The role of BARS/CtBP3, a protein involved in Golgi membrane fission, in intracellular membrane transport in vivo

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The role of BARS/CtBP3, a protein involved in Golgi membrane fission, in intracellular membrane transport in vivo

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Discipline: Life Sciences
Sponsoring establishment: Consorzio Mario Negri Sud

Thesis submitted in accordance with the requirements of the Open University for the degree of Doctor of Philosophy
May 2005
To my grandparents

“E quindi uscimmo a riveder le stelle”

(Dante; Divina Commedia; Inferno: Canto XXXIV)
In the context of intracellular membrane transport the main focus of interest has been concentrated on the processes regulating fission and fusion of membranous transport carriers. If on one hand there seems to be a unique, conserved machinery regulating fusion of all transport intermediates with their respective acceptor compartments, at the level of membrane fission there still remains much uncertainty. At least the Dynamin family of proteins has been identified as a regulatory component for a number of fission events but this is certainly not universal. There are a number of fission steps in the transport pathway that are clearly independent of dynamin and this raises the possibility that there are multiple machineries regulating membrane fission in different transport pathways. Based on the observation that the protein CtBP3/BARS (BARS) is able to induce fission, \textit{in vitro}, of Golgi-derived membranous tubules, the present study aims to characterise the relevance of this effect on multiple intracellular transport steps in the intracellular environment. BARS is found to be involved in post-Golgi protein transport to the basolateral plasma membrane and also in fluid phase endocytosis and this confirms the existence of a novel fission-inducing machinery. In addition, since the selective inhibition of either one of these two pathways (i.e. those regulated by dynamin or BARS) has no effect on the operation of the other, it is concluded that they operate independently.
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ABBREVIATIONS

3D  Three dimensional
aa  Aminoacids
AP  Adaptor protein
ARF ADP-ribosylation factor
ATTC American tissue type collection
BARS Brefeldin A-Ribosylated Substrate
BFA Brefeldin A
BHK Baby hamster kidney
bp  Base pair
BSA Bovine serum albumine
CCP Clathrin-coated pits
CFP Cyan fluorescent protein
CGN Cis-Golgi network
CI-MPR Cation-independent mannose-6-phosphate receptor
Cos Cercopithecus aethiops
CtBP C-Terminal Binding Protein
CVEM Correlative video electron microscopy
CVIF Correlative video immuno fluorescence
DAG Diacylglycerol
DGK Diacylglycerol kinase
DMEM Dulbecco’s modified Eagles medium
DMSO Dimethysulfoxide
DTT  DL-dithiothreitol
Dyn2  Dynamin2
EDTA  Ethylenediaminetetraacetic acid
EM  Electron microscopy
EMS  Electron Microscopy Science
EMT  Epithelial-mesenchimal transition
Endo H  Endoglicosidase H
ER  Endoplasmic reticulum
ERGIC  Endoplasmic reticulum-Golgi intermediate compartment
FCS  Fetal calf serum
FITC  Fluorescein Isothiocyanate
FRAP  Fluorescence recovery after photobleaching
GAP  GTP-ase activating protein
GAPDH  Glyceraldehyde-3-phosphate dehydrogenase
GBP  Guanylate-binding proteins
GDP  Guanosine diphosphate
GED  GTP-ase effector domain
GEF  Guanine nucleotide exchange factor
GERL  Golgi endoplasmic reticulum lysosomes
GFP  Green fluorescent protein
GPC  Golgi-to-plasma membrane carriers
GPI  Glycosylphosphatidylinositol
GST  Glutathione-S-transferase
GTP  Guanosine triphosphate
h  Hours
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HeLa</td>
<td>Henrietta Lacks</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxy-ethyl)-piperazine-1-ethane-sulfonic acid</td>
</tr>
<tr>
<td>HF</td>
<td>Human fibroblast</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan sulphate proteoglycans</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LMB</td>
<td>Leptomycin B</td>
</tr>
<tr>
<td>LPA</td>
<td>Lysophosphatidic acid</td>
</tr>
<tr>
<td>LPAAT</td>
<td>Lysophosphatidic acid acyltransferase</td>
</tr>
<tr>
<td>LSM</td>
<td>Laser scanning microscope</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MOPS</td>
<td>Morpholino-propane-sulfonic acid</td>
</tr>
<tr>
<td>MPR</td>
<td>Mannose-6-phosphate receptor</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide-adenine dinucleotide</td>
</tr>
<tr>
<td>NAGTI</td>
<td>1,2-N-acetylglucosaminyltransferase</td>
</tr>
<tr>
<td>NBD</td>
<td>Nucleotide binding domain</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NRK</td>
<td>Normal rat kidney</td>
</tr>
<tr>
<td>O/N</td>
<td>Overnight</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PAK1</td>
<td>p21-activated kinase-1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pCoA</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>pIgA</td>
<td>Polymeric-Immunoglobulin A</td>
</tr>
<tr>
<td>PITP</td>
<td>Phosphatidylinositol transfer proteins</td>
</tr>
<tr>
<td>PKC</td>
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</tr>
<tr>
<td>PKD</td>
<td>Protein kinase D</td>
</tr>
<tr>
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<td>Phospholipase A&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>PRD</td>
<td>Proline rich domain</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
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<tr>
<td>SBD</td>
<td>Substrate binding domain</td>
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</tr>
<tr>
<td>sec</td>
<td>Seconds</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interference RNA</td>
</tr>
<tr>
<td>SUMO</td>
<td>Small ubiquitin-like modifier 1</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate EDTA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TE</td>
<td>Tris[Hydroxymethyl]aminomethane-Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Tf</td>
<td>Transferrin</td>
</tr>
<tr>
<td>TFnR</td>
<td>Transferrin receptor</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans-Golgi network</td>
</tr>
<tr>
<td>TRIS</td>
<td>Tris[Hydroxymethyl]aminomethane</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethylrhodamine isothiocyanate</td>
</tr>
<tr>
<td>ts</td>
<td>Temperature sensitive</td>
</tr>
<tr>
<td>VSV</td>
<td>Vesicular stomatitis virus</td>
</tr>
<tr>
<td>VSVG</td>
<td>Vesicular stomatitis virus glycoprotein</td>
</tr>
<tr>
<td>VTCs</td>
<td>Vesicular-tubular clusters</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
</tbody>
</table>
CHAPTER 1

Introduction

1.1 Membrane traffic

The term “membrane traffic” describes the formation and the movement of specific membrane-bound compartments together with their fluid-phase and protein contents (the “cargo”) between and within intracellular subcompartments, or organelles (Fig. 1.1). Examples include the transfer of newly formed proteins from the endoplasmic reticulum (ER) to the Golgi apparatus and then between the various compartments of the Golgi apparatus (intra-Golgi transport). We include the concurrent movement of the membrane lipids. Although membrane traffic can be divided into a large number of separate steps, there are a number of common aspects that include the formation of cargo-containing membranous carriers from donor compartments, the anchoring of these carriers to cytoskeletal elements by molecular motor proteins, and the fusion of these carriers with their acceptor compartments (Fig. 1.2; Burger 2000).

