Functional and molecular characterization of CtBP3/BARS, a protein involved in the control of the Golgi complex

Thesis

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Functional and Molecular Characterization of CtBP3/BARS, a Protein Involved in the Control of the Golgi Complex

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Discipline: Life Sciences

Sponsoring establishment: Consorzio Mario Negri Sud

Thesis submitted in accordance with the requirement of Open University for the degree of Doctor of Philosophy

December 2001
Abstract

One of the goals of the research in the field of cell biology is the understanding of the physiology of intracellular membrane trafficking. In this respect, the present study is aimed at the comprehension of the molecular mechanisms that govern membrane trafficking, with emphasis at the events that occur at the level of the Golgi complex. Fundamental insights into the organization and the function of the Golgi complex have been provided by the use of pharmacological agents. Studies on the effects of Brefeldin A (BFA), a fungal toxin that potently inhibits membrane traffic and causes an extremely rapid disruption of the Golgi architecture and function, led to the identification of a 50-kDa cytosolic substrate of BFA-induced-ADP-ribosylation (BARS) and several lines of evidence indicated BARS as a possible regulator of the structure and function of the Golgi complex. In order to understand the molecular mechanisms that regulate the Golgi organization, the identification and characterization of BARS was undertaken. In this thesis the cloning and sequence analysis of BARS is reported, together with its functional characterization. BARS is shown to belong to the CtBP family of proteins and thus renamed CtBP3/BARS. It induces membrane fission of the Golgi tubules in vitro and catalyses the conversion of lysophosphatidic acid (LPA) into phosphatidic acid (PA), a reaction that appears essential for membrane fission at the Golgi complex as well as at the plasma membrane. This study also shows that CtBP3/BARS is localized on the Golgi and other cellular membranes. The identification of molecular interactors of CtBP3/BARS is also reported and their possible roles in membrane fission discussed.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>2D</td>
<td>Bidimensional</td>
</tr>
<tr>
<td>aa</td>
<td>Aminoacids</td>
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<tr>
<td>acyl-CoA</td>
<td>Acyl-coenzyme A</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AP</td>
<td>Adaptor protein complex</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>ARF</td>
<td>ADP-ribosylation factor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATTC</td>
<td>American Tissue Type Collection</td>
</tr>
<tr>
<td>BARS</td>
<td>BFA-ADP-ribosylated substrate</td>
</tr>
<tr>
<td>BFA</td>
<td>Brefeldin A</td>
</tr>
<tr>
<td>BLAST®</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cc</td>
<td>coiled-coil</td>
</tr>
<tr>
<td>CALM</td>
<td>Clathrin assembly lymphoid myeloid leukemia</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CGN</td>
<td>Cis-Golgi network</td>
</tr>
<tr>
<td>COP</td>
<td>Coat protein complex</td>
</tr>
<tr>
<td>CS</td>
<td>Calf serum</td>
</tr>
<tr>
<td>CtBP</td>
<td>C-terminal binding protein</td>
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<tr>
<td>CTN</td>
<td>Cis-tubular network</td>
</tr>
<tr>
<td>dCTP</td>
<td>Deoxyctydine 5’-triphosphate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagles Medium</td>
</tr>
<tr>
<td>DMP</td>
<td>Dimethylpimelidate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
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<tr>
<td>dNTP</td>
<td>Deoxynucleoside 5’-triphosphates</td>
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<td>DTT</td>
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</tr>
<tr>
<td>EC</td>
<td>Enzyme commission</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EEA1</td>
<td>Early endosome antigen 1</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(beta-aminoethyl ether)-N,N,N’,N’-tetraacetic acid</td>
</tr>
<tr>
<td>EMBL</td>
<td>European Molecular Biology Laboratory</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERGIC</td>
<td>ER-Golgi intermediate compartment</td>
</tr>
<tr>
<td>EST</td>
<td>Expressed-sequence tag</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FTCD</td>
<td>Formininotransferase cyclodeaminase</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3 phosphate dehydrogenase</td>
</tr>
<tr>
<td>GCG</td>
<td>Genetics Computer Group</td>
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<td>GDP</td>
<td>Guanosine diphosphate</td>
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<td>GED</td>
<td>GTPase-effector domain</td>
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<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
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<tr>
<td>GTPγS</td>
<td>Guanosine 5’-O-(3-thiotriphosphate)</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxy-ethyl)-piperazine-1-ethane-sulfonic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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<tr>
<td>HRP</td>
<td>Horse-Radish Peroxidase</td>
</tr>
<tr>
<td>IDI</td>
<td>Istituto Dermopatico dell’Immacolata</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectrofocusing</td>
</tr>
<tr>
<td>IFAP</td>
<td>Intermediate filament associated protein</td>
</tr>
<tr>
<td>IgGs</td>
<td>Immunoglobulins G</td>
</tr>
<tr>
<td>INX</td>
<td>α-internexin</td>
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<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>IQ</td>
<td>Ilimaquinone</td>
</tr>
<tr>
<td>LB</td>
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<td>LMB</td>
<td>Leptomycin B</td>
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<td>LPA</td>
<td>Lysophosphatidic acid</td>
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<td>LPC</td>
<td>Lysophosphatidylcholine</td>
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<tr>
<td>mADPRT</td>
<td>Mono-ADP-ribosyltransferase</td>
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<tr>
<td>MAP</td>
<td>Microtubule associated protein</td>
</tr>
<tr>
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<td>Minutes</td>
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<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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<tr>
<td>NAD</td>
<td>Nicotinamide-adenine dinucleotide</td>
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<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
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<tr>
<td>NF</td>
<td>Neurofilament</td>
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<td>NRK</td>
<td>Normal rat kidney</td>
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<td>Src homology</td>
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<tr>
<td>SLO</td>
<td>Streptolysin O</td>
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<tr>
<td>SNARE</td>
<td>Soluble NSF attachment protein receptor</td>
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<tr>
<td>TAC</td>
<td>Tip attachment complex</td>
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<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethyl-ethylenediamine</td>
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<tr>
<td>TGN</td>
<td>Trans-Golgi network</td>
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<tr>
<td>Tris</td>
<td>Tris[Hydroxymethyl]aminomethane</td>
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<tr>
<td>TTN</td>
<td>Trans-tubular network</td>
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<tr>
<td>v/v</td>
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<tr>
<td>VSVG</td>
<td>Vesicular Stomatitis Virus G protein</td>
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<td>w/v</td>
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CHAPTER 1

Introduction

1.1 Intracellular membrane traffic

1.1.1 Overview

Eukaryotic cells have developed a complex internal membrane system that is involved in the uptake and processing of external macromolecules (endocytic pathway) and in the maturation and proper delivery of internally synthesized macromolecules (biosynthetic-secretory pathway; Fig. 1.1). Proteins destined for secretion, plasma membrane or membranous intracellular organelles are synthesized at the endoplasmic reticulum (ER), which is the entrance of the biosynthetic-secretory pathway, then undergo processing and maturation in the Golgi complex by sequential addition or removal of specific carbohydrate units, and, finally, are delivered to their destinations. Similarly, lipids, that are mainly synthesized in the ER but have to reach all the membranous compartments and in some cases have to be processed in the Golgi for acquiring glycosidic chains, are transferred through different compartments of the secretory and endocytic pathways. Extracellular macromolecules are internalized and eventually degraded or recycled through different compartments of the endocytic pathway: plasma membrane, early, recycling and late endosomes, lysosomes and trans-Golgi network.
Fig. 1.1. General scheme of intracellular membrane traffic. The major organelles involved in the transport pathways are represented. Red arrows indicate membrane movements along the biosynthetic-secretory pathway; green arrows indicate membrane movements along the endocytic pathway. ER: endoplasmic reticulum.
1.1.2 Structure and function of the secretory pathway

The membrane system involved in the biosynthetic-secretory pathway is made up of several distinct organelles, including ER, Golgi, plasma membrane, secretory granules, and of tubulo-vesicular transport intermediates that mediate trafficking between organelles.

1.1.2.1 ER to Golgi transport

As already mentioned, the newly synthesized proteins enter the secretory pathway at the level of ER, where they are folded and eventually assembled in oligomers due to interaction with molecular chaperons (Ellgaard and Helenius, 2001). After correct folding, in order to be transported to the Golgi complex, proteins are selectively separated from ER resident proteins and concentrated in specialized sites, called ER exit sites or transitional ER, which are scattered over the surface of the ER (Bannykh et al., 1996). This selective event requires the assembly of the COPII protein complex, that is initiated by Sec12-mediated nucleotide exchange of GTP onto Sar1, with cargo molecules and cargo receptors potentially triggering this exchange. Sec23/24 and Sec13/31 heterodimers are sequentially assembled onto the cytoplasmic leaflet of the activated ER membrane to form COPII coated buds (Schekman and Orci, 1996). The concentration of the cargo depends on the presence of di-acidic sorting signals on the cytoplasmic tail of transmembrane proteins that have to be transported (Nishimura et al., 1999). The ER exit sites are adjacent to complex structures, that consists of clusters of vesicles and interconnected tubules (Bannykh et al., 1996), called vesicular-tubular clusters (VTCs) or ER-Golgi intermediate compartment (ERGIC). Although the role of COPII complex and the small GTPase Sar1 in membrane
budding at the ER exit sites and in ER to Golgi transport is well established (Bannykh et al., 1998; Klumperman, 2000b; Stephens and Pepperkok, 2001), the mechanism through which proteins move from ER exit sites to ERGIC and the process of formation of ERGIC are still unclear (see models in Fig. 1.2): one possibility is budding and detaching of small vesicles which will later fuse to form bigger structures (Bannykh et al., 1998); another possibility is that the budding structures on the ER exit sites give rise directly to large pleiomorphic intermediates forming the ERGIC (Lippincott-Schwartz et al., 2000). Anyway, the most accepted view considers the ERGIC as a very dynamic, mobile compartment, that coordinates anterograde movement of the cargo and recycling to ER of molecular components involved in ER to Golgi transport (Lippincott-Schwartz et al., 2000; Stephens and Pepperkok, 2001; Fig. 1.2 B). The retrograde transport of membrane components from the ERGIC to the ER, that is necessary to compensate for the anterograde movement of membranes, as well as to salvage escaped ER resident proteins and the transport machinery proteins, is believed to be mediated by the assembly of a second coat complex, COPI (Cosson and Letourneur, 1994; Letourneur et al., 1994; see also section 1.1.3.1.1). Visualization of transport in living cells by fusing the green fluorescent protein to the cargo indicated the timing of action of COP complexes in ER to Golgi transport (Presley et al., 1997b; Scales et al., 1997). Scales et al. (1997) found that COPII and COPI act sequentially in this process: COPII complex associates to carrier membranes before COPI complex and only when COPII complex dissociates, these structures, still bound to COPI, translocate through the cytoplasm reaching the Golgi complex. The movement of ERGIC structures was shown to occur along microtubules (Presley et al., 1997b; Scales et al., 1997) and to be mediated by the minus-end directed motor protein dynein (Presley et al., 1997b).
Fig. 1.2. Models describing ER-to-Golgi transport (modified from Stephens and Pepperkok, 2001). Panel A: ERGIC is viewed as a stable compartment at which COPII-coated vesicles arrive and fuse; COPI-coated vesicles are responsible for retrograde traffic in both ERGIC-to-ER and Golgi-to-ERGIC steps. Panel B: ERGIC is represented as the long-range ER-to-Golgi transport carrier itself and not a static membrane compartment. COPII-coated buds give rise to large tubular-vesicular structures either by homotypic fusion of vesicles or by direct formation of large pleiomorphic structures. Exchange of COPII for COPI triggers recycling to the ER mediated by COPI-vesicles and allows cargo to move along microtubules toward the Golgi complex. This model is the most consistent with the existing experimental data.
1.1.2.2 Structure of the Golgi complex in interphase cells.

The Golgi complex occupies a central function in the secretory pathway. In interphase cells it is usually centred around the microtubule-organizing centre (MTOC) and it is actively maintained there (Lippincott-Schwartz, 1998). The structure of the Golgi complex was revealed for the first time at the electron microscope by Dalton and Felix in 1954, who showed that it was composed of stacks of closely apposed lamellae which were later shown to be flattened saccules or cisternae. In the following decades fundamental morphological studies led to precise definition of the ultrastructure of the Golgi based on the analysis of 15 different types of cells (reviewed in Rambourg and Clermont, 1997). The Golgi complex is a single organelle that has the configuration of a network (Fig. 1.3 A). In some cells it extends over a large area of the cytoplasm, in other cells it is juxtanuclear and spheroidal. In both cases it resembles a ribbon, with a non-homogeneous structure. Some portions of the ribbon are rather compact and mainly composed of closely apposed cisternae. Other segments of the ribbon are less compact and consist of either highly fenestrated elements or membranous tubules bridging the cisternae of adjacent compact regions (Fig. 1.3 B). The relative proportion of the compact and non-compact regions is very variable from one cell to another and appear to be a characteristic of a given cell type. The non-compact regions of the Golgi are not easily identified in conventionally stained thin sections for EM, where they usually appear as vesicles surrounding isolated Golgi stacks. The Golgi ribbon is polarized, and this polarization appear to be both functional (the cis-face being the face accepting material from ER and the trans-face being where material exit en route to different destinations) and morphological (Figs. 1.3 B and 1.4).
Fig. 1.3. Diagrams of the structure of the Golgi apparatus of a spinal ganglion cell (modified from Rambourg and Clermont, 1997). Panel A shows a low-magnification diagram of the Golgi apparatus (GA) forming a network or ribbon around the nucleus (N). A small portion of the Golgi network, delimited by a square, is shown at higher magnification in the three-dimensional diagram depicted in panel B. This diagram represents the structure of the various elements that compose the Golgi ribbon. In particular, there are two types of regions along the Golgi ribbon: the compact regions (CR), mainly composed of stacked, poorly fenestrated saccular elements or cisternae, and non-compact regions (NCR), showing either fenestrated cisternae or tubules that bridge the cisternae of adjacent stacks. Highly fenestrated or tubular elements on the cis- or trans-most part of the compact region of Golgi apparatus constitutes the cis-tubular network (CTN) and the trans-tubular network (TTN), respectively. In the compact regions, perforation of the cisternae in register form wells (W), which are sites of formation of small vesicles (V), which are also seen at the edges of the Golgi ribbon and in the non-compact regions.
Fig. 1.4. Electron microscope images of the Golgi complex (reproduced from Rambourg and Clermont, 1997). Two high magnification electron microscope photographs showing a transverse (panel A) and an oblique (panel B) thin section through the compact region of the Golgi ribbon of a Sertoli cell. The cis-tubular network (CTN), the cisternae of the stacks (S), and the trans-tubular network (TTN) are clearly visible in both photographs. Cisternae of the endoplasmic reticulum (ER) and mitochondria (M) are also visible. Magnification: x 60000.
Three compartments, cis-, mid- and trans-compartments, are recognizable by EM both by exclusive structural criteria and by immunocytochemical localization of glycosyltransferases. The cis-compartment is made up of a tubular network, the cis-tubular network (CTN) or cis Golgi network (CGN), that is often adjacent to the cis-most saccular element or cisterna (see Fig. 1.3 B). The mid-compartment is formed by a variable numbers of flattened cisternae close to each other forming the stack that is the peculiar feature of the Golgi complex. The size of the lumen of the cisternae can vary and some cisternae present both flattened and distended portions. The cis-most cisternae are usually interrupted by wide fenestrations, giving rise to cavities or "wells". The non-compact regions are either composed of extensively fenestrated elements or consist of branched and anastomosed membranous tubules. Sometimes these tubules connect the cisternae of the compact region with elements of the CTN. The tubules appear to bridge cisternae of adjacent stacks all along the Golgi ribbon, not only connecting cisternae located at the same level in the stacks, but also cisternae belonging to different levels in adjacent stacks. Tubules also connect cisternae of the same stack. In the non-compact regions and in the "wells" originated by fenestrations some small vesicles (50-100 nm in diameter) are also present, that probably originate from coated buds that are visible along the tubules and the edges of the "wells", respectively (see Fig. 1.3 B). The trans-compartment is composed of three to six sacculo-tubular elements which do not remain strictly parallel to each other but, instead, show a tendency to "peel off" from the Golgi ribbon. Each one of these trans-elements consists of a central, poorly fenestrated saccular region and a peripheral zone made up of anastomosed membranous tubules which encroach into the cytoplasm on the trans-side of the Golgi. These anastomosed
tubules at the periphery of the saccular portion of the trans-elements are called 
trans-tubular network (TTN) or trans Golgi network (TGN; see Fig. 1.3 B).

Some disagreement concerning the structure of the non-compact regions,
the CGN and the TGN exists, probably arising from different methods of fixation
and different approaches used to obtain a tridimensional reconstruction of the
sample. Recently Ladinsky et al. (1999), obtained a 3D structure of the Golgi
from Normal Rat Kidney (NRK) cells by high-voltage electron microscope
(HVEM) tomography of ultra-rapid freezing, followed by freeze substitution of
specimens. In their analysis of the structure of the Golgi apparatus, while
confirming many conclusions deriving from previous studies on Golgi
organization (Rambourg and Clermont, 1997), they observed that the non­
compact regions is composed not only of bridging tubules but also of numerous
small vesicles and polymorphic membranous elements not always connected to
cisternae. Again in contrast with previous observations, they reported that the cis­
and trans-sides of the Golgi possess tubular extensions but do not display a
network architecture. In this study they also propose that polymorphic
membranous elements situated in their 3D reconstruction between the ER and the
cis-side of the Golgi are pre-Golgi elements or ERGIC, and suggest that they
could represent an intermediate stage in the formation of a new cis cisterna.

1.1.2.3 Structure dynamics of the Golgi complex in mitotic cells.

During mitosis the traffic along the secretory pathway is blocked at the
level of the ER-to-Golgi step (Featherstone et al., 1985; Farmaki et al., 1999).
Moreover, during mitosis massive changes occur at the Golgi complex in
mammalian cells. The inheritance of the Golgi complex poses particular problems
to the dividing cells. The sequence of events occurring at the Golgi complex
during mitosis is very debated (Shima et al., 1998; Zaal et al., 1999; Jokitalo et al., 2001). Some authors (Shima et al., 1998; Jokitalo et al., 2001) propose that during cell division the Golgi complex is partitioned into the daughter cells through the fragmentation and formation of numerous small units with a tubular vesicular organization; then, at the end of mitosis, these structures are subdivided at the two poles of the dividing cell in a microtubule-dependent manner. Other authors, instead, propose that during mitosis the Golgi is recycled to the ER (Zaal et al., 1999). It is widely accepted that at the onset of mitosis, between prophase and metaphase, the Golgi complex undergoes an impressive process of fragmentation. Warren and collaborators showed that from the juxtanuclear, usually pericentriolar location and ribbon organization, the Golgi is converted into numerous tubular clusters distributed throughout the cytoplasm (Shima et al., 1997). Dynamic observations of the Golgi structure in HeLa cells indicates that the mitotic process of Golgi fragmentation occurs through two distinct steps: the first occurring in 30-45 minutes involving a partial fragmentation, probably the transformation of the interphase ribbon in non-connected Golgi stacks, and a second, shorter, characterized by further fragmentation and dispersal of the mitotic Golgi (Shima et al., 1998). At the end of mitosis, during telophase, the tubular clusters are observed to move in a poleward direction, and only when the two daughter cells are formed, during G1 phase, the Golgi clusters congregate, generate short-life tubules and apparently fuse to give rise to the typical interphase Golgi apparatus (Shima et al., 1997). The fragmentation of the Golgi in mitosis has to be interpreted, according to Warren and collaborators, as a way to equally subdivide the Golgi membranes into the daughter cells. Initially, based on the vesicular model of transport (see next section), it was proposed that the
fragmentation arose from the continued budding of transport vesicles, in the absence of fusion, and that a stochastic partitioning of the mitotic Golgi fragments and vesicles could be responsible for the proper inheritance of the Golgi (Warren, 1985). But given that the accuracy of partitioning appears to be higher than what it would be if only a stochastic partitioning existed, it was concluded that other cellular mechanisms should control the inheritance of the Golgi apparatus (Shima et al., 1997). Detailed morphological analysis of the Golgi structure and microtubule dynamics during mitosis suggested a role for mitotic microtubules in the control of Golgi fragmentation and reformation (Shima et al., 1998). In fact, starting from prometaphase, the majority of mitotic Golgi membranes are organized around the developing spindle asters. Later, in metaphase, anaphase and telophase, part of the Golgi fragments are arranged around the spindle poles and part are associated to peripheral microtubules (Shima et al., 1998). Moreover, treatment of prometaphase cells with nocodazole, a microtubule-depolymerising agent, led to the dispersion of Golgi clusters from the spindle asters to the cell periphery, further implicating an involvement of microtubules in spatial organization of Golgi membranes during mitosis (Shima et al., 1998). A completely different interpretation of the events occurring at the Golgi complex during mitosis was proposed by Lippincott-Schwartz and collaborators (Cole et al., 1996a; Zaal et al., 1999), who indicated that during mitosis, as a consequence of the block of anterograde transport from ER (Featherstone et al., 1985; Farmaki et al., 1999), the Golgi resident proteins are redistributed to the ER. They suggested that the redistribution of the Golgi content to the ER during mitosis is the mechanism for the correct partitioning of the Golgi complex heritage into the daughter cells (Zaal et al., 1999). Notably, the redistribution of Golgi resident
proteins to the ER has also been proposed to be the basis for the fragmentation of the Golgi apparatus and the peripheral dispersion of Golgi fragments that occurs when cells are treated with nocodazole. The primary effect of nocodazole is the disruption of the microtubular cytoskeleton (De Brabander et al., 1976), but prolonged treatment with this compound also leads to the dispersal of the juxtanuclear Golgi to peripheral sites, producing a morphological pattern apparently very similar to mitotic pictures (Rogalski and Singer, 1984; Cole et al., 1996a; Storrie et al., 1998).

1.1.2.4 Intra-Golgi traffic

Cargo-transporting intermediates derived from the ER deliver their contents to the cis face of the Golgi complex. The cargo molecules (lipids and proteins) then move through the polarized stacks of flattened cisternae containing the resident enzymes responsible for processing and maturation of glycoproteins and glycolipids. These include glycosyltransferases and glycosidases responsible for synthesizing the huge diversity of complex oligosaccharide chains that are attached on glycoproteins and glycolipids (reviewed in Roth, 1997) and enzymes responsible for sulphation (reviewed in Farquhar and Hauri, 1997). Finally, glycolipids and glycoproteins reach the trans Golgi network (TGN), where they are packaged and targeted to their different destinations (plasma membrane, endosome/lysosomes, secretory granules). The mechanism by which cargo moves across the Golgi stack has been debated over 40 years (reviewed in Farquhar and Palade, 1998). Two main models have been proposed during this period. The first was the "cisternal progression model" introduced in the 1950s (Grassé, 1957): cisternae are formed on the entry or cis face and move with their cargo content sequentially towards the exit or trans face (Morre et al., 1979). The second was
introduced in 1981 (Farquhar and Palade, 1981), mostly based on the discovery that different regions of the Golgi stack contained different complements of carbohydrate-modifying enzymes and other proteins. The model proposes that each cisterna or set of cisternae constitutes a separate and stable compartment of distinctive composition and that transport from one cisterna to another occurs through vesicles. This model, commonly referred to as “vesicular transport model” (see Fig. 1.5), has dominated the field for over 15 years. However, the discovery of a retrograde transport mediating the retrieval of lumenal and membrane proteins from the Golgi complex to the ER (Sonnichsen et al., 1996; Orci et al., 1997), allowed a re-evaluation of an intra-Golgi retrograde transport and the proposal of a modified and improved cisternal progression model called “cisternal maturation-progression model” (Glick et al., 1997; Mironov et al., 1997b; see Fig. 1.5). This model predicts that each cisterna proceeds from the cis side to the trans side, progressively acquiring the features of the next cisterna due to the retrieval of enzymes from the distal cisterna. The progression and maturation of each cisterna implicates the progression and processing of the cargo. Recently the cisternal progression/maturation model seems to enjoy greater favour than the vesicular transport model. This is mainly due to the failure to explain how vesicles can selectively fuse with the proper cisterna during each step of intra-Golgi transport (Pelham, 1998) and the failure to detect enrichment of secretory proteins in Golgi vesicles (Love et al., 1998; Klumperman, 2000a) predicted by the vesicular model and to the finding that large proteins such as procollagen traverse the Golgi stack without leaving the lumen of Golgi cisternae (Bonfanti et al., 1998).
Vesicular transport model

Cisternal maturation-progression model

Endosomes

Plasma membrane

TGN

trans

medial

cis

ERGIC

= clathrin-coated vesicle

= COPI-coated vesicle

= resident Golgi enzymes

Fig. 1.5. Two models illustrating intra-Golgi transport. According to the vesicular transport model (schematised on the left), anterograde transport of cargo occurs through stable cisternae and is mediated by COPI-coated vesicles. At the TGN, cargoes are sorted into either clathrin-coated vesicles destined to endosomes/lysosomes or secretory carriers. The cisternal maturation-progression model (schematised on the right) predicts that ER-to-Golgi carriers by fusing each other originate a new cis-cisterna, which progressively acquires the feature of the trans-most cisterna, through the recycling of resident Golgi proteins via retrograde COPI-coated vesicles. Thus, in the cisternal maturation-progression model anterograde cargo matures in parallel with the cisterna. At the TGN, cargo destined to endosomes/lysosomes is included in clathrin-coated vesicles, while the remainder of the cisterna is converted into a large plasma membrane-directed carrier. Thin arrows indicate transport by dissociative carriers, while thick arrows indicate maturation events.
However, like the vesicular transport model, also the cisternal maturation model has some weakness: first of all, the fact that cisternal enzymes that should be continuously retrieved along the Golgi stack are not clearly enriched in the peri-Golgi vesicles (Sonnichsen et al., 1996). An alternative model (Mironov et al., 1997; Weidman, 1995) proposes that anterograde intra-Golgi transport, as well as ER-to-Golgi and post-Golgi transport, occurs through tubular structures. In fact, tubules emanate from the cis- and trans-most part of the Golgi and are supposed to mediate the traffic to the Golgi and from the Golgi. Thus, tubules interconnecting the Golgi cisternae described many times (Rambourg and Clermont, 1997; Cluett et al., 1993) are proposed to mediate intra-Golgi traffic (Mironov et al., 1997). Very recently, Griffiths (2000) proposed the idea that all the secretory pathway is continuous from ER to the TGN. According to his model the system would contain two main “gates”, the first between ER and Golgi, the second between Golgi and TGN. When the gates are open a continuity is established along the pathway. The tubular traffic model poses the problem that distinct membrane domains and vectorial traffic have to be maintained within a connected tubular system. The existence of membrane differentiation inside a continuous network would possibly be obtained through different mechanisms of protein retention and retrieval (Mironov et al., 1997; see also next section).

1.1.2.5 Mechanisms of protein compartmentalization in the Golgi

In the Golgi complex proteins and lipids are subjected to post-translational modifications, including glycosylation and proteolytic processing. The enzymatic activities responsible for this processing, together with the proteins involved in the maintenance of the structure of the Golgi itself, constitute the proteins that reside in the Golgi. All these proteins are specifically localized in the different
compartments of the organelle and are there retained, whereas the proteins in transit pass through. In the case of the maturation-progression model the resident proteins must necessarily be subjected to a retrograde transport (retrieval), whereas in the case of the vesicular transport or tubular continuities models, resident proteins can be localized either by retention mechanisms preventing them from leaving the Golgi, or by retrieval mechanisms. The understanding of the mechanisms determining the localization of the resident proteins is far to be completed. Different hypotheses have been presented to explain this phenomenon (reviewed in Gleeson, 1998). It is possible that retention involves association of resident proteins with cytoskeleton matrix (De Matteis and Morrow, 2000). Alternatively, or as an additional mechanism, the retention can involve lipid-based sorting of proteins (referred to as “lipid-mediated sorting” mechanism; Bretscher and Munro, 1993). This can occur, for example, when the transmembrane domain length matches the membrane thickness or the protein shape matches the membrane curvature (Mironov et al., 1997b; Munro, 1998). Indeed, the membrane bilayer of the Golgi complex is thought to be differently enriched in distinct lipids. Since ER is composed mainly of phospholipids and the plasma membrane is rich in sphingolipids and cholesterol, which order and thicken the bilayer, a gradient in membrane thickness is established in the Golgi. Thus, different lipid compositions would account for different thickness of the membranes and would accommodate selectively proteins with transmembrane domains of appropriate length (Bretscher and Munro, 1993). Another proposal envisages that Golgi enzymes are retained as a consequence of forming aggregates (“kin recognition” mechanism). According to this model, the enzymes
would give rise to multimeric complexes too large to enter transport vesicles and, therefore, retained in their location (Nilsson et al., 1994).

In many cases the protein localization was found to be dependent on the presence of specific sequences in the transmembrane domain, in its flanking sequences or in the short cytosolic N-terminal domain of Golgi enzymes (reviewed in Gleeson, 1998). These sequences could represent either retention or retrieval signals. Concerning the localization of soluble lumenal proteins, protein-protein and protein-lipid interactions could account for retention of these proteins (Mironov et al., 1997b).

1.1.2.6 Sorting and exit of proteins from the TGN

The TGN is the major sorting station for newly synthesized proteins and lipids in the biosynthetic pathway. Once arrived at the TGN, the cargo molecules are targeted either to the plasma membrane or to a number of compartments of the endosomal/lysosomal system or packaged into secretory granules. The transport toward the plasma membrane is commonly referred to as constitutive pathway, even if it is tightly regulated and may be controlled by extracellular stimuli (De Matteis et al., 1993; Buccione et al., 1996). In epithelial, polarized cells the Golgi-to-plasma membrane pathway is further distinguished into two routes, apical and basolateral. The different transport pathways departing from TGN should rely each on its own sorting machinery to generate specialized carriers (reviewed in Keller and Simons, 1997). The machinery that mediates basolateral sorting has not been identified yet. Sequence signals localized in the cytoplasmic portion of integral membrane proteins have been recognized as responsible for basolateral sorting at the TGN. The basolateral-directed carriers are large tubular-saccular structures (Polishchuk et al., 2000) that move along microtubules.
(Hirschberg et al., 1998; Toomre et al., 1999) and fuse with the plasma membrane in a Rab/NSF/SNAP/SNARE-dependent manner (Ikonen et al., 1995).

The apical pathway appears to be regulated by sphingolipid-cholesterol rafts, membrane microdomains formed at the TGN which would selectively include apical plasma membrane-destined cargo proteins (reviewed in Keller and Simons, 1997). The carriers mediating the transport of apical cargo appear to be distinct but morphologically similar to that containing basolateral cargo (Keller et al., 2001).

TGN is also the compartment where the biosynthetic/secretory pathway intersects the endocytic pathway. The proteins destined to lysosomes are sorted at TGN via clathrin-based machinery and are transferred to early endosomes. Once more, molecular signals are responsible for correct sorting. Soluble lysosomal hydrolases are recognized and bound by a specific receptor, the mannose-6-phosphate receptor, that mediates their segregation in clathrin-coated vesicles. Transmembrane lysosomal proteins are probably included in the same type of carriers generated by clathrin coat assembly (reviewed in Keller and Simons, 1997).

The proteins destined for regulated secretion are also sorted at TGN and included in the immature secretory granules. The mechanisms through which proteins are segregated in immature secretory granules are not known, but two alternative models have been formulated (reviewed in Tooze, 1998).

1.1.3 Molecular mechanisms of traffic

In the most traditional view of traffic along the secretory pathway, each transport step between membranes is regulated at least at four levels: 1) sorting (and eventually concentration) of cargo from resident proteins; 2) formation of the
carriers, occurring through budding and fission; 3) transport of the carriers; and 4) delivery of the cargo/carrier to the acceptor membranes, occurring through tethering and fusion.

1.1.3.1 Sorting of cargo, budding and fission

1.1.3.1.1 Coat protein complexes and the small G protein ARF and Sar1.

Sorting, the first molecular event in transport, seems to be controlled by the assembly of coat protein complexes, at least at the level of ER to Golgi transport. The coat protein complexes are also key factors for the budding of membranes, a fundamental event for the formation of carriers at the donor compartment (reviewed in Barlowe, 2000). The formation of a coated bud seems not to be sufficient to induce the fission of membranes, a process in which a fundamental part is played by lipids (Burger, 2000). Three classes of coat complexes have been well-characterized to date: clathrin, COPI and COPII coats. Based on our present knowledge, the assembly of each coat in the cell is initiated by the recruitment and activation of a specific small GTPase, that is also part of the final assembled coat. The recruitment of the GTPases and the assembly of the coat on the donor membrane is also controlled by specific signals contained in the cytosolic domain of the membrane proteins (reviewed in Springer et al., 1999).

Clathrin coats are involved in the formation of carriers that mediate transport occurring between TGN and endosomal/lysosomal compartments and from cell surface to endosomes. Clathrin coats are composed of clathrin and heterotetrameric adaptor protein (AP) complexes: AP1, AP2, AP3 and AP4 (reviewed in Kirchhausen et al., 1999).

The function of COPII seems to be restricted to a very early step of the secretory pathway, i.e. sorting and packaging of newly synthesized proteins en
route to the Golgi (Aridor et al., 1998; Bannykh et al., 1998; Stephens and Pepperkok, 2001; see also section 1.1.2.1). The COPII coat is generated by the assembly of two heterodimers, the Sec23/24 and Sec13/31 complexes, and the small GTPase Sar1 (reviewed in Schekman and Orci, 1996). The process of assembly is regulated by the activation of Sar1 GTPase, which in turn is controlled by the guanine-nucleotide exchange factor Sec12, a transmembrane protein localized to the ER (Barlowe and Schekman, 1993).

