicle formation

Vesicle formation in cells is driven by coat complexes that are recruited from the cytosol to generate the forces necessary to bend a relatively flat membrane into a vesicle. Several families of coat proteins exist, including COPI, COPII and clathrin, which assemble selectively on different intracellular compartments (Nickel et al., 2002; Rothman, 2002). Clathrin coats function in receptor-mediated endocytosis at the plasma membrane when they are associated with AP-2 adaptors, and in transport from the trans-Golgi network (TGN) to endosomes when they are associated with AP-1 adaptors (McMahon and Mills, 2004). The adaptor proteins recognize and bind motifs in cargo molecules. Many accessory proteins take part in a network of interactions that regulate clathrin-coat assembly. The ability of active ARF6 to recruit the cytosolic adaptor protein AP-1 and other coat proteins to membranes has been demonstrated in vitro (Austin et al., 2002; Takatsu et al., 2002). In addition, a recent study has demonstrated that ARF6 interacts with AP-2 in a GTP-dependent manner, but not with AP-1 both in vitro and in
These results suggest that ARF6 is involved in the early stage of clathrin-mediated receptor endocytosis, probably by directly controlling AP-2 assembly on the plasma membrane.

1.3.3.2 Activation of lipid modifying enzymes

ARF6-GTP acts also through the activation of lipid-modifying enzymes, such as PLD and phosphoinositide 4-phosphate 5-kinase (PIP5K). All of the ARFs can activate PIP5K in vitro, but only ARF6 is able to co-localise with PIP5K and thus to activate it in vivo (Honda et al., 1999). PIP5K is responsible for generating PI4,5P2 from phosphatidylinositol4-phosphate (De Matteis et al., 2002). Synthesis and turnover of PI4,5P2 have been implicated in a variety of cellular events including membrane trafficking, control of actin polymerisation and signal transduction, where it serves as a substrate for enzymes such as PLC and PI3K (De Matteis et al., 2002; Martin, 1997; Yamamoto et al., 2001). This pleiotropic role of PI4,5P2 illustrates the importance of ARF6-mediated regulation of PIP5K in the correct cell functionalities. ARF6 and PI4,5P2 co-localize on the plasma membrane and on endosomal structures (Brown et al., 2001), and the ARF6-regulated PI4,5P2 pool at the plasma membrane is apparently involved in regulated secretion. In MIN6 pancreatic β-cells, a dominant-negative ARF6 reduces PI4,5P2 levels and impairs the slow phase of insulin secretion (Lawrence and Birnbaum, 2003). In parallel, the expression of constitutively active ARF6 induces the trapping of PIP5K and PI4,5P2 at endosomal membranes and the consequent depletion of PI4,5P2 from the plasma membrane interrupts exocytosis from dense-core vesicles in PC12 cells (Aikawa and Martin, 2003; Brown et al., 2001). Direct activation of PIP5K by ARF6 has also been shown to stimulate clathrin-coat recruitment to synaptic membranes, thereby allowing synaptic vesicle recycling (Krauss et al., 2003). In cultured rat hippocampal neurons, ARF6 affects neuritis extension and branching, and this process apparently involves PIP5K (Hernandez-Deviez et al., 2004). Together, these data show that the
regulation of PIP5K activity and organized PI4,5P2 turnover is critical for ARF6-induced
membrane trafficking.

PLD is another important effector of ARF6. This enzyme mediates the hydrolysis
of phosphatidylcholine to generate phosphatidic acid (PA), which stimulates physiological
responses such as endocytosis (Brown et al., 1998), exocytosis (Hughes et al., 2004) and
the reorganisation of actin cytoskeleton (Cross et al., 1996). Two PLD isoforms, PLD1 and
PLD2, have been identified in mammals, each of which is expressed as two splice variants.
These have four conserved sequences (I–IV) and PH and phox homology (PX) domains,
which are implicated in phospholipid and protein binding (Sung et al., 1999). Both of these
PLDs are membrane-associated. PLD1 probably localizes to the Golgi complex or to
perinuclear vesicular structures, whereas PLD2 probably associates with the plasma
membrane (Exton, 2002). These enzymes may also be present in caveolae and may co-
localize with the actin cytoskeleton. ARF6 can bind directly to and activate PLD (Brown et
al., 1993; Toda et al., 1999), with a higher efficiency for PLD1 (up to 20-fold activation in
vitro) than for PLD2 (2-fold activation in vitro). Under in vivo condition however, it is the
PLD2 isoform that is susceptible to the dominant-active and the dominant-negative
mutants of ARF6 (Hiroyama and Exton, 2005).

PLD is currently considered as the major ARF6 effector active in exocytotic sites.
In PC12 cells, over-expression of the ARF6(N48I) mutant that is unable to stimulate PLD
activity strongly inhibits secretion, while in chromaffin cells the activation of PLD is
blocked by addition of a myristoylated peptide corresponding to the N-terminus of ARF6
(Caumont et al., 1998). The conclusion is that ARF6 is associated with secretory granule
membranes in its inactive GDP-bound conformation through a direct interaction with the
βγ subunit of heterotrimeric G proteins. Stimulation with a secretagogue triggers the
recruitment and the docking of granules at the plasma membrane, allowing the transient
interaction of ARF6 with an exchange factor (probably ARNO) (Caumont et al., 2000).
The activated form of ARF6 stimulates PLD1, which is located near to the exocytotic sites,
to produce a local increase in PA that results in the formation of the exocytotic fusion pore. Beyond regulating the secretory machinery, PA can also activate PIP5K to produce PI4,5P2, which in turn is an essential cofactor in the ARF-dependent stimulation of PLD (Hammond et al., 1995). Thus, regulation of both PLD and PIP5K by ARF6 defines a highly integrated mechanism to change the membrane-lipid composition (Donaldson, 2003).

1.3.3.3 Reorganization of actin structure

The change in lipid composition may mediate the ARF6-induced alterations in the cortical actin cytoskeleton as observed in HeLa cells in which the stimulation of over-expressed ARF6 with aluminium fluoride leads to redistribution of actin to plasma-membrane protrusions and ruffle formation (Peters et al., 1995). Additional evidence for a role of ARF6 in regulating the actin cytoskeleton is that the expression of ARNO and EFA6, two exchange factors for ARF6, induce the formation of F-actin-rich ruffles and promotes cell-spreading (Franco et al., 1999; Frank et al., 1998). Furthermore, dominant-negative ARF6 blocks bombesin-induced (Boshans et al., 2000) and EGF-induced (Honda et al., 1999) membrane ruffling.

In many cases, it appears that ARF6 changes the actin structure at the plasma membrane through the activation of Rac, a member of the Rho family implicated in membrane ruffling. In HeLa cells, a dominant-negative mutant of ARF6 blocks Rac-induced ruffling, while a dominant-negative Rac does not block ARF6-induced actin-rich membrane protrusions, suggesting that ARF6 can by itself alter cortical actin, and moreover, that ARF6 is required for Rac-dependent ruffling (Radhakrishna et al., 1999). Direct evidence that ARF6-GTP leads to activation of Rac has been obtained, although the mechanism has not been identified (Santy and Casanova, 2001). One protein that may integrate signals between Rac and ARF6 in the membrane ruffling response is POR1 (partner of Rac 1), the shortened form of Arfaptin 2. POR1 was originally isolated as a
protein that interacts with Rac1 (Van Aelst et al., 1996) and subsequently it was shown to bind both ARF6-GTP as well as Rac1 (D'Souza-Schorey et al., 1997). A deletion mutant of POR1 inhibits membrane ruffling in CHO cells induced by either activated Rac or activated ARF6. The binding of the Rac and ARF6 proteins to POR1 is mutually exclusive, indicating that they probably share at least part of the same binding site on POR1 (Tarricone et al., 2001). This suggests the possible mechanism by which POR1 directly mediates cross-talk between ARF6 and Rac GTPases. POR1, which colocalizes with Rac, binds to and effectively sequesters Rac (Van Aelst et al., 1996). The Rac-binding activity of POR1 is in turn regulated by the local concentration of activated ARF6. Activated ARF6 binds to POR1 and displaces Rac, thus releasing it for subsequent signalling activity (D'Souza-Schorey et al., 1997).

The ability of ARF6 to affect the cortical actin cytoskeleton appears to be essential for efficient cell adhesion and migration and for cancer cell invasion (Kondo et al., 2000). In contrast, the activities of other ARFs may be less closely involved in the fundamental aspects of cell migration (Mazaki et al., 2001). Overexpression of ARNO, the exchange factor for ARF6, in islands of MDCK epithelial cells selectively stimulates the activation of ARF6 and results in the formation of large lamellipodia. Moreover, expression of ARNO in peripheral cells stimulates cell motility and causes individual cells to pull away from their neighbours (Santy and Casanova, 2001). A constitutively active form of ARF6 is able to induce MCDK cell motility (Palacios et al., 2001). Conversely, a dominant-negative form of ARF6 attenuates the motility of these cells in a wound assay. Beyond affecting the actin cytoskeleton, ARF6 affects cell scattering by additional mechanisms. First, it has been shown that active ARF6 triggers the disassembly of interactions between adjacent cells known as adherens junctions (Palacios et al., 2001). ARF6-GDP has been seen to co-localise at cell junctions in polarised epithelial cells, and its activation leads to the inactivation of E-cadherin, a crucial component of adherens junctions. In particular, ARF6 regulates the spatial distribution and trafficking of the E-cadherins, as well as the
cadherin-based junctional components (Palacios et al., 2001). In conclusion, the activation of ARF6 is necessary to disassemble adherens junctions to initiate migration in polarised epithelial cells. After released from the tight adhesions the cells then still require ARF6 to extend the plasma membrane in a forward direction.

The integrins constitute yet another group of crucial proteins that are involved in cell migration and that are regulated by ARF6. The integrins exist as heterodimers, and they are composed of α and β subunits that pair up in different combinations to mediate cellular adhesion for specific matrix components (Hynes, 2002). While adhesion occurs on the cell surface, the integrins also undergo endocytic recycling in a process that may have an important role in cell motility by mediating the redistribution of the integrins to the migrating front (Bretscher, 1996). This recycling is regulated by ARF6 and requires the actin cytoskeleton. Indeed, over-expression of the ARF6(Q67L) dominant-positive mutant in HeLa cells can trap integrin β1 into ARF6(Q67L)-enriched large vacuoles (Brown et al., 2001). In line with these observations, the endocytic recycling of integrin β1, which is initiated by external stimuli (such as serum, EGF and PMA) is regulated by ARF6, and requires a role of the actin cytoskeleton (Powelka and Buckley, 2001). Consistent with these observations, mutant forms of ARF6 that affect either actin rearrangements or recycling inhibit the motility of a breast cancer cell line (Powelka et al., 2004). Collective migration of cancer cells often originates metastasis dissemination. In light of this, the role of ARF6 in tumour invasiveness was recently analysed. It appears that there is a critical role for ARF6 in the process of cell invasion through the regulation of invadopodia formation (Tague et al., 2004). Sustained activation of ARF6 significantly enhances the invasive capacity of melanoma cells as well as breast tumour cell lines, whereas the dominant-negative ARF6(T27N) abolishes basal cell invasive capacity as well as invasion induced by growth factors. Moreover, the ARF6 GTPase cycle regulates enhanced melanoma cell invasion through the activation of the extracellular-signal-regulated kinase (ERK), suggesting the existence of a link between ARF6-mediated signalling and ERK.
activation. Interestingly, the invasiveness of breast cancer cells requires the continued activation and cycling of ARF6 (Hashimoto et al., 2004). Both the hydrolysis-defective mutant ARF6(Q67L) and the GTP-binding-defective mutant ARF6(T27N) block the invasive activities of the MDA-MB-231 human cultured breast cancer cells, while cell adhesion is not affected.

1.3.3.4 Receptor endocytosis

The continued cycling of ARF6 between its GTP- and GDP-bound forms is also necessary for phagocytosis and endocytosis of receptors. In the case of Fcγ receptor-mediated phagocytosis in macrophages, both types of ARF6 mutants, that defective in GTP hydrolysis (Q67L) and that defective in GTP binding (T27N), have been shown to inhibit phagocytosis of IgG-coated erythrocytes (Zhang et al., 1998). During Fcγ receptor-mediated phagocytosis in murine macrophages, endogenous ARF6 becomes activated very early and transiently, and it has a critical role in the delivery of membrane from the recycling compartment to the forming phagosome (Niedergang et al., 2003).

The requirement of continued activation and cycling of ARF6 in the endocytic process has also been reported for the internalization of the β2 adrenergic receptor (β2AR). Expression of the constitutively-active or dominant-negative ARF6 mutants significantly attenuates β2AR internalisation (Claing et al., 2001). A marked inhibition of β2AR internalisation is also the consequence of a reduced expression of endogenous ARF6, as obtained using an RNA interference approach (Houndolo et al., 2005). Importantly, this effect is not unique to the β2AR, but affects a broad variety of GPCRs, including the angiotensin type I and vasopressin type II receptors, the endothelin type B receptor, and M2 muscarinic receptors. These data clearly support an essential and broader role for ARF6 in the agonist-promoted internalization process of GPCRs. The analysis of the mechanisms by which ARF6 modulates β2AR internalization revealed that ARF6 and its guanine-exchange factor ARNO form a complex with the β-arrestin that is recruited to the
plasma membrane following β2AR stimulation. It was thus proposed that β-arrestin functions as a scaffold to promote ARNO-dependent ARF6 activation, which facilitates β2AR endocytosis (Claing et al., 2001). When activated, ARF6 dissociates from the complex and may activate several signalling events and initiate receptor endocytosis.

The role of ARF6 in receptor internalization may be linked to luteinizing hormone/choriogonadotropin hormone LH/CG receptor desensitization (Figure 1.21) (Mukherjee et al., 2001; Mukherjee et al., 2000). The activation of ARF6 in ovarian follicles regulates β-arrestin binding to the active LH/CG receptor. In the GDP-bound form it sequesters β-arrestin in a complex that becomes inaccessible to the receptor. Upon LH/CG receptor stimulation, ARF6 becomes activated and β-arrestin is released, and becomes available to bind the activated LH/CG receptor. The activation of ARF6 is mediated by ARNO; indeed addition of nanomolar concentrations of ARNO in the absence of an agonist promotes the desensitization of the active LH/CG receptor (Mukherjee et al., 2000). Most importantly, this effect of ARNO is completely reversed by neutralising anti-arrestin antibodies that prevent interactions between the β-arrestin and the GPCRs, indicating that ARNO promotes the apparent release of β-arrestin from its membrane docking sites.

Interestingly, the activation of ARF proteins has been shown to occur upon agonist stimulation of a number of different GPCRs, including β2-adrenergic, M3 muscarinic, fMet-Leu-Phe, H1 histamine, and B2 bradykinin receptors (Bornancin et al., 1993; Houle et al., 1995; Mitchell et al., 1998; Rumenapp et al., 1995; Salvador et al., 2001). This suggests that the ARF6-mediated pathway constitutes widespread mechanism that modulates the accessibility of β-arrestin to activated GPCRs, thus regulating their desensitization.
Figure 1.21. Model of LH/CGR receptor desensitization. A, Docking of arrestin 2 at a membrane location distinct from the inactive LH/CGR receptor and in association with inactive ARF6-GDP. B, Activation of the LH/CGR receptor upon binding agonist promotes not only activation of the stimulatory heterotrimeric G protein Gs and consequent AC activation, but also activation of ARF6 and liberation of arrestin 2. ARNO or a similar ARF6 GEF promotes GDP/GTP exchange on ARF6 in response to LH/CGR receptor activation. C, Arrestin 2 binding to the active LH/CGR receptor mediates desensitization by interfering with the ability of agonist-activated receptor to activate Gs.
Chapter 2

Materials and Methods

2.1 Materials

DL-dithiothreitol (DTT), glucose, bovine serum albumin (BSA), Tris[Hydroxymethyl]aminomethane (Tris), ethylene glycolbis(beta-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), Na₂HPO₄, NaH₂PO₄, Na₂S₂O₃, sucrose, aprotinin, leupeptin, pepstatin, 1,10-phenanthroline, phenylmethylsulphonyl fluoride (PMSF), deoxycholic acid, Nonidet P40, ATP, UTP, GTP, GDP, GTPβS, α-NAD⁺, β-NAD⁺, meta-iodobenzylguanidine (MIBG), thrombin, substance P, clonidine and isoproterenol, were from Sigma-Aldrich (WI, USA). Mastoparan, mas7, mas17 and gonadotropin releasing hormone (GnRH) were from Calbiochem (CA, USA). The catalytic subunit protomer A and the inactive subunit protomer B of PTX were from Biomol (PA, USA). NaCl, HCl, NaOH, KOH, formaldehyde, acetic acid and methanol were from Carlo Erba (Italy). 4-(2-Hydroxy-ethyl)-piperazine-1-ethane-sulfonic acid (HEPES), glycerol, KCl, Mg(CH₃COO)₂, K₂HPO₄, KH₂PO₄, Na₂CO₃, AgNO₃ and MgCl₂, were from Merck (Germany). β-mercaptoethanol were from Fluka (Switzerland). Triton X-100 was from Bio-Rad Laboratories (UK). [³²P]-NAD (specific activity 1000 Ci/mmol) was from Amersham Pharmacia Biotech (NJ, USA). Pertussis toxin was a generous gift of Dr. R. Rappuoli (Chiron Vaccines, Siena, Italy). Myristoylated and non-Myristoylated peptides of ARF6 and ARF1 were from Laboratory of Biochemistry of Mario Negri Institute (Milan, Italy). Purified bovine brain βγ dimer was from A. Marchegiani in the laboratory.

Other materials will be specified for each procedure.
2.2 Cell culture

2.2.1 Materials

Chinese Hamster Ovary (CHO) cells were from American tissue type collection (ATTC, USA). CHO cells stably transfected with α-Adrenergic, β-Adrenergic, δ-Opioid and NK1 receptors were kindly provided by Dr. Costa (Istituto Superiore di Sanità, Rome, Italy). CHO cells stably transfected with the GnRH receptor were kindly provided by Dr. Roelle (Institute of Pharmacology and Toxicology, Marburg, Germany). Dulbecco's Modified Eagles Medium (DMEM), Ham F-12, penicillin, trypsin-EDTA, and L-glutamine were from GIBCO (UK). G418 was from Fluka. Puromycin was from Sigma-Aldrich. Foetal Calf Serum (FCS) was from Biochrom (Germany). All the plastic materials were from Corning (USA) or from Falcon (NJ, USA). Filters (0.45 and 0.2 μm) were from Amicon (USA).

2.2.2 Growth conditions and propagation of cell lines

CHO cells were grown using DMEM supplemented with 34 mg/ml proline, 2 mM L-glutamine, 100 U/ml penicillin and streptomycin, and 10 % FCS. CHO cells stably transfected with α-Adrenergic, β-Adrenergic, δ-Opioid and NK1 receptors were grown in DMEM-Ham F-12 (1:1 v/v) supplemented with 34 mg/ml proline, 2 mM L-glutamine, 100 Units/ml penicillin and streptomycin, and 10% FCS in presence of 200 μg/ml G418, for selection of resistance. CHO cells stably transfected with the GnRH receptor were grown in Ham F-12 supplemented with 2 mM L-glutamine, 100 Units/ml penicillin and streptomycin, and 10% FCS in presence of 6.25 μg/ml puromycin for selection of resistance. Complete media were prepared by diluting concentrated stock solutions with sterile water (Diaco, Italy) and filtering the mixture through 0.2 μm filter.

All the cell lines were grown under controlled atmosphere in the presence of 5% CO₂ at 37 °C. Cells were grown in 10 cm Petri dishes until 90% confluence. The medium was removed and 0.25% trypsin solution for wild type CHO cells and trypsin-EDTA
solution (0.05% trypsin, 0.02% EDTA) for stably transfected CHO cells was added for 2-5 minutes. The medium was added back to block the protease action, cells were collected into a plastic tube and centrifuged for 5 min at 200xg. The pellet was re-suspended in fresh medium and placed in a new plastic dish.

2.3 Cell fractionation

2.3.1 Materials and solutions

Dextran T-500 was from Amesham Pharmacia Biotech; polyethylene glycol 4000 (PEG4000) was from Merk. Hanks Balance Solution without Ca\(^{2+}\) and Mg\(^{2+}\) (HBSS\(^{−}\)) was from GIBCO, N-tris[hydroxymethyl]methyl-2-aminoethansulfonic acid (TES) was from Sigma.

Potassium phosphate buffer (PFB): 200mM PFB buffer, pH 7.4, was prepared dissolving 1.36 g K\(_2\)HPO\(_4\) and 1.74 g KH\(_2\)PO\(_4\) in 50 ml water. Protease inhibitors: 2.0 µg/ml aprotinin, 0.5 µg/ml leupeptin, 2 µM pepstatin, 0.5 mM 1,10-phenanthroline, 1 mM phenyl-methylsulphonyl fluoride (PMSF).

Hypotonic buffer: 10 mM TES, pH 7.0 and 1 mM EDTA.
Sucrose buffer: 10 mM TES, pH 7.5, containing 0.25 M sucrose.
HEPES buffer: 20 mM HEPES, pH 7.4; 1mM EDTA, 250 mM sucrose.
Cytosol-buffer: 5 mM Tris-HCl, pH 8.0, 5 mM MgCl\(_2\), 1mM EGTA.
Cell lysates-buffer: 20 mM Tris-HCl, pH 7.4, 0.83 mM MgCl\(_2\), 1mM EDTA, 20 mM NaCl, 1 mM DTT.