In general terms, membrane traffic can be seen to involve three main pathways:

(i) the secretory pathway (Section 1.2.1.), by which proteins destined for secretion are transported from their site of synthesis in the ER to their final destinations (e.g. plasma membrane; endosomes). The secretory pathway itself is generally subdivided into three main steps: ER-to-Golgi transport; intra-Golgi transport and Trans Golgi Network (TGN)-to-plasma membrane/ endosomes transport (Fig. 1.1; 1.2)
(ii) the endocytic pathway (Section 1.2.3.), by which fluid-phase solutes and membrane-bound proteins are taken up from the extracellular environment and internalised within membrane-bound structures (Fig. 1.1; 1.7; Pelkmans et al. 2003; Mellman 1996; Cossart et al. 2004);

(iii) the retrograde pathway which is the means by which viruses and bacteria enter cells (Fig. 1.1; Lord et al. 1998; Sannerud et al. 2003; Cossart et al. 2004). As the retrograde pathway does not constitute a part of the present study, it will not be described further here.
Fig 1.1. Intracellular membrane traffic. Schematic representation of intracellular membrane traffic. Newly synthesised proteins are folded within the ER and are transported to the Golgi apparatus where they undergo a series of post-translational modifications before being transported to their final destination (black line). Extracellular proteins and solutes are uptaken by cells within endosomes (En) and transported along the endocytic pathway (red lines). Bacterial proteins and toxins enter cells by endocytosis but follow a retrograde transport pathway (dashed black line) through the Golgi to the ER and from there to the cytoplasm where they exert their toxic effects.
1.1.1 The secretory pathway

The term "secretory pathway" defines a network of protein-protein, protein-lipid and lipid-lipid interactions that regulate the processes of post-translational protein modification, sorting, packaging, and transport between two non-contiguous cellular compartments (Fig. 1.2; Corda et al. 2002; Burger 2000). The mechanisms by which proteins are synthesised or modified in one intracellular compartment and then transported to another intracellular organelle constitute one of the most challenging aspects of intracellular transport research. Each step in the secretory pathway is characterised by a plethora of specific machineries that regulate similar processes while providing temporal and spatial resolution. At the same time, alongside the transported cargo, a large amount of membrane material is transferred from one cellular compartment to another, thus implying the existence of a highly regulated compensatory system of membrane recycling that can maintain the identity of each organelle in terms of its lipid and protein composition (Fig. 1.2; Wiedemann et al. 1998; Hsuan et al. 2001).

The first step in the formation of transport intermediates, namely the formation of membrane buds relies on local lipid rearrangements within the membrane. In this context the coat proteins (COPI and COPII; see section 1.3) have a pivotal role (Corda et al. 2002; Kozlov et al. 2002; Cockcroft et al. 2001; Kooijman et al. 2003). For the fission of the transport carrier precursors, both lipid-modifying enzymes and mechano-proteins are involved (Huttner et al. 2001; Corda et al. 2002; Athenstaedt et al. 1999; Praefcke et al. 2004; McNiven et al. 2000). Similar mechanisms, essentially acting in the reverse order, regulate the docking and fusion of the membranous carriers with acceptor membranes and the integration of the membranous components of these carriers into those of the acceptor
membrane (Corda et al. 2002; Kozlov et al. 2002). For the purpose of this thesis the focus will mainly be upon the Trans Golgi Network (TGN; see section 1.1.1.4 and Fig. 1.4; 1.5) and the mechanisms regulating the formation and fission of membranous transport carriers at this final Golgi complex stage in mammalian cells.
Fig 1.2. The secretory pathway. Schematic representation of the secretory pathway. Proteins synthesised within the ER are transported to the Golgi where they undergo post-translational modifications. At the trans-Golgi proteins are sorted and packaged into membranous carriers destined to different cellular compartments and organelles such as endosomes, secretory granules and the apical and basolateral plasma membrane.
1.1.1.1 ER-to-Golgi transport

Newly synthesized proteins undergo their initial folding in the ER before they enter the secretory pathway, a process that requires their assembly into heterooligomeric structures. Protein folding is triggered by a class of ER-resident proteins named chaperones that mediate both the folding and the retention of misfolded proteins within the lumen of the ER. Among chaperones, the better characterised are the lectins calnexin, calreticulin and other enzymes such as BiP and protein disulphide isomerase (PDI; Ellgaard et al. 2003).

Once properly folded, the proteins are sorted from the ER-resident proteins into specialised zones known as ER exit sites. These are distributed over the surface of the ER and mediate the local accumulation of protein that is required for transport to the Golgi complex (Bannykh et al. 1997). Concentrating the cargo at these exit sites depends on the presence of sorting signals, specific amino acid sequences on the cytoplasmic tails of the transmembrane proteins that are to be transported (Nishimura et al. 1999). These signals are recognised by proteins of the COPII complex that forms at the ER exit sites and which are located adjacent to complex structures that appear as clusters of vesicles and interconnected tubules (Bannykh et al. 1997). These make up what are known as the vesicular-tubular clusters (VTCs) and the ER-Golgi intermediate compartment (ERGIC).

Although the role of the COPII complex in the processes of cargo accumulation and membrane budding at the ER exit sites and in ER-to-Golgi transport has been well established (Bannykh et al. 1998; Klumperman 2000), the mechanisms that regulate protein transport from the ER to the ERGIC and from the ERGIC to the Golgi complex are still unclear. Two potential schemes have been proposed: (i)
the budding and detachment of small vesicles is followed by their fusion to form larger membranous structures (Bannykh et al. 1998; Marra et al. 2001); and (ii) the budding of structures at the ER exit sites gives rise to large pleiomorphic intermediates that themselves form the ERGIC (Lippincott-Schwartz et al. 2000). Despite these doubts, it is apparent that the ERGIC is a very dynamic and mobile compartment that coordinates the anterograde movement of cargo and the recycling to the ER of the molecular components involved in ER-to-Golgi transport (Lippincott-Schwartz et al. 2000; Stephens et al. 2001). In parallel to anterograde transport, the COPI-mediated retrograde transport of membrane components from the ERGIC to the ER is necessary to compensate for the anterograde movement of membranes and proteins that need to be cycled back to the ER (Cosson et al. 1994; Letourneur et al. 1994).

1.1.1.2 The Golgi complex and intra-Golgi transport

Once sorted from the ER into the Golgi complex, the large majority of proteins must undergo a series of post-translational modifications (see below) before they can be transported to their final destinations. First described in 1850 by Camillo Golgi as the “apparato reticolare interno”, the existence and role of the Golgi complex was debated for over 100 years. Then with the development of electron microscopy (EM) in the 1950s, the detailed analysis of intracellular structures at high resolution became possible (Grassé 1957; Marsh et al. 2002; Griffiths 2000). Since then, a large number of morphological analyses have been aimed at providing detailed descriptions of this complicated organelle (Farquhar et al. 1981; Ladinsky et al. 1999).
Fig 1.3. The Golgi complex. A. Diagram of the Golgi complex showing three cisternal stacks connected by non-compact, tubular zones to form a Golgi ribbon (from Kristic 1972). B. Immunofluorescent staining of the Golgi complex in different cell lines (Cyan shapes indicate the nucleus). C. Micrograph illustrating two Golgi stacks (1 & 2) connected by the non-compact zone (3; from Polishchuk et al. 2004).
Although its overall structure varies among different eukaryotic cell types, the Golgi complex presents a characteristic organisation of conserved compartments. The main structural elements consist of multiple stacks of planar membranous compartments (cisternae; Fig. 1.3A, C) connected by less organised membranous areas that form the tubular networks known as the non-compact zones (Fig. 1.4A, C; Farquhar et al. 1981; Ladinsky et al. 1999; Polishchuk et al. 2004). Each Golgi stack represents an operational unit for the processing of proteins, although the number of cisternae that form a single Golgi stack can be variable. Since these initial observations were made, it has become clear that each Golgi stack has a recognizable polarity. The side in contact with the ER (the cis-Golgi) is associated with small pleiomorphic membranous compartments, while the trans-Golgi side is generally associated with secretory granules and elements of the late endocytic pathway (Farquhar et al. 1981; Novikoff, et al. 1971). All of these ultrastructural features appear to be common to all types of animal cells although with immunofluorescence (IF) using typical Golgi complex markers, the organisation of the Golgi complex appears quite variable. Human fibroblasts (HFs) have a large network that extends from the nucleus towards the PM., normal rat kidney (NRK) and HeLa cells have complexes that appear predominantly as a ring in close association with the nucleus. In Madin-Darby canine kidney (MDCK) and Cos cells the appearance of the Golgi complex is ring-like and in a juxtanuclear position (Fig. 1.3B). This variability in size and shape is thought to depend on the kind of protein transport each cell type has to sustain (Clermont et al. 1995).