The role of COPI is much more debated. Although its molecular composition and its association to the membrane in vitro is well described, its precise role in living cells remains to be understood. Initially, experiments performed in an in vitro transport system, indicated a role for COPI in the regulation of anterograde transport from VTCs to the Golgi and through the Golgi stacks (Balch et al., 1984; Malhotra et al., 1989). This idea was generated essentially by the experimental observation that GTPγS, a non-hydrolyzable analogue of GTP that gave a strong inhibition of transport, also produced an accumulation of COPI-coated vesicles (Melancon et al., 1987; Malhotra et al., 1989). On the other hand, the model that envisages COPI mediating forward intra-Golgi traffic appears to be ruled out by more recent observations showing that the cargo is not enriched in COPI-coated vesicles (Sonnichsen et al., 1996; Martinez-Menarguez et al., 1999). There is large consensus on the fact that COPI mediates the retrograde transport from the Golgi to ER (Bannykh et al., 1998; Klumperman, 2000b; Stephens and Pepperkok, 2001). This conclusion is essentially based on the ability of coatomer to bind proteins that contain the retrieval motif KKXX (Cosson and Letourneur, 1994; Letourneur et al., 1994) and the KDEL receptor, a ER-Golgi recycling protein, involved in the retrieval of
ER proteins containing the signal sequence KDEL (Griffiths et al., 1994; Sonnichsen et al., 1996). Recent studies also indicate that COPI is required, at least indirectly, for anterograde traffic from ER to the Golgi (Scales et al., 1997; Stephens et al., 2000). Probably the importance of COPI in forward traffic to the Golgi is in the concentration of the anterograde cargo occurring at VTCs by exclusion from COPI-coated buds (Martinez-Menarguez et al., 1999). COPI has also been proposed as a negative regulator of tubule formation in the Golgi, based on the fact that several experimental conditions that inhibit COPI binding to the membranes, including treatment with the drug brefeldin A (BFA; see section 1.2), induce tubulation of the Golgi complex (Klausner et al., 1992; Cluett et al., 1993; Sciaky et al., 1997).

COPI is a 600 kDa complex composed of seven subunits (α-, β-, β'-, γ-, δ-, ε-, ζ-COP), that is present as a preassembled complex in the cytosol (Waters et al., 1991). Its recruitment to the membranes was suggested to be the key event for vesicle budding (Orci et al., 1993) and is mediated by the activated ADP-ribosylation factor (ARF; Serafini et al., 1991; Donaldson et al., 1992a). ARF, originally identified for its ability to enhance the ADP-ribosylation of the stimulatory G protein by cholera toxin (Kahn and Gilman, 1984; Tsai et al., 1987; Price et al., 1988), is a small GTPase of the Ras superfamily. The ARFs constitute a family of regulators of traffic. Six ARFs were found in mammals and three ARFs are expressed in the yeast Saccharomyces cerevisiae (reviewed in Roth, 1999). The six mammalian ARFs can be subdivided in three classes based on sequence homology. The class-I ARFs (ARFs 1-3) are currently the best understood and appear to be functionally redundant. These proteins have been shown to regulate not only COPI assembly, but also clathrin-AP1 assembly on the
TGN (Stamnes and Rothman, 1993) and clathrin-AP3 assembly on endosomes (Ooi et al., 1998). The class-II ARFs (ARF4 and ARF5) are not well known. The class-III comprises only ARF6 that is located on the plasma membrane and on endosomes and probably controls endocytosis. Yeast ARF1 and ARF2 are functionally redundant and are required for maintenance of the organization of the secretory and endocytic pathways, while the yeast ARF3, the homologue of the mammalian ARF6, is not essential for viability. The regulatory activity of ARF, similarly to other small GTPases in the cell, is fulfilled through the continuous switching between a GTP- and a GDP-bound form. In its GTP-bound state, ARF is active and membrane-bound and mediates the assembly of the coats on the membranes. In its GDP-bound state, ARF is inactive, dissociates from the membranes and the coats are thus disassembled (reviewed in Springer et al., 1999; Barlowe, 2000). The activity of ARF is in turn regulated by two factors that determine the rate of guanine nucleotide exchange (guanine nucleotide exchange factors, GEFs) and the rate of hydrolysis of GTP (GTPase activating proteins, GAPs), respectively (reviewed in Donaldson and Jackson, 2000 and Jackson and Casanova, 2000). ARF per se has very low exchange and hydrolysis activities. The first exchange activity for ARF was discovered as a Golgi membrane-associated activity and was shown to be the Brefeldin A sensitive element for membrane recruitment of ARF and COPI (Donaldson et al., 1992b; Helms and Rothman, 1992). Since then several proteins with ARF-GEF activity have been identified (reviewed in Jackson and Casanova, 2000) and some of them were shown to be inhibited by BFA (Peyroche et al., 1996; Morinaga et al., 1997; Sata et al., 1998), whereas others were shown to be BFA resistant (Chardin et al., 1996; Meacci et al., 1997; Claude et al., 1999). All the ARF-GEFs identified up
to now share a conserved 200-aminoacid domain called Sec7 domain, that is alone sufficient for the exchange activity (Peyroche et al., 1999). The sequences flanking the Sec7 domain are different, and probably confer substrate specificity, either directly or indirectly by targeting individual GEFs to specific membrane sites. Sequence comparison allowed to group the eukaryotic ARF-GEFs in 5 subfamilies (see Fig. 1.6; Donaldson and Jackson, 2000; Jackson and Casanova, 2000). ARF-GEFs are the target of the drug brefeldin A (BFA; see section 1.2) and their sensitivity to BFA is another criterion for their classification (Chardin and McCormick, 1999). As will be discussed in section 1.2.3.2, differences in BFA sensitivity are largely due to differences in sequences within the Sec7 domain (Peyroche et al., 1999; Sata et al., 1999).

ARFs are myristoylated at the amino-terminus and, when ARF is bound to the membranes, the lipid chain inserts into the lipid bilayer. The interaction with lipids appears to be essential for ARF activation. The crystal structures of GTP-bound ARF (Goldberg, 1998), GDP-bound ARF (Amor et al., 1994; Greasley et al., 1995) and nucleotide-free ARF bound to a Sec7 domain (Goldberg, 1998) have been resolved, allowing to propose a model for the mechanism of ARF activation. The conformational change that is responsible for ARF activation appears to be a consequence of ARF interaction with the Sec7 domain, and to be stabilized by the entry of GTP. Moreover, this transition controls the exposure of the myristoylated amino terminus, explaining how ARF couples the GDP–GTP conformational switch to membrane binding (Goldberg, 1998).
### Subfamilies

<table>
<thead>
<tr>
<th>Subfamilies</th>
<th>Activity is BFA</th>
<th>Localization</th>
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<tbody>
<tr>
<td>I Gea1/2 (S. cerevisiae)</td>
<td>Sensitive</td>
<td>Golgi</td>
</tr>
<tr>
<td>GBF1 (H. sapiens)</td>
<td>Resistant</td>
<td>Golgi</td>
</tr>
<tr>
<td>GNOM/Emb30 (A. thaliana)</td>
<td>Sensitive</td>
<td>ND</td>
</tr>
<tr>
<td>II Sec7p (S. cerevisiae)</td>
<td>Sensitive</td>
<td>Golgi</td>
</tr>
<tr>
<td>BIG1/2 (H. sapiens)</td>
<td>Sensitive</td>
<td>Golgi</td>
</tr>
<tr>
<td>III Syt1p (S. cerevisiae)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IV ARNO (H. sapiens)</td>
<td>CC PH</td>
<td>PM</td>
</tr>
<tr>
<td>Cytohesin-1 (H. sapiens)</td>
<td>Resistant</td>
<td>PM</td>
</tr>
<tr>
<td>GRP1/ARNO3 (M. musculus/H. sapiens)</td>
<td>Resistant</td>
<td>PM</td>
</tr>
<tr>
<td>Cytohesin-4 (H. sapiens)</td>
<td>Resistant</td>
<td>ND</td>
</tr>
<tr>
<td>V EFA6 (H. sapiens)</td>
<td>PH CC pr pr pr</td>
<td>PM</td>
</tr>
</tbody>
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**Fig. 1.6. The Sec7 family of ARF-GEFs (from Donaldson and Jackson, 2000).**

The Sec7 domain which catalyses the GTP/GDP exchange on ARF is represented in black. The DCB domain (for dimerisation and cyclophilin binding), which mediates interactions between GNOM monomers, is represented in grey. In the lower molecular weight GEFs, the pleckstrin homology (PH) domains are indicated as vertical stripes boxes, and the coiled-coil (CC) regions as diagonal stripe boxes. EFA6 contains proline-rich regions (pr). Brefeldin A (BFA) inhibits the *in vitro* ARF exchange activity of certain GEFs (indicated as Sensitive) but not of others (indicated as Resistant). The main subcellular localization of each GEF as determined by immunofluorescence is indicated. PM: plasma membrane. ND: not determined.
Also the mechanism of deactivation of ARF has been explored through the analysis of tridimensional structures (Goldberg, 1999). The crystallization and structure resolution of the complex formed by ARF1 and the catalytic domain of ARF-GAP revealed that the effector-binding site and the GAP-binding site do not overlap in the ARF molecule, in contrast to what occur for other small GTPases of the Ras superfamily. Furthermore, addition of coatomer to ARF and ARF-GAP in vitro, enormously accelerates the GTPase reaction, indicating a fundamental role of the coatomer in the inactivation of ARF and, thus, in the control of the ARF regulatory cycle (Goldberg, 1999).

1.1.3.1.2 Dynamins, dynamin-related proteins and their role in membrane fission

Another family of proteins involved in the regulation of transport intermediate formation is the dynamin family. Dynamins are 100 kDa GTPases with a multidomain structure (Fig. 1.7 A). In mammals dynamins are expressed from three distinct genes with different tissue expression: dynamin I was originally isolated from rat brain as a microtubule-binding protein (Obar et al., 1990) and shown to be a neuron-specific isoform (Cook et al., 1994); dynamin II is an ubiquitous protein (Cook et al., 1994; Sontag et al., 1994); and dynamin III is expressed in brain, testis and lung (Nakata et al., 1993; Cook et al., 1996). Each gene produces different splicing variants (Fig. 1.7 B), so that at least twenty-five different dynamin mRNAs are transcribed in brain (Cao et al., 1998). Dynamins contain five characterized domains (see Fig. 1.7 A): an N-terminal tripartite highly-conserved GTP-hydrolysis domain, a middle domain, a pleckstrin homology (PH) domain responsible for interaction with phosphatidylinositol-(4,5)-bisphosphate (PI4,5P2) and membrane targeting, a coiled-coil region that acts as a GTPase-effector domain (GED) and as a GAP domain, and a C-terminal proline-arginine-rich domain (PRD) containing several SH3-domain binding sites (reviewed in Hinshaw, 2000).
Fig. 1.7. Dynamin structure and members of the mammalian dynamin superfamily (reproduced from McNiven et al., 2000). **Panel A:** structural domain organization of dynamins. The different conserved regions of dynamins include a tripartite GTPase domain, highly conserved N- and C-middle domains, a pleckstrin homology (PH) domain, a coiled-coil (CC) domain and a proline-arginine-rich domain (PRD). **Panel B:** the mammalian dynamin superfamily include: three conventional dynamin genes (Dyn I, Dyn II and Dyn III) and the related dynamin-like protein (DLP1). Colors denote different conserved or spliced regions. Blu, splicing insertion or deletion sites; yellow, GTPase domain; red, PH domain; orange, CC domain; green, PRD.
The involvement of dynamins in intracellular membrane transport was initially suggested by the findings that the *Drosophila* protein *shibire*, whose mutation caused an altered endocytic phenotype with a block of conversion of coated pits to coated vesicles (Kosaka and Ikeda, 1983), was a homologue of dynamin (van der Bliek and Meyerowitz, 1991). More recent studies substantially supported the role of dynamin in formation of transport intermediates, in particular in membrane pinch-off, in different steps of endocytic pathways, and indicated the possibility that dynamin localizes and acts also in the secretory pathway. Indeed, different dynamin isoforms have been implicated so far in early and late events along the endocytic pathway and also in sorting events from the *trans*-Golgi network along the secretory pathway. Dynamins were shown to be involved in clathrin-coated vesicle formation from the plasma membrane (Kosaka and Ikeda, 1983; Herskovits *et al.*, 1993; Damke *et al.*, 1994), and also to play a role in caveolae-mediated internalization (Henley *et al.*, 1998; Henley *et al.*, 1999), and in phagocytosis (Gold *et al.*, 1999). Moreover, dynamin II was also shown to be localized in late endocytic compartments (Nicoziani *et al.*, 2000) and TGN (Henley and McNiven, 1996; Maier *et al.*, 1996; Nicoziani *et al.*, 2000) and to participate in vesicle formation from late endosomes (Llorente *et al.*, 1998; Nicoziani *et al.*, 2000) and from TGN (Jones *et al.*, 1998b). At least two groups showed an involvement of dynamins in the transport from the TGN. McNiven and collaborators reported that dynamin II inhibitory antibodies (Jones *et al.*, 1998b) and dynamin II mutants (Cao *et al.*, 2000) are able to interfere with transport from the Golgi complex and with the Golgi morphology, and Kreitzer *et al.* (2000) showed that dynamin II mutants blocked the formation of transport intermediates from the TGN to the plasma membrane. Although other authors showed that both
dynamin I and dynamin II mutants affect only endocytic transport without interfering with biosynthetic transport from the Golgi (Altschuler et al., 1998), the discrepancy can be reconciled considering that different dynamin II splicing variants were employed in these studies and hypothesising that each dynamin protein may act at distinct intracellular sites.

Dynamin-related proteins have been recently found in mammals. Together with dynamin-related proteins expressed in yeast, they form a subfamily of dynamin-related proteins (Yoon et al., 1998b), that includes Vps1p (Vater et al., 1992; Wilsbach and Payne, 1993) and Dnm1p (Gammie et al., 1995) from Saccharomyces cerevisiae, a dynamin-like protein from Schizosaccharomyces pombe, and the mammalian Dynamin-Like Protein 1 (DLP1 [Yoon et al., 1998b]; also called DRP1 [Imoto et al., 1998; Smirnova et al., 1998], dymple [Kamimoto et al., 1998], DVLP [Shin et al., 1997]). Members of this dynamin-related subfamily contain an N-terminal tripartite GTPase domain and a GED but do not have the PH or proline-rich domains. Mammalian DLP1 is ubiquitously expressed and, similarly to dynamin, potentially generates many splicing variants (see Fig. 1.7 B). The different dynamin and DLP1 genes present in the mammalian genome can generate a total of at least 36 differently spliced isoforms (McNiven et al., 2000). DLP1 seems to be the most related homologue of yeast Dnm1p, that has been shown to localize at the tips of dividing mitochondria as well as at mitochondrial constriction sites (Bleazard et al., 1999), suggesting that Dnm1p is involved in yeast mitochondrial fission. Similarly, DLP1 was found to participate in the maintenance of mitochondrial morphology (Smirnova et al., 1998), but also to be essential for the distribution and morphology of ER (Pitts et al., 1999).
Although dynamins are fundamental for the formation of transport carriers, the exact mechanism through which dynamin may control membrane dynamics during formation of membranous carriers is still matter of debate. Many studies support the view that dynamin works as a mechanochemical enzyme that, by hydrolysing GTP, generates the force needed to constrict and sever the membranes at the neck of clathrin-coated vesicles (Sweitzer and Hinshaw, 1998; Stowell et al., 1999; Marks et al., 2001). However, some results indicate that GTP hydrolysis is not essential for dynamin-mediated events occurring in clathrin-mediated endocytosis (reviewed in Sever et al., 2000b). In vitro studies have shown that purified dynamin is able to self-assemble in highly ordered helical structures in low-salt conditions or in the presence of GTPγS (Hinshaw and Schmid, 1995; Carr and Hinshaw, 1997). Moreover, in the absence of nucleotides, dynamin is able to transform synthetic spherical liposomes into long tubules, which have been shown by electron microscopy to be wrapped by high-order oligomers of dynamin in the form of stacks of rings resembling a helix (Sweitzer and Hinshaw, 1998; Stowell et al., 1999). In synaptic terminals GTPγS induces the formation of tubular membrane invaginations decorated by dynamin and capped by clathrin at the distal end (Takei et al., 1995). These results suggested that dynamin functions as a coat for the formation of tubules, by analogy with the roles of clathrin and COPs in forming coats for budding vesicles. Moreover, dynamin, after generating membrane tubules, is able, upon addition of GTP, to induce their constriction and fission in shorter tubules or vesicles (Sweitzer and Hinshaw, 1998; Stowell et al., 1999). Several models have been proposed to explain how dynamin works in the formation of tubules and vesicle from flat membranes (reviewed in Sever et al., 2000b and McNiven et al., 2000). The
initial idea envisaged that dynamin acted as a molecular "garrote", constricting the neck of the clathrin-coated pits following a conformational change of the helical collar, caused by GTP hydrolysis (Hinshaw and Schmid, 1995). The data from Stowell et al. (1999) indicated that the conformational change occurring during GTP hydrolysis increases the pitch of the dynamin helix, suggesting that dynamin causes release of vesicles from membranes by acting as a molecular "spring" rather than constricting. The two models are in someway reconciled by a mathematical model that, by assuming that the dynamin helix is rigid and the lipid bilayer is an elastic membrane, leads to the prediction that an increase in dynamin helix pitch gives rise to a local constriction of the tubular membrane, which would cause closure, i.e. fission (Kozlov, 1999). Another model proposes that dynamin could act as a molecular "ratchet", ratcheting one rung of a dynamin spiral along the adjacent rung through a complex multiplicity of intramolecular interactions between the GED and other internal domains, that would be controlled by GTP hydrolysis (Smirnova et al., 1999). A completely different model has been proposed recently by Schmid and collaborators, that envisages that dynamin has to be considered as a regulatory protein, like a classical GTPase (Sever et al., 1999; Sever et al., 2000a). According to their findings, the block of the dynamin-assembly-stimulated GTP hydrolysis enhances endocytosis, indicating that dynamin-GTP represents the active form of dynamin, possibly acting by recruiting downstream players involved in the formation of the clathrin-coated vesicles.

Very recently, the dynamin-related protein DLP1 has also been shown to be able to induce tubulation of membranes, in particular of ER membranes, forming a regular-spaced structure around the tubules (Yoon et al., 2001). It is
possible that each member of the dynamin superfamily specifically controls membrane dynamics and fission at distinct compartments of the cell by interacting with a subset of dynamin-interacting proteins. In this regard, dynamins are able to interact with different cellular proteins (reviewed in Hinshaw, 2000), among which many components of the protein machinery controlling clathrin-mediated endocytosis. Indeed, through its PRD dynamin is able to interact with the SH3 domain-containing proteins endophilins and amphyphysins, that were shown to play a role in the formation of clathrin-coated vesicles (Ringstad et al., 1999; Takei et al., 1999). Noteworthy, amphiphysin, like dynamin, can transform spherical liposomes into narrow tubules (Takei et al., 1999). Dynamin also binds microtubules and other cytoskeletal proteins such as cortactin, profilin and syndapin I, and these interactions suggested an essential role for the dynamin-cytoskeleton interplay in clathrin-mediated endocytosis (Witke et al., 1998), in phagocytosis (Gold et al., 1999), and in cell adhesion (Ochoa et al., 2000).

1.1.3.1.3 Other proteins involved in membrane fission

Phosphatidylinositol transfer proteins (PITPs) are soluble proteins expressed in all eukaryotic systems that have the property to transfer phosphatidylinositol (PI) or phosphatidylcholine (PC) from one membrane compartment to another (reviewed in Cockcroft, 2001). PITPs are thus responsible for the intracellular traffic of PI. PI is the major inositol-containing lipid in the cell and can be phosphorylated in several positions to generate different phosphoinositides. PITPs not only deliver PI to specific membrane sites, but also participate in phosphorylation of PI (Fensome et al., 1996). PITPs have been involved in the regulation of numerous cellular events: signal transduction, regulated exocytosis and also membrane traffic (reviewed in Cockcroft, 2001).
The importance of PITP and PI-derived lipids in intracellular traffic was initially revealed by genetic studies in yeast, where PITP (called Sec14p) is essential for formation of post-Golgi exocytic vesicles (Bankaitis et al., 1990). More recently PITPs were found to exert a powerful Golgi-vesiculating activity on mammalian Golgi membranes and was thus proposed to regulate membrane fission and formation of transport intermediates from the TGN (Jones et al., 1998a; Simon et al., 1998). Moreover, among the mammalian PITPs, the PITPβ was shown to be localized at the Golgi complex (de Vries et al., 1995). These results suggest that the regulation of the levels of phosphoinositides by PITP is fundamental for the control of membrane traffic at the Golgi complex.

Protein kinase D (PKD) has been recently proposed to regulate membrane fission (Liljedahl et al., 2001). The involvement of PKD in the fission of the Golgi had been initially suggested by studying the mechanism of action of the drug ilimaquinone (IQ). IQ is able to convert Golgi stacks into small vesicles (Takizawa et al., 1993) and this process was found to be regulated by the βγ subunit of the heterotrimeric G proteins (Jamora et al., 1997). The βγ subunit binds to the PH domain of PKD and this interaction causes vesiculation of the Golgi (Jamora et al., 1999). An inactive form of PKD produces extensive tubulation of the Golgi and inhibition of transport of the cargo from the TGN to the plasma membrane, suggesting that the function of PKD is in the regulation of the fission of the transport carriers forming at the TGN en route to the cell surface (Liljedahl et al., 2001).

1.1.3.2 Translocation of carriers and role of cell cytoskeleton in trafficking

In higher eukaryotic cells, cytoskeleton is central for both translocation of transport intermediates and for determining the characteristic, proper localization
of each intracellular organelle. Even if the ultimate importance of cell cytoskeleton in higher eukaryotic membrane trafficking is not completely clarified, the disruption of cytoskeleton, especially microtubules, affects different steps of transport and the intracellular spatial localization of different organelles. However, it has been observed that in several cases the mechanisms that govern membrane fission and membrane fusion, and not the actual carrier translocation events, seem to provide the predominant limit to trafficking (Schroer, 2000).

1.1.3.2.1 Regulation of membrane motility

Most endomembrane movements are driven by motor proteins along cytoskeleton, in particular along microtubules and actin filaments (reviewed in Rogers and Gelfand, 2000 and Schroer, 2000). Motors proteins are able to convert the chemical energy released by ATP hydrolysis directly into movement. Myosins, the actin motors, have been localized at several membranous structures. Myosin I localizes on endosomes and lysosomes (Raposo et al., 1999), myosins II (Ikonen et al., 1997; Musch et al., 1997) and myosin VI (Buss et al., 1998) have been found in association with the Golgi apparatus, and myosin V has been shown to mediate melanosome transport in melanocytes (Rogers and Gelfand, 2000).

Microtubule-based motors are classified as plus- or minus-end directed motors, where the minus ends of microtubules converge at the centrosome and the plus ends are directed toward the cell periphery (reviewed in Schroer, 2000). The predominant minus-end directed motor is dynein 1, which works with its accessory protein dynactin, a large protein complex that is required for motor activity of dynein. Perturbation of dynein function causes the redistribution of Golgi membranes and of the components of the endosome/lysosome system from their normal juxtanuclear locations toward the cell periphery (Burkhardt et al.,
1997; Harada et al., 1998). Indeed, inactivation of dynactin not only leads to disruption of the Golgi complex into scattered structures (Burkhardt et al., 1997), but also blocks the translocation of ER-Golgi transport intermediates from peripheral sites into the centrosomal region (Presley et al., 1997a), without however interfering with Golgi-to-ER traffic (Burkhardt et al., 1997). Kinesins represent a large family of proteins, many of which were clearly demonstrated to be microtubule-based motors (Schroer, 2000). The family comprises the conventional, heterotetrameric, two-headed kinesin I, the heterotrimeric kinesin II and monomeric kinesins. Kinesins have been shown to drive the outward movement of late endosomes and lysosomes (Hollenbeck and Swanson, 1990; Feiguin et al., 1994; Nakata and Hirokawa, 1995), the retrograde transport between Golgi and ER (Lippincott-Schwartz et al., 1995), and the apical plasma membrane delivery of cargo from TGN (Kreitzer et al., 2000). Microtubule-driven membrane transport might also be regulated by microtubule-associated proteins (MAPs), a family of proteins which are not motors. These proteins control microtubule stability and association of motor proteins to microtubules (reviewed in Drewes et al., 1998). Recently, overexpression of one member of the MAP family, the axon specific Tau, was found to perturb the normal distribution of mitochondria, endoplasmic reticulum and vimentin filaments (Ebneth et al., 1998; Trinczek et al., 1999). These organelles exhibited a net displacement towards the centrosome, indicating that transport toward microtubule plus-ends was impaired. Tau exerts its inhibitory effect by decreasing the frequency of movement, but not the velocities, of organelles (Trinczek et al., 1999). Also MAP2, a dendrite specific MAP, and MAP4, a non-neuronal MAP, were shown
to affect microtubule-based motility in vitro (Lopez and Sheetz, 1993) and in vivo (Bulinski et al., 1997), respectively.

Also intermediate filaments were shown to have some role in transport, especially in intracellular trafficking of lipids (Gillard et al., 1994; Gillard et al., 1998; Holwell et al., 1999). Recently intermediate filaments have been visualized in living cells through the GFP technology (Yoon et al., 1998a; Prahlad et al., 2000) and shown to exhibit continuous microtubule-dependent motility, which is inhibited by function-blocking antibodies directed against kinesin (Prahlad et al., 1998; Prahlad et al., 2000). Moreover, vimentin intermediate filaments have been recently found to interact with the Golgi complex, and rearrangements of the vimentin cytoskeleton have been shown to induce a fragmentation of the Golgi complex (Gao and Sztul, 2001).

1.1.3.2.2 Regulation of other trafficking events

In some cases the cytoskeleton function was found to be involved not only in the translocation but also in the formation of transport intermediates. In particular, actin cytoskeleton and myosin motors have been implicated in the production of transport intermediates from the TGN (reviewed in Stow et al., 1998). Myosin II (initially referred as to p200) was shown to be associated to a subpopulation of transport intermediates generated from the TGN (Narula and Stow, 1995; Ikonen et al., 1996; Musch et al., 1997) and to be required for their formation (Musch et al., 1997). Even if the exact mechanism of action of myosin II at the TGN is not known, these observations indicate that the actin cytoskeleton plays some role in the emerging of carriers from the TGN. Moreover, microtubules have been shown to be able to pull or push membranes into thin tubular extensions, a process mediated by membrane/microtubule tip attachment.
complexes (TACs) in Xenopus egg extracts (Waterman-Storer et al., 1995), suggesting that also microtubules have a role in the formation of tubular carriers.

1.1.3.2.3 Non-essential requirement of microtubules for traffic

As described in section 1.1.2.3, the depolymerization of microtubules leads to the fragmentation of the Golgi complex in numerous peripheral fragments. The nocodazole-induced peripheral Golgi structures show a polarized distribution of Golgi proteins (Iida and Shibata, 1991; Storrie et al., 1998). Moreover, cells treated with nocodazole are able to transport transmembrane and secretory cargoes to the correct destination (plasma membrane or extracellular milieu (Rogalski et al., 1984; Iida and Shibata, 1991; Cole et al., 1996a), indicating that nocodazole-induced Golgi structures are transport functional units. The appearance of peripheral Golgi membranes after exposure to nocodazole was followed in living cells (Cole et al., 1996a; Storrie et al., 1998). The structures abruptly appear at peripheral sites, and progressively accumulate Golgi enzymes. The peripheral Golgi structures are organized in stacks (also referred to as ministacks) with a polarized distribution of Golgi enzymes. The formation of ministacks depends on the integrity of the ER to Golgi transport. In fact, expression of a mutant Sar1 protein that blocks exit from ER, induces both an accumulation of Golgi resident proteins in the ER and an inhibition in the formation of ministacks (Storrie et al., 1998). All these results support the model proposing that the formation of functional ministacks in the presence of nocodazole is due to a block of the centripetal translocation of the ER-Golgi intermediates. Instead of assembling a unique central Golgi complex, cells lacking microtubules generate many Golgi units in the proximity of ER exit sites.
What is the function of microtubules in the secretory pathway? Given that the overall transport in nocodazole-treated cells seems not to be affected by this compound, several explanations could be formulated: 1) microtubule depolymerization could only initially affect the transport steps; then cells could rapidly establish a new equilibrium, based on the organization of peripheral Golgi units, which allows transport to occur in the absence of the microtubule cytoskeleton (Cole et al., 1996a). 2) a population of stable microtubules which persist in the cell after long incubation with nocodazole could be responsible for the peripheral redistribution of the Golgi complex and the trafficking of transport intermediates in the different steps of the secretory pathway (Minin, 1997; Chabin-Brion et al., 2001); 3) microtubules could be only required for the central localization of the Golgi complex, which in turn is only needed for a polarized transport to the plasma membrane in those cells in which the plasma membrane presents differentiated domains (Kreitzer et al., 2000).

1.1.3.3 Tethering and fusion

The specificity of membrane fusion is critical to preserve organelle identity and the proper flow of cargo within the secretory and endocytic pathways. Many proteins are involved in this process and ensures the specificity and precision of the fusion event. The first specific event is the Rab-mediated tethering of the transport carrier to the correct target compartment (reviewed in Zerial and McBride, 2001). Then, the pairing of SNAREs (Soluble N-ethyl maleimide-sensitive fusion protein (NSF) Attachment protein Receptors) between the two bilayers allows the occurrence of the fusion event bringing the two bilayers in close contact and generating the driving force needed to fuse lipid bilayers (reviewed in Chen and Scheller, 2001). Rab proteins constitute the largest
family of small GTPases. Eleven Rabs are expressed in yeast while 63 members have been estimated to be expressed in human, based on expressed-sequence tags (ESTs) and sequenced human genome. Several Rabs have been already characterized and shown to selectively localize to specific compartments (Zerial and McBride, 2001). Similarly to other GTPases, Rabs exert their regulatory function through the molecular switch between the GTP- and the GDP-bound conformations (Chavrier and Goud, 1999). Activated, GTP-bound Rabs bind to soluble factors that are referred to as Rab effectors. Many of them have been identified and shown in several cases to regulate tethering and fusion. Examples of well studied tethering-regulating Rab effectors are: 1) p115 and GM130, Rab1 effectors that are supposed to regulate fusion of ER-Golgi transport intermediate on the cis-Golgi compartment (Allan et al., 2000; Moyer et al., 2001); 2) the early endosome antigen 1 (EEA1), a Rab5 effector that mediates the tethering of early endosomes in the homotypic early endosome fusion (Christoforidis et al., 1999). These proteins all contain predicted coiled coil regions, structural domains involved in oligomerization and pairing of proteins, that have been proposed to bridge two different membranous structures during tethering. Other Rab effectors were also shown to regulate transport intermediate motility along microtubules. One such effector is the Rab6 effector Rabkinesin 6, a homologue of kinesins, that would regulate microtubule-dependent transport of carriers generated at the Golgi complex (Echard et al., 1998).

1.1.3.4 Role of lipids in trafficking

Lipids contained in the membranes of the secretory pathway have different roles. In part they are newly-synthesized lipids in transit, in part fundamental structural components of the membrane bilayer and in part regulatory and
signalling molecules that cooperate with proteins for the activities of the secretory pathway. Lipids generate a gradient of membrane bilayer thickness along the secretory pathway due to a gradual increase in concentration of sphingomyelin and cholesterol from the ER to the plasma membrane (van Meer, 1998; see also section 1.1.2.5). Moreover, lipids can form membrane microdomains by concentrating in specific sites, and the lipid composition of the bilayer can also regulate the shape and the geometry of the membranes, participating in this way in the segregation and sorting of proteins, as discussed in sections 1.1.2.5 and 1.1.2.6, but also in the fission and fusion of membranes (Chernomordik et al., 1995; Burger, 2000).

Fusion and fission are processes involving three different stages: membrane adhesion, semifusion and pore formation (reviewed in Burger, 2000). While proteins determine the specificity and probably participate in the induction of membrane fusion and membrane adhesion, lipids are key determinants in the formation of the semifusion intermediates that are generated during fusion and fission of the membranes. Biological lipids can be classified on the basis of their molecular shape and structure in which they organize in aqueous environment (Fig. 1.8). Conventionally, lipid molecules with an inverted cone-like shape are defined as those of positive spontaneous curvature, while the cone-shaped lipids have a negative spontaneous curvature. Indeed, as components of a membrane, lipids confer to membrane a spontaneous curvature, which describes the intrinsic tendency of the membrane to bend. A positive spontaneous curvature of a biological membrane corresponds to its bending toward the cytoplasm; conversely a negative spontaneous curvature describes the tendency of the membrane to bend towards the lumen or the extracellular medium (Fig. 1.9 A).
Fig. 1.8. Molecular shape and aggregation state of biological lipids (modified from Burger, 2000). Biological lipids are classified on the basis of their molecular shape or geometry. The aggregation state to which lipids give rise in aqueous solution is a complex function of their structure and charge. However, in most cases the type of aggregate formed by a lipid can be predicted on the basis of its geometry (Lichtenberg, 1993). Lipids with $V<s_l$ are referred to as inverted cone or type I lipids and aggregate in micellar structures (structures with positive curvature). Lipids with a molecular volume ($V$) equal to the product of the surface area of its polar group ($s$) and the length of its hydrocarbon chain ($l$), $V=s_l$, are referred to as cylindrical or bilayer-preferring lipids and aggregate in a flat surface to form bilayers. Lipids with $V>s_l$ are referred to as cone-shaped or type II lipids and form hexagonal II phases (structures with negative curvature).
Fig. 1.9. *Spontaneous curvature of lipid bilayers*. **Panel A**: schematic drawing of membrane spontaneous curvature and bending. When the cytoplasmic monolayer is enriched in cone-shaped lipids or the lumenal/external monolayer is enriched in inverted cone lipids, the bilayer acquires a negative spontaneous curvature and bends toward the organelle lumen or the outside of the cell. Conversely, when the cytoplasmic monolayer is enriched in inverted cone lipids or the lumenal external monolayer is enriched in cone-shaped lipids, the membrane acquires a positive spontaneous curvature and bends toward the cytoplasm. **Panel B**: diagrams showing contact between bilayers during the membrane fission occurring at intracellular tubules (on the left) or at the plasma membrane (on the right).
A biomembrane consists of a mixture of bilayer preferring lipids (cylindrical lipids) and type II lipids (cone-shaped lipids), whereas type I lipids (inverted cone lipids) are very rare. The semifusion intermediate has a strong net negative (concave) monolayer curvature. Type II lipids with their negative curvature preference, facilitate the formation of highly-curved, concave semifusion intermediates and stimulate membrane fusion. In contrast, type I lipids would strongly prevent membrane fusion. Membrane fusion has been studied in different biological and pure lipid model systems. Type II lipids, such as diacylglycerol or unsaturated fatty acids, were shown to induce fusion in model membranes, while type I lipids, such as lysophosphatidylcholine (LPC), were shown to inhibit biomembrane and model membrane fusion even at very low concentrations. Moreover, the ratio of bilayer to non-bilayer preferring lipids was shown to be under strict metabolic control, indicating a great importance of the existence of a spontaneous negative curvature in the biological membranes.