2.3.2 Procedures

All the steps described were performed at 4 °C and in presence of protease inhibitors. The protein concentration was measured using Bio-Rad Protein assay kit (Bio-Rad Laboratories) according to the manufacturer's instructions.
2.3.2.1 Preparation of plasma membranes

Plasma membranes were prepared following the procedure described by Gettys (Gettys et al., 1994), with some modification. Confluent CHO cells (6 x 10^8 cells for each preparation) were washed with HBSS\(^{-}\), detached with hypotonic buffer and then lysed with a Teflon/glass Potter homogeniser. Unbroken cells and nuclei were removed by a low speed centrifugation for 5 min at 400xg, and crude membranes were collected by the supernatant by a 20 min centrifugation at 48,000xg. The pelleted membranes were re-suspended in sucrose buffer, at a final concentration of ca. 100-150 mg/ml. 200 mg of membranes, and then mixed to a 14 g pre-weighted aqueous two-phase system composed of 5.12 g 20% dextran T-500, 2.56 g 40% PEG 4000, 0.4 ml 200 mM PFB, pH 7.4, 1.6 ml 1M sucrose and distilled water. Separation was achieved by a centrifugation for 20 min at 2,500xg. The upper phase was removed, and repartitioned against a fresh lower phase; while, to increase the yield of plasma membranes, the lower phase was extracted with a fresh upper phase. Finally, the two upper phases containing plasma membranes were combined, diluted 5 fold in sucrose buffer and collected by centrifugation (30 min at 48,000xg). The purified plasma membranes were re-suspended at 1 mg/ml in 25 mM HEPES, pH 7.4 containing 150 mM NaCl, 1 mM EDTA, and protease inhibitors and stored at -80 °C. Purity of plasma membrane preparation was assed by Western blotting with markers for cellular components (Table 2.1).

2.3.2.2 Preparation of total membranes

Confluent CHO cells (3 x 10^7 cells for each preparation) were washed with HBSS\(^{-}\), detached in 0.25% trypsin solution and then pelleted at 200xg for 5 min. Cells were re-suspended into 1ml HEPES buffer and broken by passage (14 times) through a 25-gauge syringe; unbroken cells and nuclei were removed by low speed spin for 5 min at 400xg. Post-nuclear supernatants were centrifuged for 15 min at 16,000xg and the pellet was re-
<table>
<thead>
<tr>
<th>Marker</th>
<th>Cellular compartment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na+/K+ ATPase (Godi et al., 1999)</td>
<td>Plasma membranes</td>
</tr>
<tr>
<td>CaBP1 (Fullekrug et al., 1994)</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>Rab5 and Rab7 (Chavrier et al., 1990)</td>
<td>Early and late endosomes</td>
</tr>
<tr>
<td>Mannosidase II (Velasco et al., 1993)</td>
<td>Golgi membranes</td>
</tr>
</tbody>
</table>

Table 2.1. Markers for cellular components used to assess the purity of plasma membrane preparations.
suspended in 20 mM HEPES, pH 7.4, containing 1 mM EDTA and protease inhibitors and stored at -80 °C.

2.3.2.3 Preparation of cytosol

Cytosol was prepared following the procedure described by Di Girolamo et al. (Di Girolamo et al., 1995) with some modification. Confluent CHO cells (3 x 10^7 cells for each preparation) were washed twice with 5 ml of HBSS*, detached in 0.25% trypsin solution and pelleted at 200xg for 5 min. The pellets were re-suspended in an equal volume of cytosol-buffer and broken by passage (14 times) through a 25-gauge syringe. Nuclei and unbroken cells were removed by centrifugation at 400xg for 5 min and supernatants were then centrifuged at 100,000xg for 90 min. The supernatant that constitutes the cytosol fraction was stored at -80 °C.

2.3.2.4 Preparation of total cell lysates

Confluent CHO cells (3 x 10^7 cells for each preparation) were washed twice with 5 ml of HBSS*, detached in 0.25% trypsin solution and pelleted at 200xg for 5 min. The pellets were re-suspended in 250 μl/Petri-dish of cell lysate-buffer and broken by passage (14 times) through a 25-gauge syringe. Nuclei and unbroken cells were removed by centrifugation at 400xg for 5 min and supernatant was stored at -80 °C.

2.4 SDS-PAGE and immunoblotting

2.4.1 Materials and solutions

Sodium dodecyl sulphate (SDS), glycine, TRIZMA base, red ponceau, polyoxyethylenesorbitan monolaurate (Tween-20), ammonium persulphate (APS), brilliant blue and N,N,N',N'-tetramethylethylenediamine (TEMED), were from Sigma-Aldrich. Acrylamide stock solution: 40% (w/v) acrylamide-bisacrylamide (37.5:1) was from Eurobio (France). Secondary antibodies conjugated with horse-radish peroxidase (HRP)
and directed against mouse or rabbit IgGs were from Calbiochem. Low molecular weight standards and ECL reagents were from Amersham Pharmacia Biotech.

Sample buffer 2X: 62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol and 0.1% (w/v) bromophenol blue. Running buffer: 25 mM TRIZMA base, 200 mM glycine, 0.1% (w/v) SDS. Transfer buffer: 25 mM TRIZMA base, 200 mM glycine, 20% (v/v) methanol. TBS: 150 mM NaCl, 20 mM Tris-HCl pH 7.5. TTBS: 0.05% (w/v) Tween 20, 150 mM NaCl, 20 mM Tris-HCl, pH 7.5. Blocking solution: 1% (w/v) BSA in TTBS.

2.4.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

2.4.2.1 Assembly of polyacrylamide gels

Two 16 x 18 cm glass plates were used for assembling a regular gel, while two 32 x 18 glass plates were used for assembling a long gel. The glasses were assembled to form a chamber using two 1.5 mm plastic spacers aligned along the lateral edges of the glass plates. The plates were then fixed using two clamps and mounted on a plastic base which sealed the bottom. All the materials were from Hoefer Scientific Instruments (NJ, USA). The ‘running’ polyacrylamide gel was prepared by mixing H₂O, 40% (w/v) acrylamide-bisacrylamide solution, 1.5 M Tris-HCl, pH 8.8, 10% (w/v) SDS, in order to have the selected concentration of acrylamide, 375 mM Tris-HCl, 0.1% (w/v) SDS. Then, 0.06% (w/v) APS and 0.06% (v/v) TEMED were added; the solution was pipetted and poured in the gap between the plates, leaving ~ 5 cm for the stacking gel. Soon after pouring, the gel was covered with a layer of 375 mM Tris-HCl pH 8.8 and left at RT for ~1 h. The Tris-HCl layer was removed after gel polymerisation. The ‘stacking’ polyacrylamide gel was prepared by mixing H₂O, 40% (w/v) acrylamide-bisacrylamide solution, 0.5 M Tris-HCl, pH 6.8, 10% (w/v) SDS, in order to have 4% (w/v) acrylamide, 125 mM Tris-HCl, 0.1% (w/v) SDS. Then, 0.1% (w/v) APS and 0.07% (v/v) TEMED were added, and the solution
was pipetted and poured onto the ‘running’ gel. Immediately, a 15-well comb was inserted between the glasses and left 1 h at RT.

2.4.2.2 Sample preparation and run

Samples were prepared by adding an equal volume of 2X sample buffer, incubating for 5-15 min at 100 °C in a Multi-Block Heater (Lab-Line, IL, USA), cooled at RT, briefly centrifuged and loaded onto the gel. One or two wells were loaded with 5 μg of molecular weight standards. The gel was then transferred into the electrophoresis apparatus (Hoefer Scientific Instruments) and the electrophoresis was carried out under a constant current of 8 mA (for O/N runs) or 40 mA (for ~4 hours runs).

2.4.2.3 Silver Staining and gel drying

After electrophoresis, the gels were incubated for 15 min with gentle shaking at RT in fixation solution (50% methanol: 5% acetic acid: 45% water v/v/v), washed with water (3 times rapidly) and finally placed in water for 1 h on a shaking platform. The gels were then incubated for 1-2 min in sensitizing solution (0.02% Na₂S₂O₃), washed twice rapidly with water, and incubated for 30 min at 4 °C with chilled 0.1% (w/v) AgNO₃ solution. The gels were then developed with 0.04% formaldehyde in 2% (w/v) Na₂CO₃ until bands became visible. The reaction was stopped by adding 12 g citric acid per 0.5 litre developing solution and the gels were washed with water before to be incubated for 45 min with 30% (v/v) methanol and 3% (v/v) glycerol. Finally, the gels were dried with a gel dryer (Hoefer Scientific Instruments) at 80°C under vacuum for 4 hours.

2.4.2.4 Comassie brilliant blue Staining

Gels were incubated in staining solution (50% methanol, 40% H₂O, 10% acetic acid and 0.1 % brilliant blue) for 2 h, washed with water and then de-stained with 30% methanol 10 % acetic acid. The gels were then dried as described above.
2.4.3 Western blotting

2.4.3.1 Protein transfer onto nitrocellulose

The polyacrylamide gel was soaked for 15 min in transfer buffer, placed on a sheet of 3MM paper (Whatman, NJ, USA) and covered with a nitrocellulose filter (Schleicher & Schuell, Germany). The filter was covered with a second sheet of 3MM paper, to form a "sandwich" which was subsequently assembled into the blotting apparatus (Hoefer Scientific Instruments). Protein transfer occurred at 500 mA for 4 h or at 125 mA O/N. At the end of the run, the sandwich was disassembled and the nitrocellulose filter was stained with 0.2% Red Ponceau (Sigma-Aldrich) and 5% (v/v) acetic acid for 5 min to visualize the protein bands, and then rinsed with 5% acetic acid to remove the excess of unbound dye.

2.4.3.2 Probing the nitrocellulose with specific antibodies

The nitrocellulose filters were cut into strips with a razor blade. The strips were incubated in blocking solution for 60 min at RT, then with the primary antibodies directed against the protein of interest, diluted at the appropriate concentration in the blocking solution. A list of antibodies used in this thesis and their working dilution is provided in Table 2.2. After a 2-3 h incubation at RT, or an O/N incubation at 4 °C, the antibody was removed and the strips washed in TTBS twice, for 10 min each. The strips were next incubated for 1 hour with the appropriate HRP-conjugated secondary antibodies (anti-rabbit: 1:20,000; anti-mouse: 1:5000) and washed twice in TTBS, for 10 min each, and once in TBS for 3 min. After washing, the strips were incubated with ECL reagents, according to the manufacturer instructions, for ECL-based detection.
<table>
<thead>
<tr>
<th>Specificity (antibody name)</th>
<th>Supplier</th>
<th>Animal source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-common subunits (T20)</td>
<td>Santa-Cruz</td>
<td>rabbit (polyclonal)</td>
<td>1:1000</td>
</tr>
<tr>
<td>ARF6</td>
<td>Santa-Cruz</td>
<td>mouse (monoclonal)</td>
<td>1:800</td>
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<tr>
<td>c-cbl</td>
<td>Santa-Cruz</td>
<td>rabbit (polyclonal)</td>
<td>1:1000</td>
</tr>
<tr>
<td>His</td>
<td>Sigma</td>
<td>Mouse (monoclonal)</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

Table 2.2. List of antibodies used in this study.
2.5. ADP-ribosylation assay

2.5.1 Procedure

The ADP-ribosyltransferase activity was measured following incorporation of radioactive ADP-ribose into membrane components using the procedure described by Lupi, with some modifications (Lupi et al., 2000). Samples (5 μg CHO plasma membranes) were incubated with 50 μl ADP-ribosylation buffer (50 mM PFB, pH 7.4, 5 mM MgCl₂, 4 mM DTT, 10 μM GTPγS, 700 μM β-NAD⁺ and 4.5 μCi [³²P]-NAD) for 60 min at 37 °C. For extensive ADP-ribosylation of the β subunit, plasma membranes were incubated in the same buffer for 6 h at 37 °C.

For experiments with MyrARF6, MyrARF1, MyrCoA, non-MyrARF6, purified ARF6 and ARF1, these were incubated for 15 min at 37 °C with plasma membranes in ADP-ribosylation buffer without β-NAD⁺. After the incubation, 700 μM β-NAD⁺ and 4.5 μCi [³²P]-NAD were added, and the samples were ADP-ribosylated for 60 min at 37 °C, as described above.

For PTX-catalysed ADP-ribosylation of the G₉α subunits, plasma membranes were incubated for 60 min at 37 °C with 100 nM catalytic subunit, or as a control with 100 nM inactive protomer B of PTX, in ADP-ribosylation buffer. When the assays were carried out using 25 μg total membranes or 80 μg total cell lysates from CHO cells, 250 ng purified bovine brain βγ dimer was used to obtain better signal detection for β subunit.

The reactions were terminated by diluting the samples with 50 μl 2x sample buffer and analysed by 12% SDS-PAGE. Proteins were electroblotted as described above, and the filters were exposed to Kodak X-Omat film (Amersham). For quantitative analysis, an Instant Imager (Canberra Packard Instruments, CT, USA) was used. In the experiments where purified bovine brain βγ dimers were added to samples, association of the exogenous βγ subunit with plasma membranes was evaluated by Western blotting of
plasma membrane pellets and supernatants (separated by centrifugation; 15 min, 12,000 x g), with at least 95% of the βγ subunit recovered in the membrane fraction.

2.5.2 Quantification of modified proteins

The amounts of mono-ADP-ribosylated β subunit were expressed in picomoles of modified protein and were calculated considering that binding between the β subunit and the ADP-ribose moieties is equimolar (Lupi et al., 2000). Starting from this assumption, and knowing the specific activity of radiolabelled nucleotide (calculated from the experimental condition used and expressed as cpm [³²P]-NAD/pmol β-NAD⁺), the amounts of mono-ADP-ribosylated β subunit were calculated after separation of the ADP-ribosylated samples by SDS-PAGE, through the conversion of cpm values measured by Instant Imager acquisition into pmol of ADP-ribosylated proteins.

2.5.3 Kinetic analysis

2.5.3.1 $V_{\text{max}}$ and $K_m$ for βγ dimer

Plasma membranes (5 μg/sample) were incubated with 50 μl ADP-ribosylation buffer (50 mM PFB, pH 7.4, 5 mM MgCl₂, 4 mM DTT, 10 μM GTPγS, 700 μM β-NAD⁺ and 4.5 μCi [³²P]-NAD) for 60 min at 37 °C in the presence of increasing concentrations of substrate (0-650 ng purified bovine brain βγ dimer) and a saturating concentration of β-NAD⁺ (1 mM). The samples were processed as described above, and the levels of β subunit ADP-ribosylation were analysed with an Instant Imager. Data were transformed to Eadie-Hofstee analysis, and the constants ($K_m$ and $V_{\text{max}}$) were determined using GraphPad PRISM software.

2.5.3.2 $V_{\text{max}}$ and $K_m$ for β-NAD⁺

Plasma membranes (5 μg/sample) were incubated with 50 μl ADP-ribosylation buffer (50 mM PFB, pH 7.4, 5 mM MgCl₂, 4 mM DTT, 10 μM GTPγS and 4.5 μCi [³²P]-
NAD) for 60 min at 37 °C in the presence of increasing concentrations of substrate (0-1 mM β-NAD⁺) and saturating concentrations of βγ dimer (500 ng) or ARF6 (500 ng). The samples were processed as described above, and the levels of β subunit ADP-ribosylation were analysed with an Instant Imager. Data were transformed to Eadie-Hofstee analysis, and the constants (Kₘ and Vₘₐₓ) were determined using GraphPad PRISM software.

2.6 De-ADP-ribosylation assay

Following the ADP-ribosylation assay, [³²P]-labelled plasma membranes were washed twice with 5mM Tris HCl, pH 8.0 and pelleted for 15 min at 16,000xg. The pellets were incubated for 30 min at 37 °C with 50 μg/sample of CHO cells cytosol in 50 μl de-ADP-ribosylation buffer (5mM Tris-HCl, pH 8.0, 10mM MgCl₂) containing protease inhibitors. The analysis of the samples was performed as described above for the ADP-ribosylation assay.

2.7 Production of [³²P]-ADP-ribose

[³²P]-ADP-ribose was prepared following the procedure described by De Matteis (De Matteis et al., 1994), with some modification. [³²P]-NAD⁺ (0.5 μM/sample) was incubated in 200 mM PFB, pH 7.4 (15 μl/sample), with 0.1 U/ml NADase from Neurospora crassa (Sigma), for 20 min at 37 °C. The mixture was used as source of ADP-ribose. After this incubation 90% of the radioactivity was in ADP-ribose and 10% in AMP; NAD was undetectable, as revealed by thin-layer chromatography (TLC) analysis (Figure 2.1; Section 2.16 for TLC protocol).
Figure 2.1. Production of $^{32}$P-ADP-ribose. $^{32}$P-NAD$^+$ (0.5 μM/sample) was incubated for 20 min at 37 °C in 200 mM PFB, pH 7.4 (15 μl/sample) and 0.1 U/ml NADase from Neurospora crassa. After this incubation, 90% of the radioactivity was in ADP-ribose and 10% in AMP; NAD was not detectable.
2.8. DNA preparation

2.8.1 Materials

Tryptone peptone and yeast extract were from Promega. RbCl, CaCl, MnCl₂, MOPS and Agar were from Sigma. QIAGEN Plasmid Maxi Kit was from Qiagen (CA, USA). A list of constructs used in this thesis is shown in Table 2.3.

LB: 1% Tryptone Peptone, 0.5% Yeast extract, 1% NaCl, autoclaved 15 min at 121 °C. LB-agar: LB plus 1.5% (w/v) agar: autoclaved 15 min at 121°C. TE buffer: 10 mM Tris-HCl, 1 mM EDTA pH 7.5.

2.8.2 Preparation of competent cells

Competent bacteria were obtained using the rubidium chloride method (Kushner, 1978). Bacteria were streaked and incubated overnight on a LB plate. Next day, one single colony was inoculated into 10 ml of LB and grown overnight at 37°C with shaking at 225 rpm. One ml of the overnight saturated culture was inoculated into 9 ml of fresh LB and grown until the optical density at 550 nm was 0.3. The bacteria were sub-cultured 1:20 into 100 ml pre-warmed LB and grown until the optical density at 550 nm reached 0.48. The cells were chilled on ice and centrifuged at 1000xg at 4°C for 5 min, the supernatant was removed and the pellet gently resuspended in 40 ml 30 mM potassium acetate, 100 mM RbCl, 10 mM CaCl, 50 mM MnCl₂, 15% glycerol pH 5.8. The bacteria were left on ice for 2 h, centrifuged and resuspended in 4 ml of 10 mM MOPS, pH 7, 75 mM CaCl, 10 mM RbCl, 15% glycerol.

2.8.3 Transformation of bacteria by heat shock

Competent cells were thawed on ice and ~10 ng of plasmid were added. After gentle mixing, the cells were left on ice for 30 min and then heat shocked for 90 seconds at 42 °C. After addition of 800 µl LB, the bacteria were grown for 45 min at 37 °C under constant shaking (200 rpm). The culture was then plated on LB agar containing the
<table>
<thead>
<tr>
<th>Protein</th>
<th>Vector</th>
<th>Resistance</th>
<th>Expression</th>
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</thead>
<tbody>
<tr>
<td>FLAG-ARF6</td>
<td>pXS</td>
<td>Ampicillin</td>
<td>eukaryotes</td>
</tr>
<tr>
<td>FLAG-ARF6(Q67L)</td>
<td>pXS</td>
<td>Ampicillin</td>
<td>eukaryotes</td>
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<tr>
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<td>pXS</td>
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<tr>
<td>NMT</td>
<td>pBB131</td>
<td>Kanamycin</td>
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</tr>
</tbody>
</table>

Table 2.3. List of constructs used in this study.
appropriate selective antibiotic and incubated overnight at 37 °C. The next day, an isolated bacterial colony was picked from the plate and used to inoculate 2 ml of LB containing the appropriate antibiotic and the culture was incubated O/N at 37 °C. Sterile glycerol was added to the bacterial culture (30 % v/v) for long-term storage at -80 °C.

2.8.4 DNA purification

A small amount of bacteria transformed with the plasmid of interest, were scraped from the glycerol stock, and used to inoculated in 2 ml of LB containing the appropriate selective antibiotic. The culture was grown at 37 °C under continuous shaking (200 rpm) for 6-8 hours. This pre-culture was then used to inoculate 500 ml of LB containing selective antibiotic. After 15-20 hours incubation, bacteria were collected by centrifugation at 4000xg for 10 min at 4 °C and processed according to the QIAGEN Plasmid Maxi Kit protocol. The DNA obtained was resuspended in TE buffer to a final concentration of 1 mg/ml and stored at 4 °C, or for long-term, at -20 °C.

2.9. Cell transfection

2.9.1 Materials

Lipofectamine-Plus and OptiMEM serum free reduced culture medium, were from GIBCO. cDNAs for FLAG-ARF6, FLAG-ARF6(Q67L) and FLAG-ARF6(T27N) were kindly provided by Dr. R. Weigert (National Institutes of Health, Bethesda, USA), cDNA for HA-EFA6 was kindly provided by Dr. R Buccione (Consorzio Mario Negri Sud, Italy), cDNAs encoding GFP-ARF1, GFP-ARF1(Q71L) and GFP-ARF1(T31N) were kindly provided by Dr. T. Daniele, (Consorzio Mario Negri Sud, Italy).
2.9.2 Lipofectamine Plus-reagent-based cell transfection

CHO cells were plated on 100 mm Petri dishes or 6 well plates, in normal culture medium, at a concentration suitable to have 50-70% confluence for transfection. The day after, the transfection mixture was prepared in a polypropylene tube: for each Petri dish, 4 μg DNA and 20 μl Plus reagent were diluted in 750 μl of OptiMEM culture medium. In separate tubes, 30 μl of Lipofectamine reagent was diluted in 750 μl of the OptiMEM. When CHO cells were grown in 6 well plates, for each well 1 μg DNA and 6 μl Plus reagent were diluted in 100 μl of OptiMEM; while, 4 μl of Lipofectamine reagent was diluted in 100 μl of the OptiMEM.

Transfection and reagent mixtures were shaken and incubated at RT for 15 min, and then combined and kept at RT for additional 15 min to allow DNA-Lipofectamine complex to form. In the meantime, cells were washed twice with OptiMEM medium. Cells were then incubated with the transfection mixture for 3 h at 37 °C in the presence of 5% CO₂. At the end of the incubation the transfection mixture was replaced by complete culture medium and cells incubated for additional 12-24 h prior to assay.