Functional investigations of the Golgi complex commenced with the realisation that there exist proteins specifically associated with the Golgi complex (Rabouille
et al. 1995) and with the development of methods of sub-cellular fractionation that allowed the isolation of this organelle from other cellular structures (de Duve 1975; Marsh et al. 2002). It soon became apparent that within every Golgi stack there is a clear differentiation in terms of the biochemical composition of the individual cisternae and that this separation is preserved both in space and time (Rabouille et al. 1995). The original idea that the Golgi complex only serves as a protein storage compartment or as a reservoir to balance the incoming and outgoing fluxes of membranes due to endocytosis and secretion was thus seen to be unlikely and a more complex and detailed role for this organelle was proposed.

The association of secretory granules with the trans-Golgi elements, together with the observation that the cargo contained in these granules was recognisable also within the lumen of the Golgi cisternae provided a clear sign that proteins that are due to be secreted have to traverse the Golgi complex before they are ready for export (Farquhar et al. 1981). Pulse-chase experiments using radioactive markers to label secretory proteins and the characterisation of the enzymes contained within the Golgi stacks led to the proposal that proteins synthesised in the ER enter the Golgi complex at the cis-side, and that they undergo post-translational modifications as they traverse the Golgi complex to the trans-side. Important among the processing enzymes within the Golgi complex are the glycosidases and glycosyl-transferases that effect the elaboration of the complex oligosaccharides that are attached both to glycoproteins on N-linked and O-linked glycan branches, and to glycolipids (Varki 1998; Rabouille et al. 1995; Mellman et al. 1992; Polishchuk et al. 2004). Most of these enzymes are type II integral membrane proteins that have small cytosolic domains, and large luminal domains that include their catalytic sites. These catalytic activities mainly involve the transfer
of different sugars (mannose, glucose, galactose, fucose or sialic acid) onto the \(-\text{NH}_2\) groups of asparagine residues in the proteins being processed. Their distributions are highly compartmentalised so as to ensure the correct sequential addition of sugar residues as the proteins pass through the Golgi complex. Of note, a number of these Golgi enzymes and other Golgi-associated proteins that are not necessarily involved in protein modification processes are commonly exploited as markers of the different Golgi complex sub-compartments (Table I).
<table>
<thead>
<tr>
<th>Golgi compartment</th>
<th>Protein</th>
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<tbody>
<tr>
<td><em>cis</em> and <em>CGN</em></td>
<td>GalNAC-transferase</td>
</tr>
<tr>
<td></td>
<td>GlcNAC Phosphotransferase</td>
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<tr>
<td></td>
<td>GM130</td>
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<tr>
<td></td>
<td>KDEL-</td>
</tr>
<tr>
<td><em>Medial</em></td>
<td>α-mannosidase I</td>
</tr>
<tr>
<td></td>
<td>GlcNAC-transferase I</td>
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<tr>
<td></td>
<td>α-mannosidase II</td>
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<td></td>
<td>Giantin</td>
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<td></td>
<td>GPP130/GiMPC</td>
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<tr>
<td></td>
<td>MG160</td>
</tr>
<tr>
<td><em>trans</em></td>
<td>β-1,4-galactosyltransferase</td>
</tr>
<tr>
<td></td>
<td>α-2,6-sialyltransferase</td>
</tr>
<tr>
<td><em>TGN</em></td>
<td>TGN-38/46</td>
</tr>
<tr>
<td></td>
<td>Furin</td>
</tr>
<tr>
<td></td>
<td>Mannose 6-phosphate receptor</td>
</tr>
<tr>
<td></td>
<td>Tyrosylprotein/sulfotransferase</td>
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**Table 1.1.** Localisation of membrane proteins to Golgi compartments in mammalian cells
Among these, GM130 is a protein that forms a part of the Golgi matrix and is involved in ER-to-Golgi transport. It is mainly located at the cis-Golgi network (CGN) in long tubular structures that emanate towards the ER (Barr et al. 2003; Short et al. 2003; Marra et al. 2001). The protein giantin is a common marker for the medial-Golgi cisternae and is an integral component of Golgi membranes that mediate the cisternal stacking through its large cytoplasmic domain (Linstedt et al. 1993; Seelig et al. 1994). At the level of the TGN, antibodies raised against the rat protein TGN38 (Luzio et al. 1990; Girotti et al. 1996) and its primate homologue TGN46 (Ponnambalam et al. 1996; Banting et al. 1997) are widely used to label the tubular networks and the post-Golgi carriers (Polishchuk et al. 2003). Although the role of these integral membrane proteins remains uncertain (Ponnambalam et al. 1996), TGN 38 and TGN46 are among the few proteins that have been identified as labels of TGN-derived tubular structures. They are not excluded from the post-Golgi carriers as are most of the other so-called Golgi-resident proteins (Polishchuk et al. 2003); rather, these proteins cycle from the TGN to the PM where they are re-internalised via clathrin-mediated endocytosis (Banting et al. 1998).

Although the protein modifications arising from the addition of terminal sugars are still under investigation, what still puzzles those working in the field of cellular transport is how this all happens. The first studies on the “dynamics” of the Golgi complex were essentially static in that the technology available at the time did not allow cargo proteins or Golgi-resident proteins to be followed in living cells. This resulted in conclusions being drawn from still images that represented somewhat random glimpses of different aspects of the whole process.
The original proposals depicted the Golgi complex as a whole maturing organelle that acquired membranes from the ER and gradually transformed them into components of the cis-, medial- and trans-Golgi. Eventually, these compartments would fragment to form the secretory carriers. For this reason, the cis-face was initially thought of as “immature” and the trans-face, as “mature” (Grassé 1957), with the Golgi cisternae as isolated compartments for the passage of newly synthesised proteins. The major limitation in this description lay precisely in the fact that the Golgi complex is a dynamic system and that there were no tools then available to confirm or deny the idea (Storrie et al. 2002). It also suggested that the synthesis of new proteins in the ER was itself the driving force for intracellular transport, a belief that was not contradicted until 1968, when Jamieson and Palade showed that the two processes are independent (Jamieson et al. 1968). Also, it was not clear how a forming cisterna would then develop the biochemical characteristics of the subsequent cisterna, and so on through the Golgi stack. Similarly, the fate of the Golgi-resident proteins of the ultimate trans-cisterna following its fragmentation into post-Golgi carriers was not known.