Membrane fission could be considered to a certain extent, as a membrane fusion in which the membrane leaflets that initially contact are the plasma membrane external (or intracellular lumenal) leaflets rather than the cytosolic leaflets (see scheme in Fig. 1.9 B). However, the different topology implies big differences: 1) cytosolic factors can only act on the leaflet opposite to the one that initially interact and semifuse; 2) the close membrane contact that is the first event of the process requires a strong membrane bending and the formation of highly constricted neck (Burger, 2000). The extreme curvature of the neck can be generated by a difference in lateral pressure between the two membrane leaflets (transbilayer area asymmetry), that results in membrane deformation, or by a difference in spontaneous monolayer curvature between the two membrane
leaflets (transbilayer curvature asymmetry). Transbilayer area asymmetry can be generated by insertion or modification of lipids or, also, insertion of proteins. Lipid translocation, lipid metabolism, or spontaneous transbilayer movement (flipflop) of phospholipid-breakdown products, such as ceramide and diacylglycerol, can produce transbilayer area asymmetry or transbilayer curvature asymmetry, by selectively changing the area or the shape of lipid components of one leaflet. Thus, proteins controlling the movement of lipids or their metabolism could play an essential role in controlling membrane fission. This has been shown for several proteins: PITP (see section 1.1.3.1.3), that controls the scission of COPI-coated vesicles and uncoated vesicles formed at TGN, probably through the delivery of PI to specific sites of TGN membranes and increasing the amounts of specific PI metabolites required for fission (Jones et al., 1998a; Simon et al., 1998); endophilin (Schmidt et al., 1999) and BARS (see chapter 3 of this thesis and Weigert et al., 1999), that induce fission at the plasma membrane and at the Golgi complex respectively, possibly through the conversion of lysophosphatidic acid into phosphatidic acid.

1.2 Brefeldin A

1.2.1 Overview

Brefeldin A (BFA) is a fungal macrocyclic lactone (Harri et al., 1963) that induces a rapid and reversible block of secretion (Misumi et al., 1986; Fujiwara et al., 1988). For the last 15 years this toxin has been widely used to try to elucidate the molecular mechanisms of transport. The best known effect of BFA is a dramatic morphological reorganization of the Golgi apparatus and the redistribution of both resident and cargo proteins from Golgi to ER (Fujiwara et
al., 1988; Doms et al., 1989; Lippincott-Schwartz et al., 1989; Sciaky et al., 1997). However, BFA also affects the morphology and function of the endosomal/lysosomal compartments (Lippincott-Schwartz et al., 1991; Klausner et al., 1992).

1.2.2 Effect of BFA on the Golgi morphology

The redistribution of the Golgi proteins induced by BFA is very rapid, occurring in a few minutes, and is an energy-dependent process (Doms et al., 1989). Moreover, the effect of BFA is completely reversible, and recovery of both Golgi morphology and ability to transport occurs in a few minutes after removal of the drug (Doms et al., 1989). An ultrastructural analysis of the alterations induced by BFA was performed both in vitro (Orci et al., 1991) and in vivo (Lippincott-Schwartz et al., 1990). By using cell-free Golgi membranes, Orci et al. (1991) found that BFA inhibited the formation of COPI-coated vesicles and transformed the normal Golgi stacks into an anastomosing network of tubules (Fig. 1.10). In vivo, in the presence of intact microtubules, BFA induces the complete redistribution of Golgi into ER, through the early formation of tubular processes extending out of the Golgi along microtubules (Lippincott-Schwartz et al., 1990). When the BFA-mediated Golgi redistribution to ER was analysed in living cells through time-lapse microscopy (Sciaky et al., 1997), it became clear that the process occurs in two steps: 1) the formation of an extensive network of tubules departing from the Golgi toward the cell periphery, and 2) a sudden delivery of Golgi materials into the ER.
Fig. 1.10. Golgi-derived, BFA-induced tubular networks (reproduced from Orci et al., 1991). Isolated Golgi membranes incubated with 150 μM BFA for 20 minutes were analyzed by electron microscopy. In A a conventional Epon section is shown, while in B a Lowicryl section was immuno-labelled for the secretory protein VSVG. The pictures show the tubular- reticular network (labelled by gold particles in B) into which the Golgi is converted following the treatment with BFA.
The Golgi disassembly process was recently reproduced in permeabilized CHO cells in order to define the minimal requirement for each step (Kano et al., 2000). The first step, consisting in tubule extension from the Golgi, requires microtubule integrity or exogenously added cytosol; the second, that is the fusion of the tubules with ER, requires cytosol and the active fusion factor NSF, but is independent of microtubules. In intact cells, the overall process does not have an absolute requirement for microtubules, but is significantly slowed in their absence, suggesting that the emission of tubules that brings Golgi and ER membranes in contact increases the probability of fusion between these membranes (Sciaky et al., 1997).

The tubules that emanate from the Golgi after treatment with BFA, appear not to be a unique feature of BFA-treated cells. In fact, in living cells transfected with chimeric Golgi proteins fused to GFP, tubules emanating from the Golgi, containing both recycling proteins, resident proteins and lipids were observed (Sciaky et al., 1997). These tubules appeared as very dynamic: they extended rapidly from the Golgi, broke off, and often continued to move toward the cell periphery. They moved along curvilinear tracks at average rates of 0.6 μm/s, and were dependent on the presence of intact microtubules (Sciaky et al., 1997). Tubules budding from the Golgi cisternae in BFA-treated cells, analysed dynamically, were shown to be not very different in terms of rate of extension from normal tubules observed in untreated cells. Nevertheless, BFA-treated cells showed an increased rate of tubule formation, which appeared more numerous, stable and long (Sciaky et al., 1997). These studies confirmed previous indications that BFA strongly enhances the physiological process of retrograde transport from Golgi to ER. Noticeably, not all cell types are equally sensitive to
BFA, and some (such as MDCK and PTK1 epithelial cells) are completely resistant in terms of both morphological effects on the Golgi complex and functional inhibition of secretory transport (Jackson, 2000).

1.2.3 Effect of BFA on the endo-lysosomal system

TGN, the most trans-part of the Golgi that is a constituent of both secretory and endocytic pathways, does not follow the fate of the rest of the Golgi fusing into the ER upon BFA treatment, but in most cases it extends long tubules that fuse with the early or recycling endosomes (Lippincott-Schwartz et al., 1991; Wood et al., 1991; Reaves and Banting, 1992). Other compartments of the endo-lysosomal system, such as lysosomes and early and recycling endosomes, are tubulated in response to BFA (Lippincott-Schwartz et al., 1991; Wood et al., 1991). However, in many cases in which the organelle morphology was clearly disrupted by BFA, endocytic function appeared unaffected (Wood and Brown, 1992). In polarized cells BFA was found to block apical targeting (Low et al., 1991) and transcytosis from the basal to the apical membrane (Hunziker et al., 1991; Low et al., 1992). Given that compartments of the endocytic pathway are naturally tubulated at least in certain cell types, BFA accentuates a normal process, and only in some cases disrupts the internal equilibrium that allows transport to occur (Jackson, 2000).

1.2.4 Molecular mechanism of action

1.2.4.1 Inhibition of COPI coat assembly

One of the earliest and clearest event induced by BFA is the detachment of the coatomer and the small G protein ARF from the Golgi membranes and the inhibition of formation of COPI-coated vesicles (Donaldson et al., 1990;
Donaldson et al., 1991; Orci et al., 1991). Redistribution of the 110-kD subunit β-COP is a very rapid event that begins after only 30 seconds, is completed after 2 minutes, and is completely reversed after removal of the drug (Donaldson et al., 1990). Thus, the inhibition of coatamer association to the Golgi membranes and the consequent inhibition of COPI-coated vesicle formation could be responsible for at least part of the effects of BFA on the transport pathways. The fact that the role of COPI is still not completely clarified complicates the interpretation of the action of BFA. As mentioned earlier, based on the effect of BFA, COPI has been proposed to control the equilibrium between tubulation and vesiculation of the Golgi membranes. The coincidence between tubulation of Golgi and detachment of COPI could indicate a specific role of COPI coat in preventing the extensive tubulation or promoting the detachment of tubules (Klausner et al., 1992; Sciaky et al., 1997).

### 1.2.4.2 Inhibition of ARF-guanine nucleotide-exchange activity

The first target of BFA to be discovered was the reaction of exchange of guanine nucleotides on ARF. Indeed, BFA was found to specifically inhibit the Golgi membrane-induced binding of GTP to ARF (Donaldson et al., 1992b; Helms and Rothman, 1992). As discussed in section 1.1.3.1.1, many ARF-guanine nucleotide-exchange factors (ARF-GEFs) have been so far identified and they have different sensitivity to BFA. Recently the molecular basis for the BFA-inhibition of ARF exchange activity and thus the bases for the different BFA-sensitivity of different classes of ARF-GEFs were clarified (Peyroche et al., 1999). Mutagenesis analysis of the GEA1 gene of *Saccharomyces cerevisiae*, showed that mutations conferring BFA resistance reside in a region of the Sec7 domain overlapping the ARF-binding site. Moreover, this region contained
residues differing between members of BFA-resistant and BFA-sensitive GEFs, but that are conserved among members within each subfamily (see Fig. 1.6). Peyroche et al. (1999) also found that BFA acts as an uncompetitive inhibitor: thus, rather than binding to the Sec7 domain of the GEF, BFA binds to the transient ternary complex formed by ARF, GDP and the Sec7 domain and stabilizes this complex. Therefore, BFA forms a stable abortive complex with ARF and its exchange factor, also subtracting exchange factor molecules for exchange reaction of other ARF molecules, i.e. makes endogenous ARF-GDP behave as a "dominant negative" (Chardin and McCormick, 1999).

1.2.4.3 Other processes affected by BFA

ARF not only controls COPI assembly, but also other events occurring in trafficking through the binding with a number of effectors (reviewed in Donaldson and Jackson, 2000). So, BFA effects on the morphology and function of the Golgi complex as well as of other compartments of the transport pathways, might in principle rely also on ARF functions different from the regulation of COPI coat assembly. Other proteins are detached from the Golgi very early upon BFA treatment. ARF effectors Arfaptins (Kanoh et al., 1997), the small GTPase of the Rho family Cdc2 (Erickson et al., 1996), spectrin (Beck et al., 1994; Godi et al., 1998), ankyrin (Beck et al., 1997) are all localized at the Golgi in an ARF-dependent manner and dissociate from the Golgi with a time-course similar to that of ARF and COPI. Moreover, AP1, AP3, AP4 are all rapidly dispersed after treatment with BFA (Hirst and Robinson, 1998), and at least for AP1 and AP3, given that their association to the membranes is due to ARF, the release is mediated by the BFA effect on ARF (Hirst and Robinson, 1998; Ooi et al., 1998). Other proteins are rapidly detached from the Golgi by BFA treatment but their
association with the Golgi is not demonstrated to directly depend on ARF. They include among others the oxysterol binding protein (Levine and Munro, 1998), the TGN-associated proteins p200/myosin II (Narula et al., 1992; Ikonen et al., 1997; Musch et al., 1997) and p230 (Gleeson et al., 1996), which are, thus, possible targets of BFA in the endocytic/secretory pathways.

An ARF- and coatomer-independent molecular event induced by BFA is the ADP-ribosylation of two cytosolic proteins of 50 and 38 kDa (De Matteis et al., 1994; Di Girolamo et al., 1995). The ADP-ribosylation of the 50 kDa protein, as will be discussed in section 1.3.5, is an essential event for the Golgi tubulation induced by BFA (Mironov et al., 1997a).

1.3 Mono-ADP-ribosylation of cytosolic proteins and its role in intracellular membrane traffic

1.3.1 Overview

Mono-ADP-ribosylation is a post-translational modification of proteins catalysed by enzymes called mono-ADP-ribosyltransferases (mADPRT; reviewed in Haag and Koch-Nolte, 1998; Okazaki and Moss, 1999; Ziegler, 2000). It consists in the transfer of an ADP-ribose residue from the substrate NAD$^+$ to a specific aminoacid of a cellular protein (Fig. 1.11). Like other post-translational modification of cellular proteins, mono-ADP-ribosylation appears to be involved in the modulation of the activity of the protein substrates.
Fig. 1.11. Enzyme activities of mono-ADP-ribosyltransferases and ADP-ribosylarginine hydrolases. ADP-ribosyltransferases transfer the ADP-ribose moiety of NAD⁺ to a specific aminoacid of an acceptor protein. In the presence of H₂O, transferases hydrolyze NAD⁺ to nicotinamide and ADP-ribose. The action of ADP-ribosylarginine hydrolases, which hydrolyze ADP-ribosylarginine and regenerate free arginine, is consistent with the presence of an ADP-ribosylation cycle.
1.3.2 Bacterial mADPRTs

The best known mADPRT are bacterial toxins (Moss and Vaughan, 1988). Pertussis toxin, the major virulence factor of *Bordetella pertussis*, the etiological agents of whooping cough, and cholera toxin, produced by *Vibrio cholerae*, that is responsible for cholera, both ADP-ribosylate the $\alpha$ subunit of the heterotrimeric GTP binding proteins and lead to an increase in cAMP levels. Pertussis toxin selectively modifies a specific cysteine residue of the $\alpha_i$ and $\alpha_o$ subunit abolishing their inhibitory interaction with adenylyl cyclase, while cholera toxin modifies an arginine residue of the stimulatory $\alpha$ ($\alpha_s$) subunit, abolishing its GTPase activity and thus leading to a constitutive activation of the effector adenylyl cyclase. Another bacterial toxin with mADPRT activity, which was the first to be discovered, is the diphtheria toxin produced by *Corynebacterium diphtheriae*, that shuts down protein synthesis in human cells by specifically ADP-ribosylating diphtamide, a modified histidine of elongation factor 2. Also *Clostridium botulinum* produces mADPRTs: the C2 toxin that ADP-ribosylates actin monomers on arginine residues, thereby inhibiting actin polymerization, and the C3 exoenzyme that modifies an asparagine residue of the small GTP-binding protein Rho, affecting the regulation of actin cytoskeleton.

1.3.3 Endogenous mADPRTs in vertebrates

Mono-ADP-ribosylation was more recently recognized as an important endogenous reaction in the regulation of eukaryotic cell functions. By incubation of cellular extracts with radiolabelled [adenylate $^{32}$P]-NAD, many protein targets for endogenous ADP-ribosylation have been identified and include, among others, integrins (Zolkiewska and Moss, 1993; Nemoto *et al.*, 1996), actin (Clancy *et al.*, 1996), among others.
1995), desmin (Huang et al., 1993), myelin basic protein (Boulias and Moscarello, 1994), fibroblast growth factor-2 (Boulle et al., 1995), the neuronal phosphoprotein GAP-43 (Coggins et al., 1993), the β subunit of heterotrimeric G proteins (Lupi et al., 2000), the 38 kDa and 50 kDa BFA-ADP-ribosylated substrates (De Matteis et al., 1994; see also section 1.3.4).

Several enzymes catalysing ADP-ribosylation in eukaryotic cells were purified in the last decade mainly from muscle and haematopoietic tissues. So far 7 related genes expressing mADPRT have been identified in vertebrates (Table 1.1). The proteins coded by these genes share a conserved catalytic domain and constitute a new family of proteins. All of the so far cloned mADPRTs have an N-terminal hydrophobic signal peptide and are thus predicted to be all either membrane bound or secreted. Five of them (ART1, ART2A, ART2B, ART3, ART4) also contain a C-terminal signal for GPI-linked anchorage and are thus predicted to be extracellularly-located, plasma membrane-anchored proteins. In all the cases in which the aminoacid specificity has been analysed, these enzymes were found to modify specifically arginine residues. A major characteristic of this family of proteins is the highly specificity of tissue expression (see Table 1.1). Since all the enzymes so far cloned are predicted to act extracellularly, but several intracellular substrates for mADPRT have been identified, other enzymes, probably not related in sequence to the family discussed above, remain to be cloned.

The reversibility of ADP-ribosylation is assured by the existence of ADP-ribosylarginine hydrolases (Okazaki and Moss, 1999) that remove the ADP-ribose moiety from ADP-ribosylated arginine residues. In analogy to phosphorylation/dephosphorylation cycles that regulates a large number of intracellular processes, a regulatory ADP-ribosylation/deribosylation cycle (see Fig. 1.11) has been proposed.
Table 1.1. Cloned vertebrate members of the mono-ADP-ribosyltransferase gene family (from Haag and Koch-Nolte, 1998)

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Alternate names</th>
<th>Species</th>
<th>Preferred site of expression</th>
<th>Predicted cellular localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>ART1</td>
<td>MART, Yac-1</td>
<td>human, mouse, rat, rabbit</td>
<td>skeletal and cardiac muscle</td>
<td>cell surface</td>
</tr>
<tr>
<td>ART2A, ART2B</td>
<td>Rt6-1, Rt6-2</td>
<td>human (pseudogene) mouse, rat</td>
<td>peripheral T cells, intraepithelial lymphocytes NK cells</td>
<td>cell surface</td>
</tr>
<tr>
<td>ART3</td>
<td>htMART, TART1</td>
<td>human, mouse, rat, rabbit</td>
<td>testis</td>
<td>cell surface</td>
</tr>
<tr>
<td>ART4</td>
<td>LART</td>
<td>human, mouse, rat, rabbit</td>
<td>lymphatic tissue</td>
<td>cell surface</td>
</tr>
<tr>
<td>ART5</td>
<td>TART2, Yac-2</td>
<td>human, mouse, rat, rabbit</td>
<td>testis</td>
<td>secreted</td>
</tr>
<tr>
<td>ART6A, ART6B</td>
<td>AT1, AT2</td>
<td>chicken</td>
<td>bone marrow, heterophils</td>
<td>secreted (granules)</td>
</tr>
<tr>
<td>ART7</td>
<td>CEAT</td>
<td>chicken</td>
<td>erythroblasts</td>
<td>secreted</td>
</tr>
</tbody>
</table>
The mono-ADP ribosylation was demonstrated to be an important regulatory process in the immune system. In lymphocytes, for example, mADPRT modulates signal transduction initiated by extracellular cross-linking of the T cell receptor, through the ADP-ribosylation of integrin receptors (Nemoto et al., 1996) and a 40 kDa protein able to regulate the activity of the tyrosine kinase p56\textsuperscript{lc}k (Wang et al., 1996).

1.3.4 BFA-dependent ADP-ribosylation

As anticipated in section 1.2.3.3, BFA induces the ADP-ribosylation of two cytosolic substrates of 38 and 50 kDa (Fig. 1.12; De Matteis et al., 1994). The 38 kDa was demonstrated to be identical to an isoform of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an enzyme with multiple cellular functions (Sirover, 1999). The 50 kDa substrate, called BARS-50 or BARS, was found to be present in the cytosol in its ADP-ribosylated form in two main complexes of 130 kDa and 170 kDa (Di Girolamo et al., 1995). The electrophoretic mobility of BARS in SDS-PAGE is markedly affected by the presence of urea. The apparent size of the protein is 46 kDa in the absence of urea and 50 kDa in the presence of 4M urea, where the protein is occasionally visible as a tight doublet. When the protein was analysed by bidimensional isoelectric focusing-SDS/polyacrylamide gel electrophoresis (2D IEF–SDS/PAGE) the tight doublet was resolved in 11 distinguishable spots with isoelectric point (pI) values ranging from 6.55 to 6.1. BARS is able to bind GTP and the BFA-dependent ADP-ribosylation of BARS is inhibited in a dose-dependent manner by the $\beta\gamma$ subunit of heterotrimeric G proteins, suggesting that it was a novel, unconventional G protein (Di Girolamo et al., 1995).
Fig. 1.12. BFA induces the ADP-ribosylation of two ubiquitous cytosolic proteins. Cytosol from rat lung (lane 1), liver (lane 2), spleen (lane 3), and brain (lane 4) were $[^{32}\text{P}]$ADP-ribosylated in the presence of BFA and membranes from brain (see also section 2.6), separated by SDS-PAGE and revealed by autoradiography. ADP-ribosylation reaction performed in the absence of cytosol is shown in lane 5.
The enzyme catalysing the BFA-stimulated modification of GAPDH and BARS was found to be membrane-bound (Weigert et al., 1997), but it has been not yet identified. The reaction was studied by employing as substrate pure GAPDH (De Matteis et al., 1994; Weigert et al., 1997). BFA activates the mono-ADP-ribosylation of GAPDH (and BARS) by increasing the $V_{\text{max}}$ but not the $K_M$ for NAD$^+$. The BFA-stimulated ADP-ribosylation is inhibited by Zn$^{2+}$ and Cu$^{2+}$, whereas other divalent cations such as Ca$^{2+}$, Mg$^{2+}$ and Mn$^{2+}$ are inactive. Inhibitors of the reaction were also identified: dicumarol, a well-known coumarin inhibitor that prevents the reduction of vitamin K by binding to the NAD(P)-binding site of the oxidoreductase that catalyse two steps of the vitamin K cycle (Ma et al., 1992), and ilimaquinone, a metabolite isolated from a marine sponge that causes a reversible breakdown of the Golgi complex (Takizawa et al., 1993; see also section 1.1.3.1.3).

1.3.5 Role of the 50 kDa BFA-ADP-ribosylated substrate in the toxic effect of BFA

BARS was demonstrated to be one of the targets of BFA for its action on the Golgi apparatus (Mironov et al., 1997a). Streptolysin-O (SLO)-permeabilized RBL cells and dialyzed rat brain cytosol were used to study the role of NAD$^+$, ADP-ribosylation and BFA-ADP-ribosylated substrates in the BFA toxic effect. First, an essential requirement for NAD$^+$ in the BFA-dependent disassembly of the Golgi complex was demonstrated. In fact, in the absence of NAD$^+$ BFA lost its activity even if used at concentrations up to 100-fold higher than the half-effective concentration in intact cells, while the addition of NAD$^+$ to the system restored the ability of the toxin to induce the redistribution of the Golgi complex,
albeit to a lower potency than in intact cells. Second, the presence of NAD$^+$ was demonstrated to be required in the BFA action exclusively in order to allow ADP-ribosylation of cytosolic substrates. Indeed, pre-ADP-ribosylated cytosol supported the BFA-induced Golgi transformation also in the absence of NAD$^+$. Finally, the role of the two cytosolic BFA-ADP-ribosylated substrates GAPDH and BARS was tested. While the addition of pure GAPDH together with pre-ADP-ribosylated cytosol to the NAD$^+$-deprived cell system did not change the BFA effectiveness in disassembling the Golgi complex, the addition of a cytosolic preparation enriched in native BARS reversed the effects of ADP-ribosylated cytosol, i.e. blocked the BFA action on the Golgi. These results can be interpreted in only one way: BARS, in the native form, acts to prevent the Golgi disassembling induced by BFA and ADP-ribosylation inactivates the protein. The cytosolic redistribution of β-COP, that is one of the early events produced by BFA, is independent of NAD$^+$, occurring to the same extent both in the absence and in the presence of NAD$^+$. This finding, together with the previous results, indicated that detachment of COPI is not sufficient to induce the redistribution of the Golgi complex. Thus, ADP-ribosylation of BARS is essential for BFA-induced tubulation, probably through the inactivation of the physiological functions of BARS. The demonstration that BARS is a fundamental target for the effect of BFA on the Golgi complex, also implicated that BARS might control the structure and possibly also the function of the Golgi complex.

1.4 Main findings of this thesis

In order to understand the molecular mechanisms that regulate the Golgi structure and function, I set out to identify BARS and characterize its function and
localization. In chapter 3 I report the cloning of BARS and its sequence analysis and show that BARS belongs to the CtBP family of proteins and for this reason is renamed CtBP3/BARS. The study of its action at the level of the Golgi membranes showed that this protein is able to induce membrane fission and to catalyse the conversion of lysophosphatidic acid (LPA) into phosphatidic acid (PA), and indicates that the acyltransferase activity is part of the molecular mechanism that leads to membrane fission. In chapter 4 I report that CtBP3/BARS is mainly localized in the cytoplasm and enriched at the Golgi complex and other membranous compartments. A similar subcellular localization was also observed for CtBP1, while CtBP2 was only found to be localized in the nucleus. To better clarify the mechanism of action of CtBP3/BARS in membrane fission, I set out to isolate CtBP3/BARS-interacting proteins with the aim to identify other molecular players acting in the CtBP3/BARS-induced membrane fission. As reported in Chapter 5, I found that both rat brain BARS and recombinant CtBP3/BARS are able to interact with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), microtubules, and intermediate filaments. The possible roles of these interactions in membrane fission are discussed.
CHAPTER 2

Materials and Experimental Procedures

2.1 Materials

Sodium dodecyl sulphate (SDS), glycine, DL-dithiothreitol (DTT), TRIZMA base, potassium acetate, magnesium acetate, Tris[Hydroxymethyl]aminomethane (Tris), piperazine-1,4-bis(2-ethane-sulfonic acid (PIPES), 2-morpholino-ethane-sulfonic acid (MES), ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), KH$_2$PO$_4$, Na$_2$HPO$_4$, NaH$_2$PO$_4$, MgSO$_4$, ammonium sulphate, sucrose, bovine serum albumine (BSA), aprotinin, leupeptin, pepstatin, 1,10-phenanthroline, phenylmethylsulphonyl fluoride (PMSF), sodium azide, sodium borate, sodium deoxycholate, ATP and GTP were from Sigma-Aldrich (WI, USA). NaCl, HCl, NaOH, KOH, NH$_4$Cl, glacial acetic acid, chlorophorm and trichloroacetic acid (TCA) were from Carlo Erba (Italy). 4-(2-Hydroxy-ethyl)-piperazine-1-ethane-sulfonic acid (HEPES), glycerol, KCl, MgCl$_2$, and CaCl$_2$ were from Merck (Germany). Triton X-100 was from Bio-Rad Laboratories (UK). Rotors and centrifuges were from Beckman (CA, USA). Other materials will be specified for each procedure.
2.2 Cloning and sequence analysis of BARS

2.2.1 Nucleotide and protein database analysis

Nucleotide and peptide sequences were analysed for similarity with all the available database sequences using the similarity search program BLAST® (Basic Local Alignment Search Tool) at the National Center for Biotechnology Information (NCBI) site (http://www.ncbi.nlm.nih.gov/BLAST). Pair alignments were produced using Gap, a program included in the Genetics Computer Group (GCG) software package (Accelrys Inc.) or using ‘BLAST 2 Sequences’ tool of BLAST at NCBI site. ProfileScan analysis of BARS was performed at the Swiss Institute for Experimental Cancer Research (ISREC) site (http://hits.isb-sib.ch/cgi-bin/PFSCAN). Multiple alignments were generated using the program ClustalW at the European Molecular Biology Laboratory (EMBL) site (http://www2.ebi.ac.uk/clustalw) and coloured or shaded for displaying using MacBoxshade 2.15.

2.2.2 Generation of probes

2.2.2.1 mRNA extraction

mRNA was obtained from 0.5 gr. of male rat brain using Quickprep Purification Kit (Amersham Pharmacia Biotech, NJ, USA) following the manufacturer's instructions.

2.2.2.2 Reverse transcriptase – Polymerase Chain Reaction (RT-PCR)

mRNA was reversed transcribed with Murine Reverse Transcriptase (M-MuLV) by using First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, NJ, USA). Random hexadeoxyribonucleotides and d(T)\textsubscript{18} primers were
both used to efficiently reverse transcribe also the 5' ends of mRNA. All the reagents, except mRNA, were included in the commercial kit. mRNA was diluted to 0.1 μg/μl with RNAse-free water, incubated at 65°C for 10 min and chilled on ice. Two distinct reactions were performed by mixing 11 μl of Bulk First-Strand Reaction Mix, 1 μl of DTT solutions and 1 μl (0.2 μg) of hexameric primers or 1 μl (0.2 μg) of d(T)_{18} primers, then adding 20 μl of heat-denatured RNA, and incubating at 37°C for 1 hour. At the end the two reactions were incubated 5 min at 90°C to inactivate the enzyme and then mixed to obtain a single pool of cDNA first strands.

The cDNA first strand resulting from reverse transcription was stored at -20°C or directly subjected to Polymerase Chain Reaction (PCR). Amplifications were performed by using as primers two pairs of partially degenerate oligonucleotides, designed on the bases of 4 peptide sequences obtained from BARS microsequencing and their alignment with CtBP (see Fig. 3.1). In order to design the primers, the aminoacid sequences were backtranslated using Backtranslate, a program included in the GCG software package (Accelrys Inc.). The degree of degeneracy was reduced by considering only the most probable codons, in such a way that the number of combinations was comprised between 36 and 108. The degenerate oligonucleotides and the peptides from which they were derived are listed in Table 2.1.
Table 2.1. Partially degenerate primers used for the amplification of BARS-specific probes.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide/peptide sequence</th>
<th>Peptide</th>
</tr>
</thead>
</table>
| Probe-1 forward | 5'-GCH ACH GTG GCH TTY TGY GA-3'      
a la thr val ala phe cys asp | 36-2    |
| Probe-1 reverse | 3'-CCB AAR TTG CAC GAV AAG ATR C-5'   
gly phe asn val leu phe tyr asp | 61      |
| Probe-2 forward | 5'-TGY GTG ACH CTS CAY TGY GG-3'       
cys val thr leu his cys gly  | 55-1    |
| Probe-2 reverse | 3'-TAD GGD CGD CGA CAC CTY CC-5'   
il e pro ala ala val glu gly   | 55-2    |

Nucleotides are indicated according to the following base codes: A=adenine; C=cytosine; G=guanine; T=thymine; R=A+G; W=A+T; S=G+C; Y=C+T; H=A+T+C; D=G+A+T; B=G+T+C; V=G+A+C.

In order to facilitate the subsequent subcloning of PCR products, the forward and the reverse primers were provided at the 5' ends of the restriction sites for ECORI and BAMHI, respectively. PCR was performed incubating 5 µl of cDNA first strand in 50 µl of 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 1 µM each oligonucleotide, 200 µM each dNTP, 1.5 U Taq DNA Polymerase. All the reagents were from Perkin Elmer, except oligonucleotides that were purchased from Gibco-Life technologies. The PCR reaction mixtures were layered with mineral oil (Sigma-Aldrich, WI, USA), and subjected to 35 temperature cycles (45 sec melting at 94°C, 1 min annealing at 60°C and 1 min elongation at 72°C) in a programmable thermal cycler (MJ Research, Inc.). PCR products were cloned in Bluescript SK+ vector after ECORI/BAMHI digestion.
(see also section 2.2.4.5), transformed in *E. coli* XL1-Blue competent cells (see section 2.2.4.7) and subjected to automated nucleotide sequencing (Nucleic Acid Facility, Istituto Dermopatico dell’Immacolata (IDI), Rome). Sequence alignment were performed using Gap algorithm (see section 2.2.1).

2.2.2.3 Radiolabelling of probes

After sequencing, probes obtained by PCR were radiolabelled incorporating \[^{32}\text{P}]\text{dCTP}\) by random priming using Prime-a-Gene Labelling System from Promega (WI, USA) following the manufacturer’s instructions. \([\alpha-^{32}\text{P}]\text{-deoxycytidine 5’-triphosphate ([}\alpha-^{32}\text{P}]\text{-dCTP})\) was from NEN-Dupont (UK). The Klenow fragment of DNA polymerase I was from Amersham Pharmacia Biotech (NJ, USA). All the other reagents were from the kit. ~25 ng of each PCR product was brought to 25 μl with nuclease free water, denatured for 2’ at 100°C, chilled on ice and then added to a mixture assembled with 10 μl nuclease free water, 5 μl of 5X labelling buffer (250 mM Tris-HCl, pH 8.0, 25 mM MgCl\(_2\), 10 mM DTT, 1 M HEPES, 26 A\(_{260}\) U/ml random hexadeoxyribonucleotides), 2 μl of non-isotopically labelled dNTP (500 μM each), 2 μl of nuclease-free BSA (10 mg/ml). Rapidly 5 μl of \([\alpha-^{32}\text{P}]\text{-dCTP}\) (10 mCi/ml, 800 Ci/mmol) and 1 μl of Klenow (5 U/μl) were added and the reaction incubated at RT for 60 min. At the end the reaction was terminated by heating at 100°C for 60 min and then chilling on ice. 2 μl of 0.5 M EDTA pH 8.0 were added and the probe was purified by passing it through a Nuctrap column (Stratagene, CA, USA) following the manufacturer’s instructions. The amount of radioactivity incorporated in the probe was quantified by counting an aliquot at the beta-counter (LS5801, Beckman, CA, USA).
2.2.3 Screening of a rat brain cDNA library

2.2.3.1 Solutions

Denaturing solution: 1.5 M NaCl and 0.5 M NaOH. Neutralizing solution: 0.5 M Tris-HCl pH 8.0 and 1.5 M NaCl. Washing solution: 0.2 M Tris-HCl pH 8.0 and 2X SSC buffer. 20X SSC buffer: 3 M NaCl, 0.3 M sodium citrate adjusted to pH 7.0 with NaOH. 10X PIPES buffer: 4.0 M NaCl, 0.1 M PIPES buffer pH 6.5. Hybridisation solution: 50% (v/v) formamide, 2X PIPES buffer, 0.5% (w/v) SDS, 100 µg/ml denatured and sonicated salmon sperm DNA (Sigma Aldrich, WI, USA). SM buffer: 25 mM Tris-HCl pH 7.5, 0.1 M NaCl, 15 mM MgSO₄, 0.01% (w/v) gelatin (Sigma Aldrich, WI, USA).