2.10 Expression and purification of recombinant proteins

2.10.1 Materials

Ampicillin, kanamicin and chloramphenicol were from Fluka. Imidazole, glutathione, isopropyl-β-D-1-thiogalactopyranoside (IPTG), and lysozyme were from Sigma Aldrich. Glutathione-sepharose resin was from Amersham Pharmacia Biotech. Ni-NTA beads were from Qiagen (CA, USA)
2.10.2 Purification of GST-GGA3\textsubscript{1-226}

2.10.2.1 Materials and solutions

pGEX4T1 vector containing GST-GGA3\textsubscript{1-226} fusion protein was kindly provided by Dr. P. Chavrier (Institute Curie, Paris).

GST-lysis buffer: 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% v/v glycerol. GST-washing buffer: 50 mM Tris-HCl, pH 8.0 containing 200 mM NaCl. GST-elution buffer: 50 mM Tris-HCl, pH 8.0, 200 mM NaCl, and 10 mM reduced glutathione.

2.10.2.2 Procedure

\textit{E. coli} strain BL21(DE3) harbouring the GST-fusion protein of interest, was grown in LB plus ampicillin (100 mg/ml) until the optical density OD\textsubscript{600 nm} was about 0.6. At this point, the fusion protein was induced for 5 h at 25 °C with 0.25 mM IPTG. The total bacteria culture was harvested by centrifugation at 4000xg for 10 min at 4 °C and stored at -80°C O/N. In parallel, one ml of the induced and non induced cultures were taken to confirm protein expression. The samples were collected by centrifugation, lysed with sample buffer, and 10-25 µl of the lysates were fractionated on a polyacrylamide gel. The gels were stained with Comassie blue dye, as described above.

The day after the bacterial pellet is thawed at room temperature and re-suspended (20 ml/l of culture) in GST-lysis buffer in the presence of protease inhibitors and 0.5 mg/ml lysozyme. The suspension was incubated 30 min on ice and then is lysed by sonication (5-7 pulses for 10 sec each). The lysate is centrifuged at 12,000xg for 15 min at 4 °C. The supernatant was recovered and added to 1 ml of glutathione-sepharose resin previously equilibrated in lysis buffer. The suspension was incubated with gentle agitation O/N at 4°C and then pelleted at 700xg for 10 min at 4 °C to sediment the matrix. The supernatant was discarded and the resin was washed twice with GST-washing buffer. Protein elution was started by the addition of GST-elution buffer. The purified protein was than dialysed.
against 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 10% v/v glycerol and 2 mM β-mercaptoEtOH and stored in aliquots at -80°C.

2.10.3 Purification of myristoylated ARF6

2.10.3.1 Material and solutions

pBB131 vector containing N-Myristoyltransferase (NMT) was provided by Dr. Gordon (Washington University, St. Louis, Missouri), pET3 plasmid containing ARF6 was provided by Dr. P. Randazzo (National Institutes of Health, Bethesda, USA).

ARF6-lysis buffer: 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM MgCl2, 1 mM DTT. Buffer A: 20 mM Tris-HCl, pH8.0, 100 mM NaCl, 1 mM MgCl2, 1 mM DTT, 10% v/v glycerol. Buffer B1: 20 mM Tris-HCl, pH8.0, 25 mM NaCl, 1 mM MgCl2, 1 mM DTT, 10% v/v glycerol, 1% Triton X-100. Buffer B2: 20 mM Tris-HCl, pH8.0, 25 mM NaCl, 1 mM MgCl2, 1 mM DTT, 10% v/v glycerol, 0.3% Triton X-100.

2.10.3.2 Procedures

The myristoylated form of ARF6 was purified following the procedure described by Randazzo (Randazzo and Fales, 2002), with minor modification. TurboCells BL21(DE3)pLysS E. coli (Gene Therapy System, CA, USA) were co-transformed with pET3 vector containing ARF6 which is under ampicillin selection, and with pBB131 vector containing yeast NMT. Positive double transformant colonies were isolated with ampicillin/kanamycin selection on agarose plates. A single colony of transformed bacteria was used to inoculate 50 ml LB containing 100 µg/ml ampicillin and 25 µg/ml kanamycin and grown O/N at 37 °C under continuous shaking (200 rpm). The day after, the culture was diluted in 1-2 l of LB with the same antibiotic selection and OD(600 nm) was monitored. When the optical density was about reached 0.6 sodium myristate is added to the culture to achieve a concentration of 50 µM, and the incubation is continued for an additional 20-30 min. Then IPTG is added to a final concentration of 1 mM and the incubation was
continued for 3-4 h. At the end the culture was chilled on ice. The cells are harvested by centrifugation (4000xg) for 10 min at 4°C and stored at -80 °C O/N. One-ml samples of the induced and non induced cultures are taken to confirm ARF6 expression. The cells are collected by centrifugation and lysed in sample buffer, and 10-25 µl are fractionated on a 15% SDS-PAGE. The gels are stained with Comassie blue dye, as described above.

The bacterial pellet is thawed at room temperature and resuspended in 10 ml of ARF6-lysis buffer containing the cocktail of protease inhibitors. The suspension is lysed by sonication on ice (5-7 pulses for 10 sec each). The lysate is centrifuged at 100,000xg for 60 min at 4 °C, and the pellet containing the myristoylated ARF6 was stored at -80°C O/N or for few days. The particulate fraction is resuspended in 10 ml of the buffer A containing protease inhibitors and centrifuged at 100,000xg for 60 min at 4 °C. Ten ml of buffer B1 were used to resuspend the pellet. This buffer contains Triton X-100 that extracts ARF6 from the particulate fraction. The suspension was sonicated on ice (5-7 pulses for 10 sec each) and centrifuged 100,000xg for 60 min at 4 °C. The supernatant was collected and GDP added to a final concentration of 10 µM. 35% of ammonium sulphate was added to precipitate proteins and the mixture was incubated on ice for 20 min. The precipitate proteins were collected by centrifugation at 16,000xg for 60 min at 4 °C and dissolved in 10 ml of buffer B2 containing protease inhibitors and 10 µM GDP. The solution was centrifuged at 20,000xg for 15 min at 4°C and the pellet was resuspended in 10 ml of fresh buffer B2 containing protease inhibitors and GDP. The solution is dialyzed twice against 1 l of buffer B2 containing 10 µM GDP and then applied to a pre-equilibrated 5-ml HiTrap Q column (Amersham) in a FPLC (Fast Protein Liquid Chromatography) system (Pharmacia Bio- Tec, UK). The material that does not adhere to the column is collected in fraction of 2.5 ml each. The column was then washed with 25 ml of buffer B2 and additional fraction are collected. Fifty µl of each fraction were analysed by SDS-PAGE and Western Blotting to control purity and integrity of the proteins (Figure 2.2). The fractions containing the protein were pooled and stored in aliquots at -80°C.
Figure 2.2. Purification of myristoylated ARF6. A, Fifty µl of the indicated fractions eluted from a HiTrap Q column were analysed on a 15% SDS-PAGE gel and the proteins revealed by silver staining. The positions of molecular weight standards are indicated on the left and the position of ARF6 on the right of the panel. B, The same samples as in A were revealed by Western blotting with an anti-ARF6 antibody.
2.10.4 Purification of ARNO

2.10.4.1 Material and solutions

pRSETc vector containing His-ARNO was provided by Dr. T. Daniele (Consorzio Mario Negri Sud, Italy). His-lysis buffer: 20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10 mM imidazole. His-washing buffer: 20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 20 mM imidazole. His-elution buffer: 20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 250 mM imidazole.

2.10.4.2 Procedures

*E. coli* strain BL21(DE3) harbouring the His-fusion protein of interest, was grown in LB containing ampicillin (100 mg/ml) until the OD$_{600}$ was ~ 0.6. Then, the bacteria were induced with 2 mM IPTG for 4 hours. At the end, the culture was chilled on ice and centrifuged at 5000xg for 10 min at 4 °C. After discarding the supernatant, the pellet was resuspended in 4 ml of His-lysis buffer containing the cocktail of protease inhibitors and stored at -80°C O/N or for a few days. The suspension was thawed by transferring it to a 4°C bath, protease inhibitors were added again and lysozyme was added to a final concentration of 1 mg/ml. The lysate was incubated with gentle agitation for 30 min at 4 °C and sonicated on ice 8 times for 15 sec. Then, 10 mM MgCl$_2$ and 10 μg/ml DNase I were added, and the lysate was incubated 15 min on ice and then centrifuged at 20,000xg for 20 min at 4 °C. The supernatant was recovered and added to 0.5 ml of Ni-NTA beads (Qiagen, CA, USA), previously pre-equilibrated in lysis buffer. The suspension was incubated with gentle agitation at 4°C for 1 hour and then washed 5 times with His-washing buffer and the protein was eluted by adding 500 μl of His-elution buffer and collecting in a clean tube. The elution and collection steps were repeated at least 3 times. The fractions containing high amounts (at least 0.2 mg/ml) of protein were pooled, dialysed twice against 1000 x volume of PBS and stored in aliquots at -80°C. Fifty μl of
each fraction were analysed by SDS-PAGE and the gels were stained with silver staining dye to control purity and integrity of the proteins (Figure 2.3).

2.11 GST-pull down assay for ARF6-GTP

Activation levels of ARF6 protein were assayed essentially as described by Niedergang (Niedergang et al., 2003) with minor modification. GnRH-CHO cells transiently transfected with the cDNA encoding FLAG-ARF6, FLAG-ARF6(Q67L) or FLAG-ARF6(T27N) were stimulated with 20 nM GnRH for 15 min upon 4 h starvation. Each 100 mm Petri-dishes were lysed with 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.4; 137 mM NaCl; 10 mM MgCl$_2$; 1% Triton X-100; 10% v/v glycerol) containing protease inhibitor. Cell lysates were clarified by centrifugation at 12,000xg for 15 min at 4 °C, snap-frozen in liquid N$_2$, and stored at -80 °C until the pull-down assay was performed. For each pull-down assay, 50 µg of the GST-GGA3 fusion protein (for purification see Section 2.10.2) were incubated with 75-80% of lysate in presence of 0.5% BSA for 15 min at 4 °C with rocking. The suspension was incubated with glutathione sepharose beads (30 µl of 1:1 v/v beads washed in lysis buffer) with gentle agitation for 60 min at 4 C. The beads were collected with centrifugation at 700xg for 5 min and washed three times with 1 ml of lysis buffer. Proteins that remained bound to the resin were eluted at 100 C for 5 min with sample buffer and subjected to SDS-PAGE and Western blotting.

2.12 Co-immunoprecipitation experiments

2.12.1. Materials and solutions

Protein-A Sepharose™ CL4B was from Amersham Pharmacia Biotech (NJ, USA). RIPA buffer: 100 mM Tris-HCl, pH 7.5, 1% Igepal, 0.5% Deoxycholate, 0.1% SDS, protease inhibitors.
Figure 2.3 Purification of His-ARNO. Eluates from Ni-NTA beads were analysed on a 10% SDS-PAGE gel and the proteins revealed by silver staining. Lane 1: 1/200 of the starting bacterial lysate after centrifugation; lanes 2-6: 1/200 of the washes; lanes 7-9: 50 μl of the eluates. The positions of molecular weight standards are indicated on the left and the position of His-ARNO on the right.
2.12.2 Co-immunoprecipitation of endogenous proteins from CHO cells

Semi-confluent CHO cells were incubated with RIPA buffer (1 ml/100 mm Petri dish) for 30 minutes at 4 °C with constant shaking. Cells were scraped off the plate and broken by passage (14 times) through a 25-gauge syringe. The lysates were collected and centrifuged at 12,000xg for 45 min at 4 °C. The supernatants were incubated with 2 μg of anti-Gβ antibody or anti-c-cbl antibody O/N at 4 °C with constant shaking. The day after, the samples were incubated with 40 μl of a 50% slurry protein-A sepharose resin for 1h at 4 °C with constant rotation. The resin was washed three times with RIPA buffer, boiled in loading buffer and resolved by Western blotting using antibodies raised against c-cbl and β subunit.

2.12.3 Co-immunoprecipitation assay of endogenous proteins from CHO cells plasma membranes

Twenty-five μg of CHO plasma membranes were extensively ADP-ribosylated for 6 hours in presence of α-NAD (1 mM) or β-NAD (1 mM). Modified and unmodified plasma membranes were solubilized with RIPA buffer containing protease inhibitors at 4°C on rotating weal for 30 minutes. The mixtures were briefly sonicated and centrifuged at 12,000xg for 45 min at 4°C and the supernatants were collected and incubated with 2 μg of anti-Gβ antibody overnight at 4°C with constant shaking. Then the samples were incubated with 40 μl of a 50% slurry of protein-A sepharose for 1hr at 4°C with constant rotation. The resin was washed three times with RIPA buffer, boiled in loading buffer and resolved by Western blotting using antibodies raised against c-cbl and β subunit.
2.13. Cell permeabilization with Tetanolysin

2.13.1. Materials and Solutions

Tetanolysin and Trypan blue were from Sigma-Aldrich. PIPES was from Calbiochem. Potassium glutamate was from Fluka.

Tetanolysin solution: Tetanolysin was dissolved in sterile water to a final concentration of 1 μg/μl. PBS: 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄, 2.7 mM KCl, 137 mM NaCl, pH 7.4. HGI buffer: 20 mM PIPES, 2 mM NaATP, 4.8 mM Mg(CH₃COO)₂, 150 mM potassium glutamate, 2 mM EGTA, 1 mM DTT (added just before the experiment) and KOH to obtain pH 7.0.

2.13.2 Procedures

The permeabilisation of CHO cells with tetanolysin follows the procedure described by Riese (Riese et al., 2002) with some modifications. Semi-confluent CHO cells (6-well plates) were washed with 2 ml/well of PBS and incubated in for 10 min on ice with 1 ml/well of ice-cold HGI buffer containing 0.5 μg/well of tetanolysin. The tetanolysin binds to membrane cholesterol in the cold without affecting membrane integrity and form pores of 20-40 nm diameter when the temperature is raised. The cells were gently washed with 500 μl ice-cold HGI buffer, before 1 ml of HGI buffer containing 4.5 μCi [³²P]-NAD and stimuli (1 U/ml thrombin, 5 nM pertussis toxin, 25 μM Mas7) were added. The plate was transferred to a 37 °C water bath and after 5, 10, 25, or 60 min incubation, CHO cells were washed twice with PBS buffer and harvested directly into 125 μl of sample buffer 1X, boiled and processed for SDS-PAGE. The level of cell permeabilization was quantified by the penetration of trypan blue (4 mg/ml). Permeabilised CHO cells were incubated for 5 min with 5ml HGI buffer containing trypan blue, and then washed three times with PBS and counted under an inverted light microscope. Non permeabilized cells incubated with the dye served as controls. Figure 2.4A shows that under these experimental condition about 90% of the cells are permeabilized. Moreover, under these permeabilization
Figure 2.4. Permeabilization with tetanolysin. A, Levels of CHO cell permeabilization quantified by dye penetration. Trypan blue (4 mg/ml) was added to permeabilised CHO cells. After 5 min, it was removed and the cells were washed twice with PBS and their permeabilisation quantified under light microscopy. B, Evaluation of ADP-ribosyltransferase activity as a marker for cytosolic protein content in permeabilised cells. $[^{32}P]$-ADP-ribosylated plasma membranes were incubated with cytosol from non-permeabilised and permeabilised CHO cells for 30 min at 37 °C. The levels of ADP-ribosylated β subunit were quantified using an Instant Imager. The data shown represent means (±SD) of five independent experiments, each performed in duplicate (n=10). * p < 0.01, significantly different from relevant control.
conditions, only the 25% of intracellular ADP-ribosylhydrolase activity (taken as a marker for cytosolic proteins) was lost in the supernatant (Figure 2.4B). Indeed, ADP-ribosylated plasma membranes were incubated with cytosol obtained from permeabilized and un-permeabilized CHO cells. The cytosol obtained from treated cells is able to induce a 30% of de-ADP-ribosylation respect the untreated cells. This result indicates that upon permeabilization with tetanolysin the cellular structure is not completely destroyed.

1.14. GTPγS binding assay

2.14.1 Materials

GTP-γS, triethanolamine, and 2(N-morpholino)ethansulphonic acid (MES) were from Sigma-Aldrich (WI, USA). [35S]GTPγS (specific activity 1250 Ci/mmol) and Filter-Count Solution were from PerkinElmer (NJ, USA). Cellulose nitrate membrane filters were from Whatman (UK). β-counter LS 6500 was from Beckam (USA). Filtration apparatus with vacuum pump was from Millipore (MA, USA).

2.14.2 Procedures

The assay was performed according to the procedure described by Wieland and Jakobs (Wieland et al., 1994). Plasma membrane of CHO cells (5 μg) were incubated for 30 min at 30°C in a buffer containing: 50 mM triethanolamine pH 7.3, 5 mM MES, 1 mM EDTA, 5 mM MgCl2, 143 mM NaCl, 0,16% BSA, 10 μM GTP, 4 nM [35S]GTPγS (2 x10^5 cpm/sample); in the presence of 200 nM GnRH in a final volume of 100 μl. The non specific binding was measured adding to the sample 10 μM GTPγS. The reaction was stopped by adding 2.5 ml of ice cold washing buffer (50 mM TRIS-HCl pH7.3, 1 mM EDTA and 5 mM MgCl2). Then the samples were passed through 0.45 μM cellulose nitrate filters and washed 3 times with 2.5 ml of wash buffer in vacuum apparatus. Finally the filters were collected, dissolved by filter count solution and counted for 4 min with β-scintillation counter. The results are plotted as % increase over the untreated samples.
2.15 HPLC analysis

The contents of NAD+ and NADH in ADP-ribosylated plasma membranes were measured using anion exchange HPLC analysis by Cristiano Iurisci (Consorzio Mario Negri Sud, Italy). The samples were analysed on a Partisil 10 SAX column (4.6 mm x 25 cm; Whatman), using water for the first 5 min, followed by a linear gradient of 0-1 M ammonium phosphate, pH 3.35, (5-45 min), and of 1-0 M ammonium phosphate (45-65 min). Radioactivity associated with the labelled compounds was analysed by an on-line flow detector (Packard FLO ONE A-525).

2.16 Thin-layer chromatography

Samples were chromatographed as described by Cassel (Cassel and Pfeuffer, 1978). After ADP-ribosylation reaction, samples (50 µl) were diluted to 500 µl with 50 mM PFB, pH 7.4. Three µl of each sample were added with 1 µl of nucleotide standards (20 mM NAD+, ADP-ribose, ADP and AMP in water) and applied to polyethyleneimine cellulose sheets (PEI-cellulose F, 20 x 20 cm, 0.2 mm thick) (Merck). Prior to use, cellulose sheets were scraped from the lateral margin (1 cm) and samples loaded 1 cm from the bottom. As a standard 2 x 10^4 cpm of [32P]-NAD+ were used. As elution buffer a solution of 150 mM NH₄HCO₃ was prepared and left in a sealed tank for at least 2 h prior to running. TLC plates were placed in the tank and allowed to develop for about 1 h before being removed, dried and analysed by Instant Imager.

2.17 Statistical analysis

Data are expressed as means ± SD. Statistical analysis was performed using GraphPad PRISM software to calculate paired Student’s t test (p < 0.05 was considered significant).
Chapter 3

GPCR agonists modulate mono-ADP-ribosylation of the $\beta\gamma$ subunit

3.1 Introduction

Endogenous mono-ADP-ribosylation of the $\beta$ subunit of heterotrimeric G proteins modifies the Arg129 situated in its common effector-binding surface, thereby the $\beta\gamma$ heterodimer loses its ability to modulate downstream effectors, such as type 1 AC, PI3K-\(\gamma\) and PLC\(\beta2\). Thus this reaction might constitute a novel cellular mechanism to modulate G-protein-mediated-signal transduction. To further the understanding of the physiological role of this reaction, it would be useful to elucidate the mechanisms by which it can be modulated.

We have demonstrated that mono-ADP-ribosylation of the $\beta$ subunit occurs only when it is activated. It is well accepted that the active form of the $\beta\gamma$ dimer originates from heterotrimeric G proteins when an activated GPCR causes GDP/GTP exchange on the $\alpha$ subunit (Hamm, 1998; Lambright et al., 1996). To find out whether mono-ADP-ribosylation of the $\beta\gamma$ dimer is regulated by GPCR stimulation, two experimental approaches were taken:

i) *In vitro* assay of mono-ADP-ribosylation of the $\beta$ subunit. Here, purified plasma membranes from CHO cells were stimulated with direct activators of G proteins (i.e. mastoparan) and agonists for GPCRs;

ii) *In vivo* analysis of hormonal modulation of mono-ADP-ribosylation of the $\beta$ subunit using intact and permeabilized CHO cells.
3.2 Hormonal modulation of mono-ADP-ribosylation \textit{in vitro}

3.2.1 Mastoparan induces an increase in mono-ADP-ribosylation of $\beta$ subunit

To address whether the activated $\beta\gamma$ dimer is a substrate for endogenous mono-ADP-ribosylation, the [$^{32}$P]-ADP-ribose incorporation was evaluated following treatment of plasma membranes with mastoparan. In this system, the membranes constituted the source of both the $\beta$ subunit and the enzymatic activity.

Mastoparan (Ile-Asn-Leu-Lya-Ala-Leu-Ala-Leu-Ala-Lys-Lys-Ile-Leu-amide; derived from wasp venom) is the prototype of a wide range of amphipatic, cationic peptides that can activate G proteins. As with GPCR activation, mastoparan accelerates guanine nucleotide exchange on the $\alpha$ subunit in the presence of $\text{Mg}^{2+}$; it does not alter the rate of hydrolysis of bound GTP and its action is markedly enhanced by $\beta\gamma$ dimers and blocked by PTX-catalysed ADP-ribosylation of the $\alpha$ subunit. Mastoparan is selective in its activation of different G proteins, accelerating nucleotide exchange on $G_0$ more than 30-fold, and it is almost as active on $G_i$, but less potent with $G_s$, $G_o$, $G_\omega$, $G_q$ and $p21^{ras}$. In parallel, synthetic analogues of mastoparan display diverse maximal activities and patterns of selectivity among the different G proteins. Mastoparan has been used widely as a specific probe for manipulating cellular signalling pathways in which G proteins are involved. Thus, I used mastoparan in a previously described \textit{in vitro} assay (Lupi et al., 2000) to determine if the activated $\beta\gamma$ dimer is a substrate of endogenous ADP-ribosylation. Briefly the samples (5 $\mu$g CHO plasma membrane protein) were incubated at 37 °C for 60 min in 50 mM potassium phosphate buffer (pH 7.4) with 30 $\mu$M $\beta$-NAD$^+$ and 1-2 $\mu$Ci [$^{32}$P]-NAD (specific radioactivity, 1000 Ci:mmol) in the absence and presence of mastoparan. The samples were then separated by SDS-PAGE and the [$^{32}$P]-ADP-ribosylation of the $\beta$ subunit was quantified using an Instant Imager.