This “dynamic model” was sustained from the time of the first electron microscopic investigations in 1957 (Grassé 1957) up to the mid 1960s, when a totally different transport model was proposed. Since the Golgi complex preserves a clear separation in terms of its protein composition and, to a certain extent, a stable morphological structure throughout intra-Golgi protein transport, it was postulated that the stack itself provides a platform where the cisternae represent a number of different “rooms” through which proteins enter and exit while undergoing sequential modifications. According to this new model, first proposed
by Palade in 1966 (Jamieson et al. 1966), the nature of the Golgi stacks changed from a dynamic system to a static scaffold and attention was focussed on the numerous vesicles that had been seen to be associated with the stacks. These vesicles thus became the “moving elements” that shuttle proteins from one cisterna to the other on the background of a static Golgi complex (Fig. 1.5; Balch et al. 1984; Rothman et al. 1980; Rothman et al. 1980; Orci et al. 1989; Malhotra et al. 1989; Nickel et al. 2002; Storrie et al. 2002). This proposal found support with the characterisation of the coatomer protein complexes I and II (COPI and COPII) that were shown to be associated both with newly synthesised proteins exiting the ER and with the different populations of vesicles surrounding the Golgi stacks (and hence the name COP-coated vesicles; Orci et al. 1986; Barlowe 2000; see section 1.3). This correlation between cargo proteins and coat proteins provided the perfect evidence for the corroboration of this new model. Furthermore, using electron microscopy, it was now possible to visualise the presence of the cargo proteins within the lumen of these Golgi-associated “vesicles” (Orci et al. 1986).

This vesicular transport model remained unchallenged for more than 20 years. However, with the development of techniques that allow three-dimensional analysis of EM samples by serial sectioning it became apparent that the large majority of these two-dimensional, cargo-containing, “round profiles” do not represent isolated spherical structures (vesicles), but are instead complex pleiomorphic structures that are often connected with the rims of the Golgi cisternae.
At about the same time, experiments using perturbants of secretory transport gave rise to the idea that the Golgi complex is a stable and independent intracellular compartment. Of particular importance in this respect was the use of the fungal metabolite brefeldin A (BFA; Fujiwara et al. 1988; Lippincott-Schwartz et al. 1989). BFA interferes with guanosine triphosphate (GTP) cycling on the small GTP-binding protein ADP-ribosylation factor (ARF) that is involved in the formation of the COP coats on membranes. Interference by BFA induces the complete disassembly of the Golgi complex and the redistribution of its resident enzymes into the ER. Upon BFA washout the whole Golgi complex becomes reconstituted around small preserved “platforms” that are known as the Golgi remnants. Thus the position, organisation and function of the Golgi complex are spontaneously re-established in a short time through the retrieval of membranes from the ER. Clearly, these new observations were difficult to reconcile with the model of the Golgi complex as a static structure that is independent in its organisation from other cellular organelles.

The next big shake-up for the vesicular transport model was the observation that large cargo molecules, such as procollagen, are unable to enter COP-coated vesicles (which are operationally defined as membranous compartments of up to 100 nm in diameter), but are nonetheless transported from the ER to the plasma membrane via the Golgi complex (Bonfanti et al. 1998). Obviously such large protein aggregates require an alternative to the small COP-coated vesicles for their transport. Fine morphological studies have now shown that procollagen is not transported through the Golgi stacks in vesicles, but in large distensions that
originate from the ends of the cisternae (Volchuk et al. 2000) and that always remain connected to at least one cisternal rim (Mironov et al 2001).

This thus begs the question: is it possible that different transport mechanisms exist for different cargoes? A small cargo could be transported in small dynamic vesicles and large protein aggregates could be contained in larger carriers that originate and move by a totally different means. How can this be rationalised with the observations that large membranous distensions are detectable only when a large cargo is being transported (Volchuk et al. 2000) and that when this happens, small cargo molecules (such as the vesicular stomatitis virus glycoprotein; VSVG) can move at the same rate through the same compartment (Mironov et al. 2001)?

The vesicular transport model was by now in crisis and as for every dogma facing its demise, there were a number of non-believers who had held silent for many years and who were now ready to push forward with new ideas. If it is not to be vesicular transport and not even cisternal maturation as described in 1957, how does a cargo that is transported from one side of a Golgi stack to the other do so without ever leaving the cisternal lumen? More than this, the cargos manage to undergo multiple modifications before eventually reaching the TGN for sorting and delivery to their final destinations. Clearly it was not (or not only) the cisternae that were maturing, but also the cargo itself, which led to the definition of cargo-maturation within a new potential model of intra-Golgi transport (Fig. 1.5; Bannykh et al. 1997; Glick et al. 1997; Mironov et al. 1997).
Even if it appears that the cisternal/cargo maturation models are currently the most favoured, those in the field of intracellular transport have learned not to take such models and schemes for granted. For this reason, the list of the hypotheses of intra-Golgi transport, each with its own pros and cons that are now under consideration could fill a telephone directory. It is quite clear that more than 200 years following its discovery, the way that the Golgi complex carries out its functions remains an open question. We can thus only now try to answer this question by integrating different approaches, such as light microscopy (Presley et al. 1998), EM/tomography (Ladinsky et al. 1999; Mogelvang et al. 2003; Trucco et al. 2004) and bio-informatics.
Fig 1.4. Dissection of the Golgi complex and transport models. A-C. Tomogram of a Golgi ribbon is shown including the surrounding vesicular structures or in the absence of such membranes so that the organisation of the ribbon is more evident. In C the non compact zone is illustrated with the vesicular component. D. One single stack (from the boxed region on the left) is separated into single cisternae according to their structure and function in terms of intra-Golgi transport (from Marsh et al. 2002).
1.1.1.3 The “vesicle problem”

Since the first EM studies in the mid-1950s (Grasse 1957), the vesicles that are seen to be positioned around the Golgi stacks have been considered as a component of the Golgi complex having a role in the transport of proteins along the secretory pathway (Rothman et al. 1980; Rothman et al. 1980; Orci et al. 1989; Nickel 2003; Storrie et al. 2002). However, when the model of cisternal maturation was presented, it was found that cargo proteins are actually transported through the Golgi complex without ever leaving the lumen of the cisternae. As the Golgi cisternae “mature” from the cis- to the trans-side of the stack the set of cis-cisterna enzymes is eliminated while the enzymes typical of the medial-cisterna are accrued, and so on. Since most Golgi enzymes are transmembrane proteins this necessarily involves the excision of discrete membrane patches which could well be in the form of vesicles that can then fuse with another cisterna. The accretion of the new set of enzymes could follow a similar principle. Thus the peri-Golgi vesicles are the effectors of this maturation process that is driven by a system of membrane and protein recycling (Bannykh et al. 1997; Allan et al. 1999; Mironov et al. 1997; Martinez-Menarguez et al. 2001). This hypothesis is still under debate but the recent observation that Golgi enzymes are absent from the COP-coated vesicles that surround the stacks now sheds some doubt on this idea (Kweon et al. 2004; Cosson et al. 2002).

As the main interest in peri-Golgi vesicles derives from their abundance around the stacks and from the knowledge that their formation involves the consumption of GTP for the formation of their COP coats, it should also be noted that with the
development of EM tomography, the number of peri-Golgi vesicles has been shown to be far less than expected (Ladinsky et al. 1999; Trucco et al. 2004).