2.2.3.2 Procedure

Radiolabelled probes were used to isolate the full length cDNA in a λZAPII rat brain cDNA library produced from Sprague Dawley, male, 3-months-old rats (Stratagene, CA, USA). The library was amplified once by the producer and declared to contain 2 x 10⁶ primary clones whose average size was 1.5 kb (>0.5 Kb). The bacterial host was the XL1-Blue E.Coli strain, provided with the library. The library was titered and plated according to the manufacturer’s instructions. The titre was 3 x 10⁹ (plaque forming units (pfu)/ml. The library was plated at a density of 10⁵ plaques/150-mm plate. The plaques were transferred to nylon filters (Hybond-N+, Amersham Pharmacia Biotech, NJ, USA). Each plate was transferred to two filters: the first was incubated on the plates for 2 min, the second for 4 min. The plates were stored and the filters were denatured by submerging them in denaturing solution for 2 min, neutralized by submerging them in neutralizing solution for 5 min and rinsed for 30 sec in washing solution.
The filters were baked at 80°C for 2 hours, prehybridised 2 hours at 42°C in the hybridisation solution and hybridised O/N at 42°C with $1 \times 10^6$ cpm/ml of one or the other probe (see section 2.2.2.2) in hybridisation solution. Filters were rapidly washed once with 0.1X SSC and 0.1% (w/v) SDS at room temperature and then twice with the same buffer for 10 minutes at 37°C. Filters were exposed to autoradiography films (X-OMAT, Kodak, NY, USA). Positive plaques were identified by aligning film and original plates. ~ 1 square cm of agar corresponding to each radioactive spot was cut off and put in SM buffer plus 20 μl of chlorophorm. An subsequent screening of this mixture (secondary screening) was performed by titering, plating (at a density of 1000 plaques/plate) and hybridising, as indicated before. A further screening (tertiary screening) of the plaques isolated in the secondary screenings was performed. All the tertiary-screening plaques were positive. Inserts were subcloned in Bluescript SK+ after ECORI digestion (see also section 2.2.4) and subjected to automated nucleotide sequencing (Nucleic Acid Facility, IDI, Rome).

### 2.2.4 Subcloning of DNA

#### 2.2.4.1 Materials

Restriction enzymes were from Amersham Pharmacia Biotech (NJ, USA). T4 DNA ligase, DNA molecular size standards were from Gibco/BRL (NY, USA). "QIAprep Spin Miniprep Kit" and "QIAGEN Plasmid Maxi Kit" were from Qiagen (CA, USA). Tryptone Peptone, Yeast extract and agar were from Difco, Becton Dickinson (MD, USA). 3-Morpholino-propane-sulfonic acid (MOPS), RbCl and MnCl$_2$ were from Sigma Aldrich (WI, USA).
2.2.4.2 Solutions and media

LB: 1% (w/v) Tryptone Peptone, 0.5% (w/v) Yeast extract, 1% (w/v) NaCl; autoclaved 15 min at 121°C. LB-agar: LB plus 1.5% (w/v) agar: autoclaved 15 min at 121°C. Tfbl: 30 mM potassium acetate, 100 mM RbCl, 10 mM CaCl$_2$, 50 mM MnCl$_2$, 15% (v/v) glycerol pH 5.8. Tfb2: 10 mM MOPS, 75 mM CaCl$_2$, 10 mM RbCl, 15% (v/v) glycerol pH 7. TE buffer: 10 mM Tris-HCl, 1 mM EDTA pH 8. 50 X TAE buffer (1 litre): 242 gr. TRIZMA base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA.

2.2.4.3 DNA agarose gel

Agarose gels were prepared by dissolving Agarose (Bio-Rad Laboratories, UK) in 1X TAE buffer and heating by a microwave oven. 0.5 µg/ml of ethidium bromide were added and the gel was poured and run on a agarose gel apparatus from Bio-Rad Laboratories (UK). 0.3-0.5 µg of DNA standards were loaded and often used as a reference to give an approximate estimation of the amounts of DNA samples.

2.2.4.4 PCR amplification of DNA inserts

To amplify specific regions of DNA inserts, PCR was performed incubating 10 ng DNA-plasmid as template in 50 µl of 20 mM Tris-HCl pH 8.8, 10 mM KCl, 2 mM MgSO$_4$, 10 mM (NH$_4$)$_2$SO$_4$, 0.1% Triton X-100, 0.1 mg/ml nuclease free BSA, 1 µM each oligonucleotide, 200 µM each dNTP, 2.5 U Pfu Turbo DNA Polymerase. All the reagents, except DNA and oligonucleotides, were from Stratagene (CA, USA). Oligonucleotides were purchased from Gibco-Life technologies or from Sigma-Genosys. The PCR reaction mixtures were layered with mineral oil (Sigma-Aldrich, WI, USA), and subjected to 25
temperature cycles in a programmable thermal cycler (MJ Reasearch, Inc.). Melting-, annealing-, and elongation-temperatures were adjusted according to the features of template and primers. In order to facilitate the subsequent subcloning of PCR products, the forward and the reverse primers were provided with restriction sites at their 5’ ends.

2.2.4.5 Restriction and ligation

DNA (vectors and inserts) were cut with 5 U/μg of appropriate restriction enzymes in the buffer supplied with each enzyme by Amersham Pharmacia Biotech (NJ, USA). After restriction, enzymes were usually inactivated by incubating at 65-75°C for 10-20 min, according to the manufacturer’s instructions, and loaded on 1-1.4% agarose gel. The bands of interest were cut off from the gel with a sterile scalpel, and DNA was extracted from the gel with Qiaex II extraction kit (Qiagen, CA, USA), according to the manufacturer’s instructions. DNA was eluted in Tris-HCl 10 mM pH 8.0. To ligate vector and insert, ~100 ng of vector and ~ 3-fold molar amounts of insert were incubated with 1U T4 DNA ligase in T4-DNA-ligase buffer (GIBCO BRL, UK) 10 min at RT.

2.2.4.6 Preparation of competent bacteria

A single colony of XL-1Blue E. Coli bacteria was picked from a LB-agar plate and used to inoculate 10 ml of LB. Bacteria were grown O/N, the culture was diluted in 190 ml of fresh LB and incubated at 37 °C until the optical density reached 0.5 (at 600 nm). Bacteria were harvested by centrifugation at 4000 xg for 10 min at 4 °C. The bacterial pellet was resuspended in 64 ml of Tfb1 and left on
ice for 1 to 2 hours. After centrifugation and resuspension of the pellet in 8 ml of Tfb2 cells were frozen in liquid nitrogen and stored at -80°C in 400 µl aliquots.

2.2.4.7 Transformation of bacteria

The DNA plasmid of interest (10 ng of uncut plasmid or half of a ligation reaction) was added to 200 µl of competent bacteria (see previous section) previously thawed on ice. After gentle mixing, the cells were left on ice for 30 min, heat shocked for 45 sec at 42°C, and after addition of 800 µl LB, incubated under shaking (200 rpm) at 37°C for 45 min. Bacteria were plated on LB agar containing the appropriate selective antibiotic and incubated O/N, at 37 °C. The next day, an isolated bacterial colony was picked and used to inoculate 2ml of LB containing the appropriate antibiotic. The culture was incubated at 37 °C O/N. 300 µl of 50% (v/v) sterile glycerol were added to 700 µl of the bacterial culture and stored at -80 °C.

2.2.4.8 Small-scale preparations of plasmid DNA (miniprep)

The clones obtained after transformation of ligation reaction were usually screened by miniprep and subsequent restriction analysis. Isolated bacterial colonies were picked and inoculated in 5 ml of LB containing the appropriate antibiotic. After O/N growing at 37°C under continuous shacking (200 rpm), 700 µl of the culture was mixed with 300 µl 50% (v/v) sterile glycerol and stored at -80°C, while the rest of the culture was chilled on ice and centrifuged 10 min at 4000 xg. The DNA was extracted using the “QIAprep Spin Miniprep Kit” (Qiagen, CA, USA), according to the manufacturer’s instructions and analysed by restriction analysis and separation on agarose gel.
2.2.4.9 Large-scale preparation of plasmid DNA (maxiprep)

A small amount of bacteria transformed with the plasmid of interest, were scraped from the glycerol stock, inoculated in 2 ml of LB containing the appropriate selective antibiotic and grown at 37°C under continuous shacking (200 rpm) for 6-8 hours. This pre-culture was used to inoculate 200 ml of LB containing selective antibiotic. After an O/N incubation, bacteria were collected by centrifugation at 6000 rpm in a JA14 rotor for 10 min at 4 °C and processed according to the maxi-plasmid purification protocol of the “Qiagen Plasmid Maxi Kit”. The DNA obtained was resuspended in TE buffer and stored at -20 °C.

2.3 General biochemical procedures

2.3.1 Materials

4-chloro-1-naphtol (CN), red ponceau, polyoxyethylenesorbitan monolaurate (Tween-20), ammonium persulphate (APS), N,N,N',N'-tetramethyl-ethylenediamine (TEMED), sodium carbonate, hydrogen peroxide (30%), and citric acid were from Sigma-Aldrich (WI, USA). AgNO₃ and β-mercaptoethanol were from Fluka (Sigma-Aldrich WI, USA). Methanol, formaldehyde, butanol, and acetic acid were from Carlo Erba (Italy). Secondary antibodies conjugated with Horse-Radish Peroxidase (HRP) and directed against mouse or rabbit IgGs were from Calbiochem (CA, USA).

2.3.2 Solutions

Acrylamide stock solution: 40% (w/v) acrylamide-bisacrylamide (37.5:1) (Eurobio, France). Running buffer: 25 mM TRIZMA base, 0.2 M glycine, 0.1% (w/v) SDS. SDS-sample buffer: 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. Transfer buffer: 25 mM TRIZMA base, 0.2 M 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. Western blot developing solution: 10 mg CN dissolved in 3 ml ice-cold methanol, brought to 20 ml with TBS, plus 30 µl 30% H₂O₂.

2.3.3 Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE)

2.3.3.1 Assembling of polyacrylamide gels

Two 16 x 18 cm plates were used for assembling a regular gel, while two 32 x 18 glasses were used for assembling a long gel. The plates were assembled to form a chamber using two 1.5 mm plastic spacers aligned on the lateral edges of the 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 (Germany). 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) of ammonium persulphate 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 butanol and left at RT for ~1 hour. To prepare a 4-15% gradient gel, two mixtures containing 4 and 15% (w/v) of acrylamide were mixed by using a gradient maker device (Hoefer Scientific Instruments, NJ, USA). The butanol layer was removed. The ‘stacking’
polyacrylamide gel was prepared by mixing H\textsubscript{2}O, 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)
ammonium persulphate and 0.07% (v/v) TEMED were added, the solution
pipetted and poured on the ‘running’ gel. Immediately, a 15-well comb was
inserted between the glasses and left 1 hour at RT.

2.3.3.2 Sample preparation and run

Samples were prepared by adding SDS-sample buffer, incubating at 100°C
for 5-15 min in a Multi-Block Heater (Lab-Line, IL, USA), cooled at RT, briefly
centrifuged and loaded on the gel. One or two wells were loaded with 3 µl of
Rainbow recombinant protein molecular weight markers (Amersham Pharmacia
Biotech, NJ, USA) in the case of a 4-15% gradient gel and with 5 µg of Low
Molecular Weight Standards (Bio-Rad Laboratories, UK) in the case of a 10%
gel. The gel was then transferred into the electrophoresis apparatus (Hoefer
Scientific Instruments, NJ, USA) and the electrophoresis was carried out under a
constant current of 8 mA (for O/N runs) or 40 mA (for ~ 4-hours runs).

2.3.3.3 Silver staining and gel drying

The gels were incubated with gentle shaking at RT in fresh 50% (v/v)
methanol solution (~ 0.5 litre per one regular gel) twice for 15 min, in 5% (v/v)
methanol solution for 10 min, rapidly washed with H\textsubscript{2}O, incubated with 10 µM
DTT for 20 min, with 0.1% (w/v) AgNO\textsubscript{3}, rapidly washed twice with H\textsubscript{2}O. The
gels were then incubated with the developing solution (0.28 M sodium carbonate
and 0.02% formaldehyde) until appropriate staining of protein bands. Then the
reaction was stopped by adding 12 gr citric acid per 0.5 litre developing solution
as powder and incubating 30 min. The gels were then washed with H₂O and 10% polyacrylamide gels were briefly incubated with 4% (v/v) glycerol, 4-15% gels were incubated with 30% (v/v) methanol and 3% (v/v) glycerol for 45 min. The gels were then dried with a gel dryer (Hoefer Scientific Instruments, NJ, USA) at 80°C under vacuum for 4 hours.

2.3.4 Western Blot

2.3.4.1 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 by a nitrocellulose filter (Schleicher & Schuell, Germany). The filter was covered by a second sheet of 3MM paper, to form a "sandwich" which was subsequently assembled into the blotting apparatus (Hoefer Scientific Instruments, NJ, USA). Protein transfer occurred at 500 mA for 4 hours or at 125 mA O/N. At the end of the run, the sandwich was disassembled and the nitrocellulose filter was soaked in 0.2% red ponceau (Sigma-Aldrich, WI, USA) 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.

2.3.4.2 Probing nitrocellulose with specific antibodies

The nitrocellulose filters were cut into strips with a razor blade. The strips containing the proteins of interest were incubated in blocking solution (for CN-based detection) or in blocking solution plus 5% milk (for ECL-based detection) for 1 hour at RT, and then with the primary antibody diluted at its working concentration in blocking solution (see Table 2.2 for the list of antibodies used in this thesis).
Table 2.2. List of antibodies used in Western blot experiments (except those reported in Tables 5.1 and 5.2).

<table>
<thead>
<tr>
<th>Specificity (antibody name)</th>
<th>Owner (Company or Group's Head)</th>
<th>Animal source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>BARS (SN1)</td>
<td>Our laboratory</td>
<td>rabbit</td>
<td>1:100 of affinity-purified Ab</td>
</tr>
<tr>
<td>BARS (p502)</td>
<td>Our laboratory</td>
<td>rabbit</td>
<td>1:1000 of affinity-purified Ab</td>
</tr>
<tr>
<td>BARS (AA5.3)</td>
<td>Our laboratory/ D. Piccini (IFOM, Italy)</td>
<td>mouse (monoclonal)</td>
<td>1:50</td>
</tr>
<tr>
<td>α-tubulin (clone DM 1A)</td>
<td>Sigma-Aldrich (WI, USA)</td>
<td>mouse (monoclonal)</td>
<td>1:1000</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Our laboratory</td>
<td>rabbit</td>
<td>1:1000</td>
</tr>
<tr>
<td>α-internexin (catalog no. MAB1525)</td>
<td>Chemicon International Inc. (CA, USA)</td>
<td>mouse (monoclonal)</td>
<td>1:1000</td>
</tr>
<tr>
<td>MAP2 (clone HM-2)</td>
<td>Sigma Aldrich (WI, USA)</td>
<td>mouse (monoclonal)</td>
<td>1:1000</td>
</tr>
<tr>
<td>actin</td>
<td>Sigma Aldrich (WI, USA)</td>
<td>mouse (monoclonal)</td>
<td>1:5000</td>
</tr>
<tr>
<td>Dynamin II (Dyn2)</td>
<td>M.A. McNiven (Mayo Clinic and Foundation, MN, USA)</td>
<td>rabbit</td>
<td>1:5000</td>
</tr>
</tbody>
</table>

After 2-3 hour incubation at RT, or O/N incubation at 4°C, the antibody was removed and the strips washed in TTBS twice for 10 min. The strips were next incubated for 1 hour with the appropriate HRP-conjugated secondary antibody diluted in blocking solution (anti-rabbit: 1:1000 for CN-based detection and 1:20000 for ECL-based detection; anti-mouse: 1:1000 for CN-based and 1:5000 for ECL detection) and washed twice in TTBS for 10 min and once in TBS for 3 min. After washing, the strips were incubated with the western blot developing solution for CN-based detection or with ECL reagents from
Amersham Pharmacia Biotech (NJ, USA), according to the manufacturer’s instructions, for ECL-based detection.

2.3.5 Evaluation of protein concentration

Protein concentration was evaluated using a commercially available protein assay kit (Bio-Rad Laboratories, UK) according to the manufacturer’s instructions.

2.4 Preparation of antibodies

The antibodies raised against BARS (see Table 2.3) and GAPDH were obtained through immunisation of rabbits by Giusy Fiucci, Maria Giuseppina Silletta and Claudia Cericola (Laboratory of Molecular Endocrinology - Department of Cell Biology and Oncology - Consorzio Mario Negri Sud).

2.4.1 Immunisation of the rabbit

One mg of the specific antigen was resuspended in 2 ml of PBS (1.5 mM KH$_2$PO$_4$, 8 mM Na$_2$HPO$_4$, 137 mM NaCl and 2.7 mM KCl, pH 7.4). Two ml of complete Freund’s adjuvant (Sigma Aldrich, WI, USA) were added, and this mixture used to immunise one rabbit (New Zealand strain). The rabbits were boosted after 21 and 42 days with 1 mg of antigen containing the same volume of incomplete Freund's adjuvant. The rabbits were bled and the serum prepared as described below.

2.4.2 Serum preparation

After collection, blood was allowed to clot at 37°C for 60 min, and then kept overnight at 4°C in order to make the clot contract. The serum was removed from
the clot and the insoluble material by centrifugation at 10000 xg for 10 min at 4°C, and stored at -80°C in aliquots.

Table 2.3. List of the anti-BARS antibodies used in the course of this thesis.

<table>
<thead>
<tr>
<th>Antibody name</th>
<th>Antigen: peptide sequence or recombinant protein (extension of aa sequence)</th>
<th>Animal source</th>
<th>Affinity purified on</th>
</tr>
</thead>
<tbody>
<tr>
<td>BARS/9</td>
<td>VGQAVALR (from 174 to 181)</td>
<td>rabbit</td>
<td>the same peptide</td>
</tr>
<tr>
<td>BARS/SN1</td>
<td>SVEQIREVASGAARIR (from 147 to 162)</td>
<td>rabbit</td>
<td>the same peptide</td>
</tr>
<tr>
<td>BARS/SN2</td>
<td>DAQSTQEIKEKVLN (from 44 to 58)</td>
<td>rabbit</td>
<td>—</td>
</tr>
<tr>
<td>BARS/61</td>
<td>AFGFNVLFYDPYLSDGIER (from 184 to 192)</td>
<td>rabbit</td>
<td>—</td>
</tr>
<tr>
<td>BARS/p50₁</td>
<td>GST-BARS</td>
<td>rabbit</td>
<td>His-BARS</td>
</tr>
<tr>
<td>BARS/p50₂</td>
<td>GST-BARS</td>
<td>rabbit</td>
<td>His-BARS</td>
</tr>
<tr>
<td>AA5.3 (1)</td>
<td>GST-BARS</td>
<td>mouse (monoclonal)</td>
<td>—</td>
</tr>
</tbody>
</table>

(1) This anti-BARS monoclonal antibody was produced by Daniele Piccini (Istituto FIRC Oncologia Molecolare (IFOM), Milano, Italy).

2.4.3 Protein-A purification of total IgGs

Protein-A-Sepharose beads (500 mg), suspended in 5 ml of distilled water, were packed into a column and washed with 100 ml of distilled water under constant flow. The packed beads were washed with 20 ml of PBS, and 2 ml of antiserum were loaded onto the column at 0.5 ml/min in a FPLC (Fast Protein Liquid Chromatography) system (Pharmacia Bio-Tec, UK). The beads were washed with 30 ml of PBS and the retained IgGs were eluted using 15 ml of 0.1 M glycine pH 2.5. Fractions of 0.5 ml were collected and their protein content quantified by spectrometry (by the FPLC system). The six fractions containing the
highest concentration of protein were pooled and the pH neutralised with 1 M Tris, pH 11. The protein concentration of the pooled fractions was evaluated.

2.4.4 Affinity-purification of anti-BARS IgGs

Affinity-purified antibodies were obtained by passing purified IgGs on a column covalently linked to the antigen. Peptides or His-BARS were covalently coupled to a Hi-trap NHS-activated matrix (N-Hydroxysuccinimid) matrix (Amersham Pharmacia Biotech, NJ, USA), according to the manufacturer's instruction. Specific IgGs were purified through the column with a procedure identical to that described for total IgGs in section 2.4.3. Around 50 μg of specific IgGs were obtained from each ml of serum. Their specificity was evaluated by Western blot on rat brain cytosol.

2.5 Preparation of cell and tissue extracts

2.5.1 Preparation of cytosolic extract from rat brain

Cytosol from rat brain was usually prepared by Claudia Cericola (Laboratory of Molecular Endocrinology - Department of Cell Biology and Oncology - Consorzio Mario Negri Sud).

2.5.1.1 Solutions

Buffer 1: 50 mM HEPES pH 7.4, sucrose 320 mM. Buffer 2: 50 mM HEPES pH 8, 250 mM sucrose, 500 mM KCl, 2.0 μg/ml aprotinin, 0.5 μg/ml leupeptin, 2 μM pepstatin, 0.5 mM 1,10-phenanthroline, 1 mM PMSF. Buffer 3: 50 mM HEPES (pH 8), 50 mM KCl.
2.5.1.2 Procedure

Six Sprague-Dawley male rats (200-250 g each) were killed by decapitation. The brains were removed, placed in ice-cold buffer 1, and washed extensively. Brains were placed in ice-cold buffer 2 and homogenized in a total volume of 12 ml using an Ultraturrax homogeniser (Janke & Kunke, Germany; 4 strokes 20 sec each, at medium speed). The homogenate was centrifuged at 5000 x g for 30 min at 4°C to eliminate large fragments, and the supernatant was ultracentrifuged at 60000 rpm in TL100.1 (150000 xg) for 90 min at 4°C. The resulting supernatant was dialysed against buffer 3 for 4 hours using dialysis membranes with a molecular-weight cut off of 3000 Da (Spectrum Laboratories, CA, USA). The precipitate formed during dialysis was removed by centrifugation for 60 min at 60000 rpm in TL100.1 at 4°C. The supernatant obtained was divided into aliquots, frozen in liquid nitrogen and stored at -80°C.

2.5.2 Preparation of membranes from rat brain

Membranes were prepared by Maria Giuseppina Silletta (Laboratory of Molecular Endocrinology - Department of Cell Biology and Oncology - Consorzio Mario Negri Sud).

2.5.2.1 Solutions

Homogenisation buffer: 0.32 M sucrose, 4 mM HEPES, 1 mM EDTA, pH 7.3. PBS: 1.5 mM KH2PO4, 8 mM Na2HPO4, 2.7 mM KCl, 137 mM NaCl.

2.5.2.2 Procedure

Six Sprague-Dawley male rats (200-250 g each) were killed by decapitation. The brains were removed, and washed in 40 ml ice-cold
homogenisation buffer. Brains were homogenised in 48 ml of ice-cold homogenisation buffer by 12 passes in a Dounce tissue grinder (Wheaton Science Products, NJ, USA). The homogenate was centrifuged at 1000 xg for 10 min at 4°C. The supernatant was collected and the pellet was resuspended in 10 ml ice-cold homogenisation buffer and centrifuged as before. The last supernatant was collected, mixed with the first supernatant, and centrifuged at 150000 xg for 90 min at 4°C. The pellet was suspended in 15 ml of ice-cold PBS, subdivided in aliquots and stored at −80°C.

2.5.3 Preparation of cytosolic extract from COS7 cells

COS7 cells were seeded on 6-well plates and transfected as described in section 2.10.2.2. Forty-eight hours after transfection cells were detached as described in section 2.10.1.3, washed with PBS and resuspended in 200 μl hypotonic buffer (5 mM Tris-HCl pH 8.0, 5 mM MgCl₂, 1 mM EGTA, 2.0 μg/ml aprotinin, 0.5 μg/ml leupeptin, 2 μM pepstatin, 0.5 mM 1,10-phenanthroline, 1 mM PMSF). The suspension was sonicated on ice 3 times for 15 sec and centrifuged 15 min at 1000 xg at 4°C. The supernatant was centrifuged at 150000 xg 60 min at 4°C. The supernatant obtained from the last centrifugation (cytosol) was recovered and subjected to ADP-ribosylation or stored at −80°C.

2.5.4 Total cell lysates

Total cell lysates were obtained by resuspending cells from the plates in SDS-sample buffer (at a concentration of 10⁶ cells/ml) and boiling for 10 min.
2.6 ADP-ribosylation of cytosolic proteins

2.6.1 Materials

Adenosine triphosphate (ATP), guanosine triphosphate (GTP), brefeldin A (BFA) and NAD+ were from Sigma-Aldrich. [32P]-NAD+ (specific activity, 800 Ci/mmol) was from Amersham Pharmacia Biotech (NJ, USA) or from NEN-Dupont (UK). Dimethylsulfoxide (DMSO) was from Carlo Erba (Italy).

2.6.2 Solutions

2X potassium phosphate buffer (PFB): 100mM potassium phosphate buffer, pH 7.5, 2.5 mM MgCl₂, 1mM ATP, 1mM GTP, 10 mM thymidine, stored in aliquots at -20°C. BFA was dissolved at 10 mg/ml in DMSO and stored in aliquots at -20°C. DTT was dissolved in H₂O at 1M and stored in aliquots at -20°C.

2.6.3 Procedure

The ADP-ribosylation assay was carried out incubating the reported amount of cytosol (as substrate source) in the presence of 1.5 mg/ml of rat brain membranes (as enzyme source), 60 µg/ml BFA, 30 µM NAD+ and 3 µCi [³²P]-NAD+ in 50 µl of 1X PFB and 5 mM DTT, using the following procedure. A first mixture (40 µl/sample) was prepared by mixing H₂O, 2X PFB, 10 mg/ml BFA and 1M DTT and adding membranes and cytosol at the end. A second mixture (10 µl/sample) was prepared by mixing H₂O, 2X PFB, 3 mM NAD+, and [³²P]-NAD+ (specific activity, 800 Ci/mmol); the second mixture was rapidly added to the first and incubation was carried out for 2 hours at 37°C.
2.7 BARS purification

BARS purification was performed by Maria Giuseppina Silletta (Laboratory of Molecular Endocrinology - Department of Cell Biology and Oncology - Consorzio Mario Negri Sud).

2.7.1 Solutions

Buffer A: 25 mM HEPES pH 8, 0.5 M ammonium sulphate, 5% glycerol, 1 mM DTT. Buffer B: 25 mM HEPES pH 8, 5% glycerol, 1 mM DTT. Buffer C: 25 mM HEPES pH 8, 5% glycerol, 1 mM DTT, 0.2 M sodium phosphate. Buffer D: 25 mM HEPES pH 8, 5% glycerol, 1 mM DTT, 150 mM NaCl.

2.7.2 Procedure

Cytosol was prepared from rat brain as described in section 2.5 except that compositions of buffer were the followings: buffer 1: 25 mM Tris-HCl pH 7.4, sucrose 320 mM; buffer 2: 25 mM Tris-HCl pH 8, 250 mM sucrose, 500 mM KCl, 1 mM DTT, 2 mM EGTA, 2.0 μg/ml aprotinin, 0.5 μg/ml leupeptin, 2 μM pepstatin A, 0.5 mM 1,10-phenanthroline, 1 mM PMSF; buffer 3: 25 mM Tris-HCl (pH 8), 50 mM KCl, 1mM DTT. 240 mg of cytosol were ADP-ribosylated as in section 2.6 except that cytosol was 5 mg/ml, NAD⁺ was 250 μM and [³²P]-NAD was 480 μCi/ml. An aliquot was separated on 10% SDS/PAGE followed by autoradiography (Instant imager, Packard Instrument, CT, USA) to calculate the amount of BARS present at each purification step (assuming a stochiometric 1:1 reaction). All purification steps were performed at 4°C by Fast Protein Liquid Chromatography (FPLC; Amersham Pharmacia Biotech, NJ, USA). The ADP-ribosylated cytosol was precipitated by 35% ammonium sulphate (step 1; 3 fold enrichment in BARS with a 100% yield), and then dissolved in buffer A to reach...
the same conductivity of buffer A at a final concentration of 3 mg/ml. The solution was then applied to a hydrophobic column (Phenyl Sepharose H.P., Amersham Pharmacia Biotech, NJ, USA) pre-equilibrated with buffer A at 0.25 ml/min (step 2). The column was washed with 10 bed volumes of buffer A and BARS was eluted with 20 bed volumes of a linear gradient of ammonium sulphate (0.5-0 M) in buffer B. BARS eluted at approximately 0.3-0.15 mM of ammonium sulphate and was enriched about 15-fold with a 70-80% yield. The BARS fractions were pooled, diluted 1:1 with buffer B and applied on a hydroxylapatite column (Bio-Gel HT; Bio-Rad Laboratories, UK) pre-equilibrated with buffer B (step 3). The column was washed with 3 bed volumes and eluted with a 10 bed volumes of a linear gradient of sodium phosphate (0-0.2 M) in buffer C; BARS eluted at approximately 50-100 mM as a single peak and was enriched about 5-fold with a yield of 70%. Fractions from step 3 were pooled and concentrated with centricon (10,000 MW cut-off, centriplus concentrators Amicon, MA, USA), then loaded on a gel filtration column (Superose 12, Amersham Pharmacia Biotech, NJ, USA) pre-equilibrated with buffer D and calibrated with molecular weight standards (Amersham Pharmacia Biotech, NJ, USA) (step 4). Elution was with buffer D at a flow rate of 0.4 ml/min. BARS eluted at an apparent molecular mass of 180 kDa, with a yield of 80% and a 10 fold enrichment. The BARS containing fractions from step 4 were pooled and concentrated as described above and subjected to 2D IEF-SDS/PAGE (step 5). The spots stained by coomassie blue that coincided with radiolabelled spots were excised and subjected to in situ tryptic digestion. Peptides were separated by reverse-phase HPLC and sequenced by the Protein Structure Laboratory, University of California, Davis (Young Moo Lee, Director).
2.8 Expression and purification of recombinant proteins

2.8.1 Materials

Tryptone Peptone and Yeast extract were from Difco (Becton Dickinson, MD, USA). Ampicillin and chloramphenicol were from Fluka (Sigma-Aldrich, WI, USA). Imidazole, isopropyl-β-D-1-thiogalactopyranoside (IPTG), DNase I and lysozyme were from Sigma Aldrich (WI, USA).

2.8.2 Solutions

LB: 1% Tryptone Peptone, 0.5% Yeast extract, 1% NaCl (autoclaved 15 min at 121°C). GST-lysis buffer: 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA. GST-elution buffer: 100 mM Tris-HCl pH 8.0, 20 mM glutathione, 5 mM DTT. Protease inhibitors (PrIs): 2.0 µg/ml aprotinin, 0.5 µg/ml leupeptin, 2 µM pepstatin, 0.5 mM 1,10-phenanthroline, 1 mM PMSF. Phosphate buffered saline (PBS): 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄, 2.7 mM KCl, 137 mM NaCl. GST-elution buffer: 100 mM Tris-HCl pH 8.0, 20 mM glutathione, 5 mM DTT. His-lysis buffer: 50 mM sodium phosphate buffer pH 8.0, 300 mM NaCl, 10 mM imidazole. His-wash buffer: 50 mM sodium phosphate buffer pH 8.0, 300 mM NaCl, 20 mM imidazole. His-elution buffer: 50 mM sodium phosphate buffer pH 8.0, 300 mM NaCl, 250 mM imidazole.

2.8.3 Expression and purification of GST-BARS

2.8.3.1 Generation of the DNA construct

After sequencing, the full-length clone 3C was amplified by PCR (see section 2.2.4.4) with the following primers: 5’-GCGGAATTCCATGTCAGGCAGGTCCGACCTC-3’ (forward primer), and 5’-
GATGCGGCCGCCTACAACTGGTCAGTCGTATG-3' (reverse primer). The PCR product was digested with ECORI and NOTI and subcloned in ECORI/NOTI-digested pGEX4T1 (Amersham Pharmacia Biotech, NJ, USA; see also sections 2.2.4 for general subcloning procedures) generating the pGEX4T1-BARS plasmid. Glycerol stocks were established and stored at -80°C.

2.8.3.2 Expression and purification procedure

The following procedure led to about 4-5 mg of recombinant GST-BARS. Bacteria transformed with the empty vector pGEX4T1 were usually processed in parallel and led to the production of about 10 mg of GST. A small amount of bacteria were scraped from the glycerol stock, inoculated in 2 ml of LB containing 60 μg/ml ampicillin and grown at 37°C under continuous shaking (200 rpm) for 6-8 hours. Two ml of this culture were inoculated in 100 ml of the same medium and grown in the same conditions O/N. The O/N culture was diluted 1:20 in 1 litre of the same medium and O.D. (600nm) was monitored until it reached 0.4. Then, the bacteria were induced with the addition of 0.1 mM IPTG for 2 hours. At the end, the culture was chilled on ice and centrifuged at 6000 rpm in JA10 rotor (4000 g) for 10 min at 4°C. After discarding the supernatant, the pellet was resuspended in 50 ml of GST-lysis buffer containing the cocktail of protease inhibitors (PrIs) and lysozyme and Triton X-100 were added to a final concentration of 1 mg/ml and 1%, respectively. The suspension was incubated with gentle agitation at 4°C for 30 min, sonicated on ice 8 times for 15 sec and centrifuged at 18000 rpm in JA20 rotor (20000 g) for 20 min at 4°C. The supernatant was recovered and added to 2 ml of Glutathione Sepharose 4B matrix (Amersham Pharmacia Biotech, NJ, USA), previously equilibrated in lysis buffer. The suspension was incubated with gentle agitation at 4°C for 30 min and then
centrifuged at 700 xg for 5 min to sediment the matrix. The matrix was washed 5 times with 50 ml of PBS (centrifuging as indicated above). After washing, the matrix was packed into a 10 ml chromatography column (Bio-Rad Laboratories, UK), drained, and the bottom cap of the column was replaced. The protein was eluted by adding 2 ml of GST-elution buffer, incubating for 10 min at room temperature, removing the bottom cap, and collecting the eluate. The elution and collection steps were repeated at least 5 times (Fig. 2.1). GST-BARS usually peaked in the first or in the second fraction. The fractions containing high amounts (at least 0.2 mg/ml) of protein were pooled, dialysed twice against 1000 x volumes of PBS and stored in aliquots at -80°C.

2.8.4 Expression and purification of His-BARS

2.8.4.1 Generation of the DNA construct

The full-length clone 3C was amplified by PCR (see section 2.2.4.4) with the following primers: 5’-GCTCATATGTCAGGCGTCCGACCTC-3’ (forward primer), and 5’-GCGTGATCACTACAACTGGTCAGTCG-3’ (reverse primer). The PCR product was digested with NDEI and BCLI and subcloned in NDEI/BCLI-digested pET1ld-His derived from the addition of a sequence encoding a polyhistidine sequence in PET1ld (Novagen, WI, USA), generating the pET1ld-His-BARS plasmid (see also sections 2.2.4 for general subcloning procedures). pET1ld-His-BARS plasmid was transformed in BL21-pLysS competent cells (see sections 2.2.4.6 and 2.2.4.7), and glycerol stocks were established and stored at -80°C.
Fig. 2.1. Purification of GST-BARS and GST. Eluates from GST-BARS and GST column were analysed on a 10% SDS-PAGE gel and proteins revealed by silver staining. Lane 1-4: 10 μl of the first to the fourth fractions eluted from the GST-BARS column. Lane 5-8: 10 μl of the first to the fourth fractions eluted from the GST column. The positions of molecular weight standards are indicated on the left and the positions of GST-BARS and GST on the right of the panel.
2.8.4.2 Expression and purification procedure

The following procedure led to about 2 mg of recombinant His-BARS. Bacteria transformed with the empty vector pET11d-His were usually processed in parallel and the final eluate used as negative control in the His-BARS pull-down experiments.