As shown in Figure 3.1, mastoparan treatment resulted in a large stimulation (from two- to three-fold, depending on the plasma membrane preparation) of [$^{32}$P]-mono-ADP-ribosylation.
Figure 3.1. The G-protein β subunit ADP-ribosylation induced by the mastoparans.

The levels of ADP-ribosylated β subunit induced by 25 µM mastoparan and its analogues mastoparan 17 and Mas7 incubated for 60 min at 37 °C with CHO plasma membranes and [\textsuperscript{32}P]-NAD, as quantified using an Instant Imager. The data shown represent the means (±SD) of five independent experiments, each performed in duplicate (n=10). *p < 0.01, significantly different from the control.
ribosylation of \( \beta \) was induced. To exclude any non-specific effects of mastoparan due to its amphipatic properties, different mastoparan derivatives were used as positive and negative controls. Under the same experimental conditions, mastoparan 17, an inactive mastoparan analogue, was inactive. On the other hand, mastoparan 7 (Mas7), known to be the most active of the mastoparan derivatives, caused a more pronounced \( \beta \) subunit mono-ADP-ribosylation (190 ± 30 percent increase as compared to control).

These data support the concept that activation of the \( \beta\gamma \) heterodimer modulates its own endogenous mono-ADP-ribosylation.

3.2.2 Agonists for endogenous GPCRs modulate mono-ADP-ribosylation of the \( \beta \) subunit

Once the in vitro activation of \( \beta\gamma \) dimers had been shown to modulate endogenous mono-ADP-ribosylation, the possibility of hormonal modulation of the reaction was investigated by treating plasma membranes from CHO cells with agonists for GPCRs that are endogenously expressed on the surface of these cells. Thus, I used agonists for the \( \mathrm{P}_2\mathrm{Y}_2 \) purinergic receptor, the thrombin receptor (PAR1), the cholecystokinin (CCKA) receptor and the serotonin (5-HT 1B-like) receptor.

As shown in Figure 3.2, when CHO plasma membranes were stimulated with UTP, CCK8 and 5-HT, there was a 50% average increase in \( \beta \) subunit \([^{32}\text{P}]\)-ADP-ribosylation. Under the same conditions, thrombin stimulation induced an 80% increase, while treatment with angiotensin II and EGF, the receptors for which are not expressed on CHO cells, had no effects.

This demonstrates that mono-ADP-ribosylation of the \( \beta \) subunit can be modulated by stimulation of GPCRs with their specific agonists. Since the \( \mathrm{P}_2\mathrm{Y}_2 \) purinergic receptor, the thrombin receptor, and the CCKA cholecystokinin receptor are all coupled to \( G_q \), while the 5-HT receptor is coupled to \( G_i \), it can be assumed that \( \beta\gamma \) dimers from different classes of G proteins are substrates for the endogenous ADP-ribosylation reaction. Thus, there
Figure 3.2. The G protein β subunit ADP-ribosylation induced by receptor activation.

The level of ADP-ribosylated β subunit induced by agonists (1 μM CCK8, UTP, 5-HT and angiotensin II; 1 μg/ml EGF; 10 IU/ml thrombin) incubated for 60 min at 37 °C with CHO plasma membranes and [32P]-NAD, as quantified using an Instant Imager. The data shown represent the means (±SD) of five independent experiments, each performed in duplicate (n=10). * p < 0.01, significantly different from the control.
appears to be little or no specificity in the modulation of the reaction arising from specific classes of G protein.

By evaluating of the number of picomoles of β subunit that are modified in plasma membrane preparations, I have also characterised the reaction in a quantitative fashion (see Section 2.5.2 for details of the calculation). Under basal conditions, 0.012 ± 0.004 picomoles of the β subunit were mono-ADP-ribosylated, which corresponds to 1-2% of the total amount of β present in 5 μg of plasma membranes (1 picomol/5 μg plasma membrane). Under thrombin stimulation, this approximately doubled (0.024 ± 0.007 picomoles). In both cases, the amount of the β subunit modified was very low and this could represent a problem for further studies on the mechanisms that regulate this hormonal modulation. Therefore, the experimental conditions for the in vitro assay of ADP-ribosylation were modified with a view to maximising the enzyme activity. The first parameter was the concentration of NAD+. All ADP-ribosyltransferases, both bacterial and mammalian, also possess NADase activities, although these are significantly less than the maximal transferase activities. The balance between these two activities is influenced and regulated by the concentration of NAD+. Lieberman (Lieberman, 1957) first observed NAD-dependent inhibition of cellular NADase activity and the recovery of the enzyme activity following the removal of NAD+. Further investigations have demonstrated that NAD+ not only decreases NADase activity of ART5 (ADP-ribosyltransferase 5) but also enhances its transferase activity (Weng et al., 1999). Thus, to find out if the activity of the transferase that modifies the β subunit is similarly influenced by NAD+ concentration, different amounts of NAD+ (30 μM to 10 mM) were tested in an in vitro assay of mono-ADP-ribosylation. As shown in Figure 3.3A, there is a significant increment (about 6-fold) in the amount of modified β subunit evident at 700 μM NAD+. This indicates that the transferase activity can be enhanced. It is also relevant that this concentration is comparable with the physiological concentrations of NAD+ in CHO cells.
Figure 3.3. The G-protein β subunit ADP-ribosylation induced by increasing concentrations of β-NAD⁺. A, picomoles of ADP-ribosylated β subunit obtained with increasing concentrations of β-NAD⁺ incubated for 60 min at 37°C with CHO plasma membranes and [³²P]-NAD, calculated as reported in Section 2.5.2. The data shown represent the means (±SD) of three independent experiments, each performed in duplicate (n=6). * p < 0.05, significantly different from the sample treated with 30 μM β-NAD⁺. B, level of ADP-ribosylated β subunit induced by 10 IU/ml thrombin in CHO plasma membranes incubated for 60 min at 37°C with [³²P]-NAD and 30 μM or 700 μM β-NAD⁺, as quantified using Instant Imager. The data shown represent the means (±SD) of four independent experiments, each performed in duplicate (n=8). *p < 0.05, significantly different from the control.
(785 ± 10 μM, Lee et al., 1994; Lupi et al., 2000) and compatible with the apparent $K_m$ value (350 ± 20 μM) calculated for endogenous βγ dimer mono-ADP-ribosylation (Lupi et al., 2000). The transferase activity was then assayed with 700 μM NAD$^+$ in the presence of thrombin stimulation. Under these conditions, the extent of stimulation mediated by activation of the PAR1 receptor was comparable to that obtained with 30 μM NAD$^+$ (Figure 3.3B), indicating that higher amounts of NAD$^+$ have no effect on the ability of the activated receptors to modulate the reaction.

In parallel, the transferase activity was tested in the presence of 10 μM GTPγS and 5 mM MgCl$_2$, to facilitate the activation of G proteins, together with 700 μM NAD$^+$ (Aoyagi et al., 1992; Cerione et al., 1986), either without or with receptor stimulation. This promoted the modification of 0.135 ± 0.02 picomoles under basal condition, and 0.238 ± 0.02 under thrombin stimulation, as shown in Figure 3.4.

Based on these findings, the following conditions were adopted (unless otherwise specified): samples (5 μg CHO plasma membrane protein) were incubated at 37 °C for 60 min in 50 μl ADP-ribosylation buffer (50 mM PFB, pH 7.4, 5 mM MgCl$_2$, 4 mM DTT, 10 μM GTPγS, 700 μM β-NAD$^+$, 4.5 μCi $^{32}$P-NAD) in the presence of buffer or agonists for the endogenous GPCRs (for further details, see Section 2.5).

Subsequently, the range of GPCRs analysed was enlarged by using plasma membranes obtained from CHO cells that had been stably transfected with the α$_2$-adrenoreceptor and β$_2$-adrenoreceptor (α$_2$AR and β$_2$AR, respectively), the gonadotropin-releasing-hormone receptor (GnRHR) and the substance P receptor (NK1R).

Stimulation of α$_2$-AR and β$_2$-AR with their agonists clonidine and isoproterenol induced 50% and 28% mean increases in β subunit $^{32}$P-ADP-ribosylation, respectively (Figure 3.5). Interestingly, the stimulation of GnRHR with GnRH and of NK1R with substance P resulted in a decrease in the amount of modified β subunit (-31% and -42%,...
Figure 3.4. The G-protein β subunit ADP-ribosylation induced by a combination of β-NAD⁺, MgCl₂ and GTP-γS. Picomoles of ADP-ribosylated β subunit obtained with 700 μM β-NAD⁺, 5 mM MgCl₂ and 10 μM GTP-γS incubated for 60 min at 37°C with CHO plasma membranes, [³²P]-NAD, in the absence and presence of 10 IU/ml thrombin, as quantified using an Instant Imager. The data shown represent the means (±SD) of three independent experiments, each performed in duplicate (n=6). *p < 0.05, significantly different from the control.
Figure 3.5. The G-protein β subunit ADP-ribosylation induced by activation of over-expressed receptors. The level of ADP-ribosylated β subunit induced by the indicated agonists (10 μM isoproterenol, 15 μM clonidine, 30 μM substance P, 200 nM GnRH) in CHO plasma membranes over-expressing the corresponding receptors, incubated for 60 min at 37 °C with [32P]-NAD, as quantified using an Instant Imager. The data shown represent the means (±SD) of five independent experiments, each performed in duplicate (n=10). *p < 0.05, significantly different from the control.
respectively; Figure 3.5). This reveals that mono-ADP-ribosylation of the βγ subunit can be modulated both up and down by GPCRs.

To check whether this positive and negative modulation are both mediated by specific receptor stimulation, plasma membranes obtained from non-transfected CHO cells were stimulated with substance P, GnRH, clonidine and isoproterenol. GnRH, clonidine and isoproterenol failed to induce any modifications in labelling of the β subunit, thus indicating that these compounds modulate the endogenous mono-ADP-ribosylation reaction through receptor activation (Figure 3.6). However, treatment with substance P still led to a decrease in the modified β subunit, even though this receptor was absent. Substance P inhibited mono-ADP-ribosylation probably through a non-specific mechanism that is not related to NK1 receptor activation. The amino acid sequence of substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂) includes an arginine that could itself be ADP-ribosylated. Thus substance P might act as a competitive inhibitor of the reaction, resulting in a decrease in the amount of modified β subunit; however, to date, there is no experimental evidence in support of this idea. This aspect was not further investigated.

3.2.3 Pertussis toxin abolishes the stimulatory effects mediated by 5-HT and clonidine

To confirm that modulation of ADP-ribosylation due to agonist stimulation of GPCRs is mediated by G-proteins, an in vitro assay was performed using plasma membranes from CHO cells that had been treated with PTX. It is well accepted that upon prolonged incubation (at least 3 h) in a wide variety of cell types, the catalytic subunit of PTX becomes internalised and catalyses ADP-ribosylation on the α subunit of the G₁o proteins. This disrupts their interactions with their associated receptors and leads to the uncoupling of their corresponding signal transduction events.

Here, semi-confluent CHO cells were incubated for 15 h with PTX (5 nM) and with the inactive protomer B of PTX as control. Plasma membranes from these cells were
Figure 3.6. The G-protein β subunit ADP-ribosylation induced by agonists for overexpressed receptors in wild-type CHO cells. The level of ADP-ribosylated β subunit induced by the indicated agonists (10 μM isoproterenol, 15 μM clonidine, 30 μM substance P, 200 nM GnRH) in plasma membranes obtained from wild-type CHO cells, incubated for 60 min at 37 °C with [32P]-NAD, as quantified using an Instant Imager. The data shown represent the means (±SD) of three independent experiments, each performed in duplicate (n=6). *p < 0.05, significantly different from the control.
then used in the \textit{in vitro} ADP-ribosylation assay. Five μg of each membrane preparation were incubated with 100 nM catalytic subunit of PTX in the presence of $[^{32}\text{P}]$-NAD to check for α subunit ADP-ribosylation (Figure 3.7A). Under these conditions, the plasma membrane preparation purified from CHO cells pre-incubated with PTX did not show any labelling of the α subunit. This indicates that the α subunit was previously ADP ribosylated during the \textit{in vivo} treatment with PTX, and therefore it was no longer available for modification with $[^{32}\text{P}]$-NAD. This confirms that under these conditions the G$\text{_{i/o}}$ proteins are in their inactive heterotrimeric conformation.

Samples were then analysed in the \textit{in vitro} assay in the presence of Mas7 (active on the G$_i$ family), serotonin and clonidine (both acting through G$_i$-coupled receptors). As anticipated, stimulation of β subunit mono-ADP-ribosylation induced by serotonin and clonidine was completely blocked by PTX (Figure 3.7B). By contrast, stimulation of β subunit mono-ADP-ribosylation by Mas7 was only partially blocked (35 ± 5% decrease, as compared to control). This indicates that only part of the effect of Mas7 was due to the activation of PTX-sensitive heterotrimeric G proteins. Mas7 might activate β subunit mono-ADP-ribosylation both by activating the heterotrimeric G proteins and through an indirect interaction with the ADP-ribosyltransferase.

Altogether, these data indicate that the activation of the βγ heterodimer through GPCRs or by mastoparan allows mono-ADP-ribosylation of the β subunit. This might be expected to modulate β subunit function.

### 3.3 In vitro hormonal modulation: mechanism of action

The results reported in the preceding Section demonstrate that the endogenous mono-ADP-ribosylation reaction is differently modulated by individual GPCRs. This suggests that it cannot be due only to the activation of the βγ subunits. With respect to the PAR1 receptor, there is the additional possibility that the mechanism of thrombin-induced
Figure 3.7. The effects of PTX treatment on β subunit ADP-ribosylation induced by receptor activation and Mas7. A, Mono-ADP-ribosylation of the G-protein α subunit induced by PTX. The CHO cells were incubated overnight at 37 °C with 5x10⁻⁹ M PTX. Purified plasma membranes (5µg/sample) from treated and untreated cells, as a control, were then incubated with the catalytic subunit of PTX for an additional 60 min at 37 °C. Autoradiography of plasma membranes from CHO cells pre-treated with PTX (lane 2) or not pre-treated (lane 1). B, The level of the ADP-ribosylated β subunit induced by the agonists and by Mas7 incubated for 60 min at 37 °C with [³²P]-NAD and plasma membranes from untreated (green columns) and PTX-treated (blue columns) CHO cells, as quantified using an Instant Imager. The data shown represent the means (±SD) of four independent experiments, each performed in duplicate (n=8). *p < 0.05, significantly different from the control.
ADP-ribosylation could involve activation of cellular ADP-ribosyltransferases. Recent reports have indicated that in addition to heterotrimeric G proteins, GPCRs can interact with a variety of other targets, including arrestin and the small G proteins Rho and ARF (Marinissen and Gutkind, 2001).

A possible cooperation between receptor and transferase might also explain the negative modulation mediated by the GnRH receptor, which down-regulated ADP-ribosylation of the β subunit, even though GnRH induced activation of βγ dimers. To clarify this point, in vitro experiments of mono-ADP-ribosylation were carried out in the presence of increasing amounts of purified bovine brain βγ subunit, under resting conditions and under stimulation with thrombin and GnRH. In parallel, in order to determine the appropriate range of βγ concentrations, the values of K_m and V_max for βγ were estimated.

CHO plasma membranes were used as the source of the ADP-ribosyltransferase together with a range of purified brain βγ dimer (0-650 ng), to evaluate the ADP-ribosyltransferase activity. The results are shown in Figure 3.8, where the picomoles of modified βγ subunit are shown as a function of the total amount of βγ present in the reaction mixture. The data were fitted to Michaelis-Menten kinetics, with the V_o expressed as picomoles of modified β subunit of membrane protein, while the substrate concentration is expressed as ng of purified βγ dimer. The data were then transformed according to Eadie-Hofstee theory and the kinetic constants were determined using SIGMA software, obtaining an apparent K_m of 139.9 ± 28.6 nM. The V_max was 685 ± 60 pmol/h/mg plasma membranes. This rate might plausibly be regulated by cytoplasmatic activators (and/or cofactors), and hence might be different in the natural intracellular milieu. Nevertheless, this kinetic analysis was necessary to further study the in vitro hormonal modulation of the endogenous β subunit ADP-ribosylation mediated by the GPCRs.
Figure 3.8. The kinetic investigation of the G-protein β subunit ADP-ribosylation. A, Picomoles of the ADP-ribosylated β subunit calculated in the presence of increasing concentrations of free βγ dimer, incubated for 60 min at 37 °C with CHO plasma membranes and [32P]-NAD. The picomoles were calculated as reported in Section 2.5.2. The data shown represent the means (±SD) of three independent experiments, each performed in duplicate (n=6). B, Determination of V_{max} and K_{m} according to the Eadie-Hofstee theory using SIGMA software.
3.3.1 Thrombin-mediated stimulation of β subunit mono-ADP-ribosylation results from activation of βγ dimers.

To clarify the mechanism by which activated GPCRs modulate the ADP-ribosylation reaction, increasing concentrations of purified βγ dimer (0 to 250 ng) were ADP-ribosylated in vitro using purified plasma membranes following their stimulation with thrombin (or buffer as control).

The results are shown in Figure 3.9. As expected, thrombin induces a significant increase in ADP-ribosylation (mean, 80%) that is counteracted when increasing concentrations of free βγ are added to the experimental mixture. It appears that the activation of the PAR1 receptor becomes ineffective when a surplus of free βγ is provided. This suggests that the stimulatory effect of thrombin in plasma membranes is due to the activation of the subset of βγ associated with the PAR1 receptor, rather than to an activation of the ADP-ribosyltransferase mediated by the receptor or by other plasma-membrane-associated co-factors. However, this does not exclude the possibility that in intact cells, cytosolic factors could cooperate with receptors to regulate the transferases. To investigate this possibility, instead of plasma membrane preparations, CHO cell lysates (prepared according to the procedure described in Section 2.3.2.4) were used in the in vitro assay. The samples (80 μg total cell lysate/sample containing 5 μg of plasma membranes) were analysed in the presence of increasing amounts of purified βγ subunit under basal conditions and under thrombin stimulation. Unfortunately, the presence of total cell lysates as a source of enzyme activity prevented [32P]-labelling of the β subunit, probably due to the presence of cytosolic ADP-ribosylhydrolases that reverse the ADP-ribosylation reaction. An analysis of the cytoplasmatic components involved in receptor-mediated activation of the endogenous mono-ADP-ribosylation of the β subunit will be possible when tools for inhibiting the reverse hydrolase reaction become available.
Figure 3.9. Thrombin-induced stimulation of the G-protein β subunit ADP-ribosylation is mediated by activation of the βγ dimer. Level of ADP-ribosylated β subunit induced by 10 IU/ml thrombin (green columns) incubated for 60 min at 37 °C with CHO plasma membranes and increasing concentrations of free purified βγ dimer in presence of [³²P]-NAD, as quantified using an Instant Imager. The data shown represent the means (± SD) of three independent experiments, each performed in duplicate (n=6).

* p < 0.01 and **p < 0.05, significantly different from the control.
To date, there are no data that allow us to conclude definitively that the stimulatory effects mediated by the thrombin receptor are due only to the activation of $\beta\gamma$ dimers.

### 3.3.2 Negative modulation of $\beta$ subunit ADP-ribosylation by GnRH

The same experimental approach was used to find out if GnRH-induced inhibition of ADP-ribosylation of the $\beta$ subunit is mediated by inhibition of cellular transferase(s). Plasma membranes from CHO cells over-expressing the GnRH receptor were stimulated with GnRH in the presence of increasing amounts of the $\beta\gamma$ subunit.

When free $\beta\gamma$ subunits were added at amounts up to 100 ng, the inhibitory effect of GnRH was maintained, while higher concentrations antagonised this effect (Figure 3.10). This suggests that higher concentrations of $\beta\gamma$ subunit antagonise the inhibitory influence of the stimulated GnRH receptor. The negative effects of GnRH are due to inhibition of the cellular transferase, which is mediated by the activation of the GnRH receptor.

### 3.4 Agonists modulate ADP-ribosylation of the $\beta\gamma$ dimer in living cells

The demonstration that ADP-ribosylation in the cell-free system is regulated by agonists for GPCRs is of interest with regard to its potential physiological relevance. In this section the possibility of hormone modulation in intact cells was explored. Two different approaches were used:

i) Examination by Western blotting of the ~500 Da increase in the $\beta$ subunit molecular weight due to the addition of ADP-ribose upon stimulation of CHO cells with thrombin,

ii) Following the level of $[^{32}\text{P}]$-ADP-ribosylated $\beta$ subunit in permeabilized CHO cells upon stimulation with mas7 and thrombin.
Figure 3.10. GnRH-induced negative modulation of the G-protein β subunit ADP-ribosylation is mediated by the inhibition of the ADP-ribosyltransferase. The level of the ADP-ribosylated β subunit induced by the 200nM GnRH (pink columns) incubated for 60 min at 37 °C with CHO plasma membranes, and increasing concentrations of purified free βγ dimers and [³²P]-NAD, as quantified using an Instant Imager. The data shown represent the means (±SD) of three independent experiments, performed in duplicate (n=6). * p < 0.05, significantly different from the control.
3.4.1 Hormonal modulation in living cells: a shift in the apparent molecular weight of the β subunit

The approach used to detect in vivo ADP-ribosylation was the identification of a shift in the apparent molecular weight of proteins in cells treated with the ADP-ribosyltransferase. This method was used to demonstrate the in vivo modification of Ras by ExoS (McGuffie et al., 1998). Thus, an assay was developed to evaluate hormonal modulation of endogenous ADP-ribosylation of the β subunit in living cells by comparing the β subunit molecular weight under basal conditions and upon stimulation with thrombin.