In contrast to this role of the Golgi enzymes in retrograde transport, another possibility is that peri-Golgi vesicles constitute a "reservoir of membrane curvature". Golgi cisternae are extended flat surfaces having a very low degree of curvature and only the rims constitute a region of curvature. To form transport carriers the membranes must form buds and membranous tubules from highly curved surfaces. Geometrically, one may demonstrate how even a single fusion event between a highly curved structure (such as a vesicle) and a flat disc-like structure (such as a Golgi cisterna) can induce a change in the three-dimensional (3D) shape of the disc-like structure (Our unpublished observations). According to this idea, fission and fusion of peri-Golgi vesicles may thus be the events that regulate the morphology of the whole of the Golgi complex.

Whatever the role of the peri-Golgi vesicles, it is now evident that observations made by thin section electron microscopy do not lead to the correct assignment of structures such as vesicles and pleiomorphic carriers, and even tubules.

1.1.1.4 The trans-Golgi network

The TGN is the main sorting station of the secretory pathway. This organelle is made up of a very complex tubular- reticular network that functions rather like a post office: the proteins that are processed within the Golgi complex are transported to the TGN, sorted into specialised carriers that derive from the TGN membranes, and delivered to their correct destinations. First described in 1976 by Novikoff and colleagues (Novikoff 1976) as a portion of the ER that is intermixed
with the \textit{trans}-face of the Golgi stack, the TGN was originally known as GERL (Golgi-ER-lysosome). This defined a region where three different compartments that are involved in protein secretion intersect, namely the Golgi, the ER and the lysosomes. In particular, the association of the TGN with ER elements arose from the observation that cytidine monophosphatase is present in both compartments (Novikoff 1976) though subsequent lines of evidence have proved this hypothesis wrong (Smith et al. 1990; Griffiths et al. 1986).

On the basis of several lines of evidence current models describe the TGN as a compartment that is independent of the Golgi stacks. First, the Golgi stacks and the TGN differ in their membrane lipid, protein and enzyme compositions (Novikoff 1976). The TGN membranes are enriched in cholesterol and in "typical" TGN proteins, such as TGN38 (Luzio et al. 1990) and furin (Molloy et al. 1994) which have never been observed in association with the Golgi cisternae. Most of these typical TGN proteins cycle between the TGN and either the plasma membrane or the endosomal/lysosomal network (Banting et al. 1997; Luzio et al. 1990; Ponnambalam et al. 1996; Molloy et al. 1994). Furthermore, although the Golgi stacks are characterised by a gradient of decreasing pH from the \textit{cis}- to the \textit{trans}-side, the pH in the TGN is particularly low (6.0) and more typical of the late endosomal compartment. In corroboration of these biochemical distinctions, the use of BFA as an inhibitor is a useful tool in our understanding of the dynamics of the Golgi complex. As indicated earlier, BFA induces the tubulation of the Golgi stacks and the rapid redistribution of the Golgi membranes and proteins into the ER (Fujiwara et al. 1988). The membranes of the TGN are also sensitive to BFA, although they undergo a different redistribution to that seen for the Golgi
membranes and their reaction is much slower. Instead of merging into the ER the TGN membranes merge with the lysosomal compartment (Lippincott-Schwartz et al. 1991; Reaves et al. 1992; Wood et al. 1991). Moreover, not all TGN-associated proteins are dispersed by BFA as some collapse into the TGN remnants in the vicinity of the centrosome (Reaves et al. 1992). Finally it important to note that when the TGN was first described its function was linked with that of the Golgi complex, and it was thought to have a role only in the functioning of the secretory pathway. Later, the connections between the TGN and elements of the endosomal compartment gained importance in terms of membrane and protein cycling and it became clear that it covers a more general role as the sorting station for multiple pathways.

The TGN exhibits a much higher morphological variability among different mammalian cell types. The reasons for this appear to depend on the kind of secretion characterising each cell type. In 1995, Clermont and colleagues (Fig. 1.5 Clermont et al. 1995) found that cells that do not produce secretory granules but that have very extended lysosomal systems also have very extended TGN networks. On the other hand, cells that produce secretory granules, particularly large granules have a reduced TGN. The size and shape of the TGN is also dependent on the activity of the secretory pathway. Under conditions where protein transport is arrested at the level of the ER the TGN is reduced in parallel to the number of post-Golgi carriers that are formed. When protein transport is arrested at the level of the TGN this organelle appears hypertrophic and the number and length of tubules is increased.
In 1999, a tomographic analysis from Ladinsky and colleagues (Ladinsky et al. 1994) illustrated the 3D reconstruction of a portion of the Golgi complex from NRK cells. This demonstrated that at the level of the trans-cisterna there are no evident reticular structures that would correspond to the TGN. The three trans-most cisternae appeared to produce a high number of membranous transport intermediates and tubular structures which were often associated with proteinaceous coat components. In contrast to the classical model that describes the TGN as an independent compartment, these observations raised the possibility that the TGN is a constitutive part of the Golgi stack in particular cell types, inducing the authors to propose the “multiple cisternae” model for TGN organisation (Ladinsky et al. 1994; Gu et al. 2001). However, the general consensus appears to be that tomographic descriptions of this sort should be extended to the whole Golgi complex rather than being limited to only a few stacks. More importantly, due to the morphological variability of the TGN, such studies need to be applied to a wider range of cell types before this multiple cisternae model can be confirmed or denied (Gu et al. 2001).
Fig 1.5. Diagrams of portions of the Golgi apparatus from different cell types. A. Golgi apparatus of a constitutive secreting cell characterised by a large TGN. B. Golgi apparatus from a cell producing prosecretory granules where the TGN is more reduced and less tubulated. C. Golgi apparatus of a secretory cell where secretory material accumulates within distensions in multiple cisternae and in saccular domains of the TGN. D. Golgi apparatus from a lactating mammary gland. Large secretory granules form from saccular cisternae and the TGN is nearly disappeared. Key: CGN, Cis-Golgi Network; MS, Middle Saccules; V, Vesicles; TGN, Trans-Golgi Network; Sg, Secretory Granule; Pg, Prosecretory Granules; RT, Residual membranous Tubules (adapted from Clermont et al. 1995).
1.1.2. Membrane traffic at the TGN

As indicated above, the TGN represents the sorting station for multiple pathways. Proteins are sorted at the TGN to be delivered either through the constitutive or regulated secretory pathways to the plasma membrane or to the late endosomes/lysosomes (Gu et al. 2001; Keller et al. 1997). A further division has been seen at the level of the constitutive secretory pathway of polarised cells where proteins are sorted into separate carriers destined to the apical or basolateral plasma membranes (Keller et al. 1997; Nelson et al. 2001). This complex sorting system implies the presence of distinct membrane domains within the TGN that segregate both the proteins that are destined for different cellular compartments and the sorting signals within the cargo proteins that allow their interactions with specific domains of the TGN membranes.