A small amount of bacteria were scraped from the glycerol stock, inoculated in 5 ml of LB containing 60 µg/ml of ampicillin and 10 µg/ml of chloramphenicol and grown O/N at 37°C under continuous shaking (200 rpm). The culture was diluted 1:20 in 80 ml of the same medium and O.D_{600nm} was monitored until it reached 0.6. Then, the bacteria were induced with the addition of 0.4 mM IPTG for 2 hours. At the end, the culture was chilled on ice and centrifuged at 8000 rpm in JA14 rotor (5000 g) 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 (Prls) and frozen by immersion in liquid nitrogen. The lysate was 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 at 4°C for 30 min and sonicated on ice 8 times for 15 sec. Then, 10 mM MgCl₂ and 10 µg/ml DNAse I were added, and the lysate was incubated 15 min on ice and then centrifuged at 18000 rpm in JA20 (20000 g) for 20 min at 4°C. The supernatant was recovered and added to 0.5 ml of Ni-NTA beads (Qiagen, CA, USA), previously equilibrated in lysis buffer. The suspension was incubated with gentle agitation at 4°C for 1 hour and then packed into a 10 ml chromatography column (Bio-Rad Laboratories, UK). The column
was washed 5 times with 10 ml of His-wash buffer and the protein was eluted by adding 0.5 ml of His-elution buffer and collecting in a clean tube. The elution and collection steps were repeated at least 5 times. His-BARS usually peaked in the first fraction. 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. The single fractions and the pooled protein were subjected to SDS-PAGE analysis to control purity and integrity of the protein (Fig. 2.2).

2.9 Isolation of BARS-interacting proteins

2.9.1 Co-immunoprecipitation procedures

2.9.1.1 Solutions

PBS: 1.5 mM KH$_2$PO$_4$, 8 mM Na$_2$HPO$_4$, 2.7 mM KCl, 137 mM NaCl.
HK buffer: Hepes 50 mM pH 8.0, KCl 50 mM. HKT buffer: HEPES 50 mM pH 8.0, KCl 50 mM, 0.1% (w/v) Triton. 5X TCA/deoxycholate stock: 50% TCA and 2 mg/ml sodium deoxycholate, prepared by adding TCA to dissolved sodium deoxycholate. Cocktail of protease inhibitors (PrIs): 2.0 μg/ml aprotinin, 0.5 μg/ml leupeptin, 2 μM pepstatin, 0.5 mM 1,10-phenanthroline, 1 mM PMSF.

2.9.1.2 Preparation of the cross-linked matrices

To generate the BARS-IgG matrix and the preimmune IgG matrix, protein A-Sepharose (Amersham Pharmacia Biotech, NJ, USA) was cross-linked to affinity-purified p502 IgGs and affinity purified preimmune IgGs, respectively, according to the following procedure. 200 to 500 μg IgGs previously purified on protein A-Sepharose were mixed with 1 ml of protein A-Sepharose in a 15 ml polypropylene tube and incubated 1 hour with gentle rocking at 4°C. The beads
Fig. 2.2. Purification of His-BARS. The different steps of the procedure of His-BARS purification were checked by running fractions from each step on a 10% SDS-PAGE gel and revealing proteins by silver staining. Lane 1: 1/1000 of the bacterial culture before induction with IPTG. Lane 2: 1/1000 of the bacterial culture after 2-hour induction with IPTG. Lane 3: 1/1000 of the supernatant after centrifugation of the lysate. Lane 4: 1/1000 of the flow-through remained after incubation with Ni-NTA beads. Lane 5: 1/200 of the first wash. Lane 6: 1/200 of the last wash. Lane 7: 4 µg eluate (purified His-BARS). The positions of molecular weight standards are indicated on the left.
were washed three times by adding 10 ml of 0.2 M sodium borate pH 9.0 at RT and by centrifuging at 1000 xg for 5 min at RT. All the further centrifugation steps were performed in the same conditions. Then, the beads were resuspended in 10 ml of 0.2 M sodium borate, 10 μl of the suspension were removed, the cross-linker dimethylpimelidate (DMP, Pierce, IL, USA) was added as powder to bring the final concentration to 20 mM, and the suspension was incubated on a rocker for 30 min at RT. At the end of the incubation 10 μl were removed, and the suspension was centrifuged, and the supernatant removed. The reaction was stopped by washing the beads once with 0.2 M ethanolamine pH 8.0 and then incubating in the same buffer for 2 hours at RT on a rocker. At the end of the incubation 10 μl were removed, and after centrifugation and removal of the buffer, the beads were washed three times with 0.1 M glycine pH 3.0, in order to remove those IgGs that were not covalently bound to the protein A molecules. At the end the cross-linked matrices were washed twice with PBS and stored in PBS with 0.02% sodium azide at 4°C. The efficiency of cross-linking was checked by boiling samples of beads removed at each step (before adding DMP, after incubation with DMP, after washing with PBS) in SDS-sample buffer, running on a 10% SDS-PAGE gel and revealing the proteins by silver staining.

2.9.1.3 Co-immunoprecipitation in the absence of Triton X-100

Co-immunoprecipitation experiments were performed as follows. Forty mg of cytosol were thawed in a 4°C bath and Prls were added. All the following steps were performed on ice or at 4°C using ice-cold solutions. Cytosol was precleared by mixing with 1 ml of the Protein A-Sepharose pre-equilibrated in HK buffer and incubating it for 90-120 min on a rocker. At the end of the incubation, the suspension was centrifuged for 5 min at 700 xg, the supernatant
was recovered and the protein A-Sepharose was washed by adding 2 ml of HK buffer with Prls, mixing and centrifuging as before. All supernatants were combined and called precleared cytosol or input. A few μl of precleared cytosol were removed and stored at 4°C until the end of the experiment. All of the remaining precleared cytosol was split in two, mixed with ~ 80-200 μl of BARS-IgG matrix or preimmune-IgG matrix (a volume corresponding to ~ 40 μg cross-linked IgGs), previously equilibrated in HK by means of two washes, and incubated on a rocker for 3 hours. At the end of the incubation, the suspension was centrifuged for 5 min at 700 xg and the supernatant was recovered and called flow-through. The matrices were washed 4 times, each time by adding at least 10 volumes of HK buffer, inverting the tube several times and centrifuging as before. At the end of the washing step, the proteins were eluted from the matrices by adding one volume of 2X SDS-sample buffer, vortexing few seconds, and incubating 10 min at 100°C in a Multi-block Heater (Lab-Line) and vortexing again few seconds in the middle of the incubation. After allowing the return to RT, the tubes were centrifuged and the supernatants were loaded on an SDS-PAGE gel. The input and each flow through were processed in the same way as the eluates, by boiling in SDS-sample buffer and loading on the same SDS-PAGE gel. Also the supernatant of the last washes and the proteins eluted from protein A-Sepharose used for preclearing (by adding 0.1 M glycine pH 3.0) were usually checked by running them on a parallel SDS-PAGE gel.

2.9.1.4 Co-immunoprecipitation in the presence of Triton X-100

In the case of co-immunoprecipitation experiments performed in the presence of Triton X-100, the procedure differed from the previous as indicated in the following. After thawing also Triton X-100 was added to the cytosol from a
10% (w/v) stock to have 0.1% (w/v) final concentration. All the matrixes were equilibrated in HKT rather than in HK. The washes were performed with HKT solution rather than with HK, and only the last wash before elution was done with HK in order to eliminate Triton X-100 from the matrix.

2.9.1.5 Scale-up of the co-immunoprecipitation procedure

Four hundred mg of cytosol were thawed as described below and Prls were added. All the following steps were performed on ice or at 4°C, using ice-cold solutions. Cytosol was brought to 0.1% (w/v) final concentration of Triton X-100 and was precleared by mixing with 2 ml of the Protein A-Sepharose pre-equilibrated in HKT buffer and incubated for 90 min on a rocker. At the end of the incubation, the suspension was centrifuged for 5 min at 700 xg, the supernatant was recovered and the protein A-Sepharose was washed by adding 5 ml of HKT buffer with PrIs, mixing and centrifuging as done before. The supernatant was combined with the previous one, collecting all the unbound material, that was called precleared cytosol or input. A few μl of precleared cytosol were removed and stored at 4°C until the end of the experiment. All of the remaining precleared cytosol was split in two, mixed with ~1 ml of BARS-IgG matrix or preimmune-IgG matrix (each prepared from 500 μg purified IgGs), previously equilibrated in HKT by means of two washes, and incubated on a rocker for 3 hours. At the end of the incubation, the matrixes were transferred into two chromatographic columns, allowed to pack and the flow-through from each column was collected. Then each column was washed twice with 10 ml, twice with 5 ml of HKT buffer and twice with 2 ml of HK buffer. In the meanwhile, 1.5-ml tubes were filled with 1.2 mg/ml BSA in PBS and incubated for 30 min at RT. BSA solution was carefully removed and tubes were washed once with HK. Finally, the proteins
were eluted from the columns by adding 0.1 M glycine pH 3.0 and collecting ~0.5-ml fractions in the tubes that were pre-treated with BSA in order to reduce loss of proteins by attachment on the plastic. 1/5000 of the input, 1/20 of the last washes, and 1/10 of each elution fraction were analysed by SDS-PAGE and silver staining. The majority of proteins eluted were in the third fractions.

2.9.1.6 TCA precipitation

10% TCA and 0.4 mg/ml sodium deoxycholate were added from a 5x TCA/deoxycholate stock to 500 μl of the third elution fractions. The mixture was vortexed and incubated on ice for 1 hour. After a 10 min centrifugation at 20000 xg at 4°C, the supernatant was collected and the pellet was washed twice, by adding cold acetone, centrifuging as indicated above and removing the supernatant. At the end, the pellet was dried under gentle aspiration, resuspended in SDS-sample buffer, incubated at 100°C for 15 min vortexing every 5 min and subjected to SDS-PAGE.

2.9.2 His-pull down

2.9.2.1 Solutions

His-lysis buffer: 50 mM sodium phosphate buffer pH 8.0, 300 mM NaCl, 10 mM imidazole. His-elution buffer: 50 mM sodium phosphate buffer pH 8.0, 300 mM NaCl, 250 mM imidazole.

2.9.2.2 Procedure

Forty mg of cytosol were thawed by transferring them to a 4°C bath and PrIs were added. All the following steps were performed on ice or at 4°C using ice-cold solutions. Cytosol was brought to 300 mM NaCl and 10 mM imidazole
by adding concentrated solutions and was precleared by mixing with 1 ml of Ni-NTA beads (Qiagen, CA, USA), pre-equilibrated in His-lysis buffer, and by incubating for 2 hours on a rocker. At the end of the incubation, the suspension was centrifuged at 700 xg for 5 min, and the supernatant was recovered. The beads were washed once by adding 2 ml of His-lysis buffer containing Prls and centrifuging as before. The supernatant was combined with the previous one, collecting all the unbound material, that was called precleared cytosol or input. A few μl of precleared cytosol were removed and stored at 4°C until the end of the experiment. The precleared cytosol was split in two and added to 100 μg purified His-BARS or to an identical volume of negative control eluate, containing a few protein contaminants also included in His-BARS preparation (see section 2.8.4.2 and Fig. 2.2). In the case of His-BARS pull-down experiments of pure GAPDH, 200 μg of pure GAPDH from rabbit skeletal muscle (Sigma-Aldrich, WI, USA) were added to the same amounts of His-BARS or negative control eluate. The mixtures were incubated O/N and then added to 100 μl Ni-NTA beads, pre-equilibrated in His-lysis buffer, and incubated for 1 hour on a rocker. At the end, the suspensions were centrifuged as before and the supernatant was collected and called flow-through. The beads were then washed 5 times by adding at least 10 volumes of His-lysis buffer, centrifuging and discarding the supernatant. In the meanwhile, clean 1.5-ml tubes were filled with 1.2 mg/ml BSA in PBS and incubated for 30 min at RT. BSA solution was carefully removed and tubes were washed once with His-lysis buffer. The proteins were eluted by adding 300 μl His-elution buffer, incubating on a rocker for 5 min, centrifuging as before and collecting the supernatant in the tubes pre-treated with BSA. The elution step was repeated twice, collecting in all 3 fractions from each matrix. The input, each
flow-through and each elution fraction were analysed by SDS-PAGE. His-BARS and interacting proteins usually peaked in the first elution fraction.

2.10 Cell culture and transfections

2.10.1 Cell-line culture

2.10.1.1 Materials

African-green-monkey COS7 cells and Normal rat kidney (NRK) cells were purchased from American Tissue Type Collection (ATTC, USA). Human fibroblasts (line P29 II) were from IDI (Rome, Italy). Canine-Kidney MDCK cells were kindly provided by J. Morrow’s laboratory (Yale University, CT, USA). Dulbecco’s Modified Eagles Medium (DMEM), Fetal Calf Serum (FCS), Calf Serum (CS), penicillin, streptomycin, trypsin/EDTA, and L-glutamine were from GIBCO (NY, USA). All the plastic materials were from Corning (NY, USA). Filters (0.45 and 0.2 μm) were from Amicon (MA, USA)

2.10.1.2 Growth media

COS7, Hela and MDCK cells were grown in DMEM supplemented with 4.5 g/L glucose, 2 mM L-glutamine, 1 U/ml penicillin and streptomycin and 10% FCS. Human fibroblasts were grown in DMEM supplemented with 2 mM L-glutamine, 1 U/ml penicillin and streptomycin and 10% CS. NRK cells were grown in DMEM supplemented with non-essential amino acids 4.5 g/L glucose, 2 mM glutamine, 1 U/ml penicillin and streptomycin and 10% FCS. Complete media were prepared by diluting concentrated stock solutions with sterile water (DIACO, Italy) followed by filtration through 0.2 μm filters.
2.10.1.3 Growth conditions

All cell lines were grown under controlled atmosphere in the presence of 5% CO\textsubscript{2} at 37°C. Cells were grown in a flask until 80-100% confluence. The medium was removed and trypsin/EDTA solution (0.05% trypsin, 0.02% EDTA) was added for 2-5 minutes. The medium was then added back to block protease action and the cells were collected into a plastic tube. After centrifugation for 5 min at 300 xg, the pellet was resuspended in fresh medium.

2.10.2 Cell transfection

2.10.2.1 DNA constructs

After sequencing, the coding sequence of the full-length clone 3C was amplified by PCR (see section 2.2.4.4) with the following primers: 5’-GCGGAATTTCATGTCAGGCGTCCGACCTC-3’ (forward primer), and 5’-GATGCGGCCGCCTACAACTGGTCAGTCGTATG-3’ (reverse primer). The PCR product was digested with ECORI and NOTI and subcloned in ECORI/NOTI-digested pcDNA3 (Invitrogen, CA, USA; see also sections 2.2.4 for general subcloning procedures) generating the pcDNA3-BARS plasmid. Glycerol stocks were established and stored at -80°C.

Human CtBP1 cDNA was generously provided by G. Chinnadurai (St. Louis University, St. Louis, MO, USA) as pReCMV-T7-30 plasmid (Schaeper et al., 1995). CtBP1 coding sequence was excised from this plasmid by ECORI digestion and inserted in ECORI-digested pcDNA3.

Mouse CtBP2 cDNA was obtained as an EST database clone (accession number AA433525) purchased from American Type Culture Collection (ATCC, VA, USA). After sequencing to verify correct sequence, the CtBP2 coding region
was amplified through PCR with the following primers: 5’-GCAGGTACCATGAACGGCCCCCTGC-3’ (forward primer) and 5’-CACGCGGCCGCTATTGCTCGTTGG-3’ (reverse primer).

The CtBP2 truncated mutant was generated with a similar procedure after amplification with the same reverse primer and the following forward primer: 5’-GCTGGTACCATGGCCCTTGTGGATAAG-3’.

VSVG-GFP was a gift of J. Lippincott-Schwartz (National Institute of Health, Maryland, USA; Cole et al., 1996b).

DNA for transfection was prepared as described in section 2.2.4.9.

2.10.2.2 Lipofectamine-based cell transfection

COS7 cells, Hela cells, NRK cells and MDCK cells were transfected with the Lipofectamine Reagent (GIBCO BRL, UK).

Cells were seeded on glass coverslips in 24-well plates, in normal culture medium, at a concentration suitable to have 50-70 % confluence for transfection. For the experiments in which cells were permeabilized or treated with nocodazole, fibronectin was layered on the coverslip before seeding the cells, by adding to the well 200 µl of 10 µg/ml fibronectin (Sigma Aldrich, WI, USA) in PBS containing 0.9 mM CaCl$_2$ and 0.5 mM MgCl$_2$, incubating 30 min at RT, removing the solution, and allowing to dry for a few minutes. 20-24 hours after seeding, a transfection mixture was prepared by first diluting DNA and lipofectamine in separate polypropylene tubes and then mixing the two solutions by pipetting. For each well, 250 ng of DNA were diluted in 25 µl of OPTI-MEM (Gibco/BRL, NY, USA) and 1 µl of lipofectamine was diluted in 25 µl of OPTI-MEM. The two solutions were mixed and incubated 5 min at RT. In the meanwhile, cells were washed with OPTI-MEM medium. At the end, the transfection mixture was
brought to 0.25 ml and added to the cells. After 5-6 hours of incubation in normal growth conditions, the transfection mixture was replaced by complete culture medium and cells incubated in normal growth conditions for additional 14-44 hours, as reported for each experiments.

2.10.2.3 Electroporation-based cell transfection

Human fibroblasts were transfected by electroporation. Exponentially growing cells were detached as described in section 2.10.1.3, and 4 x 10^6 cells were resuspended in 400 μl PBS containing 15 μg DNA for transfection. The suspension was placed in an electroporation cuvette (Bio-Rad Laboratories, UK) with a gap of 0.4 cm and kept on ice for 10 min before electroporation. For the electroporation a Gene-Pulser Electroporator (Bio-Rad Laboratories, UK) was used with the following working parameters: Voltage, 380 V; capacitor, 850 μF. After the pulse, cells were suspended in normal culture medium and plated on coverslips in 24-well plates at a density of 1.5 x 10^5 cells/well.

2.11 Cell permeabilization by Streptolysin O (SLO)

2.11.1 Materials

Streptolysin O (SLO) was from Sclavo (Siena, Italy). Trypan blue was from Eurobio (France).

2.11.2 Solutions

SLO: 25U/ml of SLO dissolved in sterile water. 2X SLO buffer: 40 mM HEPES KOH pH 7.2, 220 mM potassium acetate, 4 mM magnesium acetate, 2 mM DTT (added just before the experiment). Buffer B: 25 mM HEPES KOH pH
7.2, 75 mM potassium acetate, 2.5 mM magnesium acetate, 5 mM EGTA, 1.8 mM CaCl₂ (100 nM free calcium). All these solutions are stored in aliquot at −20°C and thawed at 37°C before use.

2.11.3 Procedure

SLO was thawed in a 37°C bath, 1 volume of 2X SLO buffer was added and the solution was incubated 5 min at 37°C in order to activate SLO. After incubation SLO was diluted at 2U/ml with 1X SLO buffer and put on ice and used within 30 min. SLO buffer, buffer B and a 24-well plate with 70-90%-confluent cells were put on ice. Cells were washed twice with 500 µl ice-cold 1X SLO buffer, and 250 µl of 2U/ml SLO were added. After 10-min incubation on ice, cells were gently washed with 500 µl ice-cold 1X SLO buffer. 500 µl ice-cold buffer B were added to the cells and the plate was transferred to a 37°C bath. After 1- to 20-min incubation, the cells were fixed and processed for immunofluorescence.

2.12 Triton X-100 extraction of cells

Cells plated on a coverslip were washed twice with PBS, then placed in MEMA buffer (100 mM MES pH 6.5, 1 mM EGTA, 0.5 MgCl₂, 1 mM sodium azide) containing a cocktail of protease inhibitors and 2% (w/v) Triton X-100 and incubated for 1 to 10 min at RT. After incubation, the mixture was gently replaced with 2% paraformaldehyde in PBS. Cells were then treated for immunofluorescence.
2.13 Cell treatments

2.13.1 Nocodazole

The stock solution of nocodazole (Sigma-Aldrich, WI, USA) was 20 mM in dimethylsulfoxide (DMSO), stored in aliquots at −20°. Nocodazole was diluted at 33 μM in complete medium and added to the cells for the times reported. Then the cells were fixed and processed for immunofluorescence.

2.13.2 Leptomycin B (LMB)

LMB was a generous gift of Minoru Yoshida (Department of Biotechnology, Graduate School of Agriculture and Life Sciences, University of Tokyo, Tokyo, Japan). LMB stock solution was 1 mg/ml in ethanol. LMB was diluted at the reported concentrations in complete medium and added to the cells for 4-8 hours. Then the cells were fixed and processed for immunofluorescence. Control cells were incubated with complete medium containing 0.1% ethanol.

2.14 Immunofluorescence procedures

2.14.1 Sample preparation

2.14.1.1 Materials

Alexa 488-, Alexa 546- and Alexa 633-conjugated goat anti-rabbit, anti-mouse, and anti-sheep antibodies, Alexa 488-conjugated chicken anti-mouse and anti-rabbit antibodies, and Alexa 488-phalloidin were from Molecular Probes (OR, USA). Cy3-conjugated sheep anti-rabbit or anti-mouse antibodies, paraformaldehyde, and saponin were from Sigma-Aldrich (WI, USA). Mowiol was from Calbiochem.
2.14.1.2 Solutions

PBS: 1.5 mM KH$_2$PO$_4$, 8 mM Na$_2$HPO$_4$, 2.7 mM KCl, 137 mM NaCl, pH 7.4. Paraformaldehyde: 8% or 4% (w/v) paraformaldehyde were dissolved in 65°C-heated PBS by adding a few drops of concentrated NaOH and brought to pH 7.4 with 1 M HCl. Blocking solution: 0.05% saponin, 0.5% BSA, 50 mM NH$_4$Cl in PBS. Mowiol: 20 mg of mowiol were dissolved in 80 ml of PBS, stirred O/N and centrifuged for 30 min at 12000 xg.

2.14.1.3 Procedures

Cells were fixed by adding 1 volume of 8% or 4% paraformaldehyde for 15 min at RT, and incubated in blocking solution for 20 min at RT. The cells were subsequently incubated with the specified antibodies diluted in blocking solution (see Table 2.4 for the list and dilutions of antibodies used in this thesis) for 2-3 hours at RT or O/N at 4°C. After incubation with the primary antibody, cells were washed three times in PBS and incubated with a fluorescent-probe conjugated secondary antibody directed against the constant region of the primary IgG molecule for 1 hour at RT. Secondary antibodies were diluted 1:400 in blocking solution. Commonly, Alexa 488- and Alexa 546- or Cy3- conjugated antibodies were used in double-labelling (rabbit/mouse) experiments in all possible combinations. In triple-labelling (rabbit/mouse/sheep) experiments, Alexa 488-conjugated anti-rabbit or anti-mouse antibodies raised in chicken rather than in goat were always used to label BARS, in order to avoid any possible cross-reaction (sometime detected) between secondary anti-sheep antibodies and secondary antibodies raised in goat. Fibrous actin was labelled by using Alexa 488-phalloidin diluted 1:400 in blocking solution. Coverslips were mounted in mowiol on microscope slides.
Table 2.4. List of antibodies used in immunofluorescence experiments.

<table>
<thead>
<tr>
<th>Specificity (antibody name)</th>
<th>Owner (Company or Group’s Head)</th>
<th>Animal source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>BARS (SN1)</td>
<td>Our laboratory</td>
<td>rabbit</td>
<td>1:100 of affinity-purified Ab</td>
</tr>
<tr>
<td>BARS (p502)</td>
<td>Our laboratory</td>
<td>rabbit</td>
<td>1:10 of affinity-purified Ab</td>
</tr>
<tr>
<td>BARS (AA5.3)</td>
<td>Our laboratory/D. Piccini (IFOM, Italy)</td>
<td>mouse (monoclonal)</td>
<td>1:20</td>
</tr>
<tr>
<td>Mannosidase II</td>
<td>K. Moreman (University of Georgia, Athens, GA)</td>
<td>rabbit</td>
<td>1:1000</td>
</tr>
<tr>
<td>Giantin</td>
<td>H.P. Hauri (University of Basel, Switzerland)</td>
<td>mouse (monoclonal)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Giantin</td>
<td>M.A. De Matteis (Consorzio Mario Negri Sud, Italy)</td>
<td>rabbit</td>
<td>1:2000 of affinity-purified Ab</td>
</tr>
<tr>
<td>GM130</td>
<td>Transduction Laboratories (KY, USA)</td>
<td>mouse (monoclonal)</td>
<td>1:100</td>
</tr>
<tr>
<td>GM130 (g95)</td>
<td>M.A. De Matteis (Consorzio Mario Negri Sud, Italy)</td>
<td>rabbit</td>
<td>1:2000 of affinity-purified Ab</td>
</tr>
<tr>
<td>TGN46</td>
<td>S. Ponnambalam (University of Dundee, UK)</td>
<td>sheep</td>
<td>1:500</td>
</tr>
<tr>
<td>α-tubulin</td>
<td>Sigma-Aldrich (WI, USA)</td>
<td>mouse (monoclonal)</td>
<td>1:500</td>
</tr>
<tr>
<td>Vimentin (clone V9)</td>
<td>Sigma Aldrich (WI, USA)</td>
<td>mouse (monoclonal)</td>
<td>1:200</td>
</tr>
<tr>
<td>Transferrin receptor (catalog no. 13-6890)</td>
<td>Zymed laboratories (CA, USA)</td>
<td>mouse (monoclonal)</td>
<td>1:250</td>
</tr>
<tr>
<td>Mannose-6-phosphate receptor</td>
<td>B. Hoflack (Institut Pasteur de Lille, France)</td>
<td>rabbit</td>
<td>1:300</td>
</tr>
<tr>
<td>Calreticulin</td>
<td>StressGen Biotechnologies (Canada)</td>
<td>rabbit</td>
<td>1:100</td>
</tr>
<tr>
<td>PDI (catalog no. SPA-891)</td>
<td>StressGen Biotechnologies (Canada)</td>
<td>mouse (monoclonal)</td>
<td>1:500</td>
</tr>
</tbody>
</table>
2.14.2 Immunofluorescence analysis by Laser Scan Confocal Microscopy (LSCM)

Immunofluorescence samples were observed by an Axiophot fluorescence microscope or an LSM 510 confocal microscope equipped with 40X and 63X objectives (Zeiss, Germany). Optical confocal sections were taken at 1 Airy unit with a resolution of 512x512 pixels and exported as JPEG files.
CHAPTER 3

Cloning, Sequence Analysis and Functional Characterization of BARS

3.1 Introduction

BARS is a cytosolic protein initially identified as the 50-kDa substrate of brefeldin A (BFA)-dependent ADP-ribosylation (De Matteis et al., 1994; Di Girolamo et al., 1995). Later, the BFA-dependent ADP-ribosylation of BARS was demonstrated to be one of the mechanisms through which BFA induces the disassembling of the Golgi complex, indicating a possible role for BARS in the control of the structure of this organelle (Mironov et al., 1997a). In order to characterize the function of this potentially novel protein, its cloning was undertaken. The protein was purified from rat brain cytosol, separated by two-dimensional electrophoresis gel, trypsin-digested and subjected to protein microsequencing. The peptide sequences obtained from microsequencing were the basis for the cloning of BARS cDNA.

The cloning and sequencing of BARS cDNA, the analysis of the primary structure and the functional characterization of the protein are the subject of this chapter of results.

3.2 Previous work

BARS had been already purified from rat brain cytosol at the beginning of my PhD program. This purification had been carried out by Maria Giuseppina
Silletta (Laboratory of Molecular Endocrinology - Department of Cell Biology and Oncology - Consorzio Mario Negri Sud). An overall 900-fold purification with a 40% yield was achieved through four chromatographic steps (Table 3.1). Prior to purification, the cytosolic BARS was ADP-ribosylated with $[^{32}\text{P}]-\text{NAD}^+$ in the presence of BFA. It was then precipitated with 35% ammonium sulphate, and passed through a series of chromatographic columns. After each step, the $[^{32}\text{P}]-\text{ADP-ribosylated protein}$ was identified by SDS-PAGE. The last step was a gel filtration column, from which the protein eluted with an apparent molecular mass of about 170 kDa. BARS was then concentrated and subjected to 2D-IEF electrophoresis. Three well resolved spots (at a molecular mass of 46 kDa and isoelectric points of 6.05, 6.10 and 6.15) that were clearly $[^{32}\text{P}]-\text{ADP-ribosylated}$ and Coomassie Blue-stained, were isolated and subjected to microsequencing after in situ trypsin digestion.

### 3.3 Results

#### 3.3.1 Analysis of peptide sequences.

Eight non-overlapping peptides were obtained from the microsequencing of BARS (Table 3.2). Each of them was compared with non-redundant nucleotide and protein databases, by using the BLAST algorithm at the NCBI site. The sequence of 7 peptides were highly similar or identical to some stretches of a human protein called C-terminus binding protein (CtBP; Fig. 3.1), deposited into Genbank database in 1995 (accession number U37408; gi1063637). CtBP was identified as a phosphoprotein able to bind the C-terminus of the adenoviral protein E1A (Boyd et al., 1993) and then cloned through a yeast two-hybrid screening, by using E1A C-terminus as a bait (Schaeper et al., 1995).
Table 3.1. Chromatographic purification of BARS from rat brain cytosol.

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>BARS (µg)</th>
<th>Purification (-fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat brain cytosol</td>
<td>240</td>
<td>36</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Amm. sulf. precip.</td>
<td>80</td>
<td>36</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>Phenyl seph. H.P.</td>
<td>5.33</td>
<td>25</td>
<td>31.5</td>
<td>70</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>1.066</td>
<td>17.5</td>
<td>110</td>
<td>49</td>
</tr>
<tr>
<td>Superose 12HR</td>
<td>0.1066</td>
<td>14</td>
<td>877</td>
<td>39</td>
</tr>
</tbody>
</table>

BARS was purified by Maria Giuseppina Silletta (Laboratory of Molecular Endocrinology - Department of Cell Biology and Oncology - Consorzio Mario Negri Sud). BARS was ADP-ribosylated with [32P]NAD⁺ prior to purification. The [32P]-labeled protein was quantified in each chromatographic fraction by SDS/PAGE followed by electronic autoradiography (Packard Instant Imager). ADP-ribosylated cytosol was subjected to precipitation with 35% ammonium sulfate, then dissolved in Buffer A (25 mM Hepes, pH 8, 5% glycerol, 0.5 M ammonium sulfate, 1 mM DTT), applied to a Phenyl Sepharose HP column, and eluted with a decreasing linear gradient of ammonium sulfate in buffer B (25 mM Hepes, pH 8, 5% glycerol, 1 mM DTT). Fractions containing BARS were applied to a hydroxylapatite column pre-equilibrated in Buffer B, and were eluted with an increasing linear gradient of sodium phosphate in buffer C (25 mM Hepes, pH 8, 5% glycerol, 0.2 M sodium phosphate, 1 mM DTT). Fractions containing BARS were pooled, concentrated and applied to a gel filtration column (Superose 12 H.R.) pre-equilibrated with Buffer D (25 mM Hepes, pH 8, 5% glycerol, 150 mM NaCl). Superose 12 H.R. was calibrated with the following molecular mass standard proteins: gamma globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) vitamin B-12 (1,350 kDa). The void volume of the column was determined with blue dextran.
Table 3.2. Peptides obtained from the microsequencing of rat brain BARS: degree of identity with CtBP.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>CtBP identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>peptide 38</td>
<td>TVEMPILT</td>
<td>87</td>
</tr>
<tr>
<td>peptide 36-2</td>
<td>DVATVAFCDAQSTOEIHE</td>
<td>100</td>
</tr>
<tr>
<td>peptide 36-1</td>
<td>IGSGFDNIDIK</td>
<td>100</td>
</tr>
<tr>
<td>peptide 36-3</td>
<td>TTWLHQALR</td>
<td>ns</td>
</tr>
<tr>
<td>peptide 61</td>
<td>AFGFNVLFYDPYLSDGIER</td>
<td>84</td>
</tr>
<tr>
<td>peptide 55-1</td>
<td>VSTLQDLLFHSDCVTLHCGLNE</td>
<td>100</td>
</tr>
<tr>
<td>peptide 19</td>
<td>IPDSLK</td>
<td>100</td>
</tr>
<tr>
<td>peptide 55-2</td>
<td>YPPGVSVAPTGIPAAVEGIVP</td>
<td>95</td>
</tr>
</tbody>
</table>

The peptide sequences obtained by microsequencing of rat brain BARS were aligned with the aminoacid sequence of human CtBP (accession number U37408; gi1063637; see also fig. 3.1). For each peptide the degree of identity was calculated as percentage of aminoacids identical to CtBP over the total length of the peptide. ns: no significant similarity.
Fig. 3.1. Alignment of BARS peptides with the aminoacid sequence of human CtBP. The peptide sequences obtained by microsequencing of rat brain BARS (names in bold and sequences in italics) were aligned with the aminoacid sequence of human CtBP (in plain text; accession number U37408; gi1063637).
The initial analysis of the homology between the microsequenced peptides and CtBP was partially compromised by a mistake in the released sequence of CtBP, that was replaced by a correct sequence in October 1998 (gi3702074). Below, if not differently specified, the correct CtBP sequence (gi3702074) will be considered. The high relationship between microsequenced peptides and CtBP aminoacid sequence raised the possibility that BARS was related to CtBP.

3.3.2 Constructing BARS probes for the screening of a cDNA library.

In order to obtain probes to screen a cDNA library, two DNA stretches were produced through RT-PCR from rat brain mRNA using as primers two pairs of partially degenerated oligonucleotides, designed on the basis of the microsequenced peptides and their alignment with CtBP (Fig. 3.1 and section 2.2.2.2). The RT-PCR reactions led to two amplification products of about 450 and 500 base pairs, respectively (Fig. 3.2). The partial sequencing of these DNA segments and their alignment with the human CtBP sequence (first release: gi1063637; Figs. 3.3 and 3.4) revealed that they were similar but not identical to the human cDNA coding for CtBP.