Fifteen semi-confluent 150-mm Petri dishes of CHO cells were stimulated for 30 min with control buffer or thrombin (1 IU/ml). The plasma membranes were then purified according to the procedure reported in Section 2.3.2.1, and the expected ~500 Da shift in the β subunit due to ADP-ribose addition was evaluated by SDS-PAGE. A limit in this type of experiment is posed by the very low abundance of the modified β subunit. Thus, to analyse detectable amounts of ADP-ribosylated β subunit, 150 µg of purified plasma membrane/sample were analysed and SDS-PAGE was performed using long gels, to allow better separation of the different β subunit species. In this way, a more precise visualization of the molecular weight shift was obtained. Figure 3.11 shows that in plasma membranes from unstimulated cells (lanes 1 and 3), the antibody against the β subunit revealed two bands, of 35 and 36 kDa, that had previously been characterised as the unmodified β1 and β2 subunits. An additional band of 36.5 kDa was revealed in the plasma membranes purified from thrombin-stimulated cells (lane 2), which co-migrated with the in vitro ADP-ribosylated β subunit (lane 4). The high amount of protein applied to the lanes caused some diffusion and distortion of the samples. Nevertheless, the three bands corresponding to the unmodified and modified β subunits could be resolved, and as expected, the modified β subunit was present at ng levels (vs. µg levels of the unmodified
Figure 3.11. The G-protein β subunit ADP-ribosylation induced by receptor activation in living cells. Western blotting with an anti-Gβ antibody of the nitrocellulose filter containing 150 µg/sample plasma membranes prepared from CHO cells stimulated (lane 2) or not stimulated (lanes 1 and 3) with 1IU/ml thrombin for 30 min at 37 °C and separated on long gels. Lane 4 shows 150 µg of CHO plasma membranes that have been pre-ADP-ribosylated in vitro for 60 min. The data shown are representative of at least three independent experiments.
protein) and appeared less laterally diffused than the other two bands. Also, it should be noted that the protein of 36.5 kDa represents only ADP-ribosylated $\beta_1$; ADP-ribosylation of the $\beta_2$ could not be evaluated due to its co-migration with unmodified $\beta_1$.

This procedure allows the visualization of thrombin-stimulated ADP-ribosylation of $\beta$ subunits under conditions in which the endogenous ADP-ribosylhydrolase is active, counteracting the action of the ADP-ribosyltransferase. Unfortunately, due to the low abundance of the modified $\beta$ subunit under basal conditions it was not possible to use this shift protocol to detect the inhibitory effects mediated by the GnRH receptor.

All together, the results in this Section demonstrate the existence of hormonal modulation of endogenous $\beta$ subunit mono-ADP-ribosylation also in living cells, which may result in the regulation of $\beta$ subunit function.

### 3.4.2 Hormone modulation of permeabilised CHO cells

To further investigate hormonal modulation of mono-ADP-ribosylation of the $\beta$ subunit in living cells, an alternative experimental approach was used. Tetanolysin was used to introduce pores in the plasma membranes of CHO cells and thus allow entry of $[^{32}P]$-NAD$^+$. The method used followed that described by Riese et al. (Riese et al., 2002), with some modifications, as described in Section 2.13. This system has the advantage of preserving cell structure whilst allowing entry of $[^{32}P]$-NAD$^+$. Semi-confluent CHO cells were permeabilized with tetanolysin and then incubated in the presence of $[^{32}P]$-NAD while being treated with PTX, Mas7, thrombin and GnRH, as described in Section 2.13.2. The $[^{32}P]$-ADP-ribosylated $\beta$ subunit was then analysed by autoradiography.

In intact (non-permeabilised) cells, PTX failed to incorporate radiolabelled ribose into intracellular proteins (Figure 3.12A, lane 7). After tetanolysin treatment, PTX catalysed the incorporation of radiolabelled ADP-ribose into a protein with the predicted molecular mass of the Go subunit (Figure 3.12A, lane 6). This indicates that tetanolysin
Figure 3.12. The G-protein β subunit ADP-ribosylation induced by Mas7 in living cells. A, Mono-ADP-ribosylation of the G-protein β subunit revealed by autoradiography in CHO cells permeabilized with tetanolysin and incubated at 37°C in presence of [32P]-NAD⁺ for the indicated times. B, Western blotting of the same samples as in A, with an anti-Gβ antibody. The data shown are from single experiments performed in duplicate, which are representative of at least six independent experiments.
had produced pores in the CHO cell membranes that allowed the entry of $[^{32}\text{P}]-\text{NAD}^+$. This system could, therefore, be used to evaluate hormonal modulation of endogenous mono-ADP-ribosylation of the $\beta$ subunit.

Analysis of permeabilized wild-type CHO cells incubated with $[^{32}\text{P}]-\text{NAD}^+$ under basal conditions reveals a radiolabelled band (Figure 3.12, lane 2) that co-migrates with the \textit{in vitro} ADP-ribosylated $\beta$ subunit (Figure 3.12, lane 1). Upon stimulation with Mas7, the amount of modified $\beta$ subunit significantly increased in a time-dependent manner (lanes 3, 4, 5). In parallel experiments, Mas17, the inactive analogue of Mas7, failed to induce any labelling of the $\beta$ subunit (Figure 3.13).

The same procedures were used to assess hormone modulation of endogenous mono-ADP-ribosylation mediated by activation of the PAR1 and GnRH receptors. Thrombin (1 IU/ml) induced an increase in the labelling of the $\beta$ subunit, with respect to unstimulated cells (Figure 3.14). This further confirms that in living cells, mono-ADP-ribosylation of the $\beta\gamma$ dimer is modulated by GPCRs. By contrast, incubation of permeabilized GnRH-CHO cells with $[^{32}\text{P}]-\text{NAD}^+$ under basal conditions revealed only a very weak labelling of the $\beta$ subunit. This was insufficient for detection of any inhibitory effects that might have been due to stimulation of the GnRH receptor. So far, it has not been possible to follow the inhibitory effect of the GnRH receptor in living cells.

3.5 Discussion

The $\beta\gamma$ complex is essential for a wide range of cellular functions, including apoptosis, chemotaxis, secretion and cell proliferation and differentiation. All of these processes are mediated by interactions between $\beta\gamma$ and various target molecules, including the ACs, PI3K, PLC-$\beta$ and ion channels. We have reported that the modification of its Arg 129 inactivates the $\beta$ subunit, potentially by impairing the interactions of this subunit with its effector molecules. This thus suggests that this modification is crucial in the regulation
Figure 3.13. The G-protein β subunit ADP-ribosylation induced by Mas17 in living cells. Mono-ADP-ribosylation of the G-protein β subunit revealed by autoradiography in CHO cells permeabilized with tetanolysin and incubated at 37°C in presence of [32P]-NAD for the indicated times. The data shown are from single experiments performed in duplicate, which are representative of at least six independent experiments.
Figure 3.14. The G-protein β subunit ADP-ribosylation induced by thrombin in living cells. A, Mono-ADP-ribosylation of the G-protein β subunit revealed by autoradiography in CHO cells permeabilized with tetanolysin and incubated at 37°C in presence of $[^{32}\text{P}]$-NAD for the indicated times. Lane 1, in vitro ADP-ribosylated plasma membranes; lane 2, permeabilized CHO cells, 60 min; lanes 3,4 and 5, permeabilized CHO cells stimulated with 1 IU/ml thrombin for 5, 15, and 30 min. B, Western blotting of the same samples as in A, with an anti-Gβ antibody. The data shown are from a single experiment performed in duplicate, which is representative of at least four independent experiments.
of βγ activity in general. The purpose of this part of my study was to determine if endogenous mono-ADP-ribosyltransferase can be regulated by agonist stimulation of GPCRs, which would indicate that this is a physiologically relevant response. Some of the agonists tested (UTP, thrombin, CCK8, 5-HT, clonidine and isoproterenol) induced significant increases in β subunit [32P]-ADP-ribosylation, indicating that the βγ subunit is linked to different classes of G-proteins, including Gq and Gi, which are thus substrates for the endogenous mono-ADP-ribosyltransferase. The actual increases in β subunit [32P]-ADP-ribosylation might appear low (ranging from 30 to 80%), but this is not surprising since only a subset of G-proteins specifically coupled with the activated receptors are involved in the reaction. Indeed, non-specific activation of G proteins by mastoparan and Mas7 resulted in a remarkable stimulation of β subunit [32P]-ADP-ribosylation. Pretreatment of CHO cells with PTX decreased the effect of Mas7 by about 40%. The PTX-insensitive component may be due to a direct action of Mas7 on the ADP-ribosyltransferase (or other plasma membrane, PTX-insensitive factors that affect the ADP-ribosyltransferase activity).

These results are of particular interest since they demonstrate that the rate of βγ ADP-ribosylation can be increased upon activation of certain GPCRs. Therefore, while activation of these receptors leads to the activation of G protein α and βγ subunits, there is a parallel inactivation of βγ function that could potentially regulate the duration of βγ signalling, independently of GTPase activity of the α subunit.

This hypothesis cannot be considered as a general paradigm for all GPCRs. Indeed, in plasma membranes from CHO cells over-expressing the GnRH receptor, GnRH causes a negative modulation of the endogenous β subunit mono-ADP-ribosylation reaction. This demonstrates specificity in the modulation of the reaction, depending on the receptor type, rather than the existence of a general paradigm valid for all GPCRs. Moreover, the opposing effects mediated by hormonal modulation suggest that the mechanism involved
cannot only be ascribed to activation of $\beta\gamma$ dimers upon receptor stimulation. Other cellular components could have roles in the regulation of this process. This aspect was investigated by analysis of the mechanisms by which activated receptors modulate endogenous mono-ADP-ribosylation. Beyond the dogma of signalling mediated by "GPCR/G-protein-associated effectors", it has become evident that activation of GPCRs can also lead to responses that are not mediated by G proteins. Indeed, a wide variety of intracellular molecules are now recognised as interacting with activated GPCRs, and this broadens the range of mechanisms by which these receptors can transduce environmental signals (Marinissen and Gutkind, 2001).

In the context of the present study, this suggests that the increase in ADP-ribosylation due to thrombin can be ascribed not only to activation of the subset of $\beta\gamma$ dimers associated with the PAR1 receptor, but also to activation (direct or indirect) of the ADP-ribosyltransferase.

My data (Figure 3.9) indicate that at the plasma membrane level, the thrombin-mediated stimulatory effect is blocked by addition of increasing amounts of free substrate. This suggests that the increase in $[^{32}\text{P}]$-ADP-ribosylated $\beta$ subunit is due to activation of $\beta\gamma$ dimers as consequence of PAR1 stimulation, and it rules out the possibility that it is a consequence of activation of the ADP-ribosyltransferase. At the same time, these results do not exclude the possibility that in intact cells, other cellular component(s) could cooperate to regulate the transferases. For the moment, however, no data are available to substantiate this hypothesis.

In contrast, the GnRH receptor appears to affect the ADP-ribosyltransferase reaction directly, or at least without the intervention of G protein activation. Indeed, it leads to activation of $\beta\gamma$ dimers, but it has a negative effect on the endogenous mono-ADP-ribosylation reaction. This aspect will be better elucidated and discussed in Chapter 4.
The *in vitro* receptor modulation of β subunit ADP-ribosylation set the basis for an analysis of the potential modulation of this reaction in living cells. The electrophoretic mobility shift of ADP-ribosylated β subunit, equivalent to 500 Da, provide a means of detecting β modification *in vivo* under basal and hormone-stimulated conditions. The shift in molecular weight is only just detectable under basal conditions and also only in some plasma membrane preparations. This is due to the low proportion of the β subunit that is modified under basal conditions (about 0.2%, as detected by metabolic labelling of the NAD pool with [3H]-adenine). This is itself due to the balance between the transferase and the "reverse" ADP-ribosylhydrolase activities. Of course, the hormone-stimulated reaction is also affected by the ADP-ribosylhydrolase, and so it is not possible to assess its maximal extent without having a good inhibitor. Thus, my data must be considered as basically qualitative, but supporting the idea of a basal and hormone-stimulated ADP-ribosylating machinery acting on the β subunit. The best estimate of endogenously ADP-ribosylated β subunit, determined by comparing the intensities of the β subunit under *in vitro* and *in vivo* conditions (Figure 3.11), indicates that thrombin treatment of intact cells induces the modification of 1% to 5% of the β subunit. It is possible that this fraction corresponds to the βγ pool that is coupled to the thrombin receptor.

In conclusion, using the shift approach, I have demonstrated that endogenous mono-ADP-ribosylation of the β subunit is modulated by agonists for GPCRs also under physiological conditions in living cells. This supports the hypothesis that this reaction represents a novel mechanism for the control of signalling pathways mediated by the βγ dimer.

To establish a more routine method to monitor the extent of hormone modulation of the β subunit ADP-ribosylation reaction, I used a tetanolysin-based cell permeabilisation procedure to allow the entry of [32P]-NAD+. Tetanolysin is a 55-kDa member of the streptolysin O family of cholesterol-dependent pore-forming toxins. These
pores allow the diffusion of small proteins and molecules and have been used in studies on cAMP- and Ca\(^{2+}\)-induced effects in T-cell signal transduction (Conti et al., 1993). This method has some advantages with respect to the more commonly used streptolysin O, since it allows control of the intracellular ionic concentration, it is protease free, and it allows the size of the pores to be controlled by varying toxin doses and times of incubation.

To establish proof-of-principle for the detection of intracellular ADP-ribosylation, I applied PTX. This specifically ADP-ribosylates only G\(\alpha_i\) subunits, and resulted in selective labelling of \(\alpha_i\) with \([^{32}P]\)-ADP-ribose. Importantly, in permeabilised cells, there was a loss of about 25% of the endogenous hydrolase (see Section 2.13.2, Figure 2.3B). This should have the effect of partially shifting the equilibrium of the cycle of ADP-ribosylation/de-ribosylation towards the ADP-ribosylated form of the \(\beta\) subunit, making analysis of endogenous mono-ADP-ribosylation in living cells easier. Thus the tetanolysin-based approach is not only a useful tool to investigate the existence of hormone modulation of \(\beta\) subunit ADP-ribosylation in living cells, but also for an analysis of the role of this hormone modulation in vivo.

In conclusion, in this Chapter, I have demonstrated that in an \textit{in vitro} system, endogenous mono-ADP-ribosylation is under hormone modulation. More importantly, this regulation occurs also in living cells under physiological conditions. This observation supports the hypothesis of a physiological role for this reaction.
Chapter 4

**ADP-ribosylating factor 6 (ARF6) inhibits mono-ADP-ribosylation of the β subunit**

### 4.1 Introduction

ADP-ribosylation of the β subunit is either enhanced or inhibited by activation of GPCRs (Chapter 3) and this suggests that cellular component(s) other than receptors are involved in the regulation of the reaction. This Chapter focuses on the identification of this (these) unknown component(s), starting from what is known about the regulation of ADP-ribosylation catalysed by bacterial toxins. As reported in Section 1.3, the ADP-ribosyltransferase activity of CTX requires the presence of cellular proteins, namely ARFs. In this light, the relationship between ARFs and endogenous ADP-ribosylation of the β subunit was explored. Particular attention was paid to ARF6 because:

- The sub-cellular localization of ARF6 differs from the other ARFs, being mostly associated with the plasma membrane;
- ARF6 can interact with Gβγ subunits, as shown by chemical cross-linking and immunoprecipitation (Galas et al., 1997);
- ARF6-mediated signalling pathways can be activated in response to stimulation of several GPCRs (Claing et al., 2001; Mukherjee et al., 2001).

Different approaches were used to investigate the potential involvement of ARF6:

i) *In vitro* assay of ADP-ribosylation in the presence of recombinant ARF6;

ii) *In vitro* assay of ADP-ribosylation using plasma membranes depleted in endogenous ARF6;

iii) *In vitro* assay of ADP-ribosylation in the presence of peptides that specifically inhibit endogenous ARF6;
iv) *In vivo* assay with permeabilised CHO cells over-expressing ARF6.

### 4.2 Recombinant ARF6 inhibits ADP-ribosylation of the β subunit in vitro

To address whether ADP-ribosylation of β subunit can be modulated by ARF6, plasma membranes were pre-incubated with recombinant ARF6 for 15 min at 37 °C (to facilitate the interaction of the protein with the lipid bilayer). ADP-ribosylation was then carried out as described in Section 2.5. The presence of ARF6 in the reaction mixture caused a marked decrease in $^{32}$P-ADP-ribose-labelling of the β subunit, as compared to the control (Figure 4.1A, lanes 1 and 2). Under the same experimental conditions, the addition of ARF1, or other small G proteins, including Rho, Rac and Cdc42, showed no effects (Figure 4.1A). This result represents the first indication that ARF6 negatively regulates ADP-ribosylation of the β subunit. This aspect was further analysed using total membrane preparations (25 μg protein/sample) obtained from CHO cells that had been transiently transfected with cDNA for ARF6, or for the dominant-positive ARF6(Q67L) and the dominant-negative ARF6(T27N) mutants that mimic the GTP- and GDP-bound states, respectively. In parallel, CHO cells were transfected with cDNA for the ARF6-specific exchange factor EFA6 (which is exclusively localised at the plasma membrane (Franco et al., 1999), or co-transfected with ARF6 and EFA6.

The transfection of ARF6(Q67L) or EFA6 decreased $^{32}$P-labelling of β (Figure 4.1B). A greater inhibition was seen with co-transfection of ARF6 and EFA6. This indicates that the activated form of ARF6 negatively modulates endogenous mono-ADP-ribosylation of the β subunit. By contrast, the level of ADP-ribosylated β subunit was unaffected by transfection of dominant-negative ARF6(T27N), even though this mutant should inhibit activation of endogenous ARF6 by trapping the exchange factors. The absence of effects is probably because the over-expressed mutant is unstable and has a tendency to self-aggregate (Macia et al., 2004). As a consequence, only a small proportion
Figure 4.1. ARF6 inhibits the G protein β subunit ADP-ribosylation in vitro. A, The level of ADP-ribosylated β subunit obtained in the presence of 1 µg of the indicated purified proteins, incubated for 60 min at 37 °C with 5 µg CHO plasma membranes and [32P]NAD, as revealed by autoradiography. The data shown are from a single experiment that was performed in duplicate, which is representative of at least four independent experiments. B, The level of ADP-ribosylated β subunit in 25 µg/sample total membranes that over-express the indicated proteins, incubated for 60 min at 37 °C with [32P]-NAD, as quantified using an Instant Imager. 250 ng of purified bovine brain were added to the experimental mixture to obtain a better signal detection for β subunit. The data shown represent the means (±SD) of three independent experiments, each performed in duplicate (n=6). *p< 0.05, significantly different from the control.
of ARF6(T27N) is able to form a stable complex with exchange factors at the plasma membrane, and thus to block endogenous ARF6.

4.3 Depletion of ARF6 from plasma membranes and the effects on ADP-ribosylation

To substantiate a role of ARF6 as a negative modulator of ADP-ribosylation of the β subunit, conditions that release ARF6 from plasma membranes were sought. Plasma membranes from CHO cells were incubated under conditions designed to strip ARF6 from the membrane described by Cavenagh (30 min at 4 °C with 500 mM Tris-HCl, pH 7, or 100 mM sodium carbonate, pH 11, or 1 M NaCl) (Cavenagh et al., 1996). As an alternative, samples were treated to modulate the distribution of ARF6 between membranes and cytosol in CHO cell extracts (30 min at 4 °C with 2.5 mM MgCl₂) (Gaschet and Hsu, 1999), or to destabilise ARF6 binding to membranes (100 μM GDPβS for 15 min at 37 °C) (Gaschet and Hsu, 1999). Finally, plasma membranes were treated for 30 min at 4 °C with Triton X-100 or Nonidet P40 at concentrations (0.1%) that do not solubilise the lipid bilayer. After these treatments, the samples were pelleted, washed twice in PBS, and the proteins were resolved by SDS-PAGE. The presence of ARF6 was revealed by Western blotting using an anti-ARF6 antibody. Upon all these treatments, only Triton X-100 caused substantial (~90 %) depletion of endogenous ARF6 from plasma membranes (Figure 4.2A). Importantly, under the same conditions, the β subunit was retained (Figure 4.2B).

Plasma membranes depleted in ARF6 were then used in assays of ADP-ribosylation. Here, a significant increase in the level of ADP-ribosylated β subunit was seen (Figure 4.3). This further confirms that ARF6 interferes with ADP-ribosylation of the β subunit. However, the treatment of membranes with Triton X-100 might remove important component(s) of the endogenous ADP-ribosylation machinery, or in parallel, it
Figure 4.2. Depletion of ARF6 from plasma membranes. A, Immunoblotting of plasma membranes treated as indicated, and revealed with an anti-ARF6 antibody. Plasma membranes were incubated as indicated, and then pelleted at 13,000xg and washed twice in PBS. The samples were resuspended directly in 50 μl PBS and resolved by SDS-PAGE. B, Immunoblot of the same samples as in A, revealed with anti Gβ-antibody. The data shown are from a single experiment, which is representative of at least three independent experiments.
Figure 4.3. G protein β subunit ADP-ribosylation in plasma membranes depleted of ARF6. The level of ADP-ribosylated β subunit induced by 0.1% Triton X-100 treatment. Plasma membranes were treated with the detergent for 30 min at 4 °C, pelleted at 13,000xg for 15 min at 4 °C, resuspended in ADP-ribosylation buffer, and incubated for an additional 60 min at 37 °C with [³²P]-NAD in the presence of control buffer or purified ARF6. The levels of modified β subunit were quantified using an Instant Imager. The data shown represent the means (±SD) of four independent experiments, each performed in duplicate (n=8). *p< 0.05, significantly different from untreated control (orange column), and from samples that were treated with 0.1% Triton X-100 and incubated with 10 and 20 ng ARF6.
might affect the three-dimensional structure of the phospholipid bilayer, thus influencing the activity of the ADP-ribosyltransferase. To test these possibilities, ARF6-depleted plasma membranes were ADP-ribosylated in the presence of recombinant ARF6 protein. This restored the inhibitory effect that was removed by Triton X-100 treatment (Figure 4.3) so demonstrating that the increase in the amount of modified β subunit is a consequence of the removal of ARF6 from the plasma membranes, and not a non-specific effect of the detergent treatment.