The presence of specialised membrane domains at the TGN has been demonstrated by the presence of membranous tubules, raft domains and coat proteins (Gu et al. 2001). The membranous tubules were first described in the 1960s, although their role was generally neglected since the transport of proteins was thought to be driven solely by vesicles, as suggested by Rothman and Orci (Rothman et al. 1996; Orci et al. 1986). Furthermore, the tubular structures are often lost when transport is examined in chemically fixed cells. Since the development of new fixation techniques and, in particular, of green fluorescent protein (GFP) chimeras for the study of protein transport in living cells, a clear role in membrane transport for these tubules became apparent.
Morphologically they are defined as cylindrically shaped membranes that have a length that is at least twice their diameter (Banta et al. 1995; Cluett et al. 1993). The diameter of a tubule can vary from 20 nm up to 200 nm, while their maximal length can reach some microns (Mollenhauer et al. 1998; Sciaky et al. 1997; van Deurs et al. 1996; Banta et al. 1995; Ladinsky al. 1994; Cluett et al. 1993). They can be arranged in several different ways, ranging from single, straight or convoluted units of different lengths (often observed protruding from the CGN and the TGN), to reticuli that extend both two- or three-dimensionally and which are often branched (as with those that constitute the TGN itself). Although they are present in many cellular compartments, these structures are particularly abundant at the level of the TGN, where in several cell types they constitute almost its entire structure. Recent studies (Polishchuk et al. 2003) show that these TGN tubules play a direct role in the formation of a large proportion of the post-Golgi carriers (at least 60%). At the level of the CGN the tubules mediate anterograde and retrograde protein transport between the ER and the Golgi complex (Marra et al. 2001). Also, short transient tubules have been observed that connect individual cisternae within single stacks of the Golgi apparatus that possibly mediate cargo transport or the retrograde movement of the resident enzymes (Trucco et al. 2004). Such tubular structures are not only associated with the Golgi complex, but are also present at the level of the sorting endosomes where they mediate the transport of molecules along the endocytic pathway (Hunziker et al. 1992; Nicoziani et al. 2000).

As mentioned above, the tubular domains within the TGN provide a means to segregate clusters of proteins into different regions of the membrane network. As
shown for VSVG transport, multiple tubular carrier precursors often emanate from a single region of the TGN (Polishchuk et al. 2003), with other regions never producing VSVG-positive tubules. This suggests that the physical segregation of cargo proteins within different TGN sub-domains occurs prior to protein transport.

The tubular structures at the level of the TGN are not the only means exploited for protein sorting. Transport from the TGN to the basolateral plasma membrane is also mediated by sorting signals on secreted proteins (Matter 2000; Mostov et al. 2000). These transport signals are quite heterogeneous and can be divided into three sub-categories:

1. Signals that rely on a critical tyrosine residue in the proximity of at least one large hydrophobic aminoacid;

2. A motif grouped around a leucine/leucine or a leucine/isoleucine pair;

3. Other signals that rely on neither tyrosine nor di-leucine.

In the case of proteins destined for the apical membrane it has been demonstrated that sphingolipid-cholesterol rafts play a role by recruiting glycosylphosphatidylinositol (GPI)-anchored proteins (Simons et al. 1997; Keller et al. 1997; Matter 2000). Protein-associated signals other than GPI anchors, such as N-glycan chains or even transmembrane domains (as in the case of influenza virus hemagglutinin), are also commonly exploited by proteins for their association with lipid rafts (Keller et al. 1997; Scheiffele et al. 1995).
Finally the proteins that form the coat complexes (see section 1.3) possess signals that are recognised by secretory proteins (Bonifacino et al. 2003). These interactions between cargo proteins and coat complex proteins are exploited to gather and to accumulate cargo at the site where post-Golgi carriers are to originate. The coat proteins are also involved in the transport steps from the TGN to the late endosomal/lysosomal compartment. Thus the adaptor protein subunits 1 and 2 (AP1, AP2) are involved in the formation of clathrin coats on membranes (see section 1.3) and have been found to interact with the cytoplasmic signals of cargo proteins destined for the late endosomal compartment (Bonifacino et al. 2003).

In addition to those signals recognised by coat proteins, the mannose-6-phosphate receptor (MPR) has an important role in TGN-to-lysosome transport (Gu et al. 2001; Rouille et al. 2000). This receptor recognises mannose-6-phosphate residues on proteins such as the lysosomal hydrolases and it mediates their transport from the TGN to the lysosomes. Here the receptor and its ligand dissociate leaving the MPR to cycle back to the TGN.

It is a matter of debate whether transport of constitutive cargo from the TGN to the plasma membrane occurs in a regulated fashion, which would involve the accumulation of secreted proteins into specialised domains, or via bulk flow independently of both cargo accumulation and the formation of coat complexes. Quantitative analysis of VSVG transport in non-polarised cells reveals no evidence for an accumulation of VSVG within specific sub-domains of the TGN. During transport, VSVG is evenly distributed both in tubular and saccular
structures at the TGN and in the Golgi cisternae, supporting the idea of bulk flow of constitutive proteins through the secretory pathway (Polishchuk et al. 2003). In contrast to the model where the transport of proteins from the TGN occurs via vesicular carriers that originate from the budding of the TGN membranes (Rothman et al. 1996; Schekman et al. 1996), others (Polishchuk et al. 2000) have shown that the carriers operating between the TGN and the plasma membrane are actually complex membranous structures of variable sizes that originate from the fission of TGN-derived tubules (Fig. 1.6 B; Polishchuk et al. 2000; Polishchuk et al. 2003). In the light of this recent evidence it is now possible to identify at least three steps that lead to the formation of constitutive post-Golgi transport carriers (Fig. 1.6 A; Polishchuk et al. 2003):

1. Segregation of the cargo proteins from the TGN-resident proteins into a "cargo domain" that generally consists of a pre-existing convoluted membranous tubule.

2. Anchoring of these cargo-containing convoluted tubules to cytoskeletal elements by means of molecular motor proteins and elongation of the convoluted tubules along microtubule tracks.

3. Fission of the tubule to generate one or more constitutive post-Golgi carriers.

The segregation of cargo proteins from TGN-resident proteins has been observed at both the ultrastructural and the immunofluorescence levels. Golgi-to-plasma membrane carriers (GPCs) exclude TGN enzymes such as mannosidase II, galactosyl-transferase and sialyl-transferase, as well as TGN-resident proteins like furin, the MPR and syntaxin 6 (Fig. 1.6 C; Polishchuk et al. 2003). The coat
proteins are also excluded from VSVG-positive carriers and this supports the idea that this transport step is independent of coat formation. As already mentioned, the only TGN marker that remains associated with the GPCs is TGN46 (Fig. 1.6 C; Polishchuk et al. 2003). Remarkably, another relevant protein that has been found associated with both GPCs and GPC precursors is the motor protein kinesin (Polishchuk et al. 2003). The presence of kinesin in association with the GPCs, taken together with the observation that cargo-containing tubules elongate along microtubules, suggests that tubules undergo a pulling force along cytoskeletal elements prior to the formation of constitutive transport carriers. Indeed, fission events are always associated with an active pulling force on these TGN-derived tubules and fission events have never been seen to occur on non-stretched, collapsed tubules (Polishchuk et al. 2003). Also, following fission that leads to the formation of a GPC, the remaining part of the tubule retracts elastically to the TGN (Polishchuk et al. 2003). In this respect, it is important to stress that these cargo-containing tubules that elongate from the TGN are not formed right before their protrusion but that they generally derive from pre-existing tubular structures that are collapsed on to the TGN when they are not anchored to motor proteins (Polishchuk et al. 2003).
Fig 1.6. Formation of post-Golgi carriers at the TGN. A. Model for the formation of post-Golgi transport carriers: a flat membrane (1; 5) segregates cargo into a “cargo domain” and initiates the budding (2); saccular domains attach to microtubules by means of motor proteins (3; 6) and are pulled away from donor membranes. Eventually fission protein separate the formed carrier from the compartment of origin (4). B. CVEM and 3D reconstruction of a forming post-Golgi transport carrier indicates the complexity of such structures. C. Forming cargo domains segregate proteins such as furin out of the forming carrier precursor (adapted from Polishchuk et al. 2003).
1.1.3. The endocytic pathway (Fig. 1.7)