3.3.3 Screening of a rat brain cDNA library and analysis of the cDNA clones.

The two DNA stretches obtained by RT-PCR were radiolabelled and used as probes to independently screen approximately $5 \times 10^5$ phage plaques of a rat brain cDNA library (Stratagene, CA, USA). About 20 clones were found to hybridise with both the probes. Five of them were isolated through secondary and tertiary screening, subcloned and sequenced (see sections 2.2.3 and 2.2.4). Their sequences were found highly similar to each other, but only one of them (3C; 2430 bp long) contained a full-length open reading frame (ORF; Fig. 3.5).
Fig. 3.2. Generation of DNA probes. Two pairs of degenerate primers constructed on the basis of rat brain BARS peptides, selectively amplified two segments of cDNA both from rat brain and FRTL5 cells. cDNA from rat brain (Rat Brain) or from FRTL5 cells (FRTL5) were subjected to PCR with the following pairs of primers: probe 1 sense primer and probe 1 antisense primer (1), designed on the basis of the sequences of peptide 36-2 and peptide 61, respectively; and probe 2 sense primer and probe 2 antisense primer (2), designed on the basis of the sequences of peptide 55-1 and peptide 55-2, respectively (see also fig. 3.1 and section 2.2.2). The size of DNA molecular weight standards is indicated in bp on the left of the picture.
Fig. 3.3. Sequence of probe 1. The single run sequence of the PCR product 1 from rat brain in fig. 3.2 (probe 1) was aligned with the nucleotide (CtBP: nt seq) and aminoacid sequences (CtBP: aa seq) of human CtBP (accession number U37408; gi1063637). n: nucleotide not identified.
Fig. 3.4. Sequence of probe 2. The single run sequence of the PCR product 2 from rat brain in fig. 3.2 (probe 2) was aligned with the nucleotide (CtBP: nt seq) and aminoacid sequences (CtBP: aa seq) of human CtBP (accession number U37408; gi1063637). n: nucleotide not identified.
Fig. 3.5. Nucleotide (in plain text) and deduced aminoacid sequence (in bold) of the cDNA clone 3C. The open reading frame is indicated in capital letters.
This ORF coded for a 430 aminoacid protein with a predicted mass of 47 kDa. The deduced amino acid sequence included all the 8 peptides obtained from microsequencing (Fig. 3.6), and also a peptide (peptide 9) that had been obtained from a previous analysis (trypsin digestion and microsequencing) of BARS, with only one mismatch at residue 175 (G instead of S).

### 3.3.4 Assessment that the cloned cDNA was the cDNA coding for BARS

To verify that the cloned rat protein was indeed BARS (operationally defined as the 50 kDa cytosolic substrate ADP-ribosylated by BFA), the cloned cDNA was transferred to a mammalian expression vector (pcDNA3, Invitrogen, CA, USA) and transfected in COS7 cells. The amount of BARS was then measured in the transfected cells. If the transfected cDNA was the cDNA of BARS, the ADP-ribosylatable 50 kDa protein should be increased after transfection. Fig. 3.7 A shows that indeed the cytosol of 3C-transfected COS7 cells contained a much larger amount of the 50 kDa protein ADP-ribosylated by BFA than that of mock-transfected controls. This indicates that the cloned protein was actually BARS.

To further confirm that the cDNA was indeed that of BARS and also for additional experiments described below, antibodies were raised against BARS peptides or against a GST-BARS fusion protein (see Chapter 2 and Table 2.3). All the antibodies were tested in Western blot of COS7 cells transfected with 3C cDNA (below indicated as BARS cDNA), of rat brain cytosol, and of ammonium sulphate-precipitated rat brain cytosol; in all cases a 50-kDa band that precisely co-migrated with the ADP-ribosylated protein was clearly and specifically recognised.
Fig. 3.6. Deduced aminoacid sequence of clone 3C. The peptide sequences obtained directly from microsequencing are yellow on blue background. The mismatch with the sequence of peptide 9 is indicated in red.
Fig. 3.7. BFA-dependent ADP-ribosylation of BARS in COS7 cells. 10 µg cytosol from COS7 cells (lane 1), 10 µg cytosol from mock-transfected (lane 2) or CtBP3/BARS-transfected (lane 3) COS7 cells, 100 µg entire rat brain cytosol (lane 4) and 100 µg rat brain cytosol precipitated with 35% ammonium-sulphate (lane 5) were [32P]-ADP-ribosylated, analyzed by SDS-PAGE, transferred to nitrocellulose, and revealed either by autoradiography (panel A) or by an anti-BARS antibody (anti-BARS/SN1) with peroxidase-based detection (panel B). The higher molecular mass band (50 kDa) is BARS, the lower molecular mass band (38 kDa) is GAPDH, the 38 kDa substrate of BFA-dependent ADP-ribosylation.
As an example, Fig. 3.7 B shows the ability of one of these antibodies (anti-BARS/SN1) to detect a 50-kDa band both in cytosol from BARS-transfected COS7 cells and in rat brain cytosol (lanes 3 and 4). The rat brain band was more evident after enrichment of BARS by ammonium sulphate precipitation of the cytosol (lane 5). That this band corresponded to BARS was indicated by the fact that it precisely co-migrated with the brain ADP-ribosylatable 50-kDa protein (compare lanes 3, 4, and 5 in panels A and B of Fig. 3.7). Moreover, the antibody raised against GST-BARS fusion protein was able to immunoprecipitate the 50 kDa ADP-ribosylatable protein from rat brain cytosol (Fig. 3.8) These immunoprecipitation experiments were performed by Maria Giuseppina Silletta (Laboratory of Molecular Endocrinology - Department of Cell Biology and Oncology - Consorzio Mario Negri Sud). Together with the previous data, the results obtained with the antibodies demonstrated that the clone 3C coded indeed for the BARS protein and represent the BARS cDNA. Thus, the nucleotide sequence and the deduced aminoacid sequence were submitted to the Genbank database with the name of BARS (GenBank Accession Number AF067795).

3.3.5 BARS sequence analysis

The sequence of the BARS cDNA was compared with the available nucleotide and protein databases. It was found to be highly similar to CtBP and also to CtBP2, a related protein that was cloned during this work (Turner and Crossley, 1998). CtBP1 and CtBP2 have been cloned both in human and mouse. At the aminoacid level (Fig. 3.9), the degree of identity between BARS and the two CtBPs was 97% with human and mouse CtBP1 (accession numbers: U37408 and AJ010483), and 79% with human and mouse CtBP2 (accession numbers: AF016507 and AF059735).
Fig. 3.8. Immunoprecipitation of rat brain BARS. This experiment was performed by Maria Giuseppina Silletta (Laboratory of Molecular Endocrinology - Department of Cell Biology and Oncology - Consorzio Mario Negri Sud). An antiserum raised against GST-BARS fusion protein (anti-BARS/p502; see Table 2.3) was used to immunoprecipitate \(^{32}\text{P}\)-ADP-ribosylated rat brain cytosol. Lane 1: \(^{32}\text{P}\)-ADP-ribosylated cytosol prior to immunoprecipitation (cytosol). Lane 2: the immunoprecipitate (P). Lane 2: the supernatant left after immunoprecipitation (SN). The higher molecular mass band (50 kDa) is BARS, the lower molecular mass band (38 kDa) is GAPDH.
Fig. 3.9. BARS belongs to the CtBP family of proteins. BARS is aligned with mouse CtBP1 (accession number AJ010483) and mouse CtBP2 (accession number AF059735). Identical residues are yellow on blue background, residues conserved in two sequences are black on cyan background.
The only significant region of diversity between CtBP1 and BARS was the N terminal stretch, where the two proteins differ in sequence and length. A specific feature of BARS is the presence of a serine in position 369 lacking in the other two proteins. This insertion might be a determinant difference in terms of secondary and tertiary structure of the three proteins.

At the nucleotide level, the BARS cDNA was 94% identical to mouse CtBP1 (86% to human CtBP1) and 72% identical to human and mouse CtBP2. Interestingly, a 192 bp long sequence at the 5' of the BARS cDNA was absent in the CtBP1 and CtBP2 cDNAs. This insertion represents the main difference among CtBP1 and BARS cDNAs, and gives rise to a novel ATG start codon. The BARS-specific insertion was present in several mouse sequences of the EST database. As an example, in the AA212717 sequence (gi1811346) a 449 bp long region (which included the 5' untranslated region, the ATG start codon and 273 bp of the BARS coding sequence) was 98% identical to the BARS cDNA. Further analysis of the EST database also revealed the presence of numerous human EST sequences which contain the 5' sequence specific for BARS cDNA and which therefore code for a human protein containing the N-terminal sequence of BARS. The EST database was also analysed searching for rat sequences potentially coding for CtBP1, but no such sequence was found. Instead, a rat sequence identical to a 377 bp long BARS cDNA fragment (which included 265 bp of the 5' untranslated region, the ATG start codon and 112 bp of the coding sequence) was more recently deposited in EST databases (gi11676671). Thus, the results from the analysis of the EST database excluded the possibility that the BARS clone was a cDNA library artefact and strongly suggested that BARS is a third form of CtBP that exists not only in rat but also in mouse and in human. BARS
and CtBP1 may be encoded by an alternatively spliced gene or by two different genes. To take into account the sequence relationship with CtBP1 and CtBP2, BARS was renamed CtBP3/BARS.

Further analysis of the EST database also revealed that one mouse EST library (NCI_CGAP_Mam6) includes a clone containing the CtBP3/BARS-specific cDNA sequence (gi 9761224) and a clone containing the CtBP1-specific cDNA sequence (gi 15579965) suggesting that at least in mouse the two proteins can be expressed at the same time in the same tissue.

The other 4 cDNA clones isolated and sequenced during this screening were incomplete, but identical to the CtBP3/BARS cDNA clone except from clone 1A in which there was a 3-nucleotide gap, that would be responsible for the lacking of a serine (residue 369 in CtBP3/BARS) in the deduced aminoacid sequence. The absence of the serine makes it possible that this clone is the incomplete cDNA coding for the rat CtBP1 or for another protein of the CtBP family, but the incompleteness of the clone at its 5' end did not allow this point to be clarified.

Besides the very high similarity with CtBP1 and CtBP2, the comparison of CtBP3/BARS sequence with all of the web-accessible databases through the BLAST algorithm revealed also sequence relationship with the D-3-phosphoglycerate dehydrogenase, the D-lactate dehydrogenase and other dehydrogenases from both eukaryotic and prokaryotic organisms. Further analysis (ProfileScan), performed against profile databases (Prosite and Pfam), unveiled that the central region of CtBP3/BARS (residues from 90 to 304) aligns with proteins belonging to the family of NAD-dependent D-isomer specific 2-hydroxyacid dehydrogenases (Pfam PF00389; Prosite PDOC00063; Grant, 1989; Taguchi and Ohta, 1991; Kochhar et al., 1992) and that the sequence from 166 to 193 matches
the pattern for their NAD-binding site (Prosite PS00065). This family of proteins includes mainly bacterial and plant dehydrogenases but also metazoan dehydrogenases, all with a stereospecificity for D-isomer substrates. Multiple alignment among bacterial representatives of this family, rat D-3-phosphoglycerate dehydrogenase and CtBP3/BARS was performed by using ClustalW algorithm and is shown in Fig. 3.10. In Table 3.3 the score of the pairwise alignment between each of these proteins is reported. The alignment scores between CtBP3/BARS and the other proteins are the same order of magnitude of the alignment scores between each of all the other proteins. This indicates that CtBP3/BARS has to be considered part of this family of proteins. Noticeably some residues conserved in the other dehydrogenases (proline and alanine corresponding respectively to residue 207 and 254 in D-LDH) are not conserved in CtBP3/BARS (Fig. 3.10).

As mentioned above, on the basis of the primary structure, CtBP3/BARS is expected to have a NAD-binding domain. Indeed, the sequence comprising aminoacids 163 to 193 of CtBP3/BARS (and also the corresponding sequence in CtBP1 and CtBP2) perfectly matches the sequence requirement for the NAD-binding site (Fig. 3.10). This is a glycine rich motif that organises into the so-called Rossmann fold, the supersecondary structure implicated in dinucleotide binding in different nucleotide binding proteins (Lesk, 1995; Bellamacina, 1996). Unexpectedly, Schaeper and others (1995) were unable to demonstrate any significant NAD-binding or dehydrogenase activity for CtBP1. Moreover, in CtBP2 mutation of a histidine highly conserved in the NAD-dependent D-isomer specific 2-hydroxyacid dehydrogenase family (residue 321 of CtBP2), essential for the catalytic activity of this family of dehydrogenases (Taguchi and Ohta, 1993), had no effect on the repression activity of CtBP2 (Turner and Crossley, 1998).
Fig. 3.10 Multiple alignment among representative members of the NAD-dependent D-isomer specific 2-hydroxyacid dehydrogenases family and CtBP3/BARS. D-lactate dehydrogenase from Lactobacillus plantarum (D-LDH; gi216746; Taguchi and Ohta, 1991), glycerate dehydrogenase from Hyphomicrobium methyllovorum (D-GDH; gi1706407; Goldberg et al., 1994), D-3-phosphoglycerate dehydrogenase from Escherichia coli (D-PGDH; gi1789279; Tobey and Grant, 1986), D-2-hydroxyisocaproate dehydrogenase from Lactobacillus casei (D-HICDH; gi2506353; Lerch et al., 1986), formate dehydrogenase from Pseudomonas sp. (FDH; gi4033692; Lamzin et al., 1992), rat D-3-phosphoglycerate dehydrogenase (rD-PGDH; gi1944614; Achouri et al., 1997), and CtBP3/BARS (gi3925357) were aligned with the ClustalW algorithm using default parameters. Identical residues are yellow on blue background, residues conserved at least in 4 out of 7 sequences are white on black background, and residues with similar physico-chemical properties are white on grey background. The most N-terminal and C-terminal portions of the proteins are not shown in the figure given the low significance of their alignment. Arrows indicate residues that are highly conserved in the family but not in CtBP3/BARS. The blue bar underlines the conserved sequence corresponding to the NAD-binding site (Rossmann fold).
Table 3.3 Alignment scores of members of the family of NAD-dependent D-isomer specific 2-hydroxyacid dehydrogenases and CtBP3/BARS.

<table>
<thead>
<tr>
<th></th>
<th>D-GDH</th>
<th>D-PGDH</th>
<th>D-HICDH</th>
<th>FDH</th>
<th>rD-PGDH</th>
<th>BARS</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-LDH</td>
<td>257 (69/285)</td>
<td>254 (76/251)</td>
<td>675 (204/330)</td>
<td>206 (62/217)</td>
<td>299 (81/259)</td>
<td>302 (87/292)</td>
</tr>
<tr>
<td>D-GDH</td>
<td></td>
<td>261 (84/282)</td>
<td>248 (76/293)</td>
<td>302 (78/251)</td>
<td>305 (78/275)</td>
<td>245 (74/241)</td>
</tr>
<tr>
<td>D-PGDH</td>
<td></td>
<td></td>
<td>265 (93/314)</td>
<td>232 (74/246)</td>
<td>431 (110/317)</td>
<td>250 (73/241)</td>
</tr>
<tr>
<td>D-HICDH</td>
<td></td>
<td></td>
<td></td>
<td>174 (61/243)</td>
<td>259 (70/253)</td>
<td>279 (86/291)</td>
</tr>
<tr>
<td>FDH</td>
<td></td>
<td></td>
<td></td>
<td>262 (86/288)</td>
<td></td>
<td>272 (79/236)</td>
</tr>
<tr>
<td>rD-PGDH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>299 (106/352)</td>
</tr>
</tbody>
</table>

Each score is the raw score obtained by aligning pairs of proteins with the BLAST 2 algorithm at NCBI site. In bracket the number of identical residues out of the total aligned residues is indicated. The reference and the abbreviations of the name of the proteins are given in Fig. 3.10.
The C-terminal region of the mammalian CtBPs (the last 100 amino acids) had no significant homology with any proteins so far identified. This might imply that the C-terminus is specifically involved in the function of the CtBPs.

The analysis of CtBP3/BARS sequence by comparison with Prosite database also revealed the presence of a number of putative phosphorylation sites for cAMP and cGMP-dependent protein kinase (PS00004; position: 130-133), for protein kinase C (PS00005; positions: 240-242, 253-255, 329-331, 335-337, 416-418), for Casein kinase II (PS00006; positions: 47-50, 71-74, 89-92, 98-101, 113-116, 211-214). The presence of phosphorylation sites in CtBP3/BARS is not unexpected, since CtBP1 was demonstrated to be phosphorylated, and its levels of phosphorylation were shown to change during mitotic cycle (Boyd et al., 1993).

3.3.6 BFA-dependent ADP-ribosylation of the CtBPs

To verify whether all CtBPs might be ADP-ribosylated in the presence of BFA, like CtBP3/BARS is, overexpression of CtBP1 and CtBP2 in COS7 cells, followed by cytosol extraction and ADP-ribosylation, was performed. High levels of the 50 kDa ADP-ribosylatable protein were found (Fig. 3.11). This indicates that indeed the known CtBPs are all in vitro substrates of BFA-induced ADP-ribosylation. From now on, I will refer to BARS as any of the three 50-kDa BFA-ADP-ribosylatable substrates in those cases in which I am not able to distinguish among them, i.e. when I refer to the protein isolated from rat brain cytosol and in immunofluorescence detection of the endogenous proteins.
Fig. 3.11. BFA-dependent ADP-ribosylation of CtBP1 and CtBP2 in COS7 cells. Control buffer (lane 1), 30 μg cytosol from rat brain (lane 2), 10 μg (lane 3) or 30 μg (lane 4) cytosol from untransfected COS7 cells, 10 μg cytosol from T7-CtBP1-transfected COS7 cells (lane 5), and 10 μg cytosol from CtBP2-transfected COS7 cells (lane 6) were \([^{32}P]\)-ADP-ribosylated and \([^{32}P]\)-ADP-ribosylated proteins were analysed by SDS-PAGE and revealed by autoradiography.
3.3.7 Expression of recombinant BARS for the functional characterization of BARS.

The cloning of BARS allowed to confirm results previously obtained (Mironov et al., 1997a; see also section 1.3.5) and to test directly the effect of BARS on isolated Golgi membranes. In order to perform these experiments, antibodies directed against BARS were produced (see section 2.4 and Table 2.3) and BARS was expressed as a recombinant protein in *E. coli*. To obtain recombinant BARS, the BARS coding sequence was subcloned in prokaryotic expression vectors and BARS was purified from bacteria as a GST-fusion protein or as a polyhistidine-tagged protein (see section 2.8 and Figs. 2.1 and 2.2). Both recombinant BARS and purified rat brain BARS were used in functional experiments that were performed by my colleagues; their findings will be discussed in the following section.

3.4 Discussion

As first step toward the characterization of BARS - the 50-kDa substrate of BFA-dependent ADP-ribosylation - the protein was purified from rat brain and cloned. The sequencing of BARS revealed that it is a member of the CtBP family of proteins, which led to the renaming as CtBP3/BARS. At the moment, the CtBP family includes three mammalian members, CtBP1, CtBP2 and CtBP3/BARS (Schaeper et al., 1995; Turner and Crossley, 1998; Criqui-Filipe et al., 1999; Postigo and Dean, 1999; Sewalt et al., 1999; Spano et al., 1999), one member from *Xenopus laevis* (Sewalt et al., 1999), two members from *Danio rerio* (Muraoka et al., 2000), two members from *Drosophila melanogaster* (Nibu et al., 1998; Poortinga et al., 1998). Mammalian CtBP1 and CtBP2 are 48 kDa proteins
that have been shown in this chapter to be substrates of BFA-dependent ADP-ribosylation, similarly to CtBP3/BARS. CtBP1 and CtBP2, isolated as interactors of a variety of transcriptional factors, were demonstrated to act as co-repressors of gene transcription (Turner and Crossley, 1998; Criqui-Filipe et al., 1999; Sewalt et al., 1999). Since they are widely expressed in mammalian tissues (Sewalt et al., 1999) and were found to interact with a number of transcriptional regulators, they are considered as general transcriptional co-repressors (reviewed in Turner and Crossley, 2001). Recently, another protein related to the CtBPs, called RIBEYE, was identified in mammals (Schmitz et al., 2000). This is a 120 kDa protein that was isolated as a major protein component of the synaptic ribbons of human, rat and bovine retina, and appears to be specifically expressed in the retina (Schmitz et al., 2000). RIBEYE is built from two domains, an N-terminal one that is unique in its sequence and a C-terminal one that is identical to CtBP2. Interestingly, CtBP2 and RIBEYE are encoded by a single gene, but are transcribed from different promoters and their messengers include different first exons (Schmitz et al., 2000).

CtBP3/BARS cloned from rat brain is nearly identical to mouse and human CtBP1. They essentially differ only in their N-termini, where CtBP1 is 11 aminoacids longer, and for the presence of a serine in position 369 in CtBP3/BARS. Nevertheless, the results of the analysis of the EST sequence database showed that: 1) cDNAs coding for a protein with the same N-terminal sequence of rat CtBP3/BARS also exist in mouse and human; 2) at least in mouse and human, both cDNAs coding for a protein with the N-terminus of CtBP3/BARS and for a protein with the N-terminus of CtBP1 exist. If the two proteins are products of alternative splicing of the same gene or are expressed from two different genes has not been defined so far. However, the analysis of the human genome
sequences revealed that a single gene, localized at chromosome 4, contains both the sequences coding for CtBP3/BARS and the sequences coding for CtBP1, suggesting that, at least in human CtBP1 and CtBP3/BARS cDNAs are generated by alternative splicing of the same gene.

The identification of BARS allowed the confirmation of results previously obtained, which indicated a role of ADP-ribosylation of BARS in mediating the Golgi disassembly induced by BFA (Mironov et al., 1997a; see also section 1.3.5). In Mironov et al. (1997a), it was shown that brain cytosol contains factors that prevent the Golgi disassembly induced by BFA, and it was proposed that BARS is one of this factors, considering the fact that the ADP-ribosylation of BARS by BFA correlated with the loss of inhibitory activity of the cytosol. To confirm this hypothesis the role of BARS and ADP-ribosylation were tested on streptolysin O (SLO)-permeabilized RBL cells using dialyzed rat brain cytosol, dialyzed BARS-immunodepleted cytosol, purified BARS and anti-BARS antibodies (Fig. 3.12; Spano et al., 1999). The BFA-induced disassembly of the Golgi was monitored at the immunofluorescence level using an antibody to mannosidase II, a resident Golgi enzyme. The experiments were performed by Alexander Mironov and Aurora Fusella (Unit of Intracellular Traffic – Department of Cell Biology and Oncology - Consorzio Mario Negri Sud). As shown in Mironov et al. (1997a), SLO-permeabilized RBL cells are able to respond to BFA treatments in the presence of cytosol, and NAD$^+$ is an essential cofactor for the activity of BFA (Mironov et al., 1997a; Fig. 3.12 B and C). In fact, in the presence of dialyzed cytosol, BFA was unable to redistribute the Golgi staining (Fig. 3.12 B), while when 400 μM NAD$^+$ was readded to this cytosol, BFA regained its activity (Fig. 3.12 C).
Fig. 3.12. BARS prevents the BFA effects on the Golgi complex in permeabilized cells. This experiment was performed by Alexander Mironov and Aurora Fusella (Unit of Intracellular Traffic – Department of Cell Biology and Oncology - Consorzio Mario Negri Sud). Intact RBL cells (A) or RBL cells permeabilized with 1 U/ml streptolysin O (B-H) were incubated for 15 min at 32°C in the presence of 3.3 μg/ml BFA and one of the followings: dialyzed cytosol (B), dialyzed cytosol and 400 μM NAD⁺ (C), pre-ADP-ribosylated and dialyzed cytosol (D), dialyzed BARS-depleted cytosol (E), dialyzed mock-depleted cytosol (F), dialyzed BARS-depleted cytosol and purified BARS (G), or dialyzed cytosol treated with the affinity-purified anti-BARS/9 antibody (H). The cells were fixed and labelled with an anti-mannosidase II antibody.
A similar disassembling effect of BFA was observed in the following experimental conditions, always in the absence of NAD⁺: 1) when dialyzed, pre-ADP-ribosylated cytosol was added to the cells (Fig. 3.12 D), confirming that NAD⁺ functioned as ADP-ribosyl donor in the ADP-ribosylation reaction; and 2) when dialyzed, BARS-depleted cytosol was added to the cells (Fig. 3.12 E; compare with the mock control in Fig. 3.12 F). The last result indicated that BARS in some way interfered with the effect of BFA on the Golgi. In particular, as the depletion of BARS supported the Golgi disassembly effect of the toxin in the absence of NAD⁺, this result showed that BARS is a factor that prevents the action of BFA on the Golgi. To confirm the role of BARS in the action of BFA, purified BARS was added to BARS-depleted cytosol at a final concentration 5-fold higher than calculated to be present in control cytosol. In the presence of this BARS-complemented cytosol, BFA was again almost completely unable to disassemble the Golgi (Fig. 3.12 G). Moreover, when the dialyzed cytosol was pre-incubated with an anti-BARS antibody, BFA potently disassembled the Golgi complex (Fig. 3.12 H). The two last results confirmed an inhibitory role of BARS on Golgi tubulation induced by BFA. The inhibitory effect of BARS on the Golgi disassembly by BFA is independent of another effect of BFA on the Golgi, the dissociation of the coatomer, as coatomer is equally dissociated from the Golgi complex both in the presence and in the absence of NAD⁺ after treatment with BFA (Mironov et al., 1997a). This indicated that BARS does not generically block the action of BFA, but rather it exerts an opposing action on the tubular-reticular transformation of the Golgi induced by BFA.

The studies performed on permeabilized RBL cells demonstrated that BARS prevents the tubular-reticular transformation of the Golgi induced by BFA.
and thus suggested that BARS is involved in the maintenance of the Golgi architecture. The characterization of the effects of recombinant or purified rat brain BARS on isolated Golgi membranes allowed clarification of the mechanism of action of BARS on the Golgi membranes (Weigert et al., 1999). The experiments were performed by Roberto Weigert and Gabriele Turacchio (Laboratory of Molecular Neurobiology – Department of Cell Biology and Oncology - Consorzio Mario Negri Sud). The effect of BARS on the Golgi was tested by adding BARS on isolated Golgi membranes visualised by a negative-staining electron-microscopy technique or by conventional electron microscopy (resin embedding and thin sectioning; Figs. 3.13 - 3.15). When Golgi membranes were exposed to either recombinant or partially purified rat brain BARS in 2-mg/ml cytosol, the tubular structures shown in Fig. 3.13 A underwent rapid disruption, resulting in the formation of vesicular fragments of irregular sizes (Fig. 3.13 B). The extent and time-course of the fission/fragmentation of Golgi tubular domains are shown in Fig. 3.13 C. Cytosol alone had similar, but much slower, effects. The fission/fragmentation was inhibited when cytosol was immuno-depleted of BARS and was regained when depleted cytosol was supplemented with recombinant or partially purified BARS. With 0.3 μg/ml of partially-purified BARS, the cytosol activity returned to control levels, with higher concentrations (3 μg/ml) the fission process was enhanced. A feature of Golgi membranes treated with BARS-enriched cytosol was the early appearance of a number of sites in which the tubule diameter was greatly reduced. Fig. 3.14 shows a selection of such sites, visualized using both negative staining and resin-embedded thin sectioned samples. Sometimes several constrictions were present in the same tubule and were spaced at regular intervals (85 ± 3 nm).
Fig. 3.13. BARS induces fission of the Golgi tubules. These experiments were performed by Roberto Weigert and Gabriele Turacchio (Laboratory of Molecular Neurobiology – Department of Cell Biology and Oncology - Consorzio Mario Negri Sud). **Panels A, B:** Golgi membranes visualized by negative staining. A: Untreated Golgi membranes. B: Golgi membranes incubated for 20 minutes with 2 mg/ml cytosol supplemented with 3 µg/ml partially-purified BARS. Identical effects occurred with 15 µg/ml recombinant BARS. **Panel C:** Time-course of BARS-induced fission. Golgi membranes were incubated with 2 mg/ml soybean trypsin inhibitor (open squares), 3 µg/ml partially purified BARS in the absence of cytosol (filled circles), 2 mg/ml control cytosol (filled squares), 2 mg/ml BARS-depleted cytosol without additions (open triangles), or supplemented with 0.3 (open circles), 1.0 (filled triangles), 3.0 (crosses) µg/ml partially purified BARS. A fission index was evaluated for each stack using four criteria: 1) disappearance of tubular-reticular structures and fenestrations; 2) number of vesicular structures (20 vesicles per stack was the control value in untreated membranes); 3) number of fission intermediates; 4) fraction of naked over tubule-decorated cisternae. Each criterion was given a score from 0 to 5 (for a total of 20). Values are means of triplicates. Scale bars: 100 nm.
Fig. 3.14. Structure of BARS-induced fission intermediates in Golgi tubules. These experiments were performed by Roberto Weigert and Gabriele Turacchio (Laboratory of Molecular Neurobiology – Department of Cell Biology and Oncology - Consorzio Mario Negri Sud). a-d, i: Negative staining of whole-mount preparations. e-h, j: Thin sections of resin embedded samples. a, e: Membranes incubated for 15 min with control cytosol; example of unconstricted tubules. b-d, f-g: Golgi membranes incubated with 3 μg/ml partially purified rat brain BARS or 30 μg/ml recombinant BARS added to 2 mg/ml cytosol. Moderate constrictions are seen in b and f and extreme constrictions in c and g. Rows of aligned vesicles, presumably resulting from clipping of a single tubule at regular intervals, are seen in d and h; series of fission intermediates in single tubules in i and j. Scale bars: 40 nm.
Fig. 3.15. Acyl-CoA are essential cofactors for BARS-induced fission of the Golgi tubules. These experiments were performed by Roberto Weigert and Gabriele Turacchio (Laboratory of Molecular Neurobiology – Department of Cell Biology and Oncology - Consorzio Mario Negri Sud). Panels A, B: Golgi membranes visualized by negative staining. Membranes were incubated for 20 min with 10 µg/ml partially purified BARS alone (A) or in the presence of 10 µM palmitoyl-CoA (B). Panel C: Quantification of the BARS fission effects. Untreated Golgi membranes (1), Golgi membranes incubated for 15 min with no additions (2), cytosol (3), cytosol supplemented with 10 µg/ml partially purified BARS (4), 10 µg/ml partially purified BARS (5), 10 µM palmitoyl-CoA (6), 10 µg/ml partially purified BARS and 10 µM palmitoyl-CoA (7), 10 µg/ml ADP-ribosylated BARS alone (8) or with 10 µM palmitoyl-CoA. Scale bars: 100 nm.
These dimensions are compatible with those of vesicles produced by fission. For this reason it was proposed that these are sites where fission occurs later, and they were called “fission intermediates”. These sites were also found in samples treated with cytosol alone, albeit rarely. To understand how BARS induced the formation of fission intermediates, the activity of BARS was assayed in the absence of cytosol and in the presence of different cytosolic factors. Strikingly, palmitoyl-CoA replaced cytosol in inducing the fission activity of partially purified BARS on the Golgi while BARS alone or palmitoyl-CoA alone had no effect (Fig. 3.15). This result indicated that BARS might be involved in acyl-CoA-dependent lipid modifications. This hypothesis was tested by incubating a series of lipids, lysolipids and their corresponding headgroups with recombinant BARS and radiolabelled palmitoyl-CoA. Lysophosphatidic acid (LPA) was the only molecule that showed a detectable activity as acyl acceptor from $[^{14}C]-$palmitoyl-CoA, resulting in incorporation of the label into a new radioactive species. This product was $[^{14}C]$-phosphatidic acid (PA), as demonstrated by co-migration with pure $[^{14}C]$-PA standard in thin layer chromatography (Fig. 3.16). Thus, BARS is an acyltransferase with a substrate selectivity for LPA. To be functionally relevant, the transferase activity of BARS should change the PA levels in Golgi membranes under the conditions used in the fission assay. Therefore, the BARS-induced increase in PA levels and the fission of the Golgi membranes were compared in parallel experiments. The results indicated that the two processes occurred within the same time interval (Fig. 3.17), suggesting that they are associated. These and other data (Weigert et al., 1999) led to the conclusion that LPA acyltransferase activity of BARS is an essential component of the mechanism by which this protein promotes fission in Golgi tubular networks.
Fig. 3.16. BARS is an LPA-acyltransferase. This experiment was performed by Roberto Weigert and Gabriele Turacchio (Laboratory of Molecular Neurobiology – Department of Cell Biology and Oncology - Consorzio Mario Negri Sud). LPA-acyltransferase activity of BARS was detected by incubating $[^{14}\text{C}]$-palmitoyl-CoA with 30 μM LPA (C18:1-LPA) in the absence (lane 1) or presence (lane 2) of 50 μg/ml recombinant BARS for 40 min. $[^{14}\text{C}]$-PA standard is in lane 3. Lipids were separated by thin layer chromatography (TLC). The positions of $[^{14}\text{C}]$-PA (PA) and $[^{14}\text{C}]$-palmitoyl-CoA (pCoA) are indicated on the right of the panel.
Fig. 3.17. PA synthesis in Golgi membranes is associated with membrane fission. Time-course of PA formation and tubule fission in Golgi membranes. This experiment was performed by Roberto Weigert and Gabriele Turacchio (Laboratory of Molecular Neurobiology – Department of Cell Biology and Oncology - Consorzio Mario Negri Sud). Membranes were incubated with 10 μM [14C]-palmitoyl-CoA in the absence (square) or in the presence (circles) of recombinant CtBP3/BARS and processed for lipid analysis or negative stain electron microscopy. PA levels calculated as percentage of total membrane lipids, are shown on the left axis (filled symbols, continuous lines); the index of fission is shown on the right axis (open symbols, dashed lines). Values are means ± s.d. of three independent experiments.
In summary, the experiments reported above (Weigert et al., 1999) showed that BARS is an essential rate-controlling component of the tubule fission-inducing activity of cytosol, the fission-inducing activity of BARS requires cytosolic acyl-CoAs, and BARS acts as a transferase that is able to convert LPA to PA. Moreover, the finding that synthesis of PA is strictly correlated to fission of Golgi tubules strongly suggests that BARS induces the fission of the Golgi tubules by changing the lipid composition of the membranes at the level of fission sites. A limiting step in fission of membranes is the extreme bending of the bilayer required for the close contact between the fusing membranes (see section 1.1.3.4). The appearance of extremely narrow tubules in the Golgi membranes incubated with BARS suggests that the activity of BARS leads to bending of membranes. Several possibilities exist to explain how the conversion of LPA into PA can induce the bending and thus trigger the fission of the membranes. PA has unique physical properties. It has a small headgroup with high charge density and, given its high tendency to form clusters (Boggs, 1987), it could form PA-rich microdomains. Moreover, if LPA is derived from a membrane lipid through the activity of a phospholipase, the overall reaction would convert an unfavourable phospholipid into PA, a bilayer-prefering lipid, that in certain conditions (low pH or high concentration of divalent cations) can acquire conic shape. Thus PA, if transferred to the luminal leaflet by a specific flippase, or converted to diacylglycerol, a lipid that undergoes spontaneous flip-flop in biological membranes, might introduce in the luminal leaflet a negative curvature favourable to fission of membranes. These scenarios make probable that other enzymes involved in lipid metabolism participate in the fission event induced by BARS.
The strong similarity between BARS and the CtBPs raises the question whether these proteins, so highly conserved at the level of their primary structures, could have different cellular functions, one in transcription and one in the Golgi complex organization. In principle, it is possible that one of the CtBPs might have evolved a radically different function than that of the other CtBPs, and that such function might be limited to the Golgi. The other possibility is that the CtBPs might have a dual role, one in the nucleus and one in the cytoplasm. There are number of examples of proteins having dual or multiple role in the cell (Jeffery, 1999). To try to clarify the point of dual role of this family of proteins, studies on the subcellular localization of the three CtBPs were carried out, as described in the next chapter.
CHAPTER 4

Subcellular Localization of CtBP3/BARS

4.1 Introduction

As reported in the previous chapter, the 50-kDa substrate of BFA-induced-ADP ribosylation was cloned, identified as the third member of the CtBP family of proteins and, thus, renamed CtBP3/BARS. CtBP3/BARS and the other two mammalian members of the CtBP family CtBP1 and CtBP2 were all found to be substrates of BFA-dependent ADP-ribosylation. Therefore in this thesis, I am referring to BARS in all the cases in which I am not able to distinguish among the three different substrates of BFA-dependent ADP-ribosylation. As discussed in the previous chapter, rat brain BARS as well as recombinant CtBP3/BARS were shown to induce the fission of tubules in isolated Golgi membranes. In the present chapter, the subcellular localization of BARS is investigated and compared to the localizations of CtBP1 and CtBP2, to address the functional relationship among the protein of the CtBP family.