4.4 Myristoylated peptides block ARF6-mediated inhibition

The involvement of ARF6 in ADP-ribosylation of the β subunit was further supported by using a myristoylated peptide that corresponds to the amino terminus (N-terminus) of ARF6 (MyrARF6). Taking advantage of its ability to prevent ARF6-mediated effects, MyrARF6 has been previously used to probe the interaction of ARF6 in the activation of PLD and PIP5K (Honda et al., 1999; Le Stunff et al., 2000) and Ca\textsuperscript{2+}-dependent exocytosis (Caumont et al., 1998; Galas et al., 1997).

MyrARF6 was pre-incubated with plasma membranes for 15 min at 37 °C to facilitate its interaction with the lipid bilayer and the samples were then used in an \textit{in vitro} assay of ADP-ribosylation. Treatment with MyrARF6 induced a dose-dependent increase in labelling of the β subunit (Figure 4.4), confirming that ARF6 acts as a negative regulator of the ADP-ribosylation reaction. By contrast, under the same conditions the corresponding non myristoylated peptide (non-MyrARF6; Galas et al., 1997) was ineffective. This rules out the possibility that the stimulatory effects of MyrARF6 seen here are due to membrane perturbations caused by the amphipathic properties of the amino-acid sequence. However, some studies have shown that defects in membrane architecture can be induced by interactions between the acyl chain of MyrARF6 and PIP\textsubscript{2} (Ge et al., 2001). Thus, to exclude such non-specific effects, myristoylated coenzyme A
Figure 4.4. G protein β subunit ADP-ribosylation in plasma membranes incubated with the myristoylated peptide of ARF6. The level of the ADP-ribosylated β subunit induced by increasing concentrations (5, 25, 50 μM) of MyrARF6 (green columns) and non-MyrARF6 (orange columns). Plasma membranes were incubated with the peptides for 15 min at 37 °C, and then for an additional 60 min at 37 °C with [32P]-NAD. The level of the modified β subunit was measured using an Instant Imager. The data shown represent the means (±SD) of four independent experiments, each performed in duplicate (n=8). *p<0.05, significantly different from the control (C).
Figure 4.5. G-protein β subunit ADP-ribosylation in plasma membranes incubated with MyrCoA and MyrARFl. A, The level of the ADP-ribosylated β subunit induced by 100 μM MyrCoA, as quantified using an Instant Imager. Plasma membranes were incubated with MyrCoA for 15 min at 37 °C, and then for an additional 60 min at 37 °C with [32P]-NAD. B, The level of ADP-ribosylated β subunit induced by increasing concentrations (5, 25, 50 μM) of MyrARFl, as quantified using an Instant Imager. Plasma membranes were incubated with MyrARFl for 15 min at 37 °C, and then for an additional 60 min at 37 °C with [32P]NAD. The data shown represent the means (±SD) of five independent experiments, each performed in duplicate (n=10).
(MyrCoA) and a myristoylated peptide of ARF1 that specifically blocks the functionality of ARF1 (MyrARF1) were tested and MyrCoA was found to be without effect (Figure 4.5A). By contrast, and unexpectedly, MyrARF1 (from 5 to 50 μM) induced an increase in β subunit ADP-ribosylation that was comparable to that obtained with MyrARF6 (Figure 4.5B). This result conflicts with my previous observation that ARF1 is not involved in the regulation of ADP-ribosylation of β subunit (Section 4.2.1) and suggests that MyrARF6 can affect ADP-ribosylation of the β subunit through a non-specific mechanism. To exclude this possibility, plasma membranes depleted of ARF6 were incubated with MyrARF6 and MyrARF1. The stimulation induced by the incubation with MyrARF6 was completely blocked by Triton X-100 treatment, while the effect of MyrARF1 was only partially inhibited (Figure 4.6). This indicates that the effect of MyrARF1 is non-specific, while MyrARF6 causes an actual block of endogenous ARF6.

**4.5 ARF6 down regulates ADP-ribosylation of β subunit in living cells**

To investigate whether ARF6 can also inhibit ADP-ribosylation in living cells, experiments were carried out with tetanolysin-permeabilised cells. CHO cells were transiently transfected with cDNAs encoding ARF6, ARF6(Q67L) or ARF6(T27N), or co-transfected with ARF6 plus its exchange factor EFA6. After 24 h of transfection, the cells were permeabilised with tetanolysin, incubated with [³²P]-NAD⁺, and analysed for the labelling of β subunit, according to the procedures described in Section 2.13. In cells transfected with ARF6 and ARF6(Q67L) or co-transfected with ARF6 and EFA6, a strong inhibition of ADP-ribosylation of the β subunit (ranging between 50-90%) was seen (Figure 4.7A). By contrast, ARF6(T27N) was without effect. In control experiments, where CHO cells were transiently transfected with cDNAs for GFP-ARF1 or the constitutively-active and inactive forms of ARF1 (GFP-ARF1(Q71L) and GFP-
Figure 4.6. G-protein β subunit ADP-ribosylation in plasma membranes treated with Triton X-100 and incubated with MyrARF1 and MyrARF6. Plasma membranes, untreated (pink columns), or treated with 0.1% Triton X-100 (green columns), were incubated for 15 min at 37 °C with 50 μM MyrARF1 or MyrARF6, and then for an additional 60 min at 37 °C with [32P]-NAD. The levels of ADP-ribosylated β subunit were quantified using an Instant Imager. The data shown represent the means (±SD) of four independent experiments, each performed in duplicate (n=8).
Figure 4.7. ARF6 inhibits G-protein β subunit ADP-ribosylation in living cells. A and B, The level of the ADP-ribosylated β subunit in permeabilised cells, as quantified using an Instant Imager. CHO cells were transfected with the indicated constructs for 24 h, permeabilised with tetanolysin, and incubated at 37 °C for an additional 60 min with [³²P] NAD and 100 µM GTPγS. The data shown represent the means (±SD) of four independent experiments, performed in duplicate (n=8). *p< 0.05, significantly different from the control.
ARF1(T31N), respectively), no differences in labelling of the β subunit were seen (Figure 4.7B). This confirms that ARF6 can also cause negative modulation of the reaction in living cells, and moreover, that this regulation is specifically mediated by the activated form of ARF6.

### 4.6 ARF6 is involved in GnRH-R-mediated inhibition of ADP-ribosylation of the β subunit

The finding that the activated form of ARF6 can inhibit ADP-riboylation of the β subunit and previous account of the activation of ARF6 by certain GPCRs (Claing et al., 2001; Mukherjee et al., 2001) led me to evaluate whether ARF6 is involved in the inhibition of ADP-ribosylation mediated by the GnRH receptor. Plasma membranes from GnRH-CHO cells were depleted of endogenous ARF6 and then ADP-ribosylated in the presence of 200 nM GnRH. Under these conditions, the inhibitory effects mediated by receptor stimulation were completely blocked (Figure 4.8). Importantly, re-addition of purified ARF6 protein restored the inhibition mediated by GnRH. This confirms that ARF6 is directly involved in the negative modulation of mono-ADP-ribosylation of β subunit mediated by the GnRH receptor. This was further confirmed using the MyrARF6 peptide (Figure 4.9). Under resting conditions, incubation with MyrARF6 induced an increase in the amount of modified β subunit that is comparable to that reached in plasma membranes of wild-type CHO cells. This indicates that stable transfection of the receptor did not influence the ability of MyrARF6 to block endogenous ARF6. More importantly, the incubation of plasma membranes with MyrARF6 completely blocked the inhibitory effect of GnRH (Figure 4.9). This confirms that negative modulation driven by the GnRH receptor involves ARF6.
Figure 4.8. Triton X-100-treatment blocks GnRH-mediated inhibition of G protein $\beta$ subunit ADP-ribosylation. The level of ADP-ribosylated $\beta$ subunit induced by GnRH in plasma membranes previously treated with Triton X-100. The samples were treated with 0.1% Triton X-100 for 30 min at 4 °C, pelleted at 13,000xg for 15 min at 4 °C, resuspended in ADP-ribosylation buffer, and incubated for an additional 60 min at 37 °C with [32P]-NAD, in the presence of 200 nM GnRH, and purified ARF6 or control buffer, as indicated. The levels of modified $\beta$ subunit were quantified using an Instant Imager. The data shown represent the means (±SD) of three independent experiments, each performed in duplicate (n=6). *p< 0.05, significantly different from the sample treated with 0.1% Triton X-100 and from sample treated with 0.1% Triton X-100, 200 nM GnRH and 20 ng ARF6.
Figure 4.9. Myr-ARF6 blocks GnRH-mediated inhibition of the G protein β subunit ADP-ribosylation. The level of the ADP-ribosylated β subunit induced by 200 nM GnRH incubated for 60 min at 37 °C with $[^{32}\text{P}]$-NAD, and with CHO plasma membranes previously treated with 50 μM MyrARF6 for 15 min at 37 °C. The levels of modified β subunit were quantified using an Instant Imager. The data shown represent the means (±SD) of four independent experiments, each performed in duplicate (n=8). *p< 0.05, significantly different from the respective untreated controls.
4.7 ARF6 is activated by the GnRH receptor

The process by which ARF6 and the GnRH receptor regulate ADP-ribosylation of the β subunit is not clear. It is reasonable to believe that the pathway initiated upon receptor stimulation passes through the activation of ARF6, which in its GTP-bound form leads to a decrease in β subunit labelling. I investigated whether the GnRH receptor activates ARF6. This was carried out using a recently described pull-down assay for ARF6-GTP (Niedergang et al., 2003) that takes advantage of the GTP-dependent interaction of ARF with GGAs (Golgi-localized, γ adaptin-ear–containing ARF-binding proteins, a family of ARF effector proteins) (Boman et al., 2000; Dell'Angelica et al., 2000; Hirst et al., 2000). To precipitate GTP-bound ARF6, a recombinant GST-fusion protein containing the ARF binding domains of GGA3 (GST-GGA3) was purified from bacteria (Section 2.10.2). The specificity of this assay was tested using cell extracts obtained from GnRH-CHO cells that were previously transfected with the ARF6(Q67L) and ARF6(T27N) mutants. Twenty-four h after transfection, cell lysates were prepared and incubated with GST-GGA3 and the protein complexes were then pulled-down with a glutathione resin (Section 2.11) and analysed using an ARF6-specific antibody. ARF6(Q67L) was recognised and bound by GST-GGA3, whereas the GDP-bound mutant, ARF6(T27N), was not (Figure 4.10A).

The same procedure was then used to evaluate the activation of ARF6 upon stimulation of GnRH-CHO cells with GnRH. The cells were transfected with cDNA encoding for ARF6 and 24 h after transfection the cells were stimulated with GnRH or control buffer. Stimulation with GnRH caused a significant increase in the amounts of ARF6-GTP compared with the control (Figure 4.10B). This result reveals a previously unknown pathway of activation of ARF6 that is mediated by the GnRH receptor that will contribute to a better definition of the regulatory mechanisms by which ARF6 controls mono-ADP-ribosylation of the β subunit.
Figure 4.10. ARF6 is activated by GnRH receptor stimulation. A, GST-GGA3 recognises the activated form of ARF6. CHO cells were transfected with ARF6(Q67L) (lanes 1, 3 and 4) and ARF6(T27N) (lanes 2, 5 and 6). Lysates (lanes 1 and 2) were prepared and incubated with GST-GGA3 (lanes 4 and 6) or GST alone (lanes 3 and 5). The bottom panel shows the immunoblot performed with an anti-ARF6 antibody, the upper panel shows the total protein pattern, as revealed by Ponceau Red staining. B, CHO cells were transfected with ARF6 and incubated with 20 nM GnRH or control buffer for 15 min at 37 °C. Lysates from unstimulated (lane 1) and stimulated (lane 2) cells were prepared and incubated with GST-GGA3. The immunoblot analysis was performed with an anti-ARF6 antibody. The bottom panel shows aliquots of total lysates. The data are from a single experiment carried out in duplicate, and are representative of three independent experiments.
The activation of ARF6 mediated by GPCRs like LH/CG and FSH receptors involves exchange factors for ARF6, such as ARNO (Mukherjee et al., 2000). To determine whether activation of ARF6 resulting from GnRH receptor stimulation also involves an exchange factor, GTP-binding assays were performed. Recombinant ARF6 and ARNO were incubated with GnRH-CHO plasma membranes and stimulated with GnRH. The rate of GTPγS binding was evaluated as described in Section 1.14, and the results are shown in Figure 4.11. Simultaneous incubation of plasma membranes with ARF6 and ARNO caused an increase in binding of GTPγS with respect to ARF6 alone, indicating that the GnRH receptor probably activates ARF6 through activation of an exchange factor. This is in line with my previous observations with membranes from transfected cells where the strongest inhibitory effect on ADP-ribosylation was achieved by co-transfection with ARF6 and EFA6 (Section 4.2.1).

4.8 Discussion

The experiments described show that endogenous mono-ADP-ribosylation of the β subunit can directly regulate signal transduction mediated by βγ dimer. To validate this hypothesis, particular attention was given to the mechanisms by which the reaction is modulated. As shown in Chapter 3, the ADP-ribosylation reaction can be differently modulated by activation of GPCRs, revealing that the regulatory mechanisms controlling the ADP-ribosyltransferase activity might involve unknown cellular component(s), other than G proteins and GPCRs. Putative candidates here are the members of the ARF family, due to their ability to modulate the ADP-ribosyltransferase activities of certain bacterial toxins (Kahn and Gilman, 1986; Lee et al., 1991). Its localisation at the plasma membrane and its ability to interact with βγ dimers make ARF6 the most plausible modulator from among the various ARFs. On this basis, the role of ARF6 on endogenous ADP-ribosylation of the β subunit was analysed using approaches aimed at enhancing or
Figure 4.11. The activation of ARF6 induced by GnRH receptor is mediated by ARNO. A, Level of $[^{35}S]GTP-\gamma S$ binding in plasma membrane of GnRH-cells incubated with the indicated purified proteins and stimulated with 200 nM GnRH, as measured using an $\beta$-counter. The data shown represent the means (±SD) of five independent experiments, performed in duplicate (n=10). *p< 0.05, significantly different from the sample incubated with ARF6.
inhibiting ARF6 activity. The activated form of ARF6 was found to modulate ADP-ribosylation of the β subunit negatively. In particular, an almost total inhibition of the reaction was obtained in vivo with co-overexpression of ARF6 and its exchange factor EFA6, while there was a weaker effect with the constitutively activated ARF6(Q67L) mutant. This is in line with several observations that demonstrate that many functions mediated by ARF6 require its continued cycling between its GTP- and GDP-bound forms, instead of the permanently activated state (Claing et al., 2001; Hashimoto et al., 2004; Zhang et al., 1998) and Section 1.3.3).

In the second part of this Chapter, I demonstrated that activated-ARF6 has a pivotal role in the negative modulation of ADP-ribosylation of the β subunit induced by GnRH. In an attempt to elucidate this point further, I found that ARF6 becomes activated as a consequence of GnRH receptor stimulation and that this pathway probably involves an exchange factor for ARF6. This is in agreement with the observation that guanine nucleotide exchange activity of ARNO is obligatory for the activation of ARF6 mediated by other receptors such as the LH/CG receptor or the FSH receptor (Salvador et al., 2001).

In conclusion, the results reported in this Chapter provide direct evidence that ARF6 is a crucial modulator of endogenous mono-ADP-ribosylation of the β subunit, and they contribute to a better definition of how this regulation is achieved. As shown in Figure 4.12, stimulation of the GnRH receptor leads to activation of ARF6 through involvement of an exchange factor. Upon activation, ARF6 can negatively modulate mono-ADP-ribosylation. As the data reported provide only a qualitative picture of the inhibitory effects mediated by ARF6, without explaining the mechanisms by which ARF6 controls ADP-ribosylation, this aspect will be further explored and discussed in Chapter 5.
Figure 4.12. Model of ARF6-mediated inhibition of endogenous mono-ADP-ribosylation of the β subunit. Stimulation of the GnRH receptor with its specific agonist leads to activation of ARF6 through the involvement of an exchange factor (ARF-GEF). Upon activation, ARF6 can negatively modulate mono-ADP-ribosylation.
Regulation of ARF6-mediated inhibition of mono-ADP-ribosylation of the β subunit

5.1 Introduction

The experiments described in Chapters 3 and 4 have demonstrated that ADP-ribosylation of the β subunit in living cells is regulated by a multifaceted and integrated pathway involving GPCRs and ARF6. The mechanism by which ARF6 exerts its inhibitory effect on the reaction will now be addressed. Different potential mechanisms need to be taken in consideration (see also Figure 5.1) since ARF6 might:

(i) reduce the β-NAD\(^+\) available for the reaction;
(ii) sequester the βγ dimer, making it inaccessible to the ADP-ribosyltransferase;
(iii) interact (directly or indirectly) with the ADP-ribosyltransferase, acting as an inhibitor of its activity.

In the next Sections these possibilities will be explored.

5.2 ARF6 does not alter availability of β-NAD\(^+\) or sequester the βγ dimers

ARF6 might induce a decrease in the amounts of β-NAD\(^+\) available to the ADP-ribosyltransferase in the following ways:

(i) enhancement of β-NAD\(^+\) degradation;
Figure 5.1. Possible mechanisms by which ARF6 modulates ADP-ribosylation of the β subunit. See text for more details.
(ii) consumption of $\beta$-NAD$^+$ through an auto-ADP-ribosylation process;

(iii) alteration in the redox equilibrium between NAD$^+$ and NADH.

To determine whether ARF6 alters the degradation pathway of $\beta$-NAD$^+$, plasma membranes were [$^{32}$P]-ADP-ribosylated in the presence of recombinant ARF6 or control buffer. After incubation, 3 μl of each sample were analysed by TLC for $\beta$-NAD$^+$ metabolites (Section 2.16), with the remaining sample analysed by SDS-PAGE and electroblotting (Section 2.4). Under these conditions, ARF6 induced a significant inhibition of labelling of the $\beta$ subunit (Figure 5.2A), while it did not affect the pattern of $\beta$-NAD$^+$ metabolites (Figure 5.2B). This demonstrates that the ARF6-mediated inhibitory effect on mono-ADP-ribosylation is not due to alterations of $\beta$-NAD$^+$ metabolism. This is supported by the direct incubation of recombinant ARF6 with $\beta$-NAD$^+$, where ARF6 caused no depletion of NAD$^+$ (Figure 5.3A). In addition, under the same conditions, any significant [$^{32}$P]-labelling of ARF6 would be revealed by the autoradiography, indicating that ARF6 does not consume $\beta$-NAD$^+$ via an auto-ADP-ribosylation reaction (Figure 5.3B).

ARF6 might also inhibit ADP-ribosylation of the $\beta$ subunit by altering the redox ratio, enhancing NADH at the expense of NAD$^+$. To test this, plasma membranes were incubated with recombinant ARF6 (or control buffer) and the nucleotide content profiles analysed by HPLC (Section 2.15). The presence of ARF6 had no effect on the concentration of NADH (Figure 5.4) so ruling out the possibility that a shift from $\beta$-NAD$^+$ to NADH could be responsible for the ARF6-mediated inhibition of the ADP-ribosylation reaction.

Finally, ARF6 might form a complex with the $\beta\gamma$ dimer, thus reducing its accessibility to the ADP-ribosyltransferase. A physical interaction between ARFs and $\beta\gamma$
Figure 5.2. ARF6 does not alter the metabolism of $\beta$-NAD$^+$ in membranes from CHO cells. A, The level of ADP-ribosylated $\beta$ subunit in the absence and presence of 2.5 $\mu$g ARF6, incubated for 60 min at 37 °C with 5 $\mu$g/sample CHO plasma membranes and [$^{32}$P]-NAD$^+$, as revealed by autoradiography. B, The degradation pattern of $\beta$-NAD$^+$ incubated in the presence of 5 $\mu$g/sample plasma membranes without or with 2.5 $\mu$g ARF6, for 60 min at 37 °C, as revealed by autoradiography of a TLC plate. Data shown are from a single experiment that was performed in duplicate, and which is representative of at least three independent experiments.
Figure 5.3. ARF6 does not degrade β-NAD⁺ and it does not catalyse auto-ADP-ribosylation. **A**, The degradation pattern of β-NAD⁺ incubated without and with 2.5 μg ARF6, for 60 min at 37 °C in ADP-ribosylation buffer, as revealed by autoradiography of a TLC plate. **B**, The level of ADP-ribosylated ARF6 obtained incubating 2.5 μg ARF6 in the presence of [³²P]-NAD⁺ and ADP-ribosylation buffer without or with 5 μg/sample plasma membranes, as revealed by autoradiography. The data shown are from a single experiment that was performed in duplicate, and which is representative of at least three independent experiments.
Figure 5.4. ARF6 does not change the equilibrium between NAD$^+$ and NADH. The contents of NAD$^+$ and NADH in 5 μg/sample plasma membranes incubated in control buffer or with 1μg purified ARF6, as determined by HPLC analysis. The nucleotide elution profile was checked by co-elution with commercially available standards. This experiment was done in collaboration with Cristiano Iurisci (Laboratory of Cell Regulation, Department of Cell Biology and Oncology, Consorzio Mario Negri Sud). The data shown are from a single experiment that was performed in duplicate, and which is representative of at least three independent experiments.
dimers has previously been demonstrated both *in vivo* and *in vitro*, and shown to be mediated by the GDP-bound form of ARF6 (Colombo et al., 1995; Franco et al., 1995; Galas et al., 1997) Section 1.1.3.6. These observations are not in line with my own in which the inhibition of ADP-ribosyltransferase activity is mediated by the activated form of ARF6 (Section 4.2.1). Therefore, it is unlikely that the mechanism by which ARF6 negatively modulates ADP-ribosylation of the β subunit arises from depletion of βγ dimer available for the enzyme.