The plasma membrane constitutes a dynamic boundary that separates the cytoplasm from the surrounding environment (Mellman 1996; Pelkmans et al. 2003; Conner et al. 2003; Nichols et al. 2001). Small molecules such as ions, sugars and aminoacids can easily traverse the plasma membrane through various transmembrane protein complexes that are embedded in the bilayer and that form channels and pumps. It is by means of this exchange of molecules that cells preserve their own identity in terms of chemical composition and electrical potential, despite the variable conditions of the surrounding environment. For the transport of macromolecules and molecular complexes through the plasma membrane, a different process of internalisation is required. Entire patches of membrane invaginate and are then pinched-off from the inner face of the plasma membrane and transported within the cell. This internalisation is referred to as endocytosis and it generally falls into two major types (Mellman 1996; Conner et al. 2003; Nichols et al. 2001): (i) phagocytosis; and (ii) pinocytosis.

The process of phagocytosis is restricted to a limited class of cells including macrophages, monocytes and neutrophils, and it is usually exploited to eliminate pathogens, dead-cell debris and deposits of fat. Briefly, phagocytosis involves a signalling cascade that is activated by specific cell-surface receptors that stimulate actin polymerisation/depolymerisation through the activation of the Rho family of GTPases (Conner et al. 2003; Cossart et al. 2004; Nichols et al. 2001). The actual internalisation process takes place through an actin-driven protrusion of plasma membrane patches that fuse with each other to engulf portions of the extracellular...
milieu. In the case of pathogens, once internalised they are destroyed in specific cell compartments by means of acids, acid hydrolases and free oxygen radicals.

Pinocytosis is instead a process common to all cell types (Pelkmans et al. 2003; Nichols et al. 2001; Conner et al. 2003; Mellman 1996) with the exception of red blood cells. It is divided into at least five classes on the basis of the molecular machineries that drive the process (Fig. 1.7; Conner et al. 2003):

1. Macropinocytosis.
2. Clathrin-dependent endocytosis.
3. Caveolae-dependent endocytosis.
4. Clathrin- and caveolae-independent endocytosis (which is further subdivided into dynamin-dependent or dynamin-independent endocytosis).

Macropinocytosis shares similarities with phagocytosis including a signalling cascade mediated by small GTPases that stimulate actin polymerisation (Dharmawardhane et al. 2000; Conner et al. 2003; Sabharanjak et al. 2002; Steinman et al. 1995). The membrane ruffles formed in this way then collapse onto the plasma membrane where they fuse together and form a macropinosome that is larger than normal endocytic vesicles. The severing of the macropinosome from the plasma membrane is independent of the activity of the fission-inducing protein dynamin (see section 1.2.2.1).

The remaining three sub-classes of endocytosis have all been well characterised and they are responsible for most pinocytic activities. They mediate the
internalisation of viruses which represents the most widely exploited tool used for the study of endocytosis (Pelkmans et al. 2003). Clathrin (often referred to as receptor-mediated endocytosis) and caveolae-dependent endocytosis are characterised by the formation of their respective coats (protein complexes that form at the site where the plasma membrane begins to invaginate; Bonifacino et al. 2003; Conner et al. 2003; Nichols et al. 2001). These coat complexes assemble into a curved rigid scaffold in tight association with the plasma membrane. They mediate the formation of endocytic carrier precursors (clathrin-coated or caveolin-coated pits) that remain connected to the extracellular milieu through constricted necks. In the case of clathrin-coated and caveolin-coated pits, dynamin effects the scission of the neck and the formation of an independent coated vesicle (Henley et al. 1998; van der Bliek et al. 1993). Typically, clathrin-mediated internalisation is exploited to internalise molecules that bind to a receptor that is exposed on the external face of the plasma membrane. This is the case for the transferrin receptor (TFnR), perhaps the best characterised in this context (Damke et al. 1995; Damke et al. 1994; Altschuler et al. 1998).

Endocytosis can also occur without the formation of a protein coat (Nichols et al. 2001; Pelkmans et al. 2003). This form of endocytosis still awaits full characterisation and it is generally referred to as clathrin- and caveolae-independent endocytosis (Skretting et al. 1999; Conner et al. 2003). Here, the formation of invaginated buds at the level of the plasma membrane appears to be driven solely by the lipid composition of the membrane patches. The preservation of these structures does not require any scaffolding protein(s) and in some cases lipid rafts are associated with this form of endocytosis (Nichols 2002; Nichols et al. 2001). Two different types of raft-mediated endocytosis have also been
identified: one that requires dynamin for the fission of the endocytic carrier and the other that is dynamin-independent (Pelkmans et al. 2003). Typically, bulk endocytosis of fluid-phase solutes follows dynamin-independent endocytic pathways which can be either raft-mediated or raft-independent (Dharmawardhane et al. 2000; Sabharanjak et al. 2002; Damke et al. 1995; Damke et al. 1994; Altschuler et al. 1998). Recent studies have shown that dynamin-independent fluid-phase endocytosis requires the activity of enzymes at the level of the plasma membrane that are involved in the organisation of the actin cytoskeleton such as: PKCα and ε (Song et al. 2002) Cdc42 (Sabharanjak et al. 2002) and p21-activated kinase-1 (PAK1; Dharmawardhane et al. 2000).
Fig 1.7. Endocytic pathways. Schematic representation of different endocytic routes classified for their dependency on Clathrin, Dynamin, lipid rafts and Caveolin. A) clathrin-dependent endocytosis; B) caveolae-dependent endocytosis; C & D) lipid-raft mediated endocytosis (fluid-phase); E) fluid-phase endocytosis (from Pelkmans et al. 2003).
1.2. Membrane fission

1.2.1. The role of lipids in membrane fission

Membrane transport from a donor compartment to an acceptor compartment is a process that occurs through a series of events some of which are specific for different pathways. The first event common to all pathways is the generation of curvature in a flat membranous surface, such as the plasma membrane or a Golgi cisterna, in order to form a bud (Fig. 1.8 A; Corda et al. 2002; Burger 2000; Brown et al. 2003). As indicated above, the formation of such curved domains can either be triggered by proteins (as in the case of coat-complex-associated buds and pits; see Section 1.3), or be driven solely by lipid rearrangements within the two leaflets of the bilayer. Since the leaflets of the bilayer are connected with each other, positive or negative surface curvature depends on the relative areas of the inner and outer leaflets. If the two surfaces are equal in size, then the bilayer will be planar whereas if the surface areas of the two leaflets are different the bilayer acquires a positive or negative curvature (Fig. 1.8 B; Corda et al. 2002; Burger 2000; Huttner et al. 2001; Scales et al. 1999; Brown et al. 2003). Membrane fission and fusion are both processes that require the generation of extreme localised membrane curvature and the ability to achieve this depends on several factors. One is the three-dimensional shape of lipids embedded in the bilayer: as the lipids are free to move within each bilayer leaflet, the clustering of lipids with a given shape in a specific area of one leaflet can induce variations in the overall curvature of membranes. Furthermore, the translocation of lipids not only occurs within the same leaflet, but also from one leaflet of the bilayer to the other by a
process known as “flip-flop”. This is mediated by specific membrane proteins (Fig. 1.8 C; Corda et al. 2002; Huttner et al. 2001; Burger 2000). Lipid metabolism is also relevant for the modulation of membrane curvature (Huijbregts et al. 2000; Corda et al. 2002; Scales et al. 1999; Brown et al. 2003; Freyberg et al. 2003): lipids are subjected to protein-induced metabolism that can change their properties and their 3D structures which can eventually affect the shape of biological membranes (Fig. 1.8C).