4.2 Results

4.2.1 Analysis of the subcellular localization of CtBP3/BARS

The intracellular localization of CtBP3/BARS was studied through indirect immunofluorescence techniques. Initially, different cell lines were labelled with anti-BARS antibodies to detect the localization of endogenous CtBP proteins. Only one of these antibodies, an affinity-purified antibody raised against the
whole protein fused to GST (p502; see also section 2.4 and Table 2.3), was able to give a detectable staining of the cells. This antibody is able to recognize all the three CtBPs in Western blot (Fig. 4.1, lanes 6-10) and presumably its signal in indirect immunofluorescence reflect the sum of distributions of all CtBPs. The affinity-purified p502 antibody strongly stained nuclei, but also labelled the cytoplasm with a clear perinuclear enrichment. In Fig. 4.2 the labelling of human fibroblasts with this antibody and Golgi markers is shown. The Golgi-associated labelling of BARS was weak but clearly detectable. The difficulty in detecting endogenous BARS localization with other antibodies could arise from the very low abundance of this protein in the cell.

Because of the unavailability of antibodies that specifically recognized CtBP3/BARS in immunofluorescence without cross-reacting with the other CtBPs, the localization of CtBP3/BARS was studied by overexpressing the protein in COS7 cells and detecting it with a polyclonal affinity-purified antibody (SN1; see also Table 2.3) or with a monoclonal antibody specific for CtBP3/BARS (AA5.3). Using standard immunoblot (Fig. 4.1, lane 2 and 10) and immunofluorescence (Fig. 4.3) procedures these antibodies were unable to detect endogenous proteins. Instead, they were very efficient in recognizing overexpressed CtBP3/BARS both in immunoblot (Fig. 4.1 A, lane 3 and 11) and in immunofluorescence (Fig. 4.3). Among CtBP3/BARS-transfected COS7 cells, there were distinguishable subpopulations with different levels of fluorescence, indicating different level of expression of the protein (Fig. 4.3). The cells expressing higher levels of CtBP3/BARS showed a spread distribution of CtBP3/BARS throughout the cell. In cells expressing lower levels of the protein, CtBP3/BARS was mostly localized in the cytoplasm and appeared usually also present in lower amounts in the nucleus.
Fig. 4.1. Specificity of anti-BARS antibodies in Western blot. 100 μg brain cytosol (lane 1) and total cell lysates from approximately $10^5$ untransfected or transfected COS7 cells (lanes 2-13) were separated by SDS-PAGE, transferred on nitrocellulose and probed with 1 μg/ml affinity-purified polyclonal SN1 antibody (lanes 1-5), 0.1 μg/ml affinity-purified polyclonal p502 antibody (lanes 6-9), or 1:50 monoclonal AA5.3 antibody (lanes 10-13). Cell lysates were obtained from untransfected COS7 cells (lanes 2, 6, 10), or from COS7 transfected with CtBP3/BARS (lanes 3, 7, 11), CtBP1 (lanes 4, 8, 12), or CtBP2 (lanes 5, 9, 13). The size of molecular weight standards is indicated in kDa on the left of panels. In COS7 cells the SN1 antibody is able to recognize only overexpressed CtBPs, whereas the p502 antibody is also able to detect endogenous CtBPs. Remarkably, the monoclonal AA5.3 antibody specifically recognizes overexpressed CtBP3/BARS, without detecting CtBP1 and CtBP2.
Fig. 4.2. Localization of endogenous BARS. Human fibroblasts were triple-labelled with 10 μg/ml affinity-purified p502 antibody (BARS), an anti-GM130 antibody (GM130) and an anti-TGN46 antibody (TGN46). Triple image overlay (triple) and double-image overlays showing superimposition of BARS and TGN46 labelling (BARS/TGN), of BARS and GM130 labelling (GM130/BARS), and of the two Golgi markers labelling (TGN/GM130) are also shown. Images are optical slices acquired at the confocal microscope with a pinhole equivalent to one Airy unit. Scale bar: 10 μm.
Fig. 4.3. Specificity of anti-BARS antibodies in COS7 cells. COS7 cells were transfected with CtBP3/BARS and, 24 hours after transfection, fixed and labelled with 1 μg/ml affinity-purified polyclonal SN1 antibody (A) or with 1:20 monoclonal AA5.3 antibody (B) in green. The cells were counterstained in red with an anti-α tubulin antibody (A') and an anti-calreticulin antibody (B'), respectively, to show also cells that do not overexpress CtBP3/BARS. Scale bars: 10 μm.
The amount of nuclear CtBP3/BARS in lower level expressing cells was variable, from being essentially undetectable to being comparable to the cytoplasmic amounts. In the cytoplasm CtBP3/BARS usually appeared to be diffused, although sometimes seemed to be distributed in a reticular pattern. Further, a) CtBP3/BARS was accumulated in the perinuclear area of the cytoplasm in roughly 70% of the overexpressing cells; and b) a slight enrichment of CtBP3/BARS at the plasma membrane was observed in about half of the overexpressing cells.

To define which cytoplasmic structures CtBP3/BARS was associated with, double labelling experiments on CtBP3/BARS-overexpressing cells were performed. CtBP3/BARS-transfected cells were stained with the polyclonal SN1 or the monoclonal AA5.3 antibodies and a series of markers for different intracellular compartments (Fig. 4.4). For this analysis Golgi, ER, endosomal, and cytoskeletal markers were selected. As Golgi markers, antibodies raised against the Golgi proteins GM130, giantin, and TGN46 were employed. As endosomal markers, antibodies raised against transferrin receptor, a plasma membrane-endosome recycling protein, and mannose-6-phosphate receptor, a TGN-endosome recycling protein, were chosen. As ER markers, antibodies directed against calreticulin and protein disulfide isomerase (PDI) were used. First, the general conditions of CtBP3/BARS-transfected cells were evaluated. The transfected cells were fixed and labelled 24 and 48 hours after transfection. In both cases the overall morphology of the cells and the spatial organization of several compartments observed were not apparently affected by the overexpression of CtBP3/BARS. In Fig. 4.4 the appearance of microtubules (A'), vimentin intermediate filaments (B'), actin microfilaments (C'), and the
Fig. 4.4. Double-labelling of CtBP3/BARS-transfected COS7 cells. CtBP3/BARS-transfected COS7 cells were fixed and double-labelled with antibodies to CtBP3/BARS (A-F) and with different markers of cytoskeleton and membranous intracellular compartments (A'-F'). CtBP3/BARS was labelled with the SN1 antibody (A-D, F) or the AA5.3 antibody (E). Microtubules (A') and
vimentin intermediate filaments (B') were labelled with monoclonal anti-α tubulin and anti-vimentin antibodies, respectively. Actin microfilaments were labelled by green-fluorescent phallodin (C'). Transferrin receptor containing compartments (endosomes and plasma membrane) were labelled with a monoclonal antibody to transferrin receptor (D'). Endoplasmic reticulum was labelled with a polyclonal antibody against calreticulin (E'). The Golgi complex was labelled with a monoclonal antibody against giantin (F'). Scale bars: 10 μm.
distribution of the endosomal marker transferrin receptor (D'), the ER marker calreticulin (E'), and the Golgi marker giantin (F') in overexpressing and non-overexpressing cells is shown. This preliminary analysis of double-labelled cells also indicated that CtBP3/BARS partially co-localized with the Golgi markers (Fig. 4.4 F and F'). To better analyse the intracellular distribution of CtBP3/BARS, the double-labelled CtBP3/BARS-overexpressing cells were analysed at the confocal microscope. As shown by an optical sectioning of a cell along the z axis (Fig. 4.5), CtBP3/BARS was indeed distributed in all the cytoplasm and clearly enriched in the Golgi area. Confocal analysis also indicated that CtBP3/BARS is never enriched in the nucleus in comparison with the cytoplasm. Rather, CtBP3/BARS appeared enriched at the nuclear envelope in roughly 60% of the overexpressing cells (Fig. 4.6 A) and was always excluded from nucleoli (see Fig 4.6 A). Moreover, as stated above, in about half of the overexpressing cells, regions of the plasma membrane showed enrichment of CtBP3/BARS; in these regions CtBP3/BARS was observed to co-localize with cortical actin (Fig. 4.6 B).

CtBP3/BARS is usually extracted from cells as a cytosolic component, and a cytosolic distribution was thought to be the reason for the high level of diffused staining in all the transfected cells observed. Thus, to try to reduce the cytosolic background in the cell, CtBP3/BARS-transfected cells were permeabilized before fixation. Protocols of permeabilization with streptolysin O (SLO) or short treatments with non-ionic detergents (Triton X-100) were used. In both cases a strong reduction of diffused cytoplasmic staining was obtained and in most of the cells (roughly 70% of the overexpressing cells) a clear Golgi localization of CtBP3/BARS was observed (Fig. 4.7 A).
Fig. 4.5. Optical sectioning of a CtBP3/BARS-overexpressing cell: accumulation of CtBP3/BARS in the Golgi area. Twenty-four hours after transfection, CtBP3/BARS-transfected COS7 cells were fixed and double-labelled with the SN1 antibody (CtBP3/BARS) and an anti-giantin antibody (giantin). Overlay of single images is also shown (merge). The cells were analyzed at the confocal microscope with a pinhole equivalent to one Airy disk diameter. Images are serial optical sections - upper images correspond to the upper slice of the cell (top), lower images to the lower slice of the cell (bottom) - acquired at 0.3-µm intervals along the z axis. Scale bar: 5 µm.
Fig. 4.6. Optical sectioning of CtBP3/BARS-overexpressing cells: enrichment of CtBP3/BARS at the nuclear envelope (A) and at the plasma membrane (B). CtBP3/BARS-transfected COS7 cells were fixed 24 hours after transfection and analyzed at the confocal microscope with a pinhole equivalent to one Airy
Panel A: cells were double-labelled with the AA5.3 antibody (CtBP3/BARS) and an anti-calreticulin antibody (calreticulin). Overlays of the single images is also shown (merge). Images are serial optical sections - upper images (top) correspond to the upper slice of the cell, lower image to the lower slice of the cell (bottom) - acquired at 0.3-μm intervals along the z axis. Panel B: cells were double-labelled with the SN1 antibody (CtBP3/BARS) and with green-fluorescent phalloidin to detect F-actin (actin). Image overlay is also shown (merge). Scale bars: 5 μm.
Fig. 4.7. Localization of CtBP3/BARS at the Golgi complex and at the microtubules. COS7 cells were transfected with CtBP3/BARS and, 24 hours after transfection, permeabilized with SLO, incubated at 37°C for 5 min and fixed with 4% paraformaldehyde. In panel A, cells were double-labelled with an anti-giantin antibody (giantin) and the SN1 antibody (CtBP3/BARS); image overlay is also shown (merge). In panel B, cells were double-labelled with an anti-α tubulin antibody (tubulin) and the SN1 antibody (CtBP3/BARS). Enlargements of the area surrounded by the white box are shown on the bottom. Images are optical slices acquired at the confocal microscope with a pinhole equivalent to one Airy unit. Scale bars: 5 μm. These experiments were done with the help of Claudia Cericola (Laboratory of Molecular Endocrinology - Department of Cell Biology and Oncology - Consorzio Mario Negri Sud).
Golgi-associated CtBP3/BARS staining was also detected in cells after 20 minutes of exposure to buffer upon SLO permeabilization. Although the cytoplasmic diffused staining was substantially decreased after SLO permeabilization, peripheral, filamentous CtBP3/BARS staining persisted and was found to co-localize in part with microtubules (Fig. 4.7 B). Nuclear envelope and plasma membrane localizations, detected in a certain percentage of cells in the absence of permeabilization (see above), also persisted after SLO permeabilization.

To confirm that the perinuclear CtBP3/BARS was indeed associated with the Golgi, the CtBP3/BARS-overexpressing cells were treated with nocodazole, a drug that is able to depolymerise microtubules and rearrange the Golgi complex in small peripheral structures, also called ministacks (Rogalski and Singer, 1984; Cole et al., 1996a). As shown in Fig. 4.8, after treatment with nocodazole CtBP3/BARS labelling was redistributed from the juxtanuclear area to peripheral spots. The redistribution of TGN46 occurred earlier than that of other central Golgi markers such as giantin, and was almost complete after 1h exposure to the drug, whereas at that time giantin was still concentrated in juxtanuclear position. After 1 hour of exposure to nocodazole, CtBP3/BARS was in part still accumulated in perinuclear position and in part distributed to big spots in close vicinity to TGN46 stained spots. After 2 hours of treatment with nocodazole also giantin staining was redistributed into peripheral spots and CtBP3/BARS appears in close association with both giantin and TGN46 spotted structures. The set of experiments described in this section established that CtBP3/BARS is mostly present in the cytoplasm of the cells and is associated with the Golgi complex.
Fig. 4.8. Redistribution of CtBP3/BARS upon nocodazole treatment. COS7 cells were transfected with CtBP3/BARS and 24 hours after transfection were
treated with 33 μM nocodazole. Cells were fixed before adding nocodazole (panel A), or 1 h (panel B) and 2 h (panel C) after addition of nocodazole. After fixation cells were triple-labelled with the SN1 antibody (CtBP3/BARS), an anti-TGN46 antibody (TGN46), and an anti-giantin antibody (giantin). Double-image (giantin/BARS; BARS/TGN; TGN/giantin) and triple-image (triple) overlays are also shown. Images were acquired at the confocal microscope with a pinhole equivalent to one Airy unit. Scale bars: 5 μm.
4.2.2 Comparison of the subcellular distributions of CtBP1, CtBP2 and CtBP3/BARS

The experiments reported in section 4.2.1 showed that CtBP3/BARS is barely present in the nucleus of overexpressing cells, while it is essentially localized in the cytoplasm. This finding was not obvious, given the high similarity between CtBP3/BARS and the transcriptional regulators CtBP1 and CtBP2. In order to clarify the functional relationship among the three mammalian CtBPs, the intracellular localizations of CtBP1 and CtBP2 were investigated in transfected cells as was done for CtBP3/BARS. CtBP1, CtBP2 and CtBP3/BARS were transfected in parallel in different cell lines and their intracellular distributions revealed by labelling with the anti-BARS SN1 antibody. This antibody is able to recognize all the three overexpressed proteins in Western blot (Fig. 4.1 lanes 3-5) as well as in immunofluorescence (Fig. 4.9). Differences in the distributions of the three proteins were clearly detected. As described above, CtBP3/BARS, in the absence of permeabilization, appeared mostly localized in the cytoplasm with a diffused or reticular distribution and often a perinuclear accumulation. In cells expressing low levels of protein, CtBP1 showed prevalently a cytoplasmic distribution similar to that of CtBP3/BARS (Fig. 4.9 B and E, insets), while in cells expressing relatively higher levels of protein (roughly 50% of the overexpressing cells), CtBP1 was localized to very long cytoplasmic ribbon-like structures (Fig. 4.9 B and E). In contrast, CtBP2 was exclusively localized in the nucleus in roughly 90% of the overexpressing cells (Fig. 4.9 C and F). Similar results, with CtBP3/BARS and CtBP1 mostly cytoplasmic and CtBP2 exclusively nuclear, were obtained in COS7 cells, HeLa cells, MDCK cells and NRK cells.
Fig. 4.9. Subcellular distribution of CtBPs. COS7 (A-C) or HeLa cells (D-F) were transfected with CtBP3/BARS (A, D), CtBP1 (B, E) or CtBP2 (C, F). Twenty-four hours after transfection the cells were fixed and labelled with 1 μg/ml affinity-purified polyclonal SN1 antibody shown in green. COS7 cells were also counterstained in red with an anti-α tubulin antibody to evidence the cell cytoplasm. In B and E, insets show cells expressing lower level of CtBP1 that do not present CtBP1-labelled ribbons. Scale bars: 10 μm.
4.2.3 Analysis of the CtBP1-labellede structures

The peculiar structures observed in CtBP1-transfected cells were subject of a brief study in order to understand their nature and origin. CtBP1-overexpressing cells were double labelled with the anti-BARS/SN1 antibody and different markers. First, double labelling experiments with cytoskeleton markers were performed in order to investigate whether the long ribbons labelled by CtBP1 were cytoskeletal structures (Fig. 4.10). The CtBP1-labelled ribbons appeared to be completely different in organization and distribution from microtubules (labelled by an anti α-tubulin antibody; Fig. 4.10 A, B, C), vimentin intermediate filaments (labelled by an anti vimentin antibody; Fig. 4.10 D, E, F), and actin microfilaments (labelled by phalloidin; Fig. 4.10 G, H, I). To further exclude that the structures labelled by CtBP1 were cytoskeletal elements of the cell, their resistance to detergent treatments was tested. When the overexpressing cells were treated with Triton X-100, in conditions that do not alter the cytoskeleton and do not affect the distribution of membrane proteins such as the Golgi integral membrane protein giantin (Fig. 4.11, compare A’ and C’), the CtBP1-labelled structures completely disappeared (Fig. 4.11, compare A and C), indicating that with high probability CtBP1-labelled structures did not belong to cytoskeleton. Moreover, CtBP1-transfected cells were labelled with a variety of markers for different intracellular membranous structures - Golgi, endosomes and ER - and in no case co-localizations between membranes and CtBP1 ribbons were found (Fig. 4.12).

The main question concerning these structures is if they are pre-existing structures on which CtBP1 is targeted after overexpression, or structures produced by the overexpression of CtBP1. The fact that these peculiar structures were never observed by labelling untransfected cells makes unlikely the first possibility.
Fig. 4.10. CtBP1-labelled structures do not colocalize with cell cytoskeleton. CtBP1-transfected COS7 cells were fixed 24 hours after transfection and double-labelled with the SN1 antibody (A, D, G) and with an anti-α tubulin antibody (B), an anti-vimentin antibody (E) or green-fluorescent phalloidin (H). Respective double-image overlays are shown in C, F and J. Scale bars: 10 μm.
**Fig. 4.11.** CtBP1-labelled ribbons are solubilised by Triton X-100 treatment. COS7 cells were transfected with CtBP1 and 24 h after transfection were treated with 2% Triton X-100 in MEMA buffer (C, C' and D) or incubated in MEMA buffer for 10 min at RT (A, A' and B). At the end the cells were fixed and labelled with the SN1 antibody (A and C), an antibody against giantin, a non-solubilised marker (A' and C'), and an antibody against p115, a solubilised marker (B and D). Scale bars: 10 μm.
Fig. 4.12. CtBP1-labelled structures do not colocalize with intracellular membranes. Panel A: COS7 cells were transfected with CtBP1 and, 24 hours after transfection, were fixed and double-labelled with the SN1 antibody (CtBP1) and an antibody against the Golgi marker giantin (giantin) or an antibody against the endosomal marker transferrin receptor (tfR); respective double-image overlays are shown on the right (merge). Panel B: COS7 cells were transfected with CtBP1 and VSVG-GFP. Six hours after transfection were transferred at 39.5°C and incubated O/N at this temperature in order to allow VSVG-GFP accumulation in the ER. The cells were then fixed and labelled with the SN1 antibody and red fluorophore (CtBP1). VSVG-GFP, restricted in the ER because of the temperature block, was detected through its spontaneous fluorescence and shown in green (ER). Scale bars: 10 μm.
Moreover, the results indicated that they are not cytoskeletal structures. Two hypotheses remain open: 1) these structures are easily solubilized, pre-existing membranous structures not explored with the markers used in this study; 2) they are structures generated by self-aggregation of overexpressed CtBP1.

4.2.4 Signals for nuclear localization of CtBP2

The nuclear localization of CtBP2 suggested that this protein has specific signals to be transported into the nucleus. The N-terminus of CtBP2 is the portion of the molecule that is more divergent from the sequences of the homologues CtBP1 and CtBP3/BARS, and, interestingly, is highly rich in basic amino acids (Fig. 4.13 A), like many nuclear localization signals (Nakielny and Dreyfuss, 1999). These considerations suggested that the N-terminus might contain the signal responsible for the nuclear localization of CtBP2. To test this possibility, a truncated mutant of CtBP2 lacking the first 25 amino acids of the protein was transfected in COS7 cells. In contrast to the wild type protein, the truncated mutant (ΔN-CtBP2) was no more able to accumulate in the nuclei of the transfected cells (Fig. 4.13 B), indicating that the CtBP2 N-terminus contains a nuclear localization signal.

4.2.5 Inhibition of nuclear export did not affect CtBP1 and CtBP3/BARS subcellular localizations

Recently, some proteins involved in endocytosis that had been always observed to be localized in the cytoplasm, were surprisingly found to shuttle between the nucleus and the cytoplasm (Hyman et al., 2000; Vecchi et al., 2001). The nucleocytoplasmic shuttling of endocytic proteins was discovered through the use of leptomycin B (LMB), a drug that specifically inhibits nuclear export.
Fig. 4.13. CtBP2 contains a nuclear localization signal at its N-terminus. **Panel A:** the N-terminal portions of the three CtBPs and the truncated CtBP2 (ΔN-CtBP2) were aligned. Basic aminoacids are shown in cyan on red background. **Panel B:** COS7 cells were transfected with CtBP2 (CtBP2) or with the truncated mutant ΔN-CtBP2 (ΔN-CtBP2) and 24 hours after transfection were labelled with the SN1 antibody. Scale bars: 10 μm.
Leptomycin B inhibits nucleo-cytoplasmic transport of proteins mediated by the soluble receptor CRM1 or exportin 1, the best characterized nuclear export pathway (Fornerod et al., 1997; Kudo et al., 1998; Kudo et al., 1999; Yoneda et al., 1999). The block of the exit from the nucleus allowed to detect nuclear accumulation of those proteins with a predominant extranuclear localization, revealing that, although mostly localized in the cytoplasm in steady-state conditions, they were subjected to nucleocytoplasmic shuttling. To investigate if CtBP1 and CtBP3/BARS could undergo a nucleocytoplasmic shuttling, CtBP1- and CtBP3/BARS-transfected COS7 cells were treated for 4-8 hours with leptomycin B up to 10 ng/ml. In no experiment was observed an increase in the nuclear localization of the two proteins (Fig. 4.14, compare A and B with D and E). In the same experiments also ΔN-CtBP2-transfected COS7 cells were treated with leptomycin B. In contrast to the other two proteins, the truncated CtBP2 mutant ΔN-CtBP2 showed an increase of nuclear localization both in terms of intensity of nuclear fluorescence (Fig. 4.14, compare C with F) and in terms of percentage of cells that showed higher levels of nuclear staining as compared to cytoplasmic staining (94% of LMB-treated cells versus 63% of control ethanol-treated cells). These results indicate that CtBP1 and CtBP3/BARS completely lack leptomycin B-sensitive nuclear export signals. However, they do not exclude the possibility that CtBP1 and CtBP3/BARS undergo a nucleo-cytoplasmic shuttling regulated by a nuclear export receptor different from CRM1. In contrast, ΔN-CtBP2 exit from the nucleus appeared to be sensitive to leptomycin B, implicating that CtBP2 localization is also regulated by nuclear export signals. It is worthy to note that also in the absence of its nuclear localization signal CtBP2 is able to enter into the nucleus, probably due to its size below the threshold of free diffusion of molecules across the nuclear pore (Weis, 1998).
Fig. 4.14. Subcellular localization of CtBP3/BARS and CtBP1 is not affected by LMB. COS7 cells were transfected with CtBP3/BARS (A, D), CtBP1 (B, E), or ΔN-CtBP2 (C, F). Twenty hours after transfection the cells were incubated with 10 ng/ml LMB (D-F) or with 0.1% ethanol (A-C) for 8 h. At the end the cells were fixed and labelled with the SN1 antibody. Scale bars: 10 μm.
4.3 Discussion

By studying the subcellular localization of CtBP3/BARS, I found that the new protein is essentially localized in the cytoplasm, and that, after removing the soluble cytosolic fraction of the protein by cell permeabilization, it appears clearly localized at the Golgi complex in about 70% of the cells. There can be several reasons for the protein not to be detected in association with the Golgi complex in all of the transfected cells. First, CtBP3/BARS might be associated with the Golgi only in a precise phase of the cell cycle. This would imply that CtBP3/BARS plays a role at the Golgi in a specific step of the life cycle of this organelle. One such step could be the fragmentation that precedes the partitioning of the Golgi occurring in mitosis (Shima et al., 1998). This hypothesis, if confirmed, would also suggest that the fission activity of CtBP3/BARS might be involved in the fragmentation of the Golgi occurring at the beginning of mitosis. A second possibility is that CtBP3/BARS association with the Golgi might be dependent on the trafficking conditions of the cell, which may again be related either to the specific cell cycle step or to other growth conditions such as density or stress. Finally, it should be considered that CtBP3/BARS association with the Golgi could be regulated by external stimuli and intracellular signalling events. In a certain percentage of cells CtBP3/BARS appeared also to be localized at the nuclear envelope and at the plasma membrane. This result suggests that CtBP3/BARS also control membrane dynamics at intracellular sites different from the Golgi tubules. After cell permeabilization CtBP3/BARS was also found in part associated with microtubules. This observation is in agreement with biochemical results described in the following chapter and will be discussed later.
The results reported in this chapter indicate that, despite their high degree of similarity, CtBP1 and CtBP3/BARS have intracellular localizations different from that of CtBP2. This suggests that they have different functions in the cell. One obvious conclusion from the data presented could be that CtBP2 is the CtBP responsible for the transcriptional regulation inside the nucleus and CtBP1 and CtBP3/BARS could be cytoplasmic proteins responsible for the regulation of membrane dynamics. Nonetheless, in some cells CtBP1 and CtBP3/BARS are also present in the nuclei, although at levels not higher than in the cytoplasm, leaving open the possibility that they also play a nuclear function, probably the regulation of transcription, which has been demonstrated for CtBP1 and CtBP2 (Turner and Crossley, 1998; Criqui-Filipe et al., 1999; Postigo and Dean, 1999; Sewalt et al., 1999; Turner and Crossley, 2001). In the present study, I was unable to detect nucleocytoplasmic shuttling of CtBP1 and CtBP3/BARS, but this does not exclude that they are transported outside of the nucleus through a machinery different from that assayed in this study using the specific inhibitor LMB. An interesting hypothesis would be that the CtBPs are responsible for the communication between nucleus and cytoplasm that allows the correct timing of Golgi fragmentation at the beginning of mitosis. The CtBPs could have a double function in the cell, as demonstrated for many other proteins (Jeffery, 1999; Hyman et al., 2000; Vecchi et al., 2001). If one or more CtBPs would be competent for both regulation of transcription and regulation of fission at the Golgi membranes, their increased availability at the Golgi upon break-down of the nuclear envelope could constitute the mechanism through which the cell obtains synchronization between nuclear envelope disappearance and the events of Golgi fragmentation and dispersal occurring during mitosis.
After overexpression of CtBP1, large and long ribbon structures were observed in cells expressing high level of the protein. The results presented in this chapter indicate that these structures are not cytoskeletal elements of the cell. Moreover, although they do not completely exclude the possibility that they are membranous structures, they make this possibility extremely unlikely. Notably, similar structures are also occasionally observed in cells overexpressing CtBP3/BARS incubated at 40°C (observation made by Matteo Bonazzi - Laboratory of Molecular Neurobiology – Department of Cell Biology and Oncology - Consorzio Mario Negri Sud). The most probable explanation for the appearance of these structures is that they are originated by self-aggregation of overexpressed proteins. This would imply that CtBP1 and CtBP3/BARS have a strong propensity to originate highly structured and ordered structures. Ribeye, a protein related to the CtBPs, is a major component of synaptic ribbons and it also has the ability to self-aggregate, a fundamental feature for the formation of stable synaptic ribbons (Schmitz et al., 2000). The ability of CtBP1 and CtBP3/BARS to self-aggregate when overexpressed may thus be a symptom of the aptitude of the protein to form multimeric aggregates also in more physiological conditions, and may indicate an importance of ordered molecule assembly in the mechanism of action of BARS.
CHAPTER 5

Identification of CtBP3/BARS-Interacting Proteins

5.1 Introduction

The results reported in the previous chapter indicated that BARS, the 50-kDa substrate of BFA-induced ADP-ribosylation, isolated and cloned from rat brain, is localized at the Golgi complex. Moreover, as reported in Chapter 3, CtBP3/BARS is able to induce the fission of the Golgi complex in an \textit{in vitro} system and to catalyse the conversion of lysophosphatidic acid (LPA) into phosphatidic acid (PA). As discussed in section 3.4, the mechanism through which CtBP3/BARS induces membrane fission could involve the modification of membrane curvature obtained by changing the lipid composition of the bilayer. In order to better elucidate the mechanism of action of CtBP3/BARS, the identification of other molecular players acting in the CtBP3/BARS-induced fission process would be fundamental. Therefore, I searched for CtBP3/BARS-interacting proteins. In this chapter the experimental systems set up to isolate CtBP3/BARS-interacting proteins and the identification of four such proteins by using two different biochemical assays are reported.
5.2 Results

5.2.1 Biochemical approaches to identify CtBP3/BARS-interacting proteins

The identification of CtBP3/BARS-interacting proteins involved in membrane fission was approached by biochemical methods. This choice came after excluding the yeast-two hybrid screening, a widely used method to detect interactions between proteins, because it had been already exploited to identify CtBP1-interacting proteins by other investigators and had led to isolation of few specific clones, coding for the novel protein CtIP and the 70-kDa subunit of the KU autoantigen involved in DNA repair (Schaeper et al., 1998). Moreover, the availability of biochemical and affinity chromatography expertise in the laboratory also prompted me to set-up biochemical systems to isolate BARS-interacting proteins from rat brain cytosol, the same source from which BARS was purified (see section 3.2).