### 5.3 ARF6 acts as a classical inhibitor for ADP-ribosylation of the β subunit

It remains to be considered whether ARF6 can modulate the activity of the ADP-ribosyltransferase by acting as a classical inhibitor. Here ARF6 might:

(i) bind the ADP-ribosyltransferase in its active site, competing with the binding of βγ (competitive inhibition);

(ii) bind the ADP-ribosyltransferase away from the active site (non-competitive inhibition);

(iii) bind the βγ-ADP-ribosyltransferase complex to produce an inactive conformation (uncompetitive inhibition);

(iv) activate another cellular component that, in turn, inactivates the ADP-ribosyltransferase.

To discriminate between these hypotheses, a kinetic analysis was carried out. As a first step, the inhibitor binding constant ($K_i$) that quantifies the strength of the complex formed between the ADP-ribosyltransferase and ARF6 was evaluated. *In vitro* assays of ADP-ribosylation were carried out in the presence of increasing amounts of recombinant ARF6 (0 to 1,000 ng), and plasma membranes as the source of the βγ dimer and the
enzymatic activity. The result is shown in Figure 5.5, where the percentage inhibition of β subunit labelling is given as a function of the increasing concentrations of ARF6. The $K_i$ for ARF6 (84 ± 20 nM) was obtained from an analysis using Graph Pad PRISMA software. Under these conditions, the maximum inhibitory effect only reaches a 35-40% decrease in labelling of the β subunit. This is lower than the inhibition that occurred in CHO cells co-transfected with cDNA for ARF6 and EFA6 or transfected with ARF6(Q67L) (Sections 4.2.1 and 4.2.4). Since the ARF6 used in the present experiments was purified in its GDP-bound form (Section 2.10.3), it is possible that it lost some of its activity on the ADP-ribosyltransferase. Probably the endogenous exchange factor present in the plasma membrane samples was insufficient to activate all the exogenous ARF6. To obtain a real estimate of $K_i$, it will be useful to carry out similar experiments using both recombinant ARF6 and recombinant exchange factor. This point is currently under investigation.

5.3.1 ARF6 acts as a competitive inhibitor

Measurements of the rates of catalysis at different concentrations of substrate and inhibitor serve to distinguish between competitive and non-competitive mechanisms. Thus, different concentrations of ARF6 were tested in an in vitro assay of ADP-ribosylation in the presence of increasing amounts of the $βγ$ dimer. Inhibition mediated by ARF6 was counteracted by addition of $βγ$ subunit (Figure 5.6), revealing a competitive mechanism, where the inhibitor can block the enzyme or can act as an alternative substrate. In my system, this latter possibility is supported by evidence that in ADP-ribosylated plasma membranes there is a significant $[^{32}P]$-labelling of a ~20-kDa protein corresponding to ARF6, as revealed by Western blotting with a specific anti-ARF6 antibody. Interestingly, when 1 µg purified ARF6 or ARF1 are ADP-ribosylated under the same conditions, strong labelling is observed only for ARF6 (Figure 5.7A). The selectivity between these
Figure 5.5. Kinetics of inhibition mediated by ARF6 on the β subunit ADP-ribosylation. The level of inhibition of β subunit ADP-ribosylation induced by increasing concentrations of purified ARF6, as quantified using an Instant Imager. Plasma membranes (5 μg/sample) were incubated for 15 min at 37 °C with ARF6, and then for an additional 60 min at 37 °C with [³²P]-NAD⁺. The data shown represent the means (±SD) of six independent experiments, each performed in duplicate (n=12).
Figure 5.6. Inhibitory effect of ARF6 is blocked by increasing concentrations of the βγ dimer. The level of inhibition of β subunit ADP-ribosylation induced by ARF6 in the presence of increasing concentrations of purified bovine brain βγ dimer. Plasma membranes (5 μg/sample) were incubated for 15 min at 37 °C with 20 ng, 100 ng or 200 ng ARF6, and then for an additional 60 min at 37 °C in the presence of [32P]-NAD+ and the βγ dimer (ranging from 100 to 500 ng). The levels of β subunit ADP-ribosylation were quantified using an Instant Imager. The data shown represent the means (±SD) of three independent experiments, each performed in duplicate (n=6). *p< 0.05, significantly different from the respective controls.
ARFs parallels the selective ability of ARF6 to inhibit the mono-ADP-ribosylation reaction. 

[^32P]-labelling also occurred in permeabilized cells over-expressing ARF6 (Figure 5.7B). Notably, only the activated form of ARF6 is labelled, while ARF6(T27N) was not. This parallels the ability of the GTP-bound form of ARF6 to inhibit endogenous ADP-ribosylation of the β subunit. Together, these observations support the idea that the activated form of ARF6 can substitute βγ in the catalytic site of the mono-ADP-ribosyltransferase.

### 5.3.2 ARF6 is ADP-ribosylated by an enzymatic activity

To confirm the hypothesis that ARF6 acts as an alternative substrate, it is necessary to verify that ADP-ribosylation of ARF6 is due to enzymatic catalysis and not due to formation of adducts with the ADP-ribose generated by cellular NADases. Thus, recombinant ARF6 was incubated in ADP-ribosylation buffer (without β-NAD⁺) in the presence of free[^32P]-ADP-ribose (Section 2.7). The absence of[^32P]-labelling of ARF6 under these conditions ruled out the existence of a non-enzymatic ADP-ribosylation reaction (Figure 5.8). Moreover, recombinant ARF6 is not modified by incubation with[^32P]-NAD⁺ in the absence of plasma membranes (Figure 5.3B) demonstrating that labelling of ARF6 is not due to non-specific binding of[^32P]-NAD⁺. Overall these results reveal that ARF6 is ADP-ribosylated by an enzyme-catalysed process. Possibly related to this is the observation (Galas et al., 1997) that in two-dimensional gel electrophoresis of purified chromaffin granule membranes, an anti-ARF6 antibody revealed the presence of two spots having different isoelectric points that are compatible with the ribosylated and un-ribosylated forms of ARF6.
Figure 5.7. ARF6 is $^{32}$P-labelled both \textit{in vitro} and \textit{in vivo}. A, Bottom panel: labelling of purified ARF6 and ARF1 incubated for 60 min at 37 °C with 5 µg/sample CHO plasma membranes and $^{32}$P-NAD$^+$, as revealed by autoradiography. The upper panel shows the protein content of the same samples, as revealed by Ponceau Red staining. B, Labelling of over-expressed ARF6, dominant-positive ARF6 (ARF6DP) and dominant-negative ARF6 (ARF6DN), and of ARF6 co-expressed with EFA6. The CHO cells were transfected with the indicated constructs for 24 h, permeabilised with tetanolysin, and incubated at 37 °C for an additional 60 min with $^{32}$P-NAD$^+$ and 100 µM GTP$_7$S. The bottom panel shows ARF6 in the same samples, as revealed by Western blotting with an anti-ARF6 antibody. The data shown are from single experiments that were performed in duplicate, which are representative of at least three independent experiments.
Figure 5.8. ARF6 is ADP-ribosylated by an enzymatic activity. Level of ADP-ribosylated ARF6 following incubation for 60 min at 37 °C with 5 μg/sample CHO plasma membranes and 3 μCi/sample $[^{32}P]$-NAD$^+$, or with 3 μCi/sample $[^{32}P]$-ADP-ribose, as revealed by autoradiography. The top panel shows the immunoblot with an anti-ARF6 antibody. The bottom two panels represent two different exposure times for the autoradiography. The data shown are from a single experiment that was performed in duplicate, which is representative of four independent experiments.
5.3.3 ARF6 is mono-ADP-ribosylated

To exclude the possibility that ARF6 is modified by attachment of a poly-ADP-ribose chain, an inhibitor for arginine-specific mono-ADP-ribosyltransferases, MIBG, was tested (Loesberg et al., 1990; Smets et al., 1990). Plasma membranes were used to ADP-ribosylate recombinant ARF6 in the presence of MIBG or control buffer. Under these conditions, ADP-ribosylation of ARF6 was strongly inhibited (Figure 5.9A), confirming that the enzyme reaction occurs on arginine and is mediated by a mono-ADP-ribosyltransferase. This was further supported by the reversal of the ADP-ribosylation of ARF6 by cytosolic ribosylarginine hydrolase, which removes single (but not poly-) ADP-ribose moieties attached to proteins (Figure 5.9B; Section 2.6). This demonstrates not only that ARF6 undergoes mono-ADP-ribosylation on arginine, but also that the complete cycle of mono-ADP-ribosylation/ de-ADP-ribosylation is active on ARF6, as it is for the β subunit.

At this point, it is important to understand whether a unique enzyme is responsible for both β subunit and ARF6 ADP-ribosylation. This aspect is complicated by the lack of characterisation of the enzyme involved. To overcome this, I tested whether the two ADP-ribosylation reactions are similarly modulated by inhibition and activation of the transferase activity. First, the inhibitory effects mediated by MIBG were quantified. Plasma membranes as a source of enzyme were used to modify purified βγ dimer and recombinant ARF6 in the presence of MIBG, which significantly inhibited ribosylation of both proteins to the same extent (Figure 5.10).

In parallel, positive modulators of β subunit ADP-ribosylation were tested. As shown earlier (Section 3.2) Mas7 and thrombin increase the [³²P]-labelling of β subunits, although through different mechanisms. The stimulation of the PAR1 receptor leads to activation of βγ dimers that become substrates of the endogenous ADP-ribosyltransferase, while Mas7 enhances the reaction through both a direct effect on the ADP-
Figure 5.9. ARF6 is mono-ADP-ribosylated by an enzymatic activity. A, The level of ADP-ribosylated ARF6 obtained in the absence and presence of 200 μM MIBG, incubated for 60 min at 37 °C with 5 μg/sample CHO plasma membranes and [32P]-NAD+, as revealed by autoradiography. The bottom panel shows the immunoblot with an anti-ARF6 antibody of the same nitrocellulose filter. B, The level of ADP-ribosylated ARF6 in the absence and presence of 50 μg cytosol, as revealed by autoradiography. Plasma membranes (5 μg/sample) were incubated for 60 min at 37 °C with [32P]-NAD+, and then for an additional 60 min at 37 °C with cytosol from CHO cells. The bottom panel shows the immunoblot with an anti-ARF6 antibody of the same nitrocellulose filter. The data shown are from single experiments performed in duplicate, which are representative of four independent experiments.
Figure 5.10. ARF6 mono-ADP-ribosylation is inhibited by treatment with MIBG. The levels of ADP-ribosylated ARF6 (green columns) and β subunit (blue columns) obtained in the absence and presence of 200 μM MIBG, incubated for 60 min at 37 °C with 5 μg/sample CHO plasma membranes and [32P]-NAD+, as quantified using an Instant Imager. The data shown represent the means (±SD) of five independent experiments, each performed in duplicate (n=10). *p< 0.001, significantly different from the respective untreated samples.
ribozyme transferase and activation of G proteins (Section 3.2.3). Thus, βγ dimer and ARF6 were ADP-ribosylated under basal condition and in the presence of thrombin or Mas7. A significant increase in mono-ADP-ribosylation of ARF6 occurred with Mas7, but not with thrombin (Figure 5.11) indicating that the activation of heterotrimeric G proteins is ineffective for ARF6 ADP-ribosylation. The strong labelling of ARF6 obtained with Mas7 can be ascribed to its ability to activate the ADP-ribosyltransferase. From a quantitative point of view, the increase in ARF6 labelling due to Mas7 (~85%) is comparable to the enhancement of labelling of β subunits insensitive to PTX (Section 3.2.3; Figure 3.7). Thus Mas7 stimulates ADP-ribosylation of ARF6 and Gβ to similar extents giving further support to the conclusion of a single enzymatic activity acting on both these substrates.

Overall, these findings support a model in which, once ARF6 is activated it can substitute for the β subunit at the catalytic site of the endogenous ADP-ribosyltransferase. If this is correct, then we can predict that stimulation of the GnRH receptor that activates ARF6 (Section 4.7) should lead to a decrease in the labelling of the β subunit, and in parallel, to an increase in ADP-ribosylation of ARF6. To test this, 15 ng purified ARF6 (a concentration that can be easily revealed by autoradiography, but that is insufficient to inhibit ADP-ribosylation of the β subunit; Figure 5.4) was ADP-ribosylated in the presence of plasma membranes from GnRH-CHO cells incubated with GnRH or control buffer. The decrease in ADP-ribosylation of the β subunit that occurred upon stimulation with GnRH corresponds to a comparable increase in labelling of ARF6 (Figure 5.12).

For final confirmation that ARF6 substitutes for the β subunit in the catalytic site of the endogenous transferase, all the experiments reported here need to be repeated using a purified preparation of the ADP-ribosyltransferase enzyme. In addition, one would predict that the K_m values for the two ADP-ribosyltransferase activities, determined for the common substrate NAD^+, should be the same if a single enzyme modifies both ARF6 and
Figure 5.11. ARF6 mono-ADP-ribosylation is modulated by Mas7, but not by thrombin. The level of ADP-ribosylated ARF6 (green columns) and β subunit (blue columns) obtained in the absence and presence of 25 µM Mas7 or 10 IU/ml thrombin, incubated for 60 min at 37 °C with 5 µg/sample CHO plasma membranes and [³²P]-NAD⁺, as quantified using an Instant Imager. The data shown represent the means (±SD) of four independent experiments, performed in duplicate (n=8). *p< 0.05, significantly different from the respective untreated samples.
Figure 5.12 ARF6 ADP-ribosylation is induced by stimulation of the GnRH receptor.

The level of ADP-ribosylated ARF6 (green columns) and β subunit (blue columns) obtained in the absence and presence of 200 nM GnRH, incubated for 60 min at 37 °C with 5 µg/sample GnRH-CHO plasma membranes and \([^{32}P]-\text{NAD}\), as quantified using an Instant Imager. The data shown represent the means (±SD) of three independent experiments, each performed in duplicate (n=6). *p< 0.05, significantly different from the respective untreated samples.
the β subunit. Measurements of the apparent $K_m$ were therefore carried out using plasma membranes as the source of enzymatic activity in the presence of increasing concentrations of $\beta$-NAD$^+$ and saturating concentrations of either ARF6 or βγ dimer. The results are shown in Figure 5.13A, the kinetic constants were determined giving apparent $K_m$ of 216.3 ±17.5 μM and 80.4 ±8.2 μM for the βγ dimer and ARF6, respectively (Figure 5.13B). This result suggests the existence of two different enzymes that catalyse ADP-ribosylation of ARF6 and the β subunit and conflicts with my previous observations that ARF6 down-regulates ADP-ribosylation of the β subunit by acting as a competitive inhibitor. However, there are alternative explanations, as discussed in next Section.

5.4 Discussion

In this Chapter, the mechanism by which ARF6 is able to inhibit endogenous mono-ADP-ribosylation of the β subunit has been investigated. I first excluded that ARF6 acts via subtraction of $\beta$-NAD$^+$ or sequestering of the βγ dimers. Instead, the data suggest that ARF6 inhibits the ADP-ribosyltransferase by displacement of the βγ dimer from the catalytic site, acting as a competitive inhibitor. This conclusion is supported by the demonstration that increasing concentrations of βγ overcome the ARF6-mediated inhibitory effect. In addition, I have shown that ARF6 is itself a substrate of an endogenous mono-ADP-ribosylation reaction that is modulated by an activator (Mas7) and an inhibitor (MIBG) of the enzyme that ADP-ribosylates β subunit. An approximate quantification of the extent of labelling of the two substrates revealed that ARF6 is preferred, being ADP-ribosylated to a level twice that of the β subunit (Figure 5.13). This is not inconsistent with the idea that a single ADP-ribosyltransferase is involved. However, this conclusion was not supported by the finding that the $K_m$ values for $\beta$-NAD$^+$ are different for the two activities. There are several possibilities that might explain these conflicting results:
Figure 5.13 Kinetic investigation of ADP-ribosylation of ARF6 and the β subunit. A, Picomoles of ADP-ribosylated ARF6 (pink triangles) and β subunit (blue rectangles) calculated in the presence of increasing concentrations of β-NAD⁺, incubated for 60 min at 37 °C with 5 μg/sample CHO plasma membranes and [³²P]-NAD⁺, as quantified using an Instant Imager. The picomoles were calculated as reported in Section 2.5.2. The data shown represent the means (±SD) of five independent experiments, each performed in duplicate (n=10). B, Kinetic parameters as calculated according to Eadie-Hofsee theory using GraphPad SIGMA software.
• the interactions of ARF6 or the β subunit with the ADP-ribosyltransferase might differently affect the ternary structure of the enzyme, thus leading to different three-dimensional rearrangements of protein domains. As a consequence, the affinity for β-NAD⁺ might change. This hypothesis is supported by studies on the ARTs, which have demonstrated that certain amino-acid sequences outside the NAD⁺-binding site can differently modulate the affinity for the substrate and the catalytic activity of the entire enzyme (Bourgeois et al., 2003). Thus the activity of my ADP-ribosyltransferase could also be influenced by such protein domains. At present there are no data to substantiate this idea.

• the catalytic mechanisms that mediate ADP-ribosylation of ARF6 and the β subunit may actually be different. Bisubstrate kinetic analyses have indicated that many enzymes using β-NAD⁺ as a substrate (such as lactate dehydrogenase, CTX, ARTs and sirtuins) exhibit sequential mechanisms of catalysis in which all the substrates bind to the enzyme to form a ternary complex with the enzyme, prior to any catalytic step (Borra et al., 2004; Larew et al., 1991; Osborne et al., 1985). Since sequential mechanisms can be ordered (in which the substrates bind the enzyme in a defined sequence) or random (in which the order of addition of substrates is random), my ADP-ribosyltransferase might use different mechanisms that depend on the substrate (ARF6 or the β subunit) resulting in different affinity for β-NAD⁺.

• the method used to calculate Km constants might provide erroneous values. Indeed, Km values were extrapolated from the picomoles of ARF6 and βγ that were modified in the presence of increasing concentrations of β-NAD⁺. The labelling of β subunits was calculated on the understanding that binding of ADP-ribose occurs only on a single arginine, as previously demonstrated in mutagenesis experiments for the β subunit (Section 2.5.2; Lupi et al., 2000). The same principle was applied in the estimation of ADP-ribosylation of ARF6. However, it cannot be excluded
that the enzyme catalyses addition of ADP-ribose on multiple arginine residues. This mono-ADP-ribosylation at multiple sites would certainly cause problems with the calculations of $K_m$ values for $\beta$-NAD$^+$.

- plasma membranes (used as the source of enzyme) might contain or lack specific components that are able to influence the affinity of the endogenous ADP-ribosyltransferase for $\beta$-NAD$^+$. In addition the simultaneous presence of ARF6 and the $\beta$ subunit in plasma membranes could also affect $K_m$ estimation.

To discriminate between these different possibilities, kinetic analyses must be repeated using purified proteins. In parallel, it will be important to achieve a better characterisation of the ADP-ribosylation of ARF6, in order to specify the arginine residue(s) that is/are modified. For the present, the $K_m$ values reported here must be taken as only indicative. By contrast, my other results are fully consistent with a model in which once activated by the GnRH receptor, ARF6 interacts with the transferase and reduces the extent of $\beta$ subunit mono-ADP-ribosylation through a competitive mechanism (Figure 5.14). An intriguing aspect that also arises is the existence of a reversible ADP-ribosylation reaction that is active on ARF6. ARF6 is modified only when it is bound to GTP and thus in the form that mediates ARF6 functions. It is plausible that the ARF6 ADP-ribosylation/de-ADP-ribosylation cycle modulates its functionality, as has been described for the $\beta y$ dimer. Thus, mono-ADP-ribosylation might represent a general mechanism for regulation of a variety of cellular proteins involved in signalling pathways initiated by GPCRs.
Figure 5.14 Model for inhibition of G-protein β subunit mono-ADP-ribosylation mediated by ARF6. Once activated by the GnRH receptor via an exchange factor, ARF6 interacts with the ADP-ribosyltransferase and down-regulates β subunit mono-ADP-ribosylation. In parallel, ADP-ribosylation takes place on ARF6. The modified ARF6 then serves as substrate for a cytosolic ADP-ribosylhydrolase, which regenerates native ARF6. The cycle of ADP-ribosylation/de-ADP-ribosylation of ARF6 might modulate its functionality, as has been described for the βγ dimer.
Chapter 6

In search of a physiological role for β subunit mono-ADP-ribosylation: the degradation pathway of the βγ dimer mediated by ubiquitylation

6.1 Introduction

In previous Chapters, I have defined a multifaceted pathway that regulates endogenous mono-ADP-ribosylation of the β subunit. The physiological significance of this reaction remains to be properly defined. My data suggest that ADP-ribosylation influences cellular functions that are mediated by βγ dimers and/or reactions or pathways in which βγ dimers act as substrates. One of these is the process of βγ degradation that occurs via the ubiquitin proteosome pathway (UPP) (see also Section 1.1.4.2). The UPP consists of a series of reactions that are catalysed by three classes of enzymes: ubiquitin-activating enzyme, ubiquitin-conjugating enzyme and ubiquitin ligase. These allow the attachment of ubiquitin chains to one or more lysines in a target protein (Miller and Gordon, 2005). The transducin γ subunit is one of the targets of the UPP and its ubiquitylation coincides with degradation of the entire βγ dimer. The process is inhibited by interaction of the βγ with phosducin, which probably acts as a protective factor for βγ upon light-dependent activation (Obin et al., 2002; Obin et al., 1994). Interestingly, a two-hybrid screening approach performed by Dr. M. Sallese in our Department has shown that a ubiquitin ligase interacts with the β subunit. These observations lead to the idea that the mono-ADP-ribosylation reaction can operate like phosducin to preserve the βγ dimer from degradation. The ADP-ribosylated form of the β subunit might be unable to form a complex with the ubiquitin ligase enzyme and therefore prevent βγ dimers from entering
the degradation pathway (Figure 6.1).

This hypothesis was tested using:

i) Co-immunoprecipitation assays, to confirm the interaction between the βγ dimer and ubiquitin ligase;

ii) Co-immunoprecipitation assays using pre-ADP-ribosylated β subunits, to evaluate the influence of ADP-ribosylation on the interaction between the βγ dimer and ubiquitin ligase.