Three different affinity precipitation systems were applied. Two of them were based on the use of recombinant CtBP3/BARS, either fused to GST or tagged with a poly-histidinie sequence. The third was based on the use of an anti-BARS antibody able to immunoprecipitate BARS (see section 3.3.4). All of the different methods were based on the possibility to recover BARS and, possibly, BARS-interacting proteins, bound to a sedimentable matrix. The use of the immunoprecipitating antibody was found to be the most useful among these approaches. In a typical experimental scheme (see Fig. 5.1), affinity purified anti-BARS IgGs or total IgGs purified from the preimmune antiserum, employed as negative control, were covalently attached to protein A-Sepharose beads.
Fig. 5.1. Scheme illustrating BARS co-immunoprecipitation experiments. Protein A-Sepharose beads were cross-linked to anti-BARS or preimmune IgGs through the use of dimethyl pimelimidate (DMP). The two IgG matrix were incubated with cytosol to allow the binding of interacting cytosolic proteins, extensively washed and the proteins bound were eluted with 0.1 M glycine pH 3.0 or by adding SDS-sample buffer and boiling for 10 minutes. After the elution step the proteins were separated by SDS-PAGE and the proteins eluted from the BARS-IgG matrix were compared with the proteins eluted from the preimmune-IgG matrix to recognize specific BARS-interacting proteins.
The IgG-protein A-Sepharose matrixes were then incubated with rat brain cytosol and, after extensive washing, the proteins retained on the matrixes were eluted with a low pH buffer or by boiling the matrixes in SDS-sample buffer. The eluted proteins were finally separated by SDS-PAGE and revealed by silver staining. In preliminary experiments the procedure was performed in the absence of detergents in order to preserve as much as possible native interactions between CtBP3/BARS and other proteins. In these conditions however, numerous proteins were also precipitated by preimmune IgGs, as shown both by silver staining (Fig. 5.2 A, see lanes 6-7) and Western blot with specific antibodies (see detection of dynamin II shown in Fig. 5.2 B, lanes 6-7). For this reason several trials to reduce immunoprecipitation by preimmune IgGs were performed. At the end, the use of 0.1% Triton X-100, effectively reduced the amounts of proteins aspecifically precipitated by IgGs, while apparently preserving both the ability of anti-BARS IgGs to immunoprecipitate BARS and the interactions existing between BARS and other proteins in brain cytosol (Fig. 5.2 A and B, lanes 8-9). In fact, several bands specifically precipitated by the anti BARS-IgG matrix could be seen on silver-stained gel. In Fig 5.2 A a silver-stained SDS-PAGE gel is shown. In addition to BARS (indicated by a red arrowhead), several bands (indicated by black arrowheads) appear in lane 9 which are not detectable in lane 8 (estimated molecular weights: 155, 105, 80, 65, and 35 kDa). Fig. 5.3 A shows that two further bands (estimated molecular weights: 125 and 70 kDa) specifically precipitated by the anti BARS-IgG matrix, could be resolved by analysing the proteins on a long gel (see section 2.3.3.1).
Fig. 5.2 Co-immunoprecipitation of BARS-interacting proteins. 20 mg dialyzed rat brain cytosol were precleared on protein A-Sepharose and then incubated with preimmune (C)- or BARS (B)-IgG matrix in the absence (-) or in the presence (Tr) of Triton X-100. At the end of incubation, each flow-through (FT) was recovered and each matrix was extensively washed. The proteins retained by the matrixes were eluted by adding SDS sample buffer, boiling the suspension and recovering the supernatant after centrifugation. 1/400 of the precleared cytosol (lane 1), 1/400 of each flow-through (lane 2-5) and 1/20 of
each eluate (lane 6-10) were analysed by 4-15% gradient SDS-PAGE. **Panel A:** SDS-PAGE gel revealed by silver staining. The arrowheads indicates proteins specifically detected in the eluate from BARS-IgG matrix, distinguishable only when the incubation was performed in the presence of Triton X-100. Estimated molecular weights are on the right. The red arrowhead indicates BARS. **Panel B:** SDS-PAGE gel transferred onto nitrocellululose, probed with the anti-dynamin II Dyn2 antibody and the anti-BARS p502 antibody, and revealed by ECL detection method. The positions of molecular weight standards are indicated on the left of the figure.
Fig. 5.3. The anti-BARS antibody did not recognize in Western blot the co-immunoprecipitated proteins. 20 mg dialyzed rat brain cytosol were treated as in Fig. 5.2. Panel A: 4-15% SDS-PAGE long gel revealed by silver staining. Lane 1, 1/10 of eluate from preimmune-IgG matrix; lane 2, 1/10 of eluate from BARS-IgG matrix. Panel B and C: an identical SDS-PAGE long gel transferred onto nitrocellulose, probed with the affinity-purified anti-BARS IgGs, and revealed by ECL detection method. Lane 1, 1/200 of precleared cytosol; lane 2, 1/10 of eluate from preimmune-IgG matrix; lane 3, 1/10 of eluate from BARS-IgG matrix. Panel C represents a longer exposition of the film as compared to panel B, in order to show the absence of as well faint bands. The positions of molecular weight standards are indicated on the left of the figure.
To establish whether the proteins co-immunoprecipitated with BARS were precipitated by direct binding to the antibody or were precipitated as a consequence of their interaction with BARS, two analogous gels were loaded with the eluted proteins: one was revealed by silver staining and the other was transferred onto nitrocellulose and probed with the same anti-BARS antibody used for immunoprecipitation (Fig. 5.3). As shown in Fig. 5.3 (panels B and C) this antibody did not recognize the proteins specifically co-immunoprecipitated with BARS (arrowheads in panel A), suggesting that they were indeed precipitated as a consequence of binding to BARS, rather than of direct recognition by the antibody.

In contrast to the immunoprecipitation approach, the use of GST-CtBP3/BARS or His-tagged CtBP3/BARS did not lead to direct identification of BARS-interacting proteins on silver-stained gel. In part, this was due to the presence of degradation products of the recombinant proteins that gave troublesome bands on the gel, making extremely difficult the detection of possible interacting proteins at the silver staining level.

5.2.2 Testing the presence of potential partners of BARS among BARS-interacting proteins

After setting up the procedure, the first step was testing whether the transcriptional regulators that had been found by others to interact with CtBP1 and CtBP2 (see Table 5.1), were present among the co-immunoprecipitated proteins and, if this was the case, co-migrated with the proteins detected at the level of silver staining. Specific antibodies directed to most of the CtBPs-interacting transcriptional factors were obtained from different laboratories and tested on the
co-immunoprecipitated proteins. Input cytosol and eluates from BARS-IgG matrix and preimmune-IgG matrix were subjected to SDS-PAGE, transferred onto nitrocellulose and probed with the available antibodies. Most of the CtBP1- and CtBP2-interacting transcriptional factors are highly specific in terms of tissue distribution, and, as expected, were not detected in brain cytosol (see Table 5.1). The antibodies giving detectable signals in brain cytosol never recognized specific bands in the BARS-immunoprecipitate (see Table 5.1), allowing me to conclude that none of the bands co-immunoprecipitated with BARS and visible in silver staining corresponded to already known CtBPs interactors.

Since CtBP3/BARS induces membrane fission and is involved in lipid metabolism, by specifically catalysing the formation of PA from LPA, one could expect that it interact with other proteins controlling lipid metabolism and/or with proteins mediating fission. Therefore, similarly to what is described above for transcriptional factors, the proteins co-immunoprecipitated with BARS were also analysed for the presence of several potential interactors, by using specific antibodies (see Table 5.2), among them: 1) phospholipase A\textsubscript{2} (PLA\textsubscript{2}), PI-4-kinase, phosphatidylinositol transfer protein (PITP) \(\alpha\) and \(\beta\), which are proteins involved in lipid metabolism; 2) dynamin I and dynamin II, proteins involved in membrane tubulation and fission (see section 1.1.3.1.2); 3) ARF, a fundamental regulator of budding of membranes (see section 1.1.3.1.1). None of these proteins appeared to be co-immunoprecipitated with BARS. Therefore, the direct identification of the isolated proteins through the application of mass spectrometry (MS) techniques was undertaken.
Table 5.1. Transcriptional factors interacting with CtBP1 and CtBP2.

<table>
<thead>
<tr>
<th>Transcriptional factor (approx. mw in kDa)</th>
<th>Interacts with</th>
<th>Detectable in brain cyt</th>
<th>IP with BARS</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1A (30)</td>
<td>CtBP1</td>
<td>NO</td>
<td></td>
<td>Schaeper et al., 1995</td>
</tr>
<tr>
<td>Net (45)</td>
<td>CtBP1</td>
<td>YES</td>
<td>NO</td>
<td>Criqui-Filipe et al., 1999</td>
</tr>
<tr>
<td>Histone deacetylase I (60)</td>
<td>CtBP1</td>
<td>YES</td>
<td>NO</td>
<td>Sundqvist et al., 1998</td>
</tr>
<tr>
<td>CtIP (125)</td>
<td>CtBP1</td>
<td>NO</td>
<td></td>
<td>Schaeper et al., 1998</td>
</tr>
<tr>
<td>BKLF (40)</td>
<td>CtBP2</td>
<td>YES</td>
<td>NO</td>
<td>Turner and Crossley, 1998</td>
</tr>
<tr>
<td>Polycomb 2 (80)</td>
<td>CtBP2</td>
<td>NO</td>
<td></td>
<td>Sewalt et al., 1999</td>
</tr>
<tr>
<td>Evi-1 (145)</td>
<td>CtBP2</td>
<td>NO</td>
<td></td>
<td>Turner and Crossley, 1998</td>
</tr>
<tr>
<td>AREB6 (pred., 125)</td>
<td>CtBP2</td>
<td>nd</td>
<td></td>
<td>Turner and Crossley, 1998</td>
</tr>
<tr>
<td>ZEB (pred., &gt;127)</td>
<td>CtBP2</td>
<td>nd</td>
<td></td>
<td>Postigo and Dean, 1999</td>
</tr>
<tr>
<td>FOG-1 (150)</td>
<td>CtBP2</td>
<td>NO</td>
<td></td>
<td>Turner and Crossley, 1998</td>
</tr>
</tbody>
</table>

The known interactors of CtBP1 and CtBP2 with their approximate molecular weight are reported (Pred.: molecular weight predicted on the base of the aminoacid composition). It is also reported if these proteins were detected on rat brain cytosol with available antibodies; anyway, they were never found among the proteins co-immunoprecipitated (IP) with BARS, possibly due to masking of BARS epitopes following interaction with these proteins. The presence of AREB6 and ZEB in rat brain cytosol was not determined (nd), due to unavailability of specific antibodies; however, they are not expected to be expressed in brain (Postigo and Dean, 1997; Ikeda et al., 1998).
Table 5.2. Antibodies tested on proteins co-immunoprecipitated with BARS and pulled-down by GST-BARS.

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Antibody name</th>
<th>Owner (Company or Group’s Head)</th>
</tr>
</thead>
<tbody>
<tr>
<td>85-kDa cytosolic PLA₂</td>
<td>cPLA₂</td>
<td>C.C. Leslie (National Jewish Medical and Research Center, Denver, CO, USA)</td>
</tr>
<tr>
<td>PITP α</td>
<td>PITP α</td>
<td>G.T. Snoek (Utrecht University, The Netherlands)</td>
</tr>
<tr>
<td>PITP β</td>
<td>PITP β</td>
<td>G.T. Snoek (Utrecht University, The Netherlands)</td>
</tr>
<tr>
<td>PI-4-Kinase β</td>
<td>PI-4-Kinase β</td>
<td>M.A. De Matteis (Consorzio Mario Negri Sud, Italy)</td>
</tr>
<tr>
<td>Dynamin I</td>
<td>Hudy-1</td>
<td>Upstate Biotechnology (NY, USA)</td>
</tr>
<tr>
<td>Dynamin I</td>
<td>Dynamin</td>
<td>Transduction Laboratories (KY, USA)</td>
</tr>
<tr>
<td>Dynamin II</td>
<td>Dyn2</td>
<td>M.A. McNiven (Mayo Clinic and Foundation, MN, USA)</td>
</tr>
<tr>
<td>Dynamins</td>
<td>MC63</td>
<td>M.A. McNiven (Mayo Clinic and Foundation, MN, USA)</td>
</tr>
<tr>
<td>ARFs</td>
<td>ARF</td>
<td>M.A. De Matteis (Consorzio Mario Negri Sud, Italy)</td>
</tr>
</tbody>
</table>
5.2.3 Scale up of the immunoprecipitation procedure

To identify the proteins co-immunoprecipitated with CtBP3/BARS, a scale-up of the immunoprecipitation procedure was performed in order to obtain enough material to be subjected to nano-electrospray MS analysis. In brief, 1 ml of protein A-Sepharose was cross-linked to 500 μg affinity purified anti-BARS IgGs, obtained from approximately 10 ml of antiserum, or to 500 μg preimmune IgGs. Each matrix was incubated with 200 mg of rat brain cytosol, then packed in a chromatography column and extensively washed. The proteins retained on the columns were eluted with 0.1 M glycine pH 3.0, collecting 0.5 ml fractions. Minor amounts of the fractions were analysed by SDS-PAGE and revealed by silver staining. The third fractions contained the majority of the eluted proteins (Fig. 5.4 A). Proteins candidate for MS analysis were selected by comparing the proteins eluted from the anti-BARS matrix (lane 2) with the proteins eluted from the control matrix (lane 1). The proteins contained in the third fractions were concentrated by precipitation, separated by a preparative SDS-PAGE gel, and revealed by silver staining. The proteins previously selected showed a staining intensity ranging between 100 and 500 ng of BSA loaded on distinct lanes of the same gel. The bands were excised from the gel and subjected to trypsin in-gel digestion and MS analysis. The trypsinization and MS analysis were entirely performed at the Molecular Structure Facility directed by Dr. Young Moo Lee at the University of California in Davis, California.
Fig. 5.4. Identification of BARS-interacting proteins. Two-hundreds mg dialyzed rat brain cytosol were precleared on protein A-Sepharose and then incubated with 1 ml preimmune- or BARS-IgG matrix. At the end of incubation, the flow-through was recovered and each matrix was packed in a chromatography column and extensively washed. Finally, proteins were eluted with 0.1 M glycine pH 3.0, collected in ~ 0.5 ml fractions, and subjected to 4-15% gradient SDS-PAGE. Panel A: long gel revealed by silver staining. Lane 1, 1/10 of the third fraction eluted from preimmune-IgG matrix (C); lane 2, 1/10 of the third fraction eluted from BARS-IgG matrix (B). Blue arrows show BARS-interacting proteins
selected for the mass spectrometry analysis. The bands identified by mass spectrometry analysis as MAP2, INX and GAPDH are indicated. **Panel B:** an analogous long gel transferred onto nitrocellulose, probed with antibodies to the proteins indicated and revealed by ECL detection method. Lane 1, 1/5000 of the precleared cytosol (I); lane 2, 1/20 of the third fraction eluted from the preimmune-IgG matrix (C); lane 3, 1/20 of the third fraction eluted from the BARS-IgG matrix (B). The positions of molecular weight standards are indicated on the left of the figure.
5.2.4 Identification of CtBP3/BARS-interacting proteins

The MS approach used for protein identification was the nano-electrospray MS/MS sequence tag searching. The MS analysis was successful for three out of the eight bands isolated, whereas the others only gave peptides resulting from keratin digestion or trypsin autodigestion, a common result in case of scarcity of protein sample. The mass and sequence of peptides obtained from the three bands is shown in Table 5.3. The 35-kDa protein resulted to be glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), a glycolytic enzyme that has also been implicated in other cellular functions (reviewed in Sirover, 1999). The 65-kDa band was identified as α-internexin (INX), a type IV neuronal intermediate filament protein (Kaplan et al., 1990). Finally, the 300-kDa protein was identified as the microtubule-associated protein 2 (MAP2), a constituent of the family of non-motor microtubule-associated protein (Garner and Matus, 1988; Drewes et al., 1998). These results were also confirmed by probing the co-immunoprecipitated proteins with specific antibodies in Western blot. In addition to GAPDH, INX and MAP2, also tubulin, the major component of microtubules (Dutcher, 2001), was detected in the BARS immunoprecipitate by Western blot analysis (Fig. 5.4 B). In contrast, other abundant cytosolic proteins, for example actin, were not co-immunoprecipitated with BARS (Fig. 5.4 B).
Table 5.3. Peptide sequences obtained from nano-electrospray MS/MS sequence tag searching analysis of BARS-interacting proteins

<table>
<thead>
<tr>
<th>Approximate protein mass (in kDa)</th>
<th>Peptide mass (in Da)</th>
<th>Peptide sequence</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>1555.80</td>
<td>GAAQNIIPASTGAAK</td>
<td>GAPDH</td>
</tr>
<tr>
<td></td>
<td>1778.78</td>
<td>LISWYDNEYGYSNR</td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>1132.60</td>
<td>FANLNEQAAAR</td>
<td>α-internexin</td>
</tr>
<tr>
<td></td>
<td>1120.58</td>
<td>EYQDLLNVK</td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>1187.61</td>
<td>LASVSADAEVAR</td>
<td>MAP2</td>
</tr>
<tr>
<td></td>
<td>1271.62</td>
<td>FAAPVQPEEER</td>
<td></td>
</tr>
</tbody>
</table>
The interactions revealed by co-immunoprecipitation were also confirmed with an independent assay, based on the use of recombinant CtBP3/BARS tagged with a poly-histidine sequence (His-BARS). His-BARS was expressed in bacteria and purified by affinity-chromatography. In parallel, a mock control for His-BARS was also produced from bacteria transformed with the empty vector (see sections 2.8.4 and 2.9.2 for more details). Cytosol was incubated with His-BARS or the mock preparation. The mixture was then incubated with a Ni\textsuperscript{2+}-NTA beads, and after extensive washing, the proteins retained by the matrix were eluted with imidazole, subjected to SDS-PAGE, transferred onto nitrocellulose and assayed in Western blot with specific antibodies directed against GAPDH, INX, MAP2, and α-tubulin. His-BARS was able to pull-down GAPDH, INX, and tubulin, as shown in Fig. 5.5, but not MAP2.

5.2.5 CtBP3/BARS interacts directly with GAPDH

GAPDH is a very abundant cytosolic protein and has been found to interact with many cellular proteins (Sirover, 1999). In particular, the ability of GAPDH to interact with microtubules was already described (Kumagai and Sakai, 1983; Huitorel and Pantaloni, 1985; Durrieu et al., 1987; Launay et al., 1989; Walsh et al., 1989; Somers et al., 1990). Thus, the possibility existed that the interaction between BARS and GAPDH was indirect, i.e. occurred as a consequence of interaction with common partners, for example microtubules. Therefore, the ability of pure CtBP3/BARS and GAPDH to interact directly was tested by pull-down experiments performed in the absence of cytosol. Fig. 5.6 shows that CtBP3/BARS was able to pull-down pure GAPDH in the absence of cytosol, indicating the existence of a direct interaction between the two proteins.
Fig. 5.5. Pull-down of BARS-interacting proteins. 20 mg dialyzed rat brain cytosol was precleared on Ni\textsuperscript{2+}-NTA beads (input), incubated with 100 µg His-BARS (BARS) or with mock control (CTR), and then incubated with Ni-NTA beads. At the end of incubations, the flow-through (FT) was recovered, the beads were extensively washed and the proteins bound to the beads were eluted with 250 mM imidazole, collecting 0.3 ml fractions. The proteins were separated on a 4-15% gradient SDS-PAGE long gel, and transferred onto nitrocellulose. His-BARS is visualized by ponceau staining. The other proteins are detected with specific antibodies and revealed with ECL detection method. Lane 1, 1/500 of precleared cytosol; lane 2 and 3, 1/500 of flow-through CTR and BARS, respectively; lane 4-7, 1/5 of fractions eluted from sample incubated with CTR (lanes 4, first fraction; lane 6, second fraction) and from sample incubated with BARS (lane 5, first fraction; lane 7, second fraction). This experiment was done with the help of Claudia Cericola (Laboratory of Molecular Endocrinology - Department of Cell Biology and Oncology - Consorzio Mario Negri Sud).
Fig. 5.6. Pull-down of pure GAPDH by His-BARS. 20 mg dialyzed, precleared rat brain cytosol (CYT) or 200 µg pure GAPDH (GAPDH) were incubated with 100 µg His-BARS (BARS) or with mock control (CTR), and then incubated with Ni-NTA beads. At the end of incubation, the beads were extensively washed and the proteins bound to the beads were eluted with 250 mM imidazole, collecting 0.3 ml fractions. CYT (lane 1), GAPDH (lane 2), the first fractions (lanes 3-6) and the second fractions (lanes 7-10) were separated on a 4-15% gradient SDS-PAGE gel, and transferred onto nitrocellulose. His-BARS and GAPDH were revealed with anti-BARS/SN1 antibody and a monoclonal anti-GAPDH antibody, respectively, using ECL detection method. Lane 1, 1/500 of precleared cytosol; lane 2, 1/500 of GAPDH; lanes 3 and 7, 1/5 of fractions eluted from sample of incubated with CYT and CTR; lanes 4 and 8, 1/5 of fractions from sample incubated with CYT and BARS; lanes 5 and 9, 1/5 of fractions from sample incubated with GAPDH and CTR; lanes 6 and 10, 1/5 of fractions from sample incubated with GAPDH and BARS. This experiment was done with the help of Claudia Cericola (Laboratory of Molecular Endocrinology - Department of Cell Biology and Oncology - Consorzio Mario Negri Sud).
5.3 Discussion

The co-immunoprecipitation and pull-down experiments reported in this chapter demonstrate that BARS 1) directly interact with GAPDH, and 2) is also able to interact with tubulin and INX, although it was not ascertained if these interactions are direct or are mediated by binding to common interactors. Co-immunoprecipitation experiments also led to the identification of MAP2 among the BARS-interacting proteins, although the interaction between BARS and MAP2 was not confirmed by pull-down experiments. In addition, others possible interactors were also isolated in this study by co-immunoprecipitation. However, their identification by MS was elusive, possibly because of their inaccessibility to trypsin digestion or their too scarce amounts. In fact, the silver staining detection of proteins is far from being quantitative, and for this reason no precise measure of the amounts of proteins that were subjected to MS analysis is available. A further scale-up of the procedure of immunoprecipitation will possibly lead to their identification.

GAPDH is a multifunctional protein. As a tetramer composed of 4 identical subunits, its role as a glycolytic enzyme (EC number 1.2.1.12) is well characterized. However, independent studies from a number of different laboratories reported a variety of different biological functions of GAPDH in different subcellular compartments (Sirover, 1999). GAPDH was shown to have nuclear functions, acting in the form of monomer as an uracyl-DNA-glycosylase in DNA repair processes (Meyer-Siegler et al., 1991), and also acting as a tRNA export protein (Singh and Green, 1993). In the cytoplasm besides acting as a glycolytic enzyme, GAPDH was demonstrated to bind mRNAs and thus proposed
to regulate translation (Nagy and Rigby, 1995). At the level of membranes, GAPDH was shown to act as a fusogen (Lopez Vinals et al., 1987; Glaser and Gross, 1995; Hessler et al., 1998). In particular, an isogenic species of GAPDH, lacking glycolytic activity, was isolated from rabbit brain as a factor catalysing rapid fusion of vesicles in vitro (Glaser and Gross, 1995). Moreover, GAPDH was found to bind microtubules, and this ability was shown to be required for correct endocytosis (Robbins et al., 1995). More recently, GAPDH was found to be associated with COPI-coated vesicles that are formed in the presence of Rab2 and GTP\(_{\gamma}\)S from ER-Golgi intermediate compartment (ERGIC) (Tisdale, 2001). Indeed, a constitutively activated form of Rab2, as well as wild type Rab2, promotes the formation of COPI-coated vesicle enriched in proteins recycling to ER (Tisdale, 1999) and also containing GAPDH (Tisdale, 2001). The author showed that when GAPDH recruitment to these vesicles was inhibited by the addition of an anti-GAPDH antibody, vesicles did form but the ER-to-Golgi transport was inhibited, suggesting that GAPDH can be involved in the translocation and/or fusion of carriers to target compartment.

The direct interaction between BARS and GAPDH could have different implications. First, the interaction reported in this chapter, together with the findings that GAPDH has fusogenic activity and is required for transport between ER and Golgi (Lopez Vinals et al., 1987; Glaser and Gross, 1995; Hessler et al., 1998; Tisdale, 2001), suggests that the two proteins participate in a complex mediating the membrane rearrangements occurring in fusion and fission. Second, given that GAPDH binds microtubules, it might mediate the interaction between BARS and cytoskeleton. As will be discussed below, the cytoskeleton is proposed to have a major role in membrane fission. The analysis of these hypotheses and
the comprehension of the precise role of the BARS-GAPDH complex in membrane transport will be matter of future studies.

It is also noteworthy to consider that GAPDH is the 38-kDa substrate of BFA-induced ADP-ribosylation. Even if this might suggest that GAPDH was in some way involved in the Golgi redistribution induced by BFA, the role of ADP-ribosylation of GAPDH in the BFA effect was ruled out by early studies on permeabilized RBL cells (Mironov et al., 1997a).

BARS was found to interact with elements of two cytoskeletal systems present in eukaryotic cells: microtubules and intermediate filaments. While the role of microtubules in membrane traffic has been addressed by numerous studies (reviewed in Lane and Allan, 1998; Lippincott-Schwartz, 1998; see also section 1.1.3.2), the role of intermediate filaments is more vague, although some data indicates the participation of intermediate filaments in traffic (Evans, 1994; Gao and Sztul, 2001).

Physical and functional links between microtubules and intermediate filaments have been ascertained (Rogers and Gelfand, 2000). Thus, it is possible that BARS directly interact with only one of the two cytoskeletal systems. Furthermore, the interactions between BARS and microtubule and intermediate filament proteins could reflect indirect interactions, for example through GAPDH, given that GAPDH interacts with microtubules (Kumagai and Sakai, 1983; Huitorel and Pantaloni, 1985; Durrieu et al., 1987; Launay et al., 1989; Walsh et al., 1989; Somers et al., 1990). Even though this possibility appears unlikely, further work will be necessary to uncover the relative order of the physical interactions.
One of the BARS-interactors identified in this screening is the neuron-specific intermediate filament protein INX. Intermediate filaments consist of more than 50 distinct proteins structurally related, that are capable of forming morphologically similar filaments in different cell types (reviewed in Fuchs and Weber, 1994). Currently, intermediate filament proteins are partitioned into 6 classes based on sequence homology, assembly properties and tissue distribution. Type IV intermediate filament proteins are expressed in neurons and include INX, the neurofilament (NF) triplet proteins NF-light chain (NF-L), NF-medium chain (NF-M) and NF-heavy chain (NF-H), peripherin and nestin (reviewed in Lee and Cleveland, 1996). Neurofilaments, initially considered composed only of the NF triplet proteins, are indeed heteropolymers whose composition can be variable and includes also other intermediate filament proteins such as INX and peripherin, but also unrelated proteins, named intermediate filament associated proteins (IFAPs). The role of IFAPs seems to be the regulation of assembly state and the interaction with the other cytoskeletal systems (Coulombe et al., 2000; Herrmann and Aebi, 2000). Intermediate filaments do not seem to have a housekeeping function in the cell, since lower eukaryotes lack an intermediate filament cytoskeleton and mutant vertebrate cells can grow in the absence of an intermediate filament network (Colucci-Guyon et al., 1994; Coulombe et al., 2000). Rather, intermediate filaments seem to have a fundamental regulatory role in extremely specialized process of the cell, such as radial growth of axon (Lee and Cleveland, 1996), sphingolipid synthesis and cholesterol metabolism (Evans, 1994; Gillard et al., 1994; Holwell et al., 1999). In particular, the defect in lipid synthesis and metabolism occurring in cells lacking an intermediate filament network appears to be caused by alteration in intracellular membrane traffic (Evans, 1994). An
involvement of intermediate filaments in membrane traffic, in particular in Golgi function, has been also indicated by a recent work by Gao and Sztul (2001), who showed that 1) the Golgi complex and intermediate filament cytoskeleton interact through the formininotransferase cyclodeaminase (FTCD), and 2) overexpression of FTCD dramatically changes the structure of the intermediate filament cytoskeleton and causes a coordinate disruption of Golgi structure. Although the function of intermediate filament cytoskeleton has to be further investigated, these findings suggest a specific role for intermediate filament cytoskeleton in the regulation of membrane traffic, probably as important as the role of microtubules and actin microfilaments (see section 1.1.3.2).

The interaction between BARS and components of the cytoskeleton may suggest a role for the cytoskeleton in fission events. An organized network of proteins might cooperate into the detachment of carriers from Golgi by contributing to generate the mechanical force required. Otherwise, given that microtubules are involved in the generation of tubules from membranes (Lane and Allan, 1998; Waterman-Storer and Salmon, 1998), my finding may suggest the existence of a physical association between the machinery that generates tubules and the machinery that induces their release from the Golgi. This issue will be further considered in the final discussion.
CHAPTER 6

Final Discussion

In the present study the 50-kDa BFA-ADP-ribosylated substrate (BARS) was cloned from a rat brain cDNA library. Its sequencing revealed that it belongs to the family of CtBP proteins, which are found to be expressed from fruit fly to human. In particular, this protein constitutes the third mammalian member of the CtBP family and was therefore renamed CtBP3/BARS. The ability to be ADP-ribosylated in the presence of BFA was found to be a feature of all the members of the family, since not only CtBP3/BARS but also CtBP1 and CtBP2 can be ADP-ribosylated \textit{in vitro} in the presence of BFA. Thus, all of the CtBPs have to be considered as BARS.

By employing a permeabilized cell system, BARS was demonstrated to prevent the tubulation and disassembling of the Golgi complex induced by BFA. This finding suggested that BARS participates in the maintenance of the structure of the Golgi complex. When the brain-purified BARS or the recombinant CtBP3/BARS were assayed on isolated Golgi membranes, they were revealed to be responsible for the fission/fragmentation activity present in the brain cytosol. In addition, recombinant CtBP3/BARS was shown to catalyse the formation of PA from LPA, and this activity was correlated to the Golgi fission/fragmentation activity: the two activities occur within the same time interval and show the same dependence on specific acyl-CoAs, strongly suggesting that the fission activity of BARS is at least in part mediated by the conversion of LPA into PA. How the formation of PA from LPA might control the membrane fission has been discussed in section 3.4. The machinery responsible for membrane fission at the
Golgi complex probably includes additional enzymatic activities, such as phospholipases, PA phosphatases or flippases, but also possibly further components, able for example to supply a mechanical force needed for the scission of membranes.

The three mammalian CtBPs share a high degree of identity (Fig. 3.9). In particular, CtBP1 and CtBP3/BARS are extremely conserved, being substantially identical (97% identity) except for the presence of an additional 11-aa N-terminal sequence in CtBP1: the possibility exists that they are product of alternative splicing of the same gene. CtBP2 diverges from the other two proteins especially in the C-terminal one hundred aa sequence, showing a 79% overall identity with both CtBP1 and CtBP3/BARS. CtBP1 and CtBP2 were shown to act as transcriptional co-repressors (reviewed in Turner and Crossley, 2001), while we showed that CtBP3/BARS induces the fission of Golgi tubules and acts as an LPA-acyltransferase. The immunofluorescence studies reported in this thesis showed that CtBP3/BARS has a cytosolic, diffused localization both at the level of cytoplasm and nucleus, but is also clearly enriched at the Golgi membranes and at other cellular membranes (plasma membrane and nuclear envelope). A similar subcellular distribution was also observed for CtBP1, although its strong propensity to form long and thick structures after overexpression complicated the analysis of its localization. In sharp contrast, CtBP2 is only localized in the nucleus. These localization data strongly support the conclusion that, despite their high degree of sequence identity, the three proteins have different cellular functions. How in principle these different functions can intersect and overlap in the cell has been already discussed in section 4.3. Here I just want to underscore that the findings that different members of the CtBP family, which have been involved in different functions, have also different subcellular localizations.
support the conclusion that the same protein or highly similar proteins are engaged in different, apparently independent, cellular processes. The existence of numerous proteins having multiple roles in the cell was discussed by Jeffery (1999) and new examples of proteins with double functions came out recently in the field of intracellular traffic. Indeed, some of the proteins participating in the clathrin-based endocytosis machinery, eps15, epsin1, and the clathrin assembly lymphoid myeloid leukemia (CALM) were found: 1) to localize in the nucleus upon nuclear export block; and 2) to be able to positively modulate gene transcription (Hyman et al., 2000; Vecchi et al., 2001). In this picture, the double function reported for the protein of the CtBP family is not surprising. Interestingly, Vecchi et al. (2001) were not able to discover any link between the two processes - endocytosis and gene transcription - in which eps15, epsin1, and CALM are involved. Concerning the question of the CtBPs, it remains to be understood whether the two processes – membrane fission and repression of gene transcription - are really independent or if cells have destined similar proteins to play different functions, with the final aim to simultaneously control them or to relate them in terms of temporal and/or spatial occurrence. A possible model connecting the nuclear localization and the Golgi fission activity of the CtBPs has been envisaged in section 4.3.

The transport intermediates that are responsible for delivering cargo to the plasma membrane are large tubular-saccular structures that emerge from the Golgi and leave the Golgi along microtubules (Hirschberg et al., 1998; Toomre et al., 1999; Polishchuk et al., 2000). The fission event – and, thus, the molecular machinery that controls it - might be of great importance for the regulation of the release of these intermediates from the Golgi. In order to better understand the mechanisms of membrane fission in the scenario of the physiology of formation
of transport intermediates from the Golgi, we decided to investigate the molecular composition of the fission machinery and to set up a biochemical method to isolate and identify BARS-interacting proteins. Some of the BARS-interacting proteins isolated in this work have been identified. They are the multifunctional protein GAPDH and the cytoskeletal proteins α-internexin (INX), tubulin and microtubule-associated protein 2 (MAP2). Other BARS-interacting proteins isolated in the course of this thesis still remain to be identified.

Although the precise physiological meaning of these interactions is still to be explored, I will make some considerations and suggestions about their possible function. Since GAPDH has been suggested to be a fusogenic protein (Lopez Vinals et al., 1987; Glaser and Gross, 1995; Hessler et al., 1998) and has also been shown to be required in intracellular traffic (Tisdale, 2001), together with BARS it might cooperate in the processes that lie beneath fusion and fission. The complex formed by the two proteins can for example be competent for the regulation of the geometry of the membranes. Even though GAPDH was shown to induce the fusion of membranes, its mechanism of action is not known. A detailed model of the action of the complex BARS-GAPDH would require more information about the molecular action of GAPDH on the membranes.

An interaction between BARS and microtubules is supported not only by biochemical data but also by observations on the subcellular localization of CtBP3/BARS. In fact, after cell permeabilization and removal of the soluble fraction of CtBP3/BARS, part of the protein appeared to be associated with the microtubule network. Microtubules were demonstrated to be required for the formation and elongation of tubules that are generated at the endoplasmic reticulum (Waterman-Storer et al., 1995; Waterman-Storer and Salmon, 1998) and at the lysosomes (Swanson et al., 1987; Swanson et al., 1992), and are
probably necessary also for the formation of tubules at the trans-Golgi network (TGN; Cooper et al., 1990; Toomre et al., 1999). At least two different mechanisms have been proposed to explain the microtubule-dependent formation of tubules: 1) sliding of membranes along microtubules mediated by a motor activity; 2) attachment of the membranes to the polymerising plus-end of microtubules (Waterman-Storer and Salmon, 1998). Independently from the type of mechanism that leads to microtubule-mediated extension of tubules, one possibility to rationalize the interaction between BARS and microtubules is that a single, complex machinery, responsible for the formation of tubular carriers acts by first extending tubules out of the TGN and then cutting them at their origin to release them in the cytoplasm. In this picture microtubules and a fission activity would be integral parts of the same machinery. Another possible interpretation of the interaction between BARS and microtubules is that the microtubule network and, possibly, a microtubule motor activity might be required to supply the mechanical force for the scission of the transport intermediates. Furthermore, the requirement of a cytoskeletal network to fulfil membrane fission would also justify the interaction between BARS and intermediate filaments; indeed, as discussed in section 5.3, intermediate filaments might have a role in membrane trafficking as important as that of microtubules.

In conclusion, in this work a fundamental component of the machinery that control membrane fission has been cloned and shown to be localized at the Golgi complex. Some of its molecular interactors have been identified, and elucidating the role that they play in membrane fission might provide new insights into the mechanisms regulating intracellular membrane trafficking.
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