6.2 βγ interacts with the ubiquitin ligase c-cbl, and this interaction is not affected by ADP-ribosylation of the β subunit

To confirm the possibility of an interaction between βγ dimers and c-cbl (a ubiquitin ligase normally expressed in CHO cells), co-immunoprecipitation assays were carried out using an anti-Gβ subunit antibody (as described in Section 2.12). Under these conditions, endogenous c-cbl was found to interact with the β subunit (Figure 6.2A). Furthermore, the β subunits co-immunoprecipitate with the anti-c-cbl antibody (Figure 6.2B), demonstrating that ubiquitin ligases are partners of the βγ dimer in living cells. If this interaction is affected by ADP-ribosylation as proposed, then a large proportion of the β subunits would have to be modified. However, in intact cells, only a low amount of the β subunit is ADP-ribosylated (Section 1.2.3.3.2) due to the presence of ADP-ribose hydrolase. By contrast, a higher degree of ADP-ribosylation (~60%) can be achieved in purified plasma membranes on prolonged incubation (6 h) with ADP-ribosylation buffer (Figure 6.3A). Thus, interactions between c-cbl and the native or ADP-ribosylated forms of the β subunit could be evaluated using plasma membranes instead of total cell lysates.
Figure 6.1. The βγ dimer as a substrate of the ubiquitin proteasome pathway (UPP).

A, The γ subunit is ubiquitylated by the UPP and the entire βγ dimer is degraded by the proteosome. B, The interaction with phosducin prevents ubiquitylation by the UPP preserving the functionality of the βγ dimer. C, Model of the possible role of ADP-ribosylation of the β subunit in the UPP-mediated degradation pathway.
Figure 6.2. Co-immunoprecipitation of the β subunit and c-cbl. A, Co-immunoprecipitation of endogenous c-cbl from CHO cells using an anti-Gβ antibody. Lanes 1 and 2, cell lysates; lanes 3 and 4, co-immunoprecipitation from CHO cells. B, Co-immunoprecipitation of endogenous β subunit from CHO cells using an anti-c-cbl antibody. Lane 1, cell lysate; lanes 2 and 3, co-immunoprecipitation from CHO cells. The data shown are from a single experiment that was performed in duplicate, and which is representative of at least three independent experiments.
and in this system, the modified and unmodified β subunits were both able to interact with c-cbl (Figure 6.3B, lanes 2 and 3). This indicates that endogenous ADP-ribosylation of the β subunit does not affect its interaction with ubiquitin ligase, and therefore, that the ADP-ribosylation reaction is not involved in the degradation pathway of the entire βγ dimer.

6.3 Discussion

In this Chapter, I approached the study of the physiological role of endogenous β subunit mono-ADP-ribosylation. Recent reports have demonstrated that βγ dimers are ubiquitylated and then targeted to the proteasome for degradation. In addition, the finding that β subunits interacts with the ubiquitin ligase c-cbl offers clues into a possible role for ADP-ribosylation in the degradation pathway of βγ. Indeed, it can be postulated that the ADP-ribosylated βγ dimer might not be recognised by ubiquitin ligase, and thus will be protected. In this case, mono-ADP-ribosylation could act to stabilize βγ dimers upon agonist stimulation of some GPCRs. Experimental evidence however ruled out this possibility, the ADP-ribosylated β subunit being able to interact with c-cbl also in its unmodified form. The ADP-ribosylated Arg129 is thus probably not included in the interaction site with cbl. The role of the interaction between βγ and c-cbl remains to be identified, and at present no data can be offered to elucidate these points.

However, a variety of other possible physiological roles of endogenous mono-ADP-ribosylation remain to be evaluated, and some of these will be considered in Chapter 7.
Figure 6.3. Effects of ADP-ribosylation on the interaction between the β subunit and c-cbl in plasma membranes. A, Time-dependence of β subunit ADP-ribosylation when 5 µg of plasma membranes were incubated for increasing times at 37 °C with β-NAD⁺ and [³²P]-NAD, as quantified using an Instant Imager. The data shown represent the means (±SD) of five independent experiments, each performed in duplicate (n=10). B, Co-immunoprecipitation of endogenous c-cbl from purified plasma membranes of CHO cells using an anti-Gβ antibody. Lane 1, untreated plasma membranes; lane 2, plasma membranes incubated with α-NAD⁺; lane 3, plasma membranes incubated with β-NAD⁺. Data shown are from a single experiment that was performed in duplicate, which is representative of at least three independent experiments.
Final Discussion

The aim of this investigation has been the identification of mechanisms that regulate the endogenous mono-ADP-ribosylation of the $\beta$ subunit of heterotrimeric G proteins.

As already discussed (Chapter 1), we previously reported that $\beta$ subunits are ADP-ribosylated in intact cells by an arginine-specific, plasma-membrane-associated enzyme (Lupi et al., 2000). This modification can be considered as a general mechanism since it occurs in several cell lines including CHO, HL60, Swiss 3T3 and FRTL5 cells (Lupi et al., 2000). From a quantitative point of view, under resting conditions $\sim$0.2% of the total complement of $\beta\gamma$ dimers is modified in intact CHO cells and it is reasonable that this fraction corresponds to a cellular pool of $\beta\gamma$ dimer that remains inactive. This is supported by the demonstration that mono-ADP-ribosylation inactivates the $\beta\gamma$ dimers by impairing their interactions with effector enzymes such as AC I, PI3K$\gamma$ and PLC$\beta$2 (Lupi et al., 2002). Thus, the reaction appears to represent a cellular mechanism that modulates G-protein-mediated signal transduction processes through a direct regulation of $\beta\gamma$ dimers. The possible physiological importance of this reaction is further supported by its reversibility; the modified $\beta$ subunit is de-ADP-ribosylated by a cytosolic ADP-ribosylhydrolase that regenerates native $\beta\gamma$ by releasing the bound ADP-ribose. Therefore, in intact cells, a cycle of ADP-ribosylation/de-ADP-ribosylation operates and this can modulate G$\beta$-mediated activities.

Starting from these observations, I analysed how this ADP-ribosylation is modulated. Previous data suggested that it preferentially occurs on the activated form of $\beta\gamma$
(Lupi et al., 2002). Considering that active βγ originates from heterotrimeric G proteins upon activation of GPCRs (Hamm, 1998; Lambright et al., 1996), I demonstrated that ADP-ribosylation of β subunits is modulated in response to stimulation of certain GPCRs in vitro, and more importantly, also in living cells. Interestingly, I demonstrated that the occurrence of hormonally induced ADP-ribosylation of the β subunit shows specificity with regard to receptor identity. Activation of some receptor leads to an increase in active βγ heterodimers, which can subsequently be inactivated by ADP-ribosylation. In this case, ADP-ribosylation is thus a signal termination mechanism for βγ-mediated functions that are independent of α subunit activity. By contrast, I found that the GnRH receptor decreases the rate of β subunit ADP-ribosylation. This might be due to a short-lived inhibition of ADP-ribosyltransferase activity, which would result in a time-extension of βγ-mediated activities. We can speculate that the endogenous cycle of ADP-ribosylation/de-ADP-ribosylation of the β subunit represents a mechanism that transiently regulates the amount of βγ that is available for cellular signalling. This conclusion is of interest because of the role of βγ dimers in the control of cell proliferation and regulation of normal cell growth and development (reviewed by Schwindinger and Robishaw, 2001). Experiments aimed at evaluating the activities of intracellular effectors that are regulated directly by the βγ dimer under conditions in which the state of the ADP-ribosylation/de-ADP-ribosylation cycle is shifted towards the ADP-ribosylated or de-ADP-ribosylated form of β will help to flesh out these ideas.

A further possibility to be considered is that ADP-ribosylation of the β subunit might represent a means to modulate Gα-mediated activities since inhibition of the βγ heterodimer through its mono-ADP-ribosylation should prolong the activation state of the α subunit. This proposal however, conflicts with our observations that modified βγ dimers retain their competence to reconstitute inactive heterotrimers with α subunits (Di Girolamo, unpublished data). It is conceivable that once the modified βγ re-forms the
inactive heterotrimer with GDP-bound Gα, de-ADP-ribosylation by the cytosolic hydrolase takes place, hence reforming the inactive heterotrimer. This will then be ready for a new round of activation.

More intriguing is that mono-ADP-ribosylation represents a molecular switch for βγ activities. One can hypothesise that the transferase serves as a scaffold protein that interacts with βγ dimers and then with other still unidentified proteins. Mono-ADP-ribosylation would switch off βγ activity, and hence G-protein signalling. At the same time it could activate other signalling pathways. In this way, the transferase could act as a link between heterotrimeric G proteins and other signalling pathways downstream of the βγ subunit. An example of this kind of mechanism is seen with the RGS proteins. In endothelial cells, p115RhoGEF switches off thrombin-activated Gα13 due to its RGS activity. In parallel, it activates RhoA due to its GEF activity, thus inducing downstream morphological changes (Fukuhara et al., 2001). The full elucidation of the physiological role of β subunit mono-ADP-ribosylation must await the molecular characterisation of the specific enzyme(s) involved in this modification.

Central to the present investigation is also the identification of a new role for ARF6 as a negative modulator of endogenous β subunit ADP-ribosylation. Although ARF6 belongs to a family of proteins originally described as essential co-factors for ADP-ribosylation catalysed by CTX, I have demonstrated that the activated form of ARF6 can inhibit endogenous ADP-ribosylation of the β subunit both in vitro and in vivo. In particular, ARF6 inhibits the β subunit ADP-ribosylation mediated by GnRH receptor simulation. In analysing this regulatory pathway, I also found that the stimulated GnRH receptor causes activation of ARF6, probably through an ARF6-GEF. This is in line with a number of studies that indicate that activation of the ARFs occurs upon agonist activation of certain GPCRs, including the M3 muscarinic, fMet-Leu-Phe, H1 histamine and B2 bradykinin receptors (Houle et al., 1995; Mitchell et al., 1998; Rumenapp et al., 1995;
Salvador et al., 2001). It is further supported by the recent finding that the A2A receptor interacts directly with ARNO/cytohesin-2 (Gsandtner et al., 2005).

My proposal for the role of ARF6 in GnRH receptor-mediated inhibition of β subunit mono-ADP-ribosylation is summarised in Figure 5.14. Upon receptor activation, ARF6 is activated though an exchange factor and is then able to recognise the ADP-ribosyltransferase. This in turn loses its ability to interact with and to modify the βγ dimer. As a consequence, the extent of β subunit modification is reduced and so its activities are favoured. Analysing the mechanisms by which ARF6 down-regulates the transferase, I found that ARF6-GTP inhibits ADP-ribosylation of the β subunit directly by acting as a competitive substrate for the endogenous transferase. This is supported by the finding that activated ARF6 is itself a substrate for ADP-ribosylation. The ADP-ribosylated form of ARF6 is de-ADP-ribosylated by a cytosolic ADP-ribosylhydrolase. This means that the endogenous cycle of ADP-ribosylation/de-ADP-ribosylation should be active on different substrates, and thus it could modulate the functionality of more than one membrane-associated protein.

Concerning the physiological role of ADP-ribosylation of ARF6, it is conceivable that once modified, it has an altered function, as has been seen for other substrates of the ADP-ribosylation reaction. The activation of ARF6 that occurs in response to receptor stimulation is involved in the desensitization and the internalization of the receptors, both being processes that regulate the duration and strength of GPCR signals (Claing, 2004; Hunzicker-Dunn et al., 2002). Thus, if ADP-ribosylation affects the functions of ARF6, it is possible that a cycle of mono-ADP-ribosylation/de-ADP-ribosylation will modulate the signalling cascade mediated by cell surface receptors. ADP-ribosylation reaction cannot be considered as simply a mechanism for the regulation of βγ-mediated signalling; rather, it represents a general mechanism that can regulate other responses to GPCR activation.
This is of some interest because of the loss- and gain-of-function alterations in GPCR-mediated signalling that have been identified as causes of a number of disease conditions, including retinal, endocrine, metabolic and developmental disorders (Maisel et al., 1990; Spiegel et al., 1993). So what is the molecular basis for these diseases? Stated most simply, receptors, G proteins and effectors function as a series of "on-off" switches that are triggered by an extracellular signal. Changes that inappropriately switch on or switch off one of the components will cause an error in one or more cellular functions. In this context, it will be fascinating to determine if the endogenous ADP-ribosylation reaction can regulate the correct "on-off" switching processes, thus influencing cell responses to external stimuli both in physiological and in pathological conditions. However, much further work is needed to confirm these suggestions and to provide the molecular foundations for the full physiological role of ADP-ribosylation. First of all, identification of the ADP-ribosyltransferase enzyme that catalyses endogenous ADP-ribosylation of the β subunit, and probably also of ARF6 is a number one priority. In parallel, understanding how the ADP-ribosylation of ARF6 is modulated will help to understand the impact of this reaction in ARF6-mediated functions such as receptor desensitisation and internalization. The answers to these questions will further the understanding of this reaction as a regulator of GPCR-mediated signalling, and they will facilitate future efforts in the elucidation of the initiation and progression of some disease states.

In conclusion, the evidence provided here contributes to the definition of the mechanisms that regulate endogenous ADP-ribosylation of β subunits and opens the doors to future studies regarding its full physiological role. Also in revealing ARF6 as a new substrate of the ADP-ribosylation reaction, I have indicated the possibility of a complex and multidimensional network of control for the regulation of signal transduction processes.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>$\beta_2$-AR</td>
<td>$\beta_2$-adrenergic receptor</td>
</tr>
<tr>
<td>$\alpha_2$-AR</td>
<td>$\alpha_2$-adrenergic receptor</td>
</tr>
<tr>
<td>$\beta$ARK</td>
<td>$\beta$-adrenergic receptor kinase</td>
</tr>
<tr>
<td>5-HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AGS</td>
<td>Activator of G protein signalling</td>
</tr>
<tr>
<td>AP</td>
<td>Adaptor protein</td>
</tr>
<tr>
<td>ARD</td>
<td>ADP-ribosylation factor domain protein</td>
</tr>
<tr>
<td>ARF</td>
<td>ADP-ribosylation factor</td>
</tr>
<tr>
<td>ARL</td>
<td>ARF like protein</td>
</tr>
<tr>
<td>ART</td>
<td>ADP-ribosyltransferase</td>
</tr>
<tr>
<td>AT$_1$-R</td>
<td>Angiotensin 1 receptor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>B$_2$-R</td>
<td>Bradykinin receptor</td>
</tr>
<tr>
<td>BFA</td>
<td>Brefeldin A</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C3</td>
<td>Clostridium botulinum toxin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic AMP</td>
</tr>
<tr>
<td>cAPK</td>
<td>cAMP-dependent kinase</td>
</tr>
<tr>
<td>CCK8</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster hovary</td>
</tr>
<tr>
<td>COP</td>
<td>Coat protein or coatomer</td>
</tr>
<tr>
<td>CTA</td>
<td>Cholera toxin, monomeric A subunit</td>
</tr>
<tr>
<td>CtBP1-s/BARS</td>
<td>BFA-ADP-ribosylated substrate</td>
</tr>
<tr>
<td>CTX</td>
<td>Cholera toxin</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DBD</td>
<td>Double zinc-finger domain</td>
</tr>
<tr>
<td>DIC</td>
<td>Dynein intermediate chain</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified minimal essential medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRAG</td>
<td>Dinitrogenase reductase-activating glycohydrolase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>DRAT</td>
<td>Dinitrogenase reductase/mono-ADP-ribosyltransferase</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>DTX</td>
<td>Diphtheria toxin</td>
</tr>
<tr>
<td>E1</td>
<td>Ubiquitin-activating enzyme</td>
</tr>
<tr>
<td>E3</td>
<td>Ubiquitin-ligase enzyme</td>
</tr>
<tr>
<td>EC₅₀</td>
<td>50% effective concentration concentration of substrate at which the reaction rate is half-maximal</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDIN</td>
<td>Epidermal cell differentiation inhibitor</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EF-2</td>
<td>Elongation factor 2</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<td>EGTA</td>
<td>thymeneglycol-bis-(2-aminoethyl ether)-N,N',N,N'-tetracetic acid</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>ERM</td>
<td>Ezrin/radixin/moesin family</td>
</tr>
<tr>
<td>ExoS</td>
<td>Exotoxin S</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
</tr>
<tr>
<td>fMLP</td>
<td>Formyl-Met-Leu-Phe</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>g</td>
<td>Gravity</td>
</tr>
<tr>
<td>Gα</td>
<td>G protein α subunit</td>
</tr>
<tr>
<td>Gβ</td>
<td>G protein β subunit</td>
</tr>
<tr>
<td>Gγ</td>
<td>G protein γ subunit</td>
</tr>
<tr>
<td>G418</td>
<td>Geneticine</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GDI</td>
<td>Guanine nucleotide dissociation inhibitor</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine 5’-diphosphate</td>
</tr>
<tr>
<td>GDPβS</td>
<td>Guanosine 5’-O-(2-thiodiphosphate)</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine-nucleotide exchange factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GGA</td>
<td>Golgi-localising gamma adaptin ear homology domain, ARF binding proteins</td>
</tr>
<tr>
<td>GIRK</td>
<td>Inward rectifier G-protein-gated K⁺ channel</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin releasing hormone</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein-coupled receptor</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>GPR</td>
<td>G protein regulatory motif</td>
</tr>
<tr>
<td>GRK</td>
<td>G-protein receptor kinase</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine 5’-triphosphate</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine triphosphatase</td>
</tr>
<tr>
<td>GTPγS</td>
<td>Guanosine 5’-0-(3-thiotriphosphate)</td>
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<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
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<tr>
<td>HBSS</td>
<td>Hanks balanced solution</td>
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<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>HL60</td>
<td>Human leukaemia</td>
</tr>
<tr>
<td>HNP-1</td>
<td>Human defensin</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin 8</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol1,4,5-trisphosphate</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>IU</td>
<td>International unit</td>
</tr>
<tr>
<td>K&lt;sub&gt;ATP&lt;/sub&gt;</td>
<td>ATP-sensitive K⁺ channel</td>
</tr>
<tr>
<td>K&lt;sub&gt;cat&lt;/sub&gt;</td>
<td>The catalytic rate of an enzyme</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>Ki</td>
<td>Inhibition constant</td>
</tr>
<tr>
<td>Km</td>
<td>Michaelis-Menten constant, measures the affinity of an enzyme for its substrate</td>
</tr>
<tr>
<td>K&lt;sub&gt;on&lt;/sub&gt;</td>
<td>Association rate constant</td>
</tr>
<tr>
<td>K&lt;sub&gt;off&lt;/sub&gt;</td>
<td>Dissociation rate constant</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Lymphocyte-function-associated antigen</td>
</tr>
<tr>
<td>LH/CG R</td>
<td>luteinizing hormone/choriogonadotropin hormone hormone receptor</td>
</tr>
<tr>
<td>LT</td>
<td>Heat-labile enterotoxin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>mADPRT</td>
<td>Mono-ADP-ribosyltransferase</td>
</tr>
<tr>
<td>MALDI-MS</td>
<td>Matrix assisted laser desorption/ionization mass spectrometry</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MIBG</td>
<td>Meta-iodobenzylguanidine</td>
</tr>
<tr>
<td>MOPS</td>
<td>4-morpholinepropanesulfonic acid</td>
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<tr>
<td>Myr-ARF6</td>
<td>Myristoylated peptide of ARF6</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADase</td>
<td>NAD glycohydrolase</td>
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<tr>
<td>NDPK</td>
<td>Nucleoside diphosphate kinase</td>
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<tr>
<td>NK1-R</td>
<td>Substance P receptor</td>
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<tr>
<td>Non-Myr-ARF6</td>
<td>Non myristoylated peptide of ARF6</td>
</tr>
<tr>
<td>NP40</td>
<td>Nonylphenoxypolyethoxy ethanol</td>
</tr>
<tr>
<td>O/N</td>
<td>Overnight</td>
</tr>
<tr>
<td>P2X7</td>
<td>Purinergic receptor X7</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAR1</td>
<td>Thrombin receptor 1</td>
</tr>
<tr>
<td>PARG</td>
<td>Poly-ADP-ribose glycohydrolase</td>
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<tr>
<td>PARP</td>
<td>Poly-ADP-ribosylpolymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology domain</td>
</tr>
<tr>
<td>PhLP</td>
<td>Phosducin-like protein</td>
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<tr>
<td>PHP</td>
<td>Protein histidine phosphatase</td>
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<tr>
<td>PI</td>
<td>Phosphoinositide</td>
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<tr>
<td>PI3,4,5-P3</td>
<td>Phosphatidylinositol 3,4,5-triphosphate</td>
</tr>
<tr>
<td>PI3,4-P2</td>
<td>Phosphatidylinositol 3,4-bisphosphate</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PI3P</td>
<td>Phosphatidylinositol 3-phosphate</td>
</tr>
<tr>
<td>PI4,5-P2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PIP5K</td>
<td>Phosphatidylinositol 4-phosphate 5-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>POR1</td>
<td>Partner of Rac 1</td>
</tr>
<tr>
<td>PTX</td>
<td>Pertussis toxin</td>
</tr>
<tr>
<td>PX</td>
<td>Phox homology domain</td>
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<tr>
<td>RGS</td>
<td>Regulator of G protein signalling</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Sir2p</td>
<td>Silent information regulator 2 protein</td>
</tr>
<tr>
<td>SIRT2</td>
<td>Human Sir2p homologue</td>
</tr>
<tr>
<td>TGN</td>
<td>trans-Golgi network</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane helix</td>
</tr>
<tr>
<td>TNKS</td>
<td>Tankyrase</td>
</tr>
<tr>
<td>TRIS</td>
<td>Tris[hydroxymethyl]aminomethane</td>
</tr>
<tr>
<td>Ubc/E2</td>
<td>Ubiquitin-conjugating enzyme</td>
</tr>
<tr>
<td>UPP</td>
<td>Ubiquitin proteasome pathway</td>
</tr>
<tr>
<td>UTP</td>
<td>Uridine 5'-triphosphate</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/volume</td>
</tr>
<tr>
<td>V_{max}</td>
<td>Maximal velocity of an enzymatic reaction at saturating substrate concentration</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight/volume</td>
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family of proteins with gamma-adaptin and VHS domains that facilitate trafficking between the

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