1.2.1.1. The role of phosphatidic acid in membrane fission

The local formation of polyphosphoinositides, diacylglycerol (DAG) and phosphatidic acid (PA) has a key role in the regulation of intracellular membrane trafficking (Fig. 1.8 C; Corda et al. 2002; Scales et al. 1999; Kooijman et al. 2003; Burger 2000; Van Lint et al. 2002; Brown et al. 2003; Athenstaedt et al. 1999). These lipids function in two different ways: (i) through the recruitment of specialized proteins that are involved in cargo segregation as well as in membrane tubulation, fission and fusion (Antonny et al. 1997; Baron et al. 2002; Maeda et al. 2001; Ktistakis et al. 2003; Manifava et al. 2001); and (ii) through a direct role in membrane bending and destabilization by virtue of their biophysical properties (Kooijman et al. 2003). PA is important in the modulation of membrane curvature that leads to fission and there is evidence that the modulation of its concentrations within cell membranes can regulate transport events (Kooijman et al. 2003; Siddhanta et al. 2000; Sweeney et al. 2002; Chen et al. 1997). Thus phospholipase D (PLD)-induced increases in PA levels can stimulate the release of secretory vesicles from the TGN (Chen et al. 1997). Conversely, if the activity of PLD is
inhibited by means of primary alcohols, the intracellular PA levels are reduced and the release of post-Golgi carriers is inhibited (Siddhanta et al. 2000).

PA-rich micro-domains can therefore directly destabilise the interactions between the two leaflets of the bilayer through their interactions with divalent cations (Kooijman et al. 2003). PA has also been seen to stimulate the lipid penetration of the membrane-destabilising protein dynamin (thereby favouring the interaction of dynamin with the constricted neck of membrane buds; Burger et al. 2000), and it has a high affinity for binding to some traffic-related proteins, including ARF, kinesin, and the β subunit of the coatmer (Ktistakis et al. 2003; Manifava et al. 2001). Finally, PA can be easily metabolised to DAG by phosphatidate phosphatases (Brindley et al. 1998), or to lysophosphatidic acid (LPA) by phospholipase A$_2$ (PLA$_2$; Brown et al. 2003).

DAG itself is another regulator of membrane transport processes. It has a strongly conical shape, and as it is uncharged, it is capable of very fast transbilayer movements (flip-flop; Corda et al. 2002 and references therein). Moreover, DAG is also important in fission events as a binding site for several traffic-related proteins, including PKD (Baron et al. 2002; Maeda et al. 2001) and proteins involved in the formation of coat complexes such as ARFGAP (Antonny et al. 1997). The opposing activities of phosphatidate phosphatases and DAG kinases (DGKs; which convert DAG to PA) regulate the relative concentrations of PA and DAG within biomembranes.

The conversion of PA to LPA is of particular significance for the geometry of membrane bilayers since the cone-shaped PA is converted to the inverted-cone-
shaped LPA, thus facilitating rapid changes in membrane curvature (Brown et al. 2003; Scales et al. 1999; Kooijman et al. 2003). The inverse reaction is mediated by lysophosphatidic acid acyl transferases (LPAATs) and involves the addition of an unsaturated fatty acid (generally arachidonate) to LPA to form PA. This latter reaction has been studied mostly in the context of synaptic-like microvesicles, where it appears to be mediated by the protein endophilin (Reutens et al. 2002; Schmidt et al. 1999; Huttner et al. 2002; Gad et al. 2000). It is interesting to note that this protein not only acts at the plasma membrane, where endophilin I was first seen to localise: recent evidence has indicated the presence of the endophilin B1b isoform at the level of the Golgi complex (Modregger et al. 2003).

All of the proteins involved in lipid metabolism discussed above form part of the large number of enzymes that co-operate in the metabolism of membrane lipids and that can promote fast and localised modulation of the curvature of lipid bilayers (Corda et al. 2002; Huijbregts et al. 2000). Overall, the majority of the enzymes involved in these processes can be grouped into the families of:

- Phospholipases (e.g. PLD, PLA₂)
- Acyltransferases (e.g. the endophilins, CtBP3/BARS)
- Kinases (e.g. PKD, PKC)
- Phosphatidylinositol transfer proteins (e.g. PITP classes I and II)
Fig 1.8. Formation of membranous transport carriers. A. Schematic representation of the main steps leading to the formation of transport carriers from cellular membranous organelles. B. The geometry of the lipids forming the bilayers can generate curvatures within membranes required for the formation of buds and fission sites. C. Enzyme-mediated lipid metabolism can drive the generation of membrane curvature at the fission site through local modulation of the geometry of the lipids (adapted from Corda et al. 2002).
1.2.2. Protein-induced membrane fission

The molecular mechanisms that regulate membrane fission have been extensively studied over the last decade. Although the role of lipid metabolism in the rearrangements of biological membranes that precede fission has been investigated, it remains unresolved whether this is sufficient by itself to drive the whole fission process. The severing of membranes can be triggered by proteins other than those forming coat complexes or catalysing lipid metabolism. For instance, there is evidence for a role for motor proteins such as the kinesins and the dyneins in membrane fission. These proteins mediate the docking of membranes onto cytoskeletal elements and induce a pulling force that stretches and destabilises membranes (Allan et al. 2002; Kreitzer et al. 2000; Gross et al. 2000; Polishchuk et al. 2003). In most cellular systems an important role is also played by proteins that have activities that are not strictly related to lipid metabolism, but rather to mechanical membrane destabilisation in the late steps of membrane fission. The most relevant proteins involved in these late phases of membrane severing are the dynamins, the dynamin-related proteins, and BARS, although for the last of these a role in lipid remodelling is still under investigation.

1.2.2.1. Dynamin

The dynamins are high molecular weight (100-kDa) monomeric GTP-ases that constitute a highly differentiated superfamily that is involved in several intracellular fission processes and in a number of actin-driven events (Praefcke et al. 2004; McNiven et al. 2000; van der Bliek 1999; McNiven et al. 2004; Orth et al. 2003). Members of the dynamin superfamily are differentiated into classical
dynamins, dynamin-related proteins, and guanylate-binding proteins-related proteins (GBP-related proteins; Praefcke et al. 2004; McNiven et al. 2000). Although the protein structures of the various members of the dynamin superfamily are highly variable (van der Bliek 1999; Praefcke et al. 2004), minimal distinguishing features are nevertheless common to all of the members. These include a large N-terminal GTP-ase domain of approximately 300 aminoacids, a middle domain, and a C-terminal GTP-ase effector domain (GED) that is involved both in the oligomerisation of the protein and in the regulation of the GTP-ase activity (Fig. 1.9 A). This basic structure is then supplemented by the presence of other domains that can vary among the different members of the protein family. Among these, there are the proline-rich domain (PRD) and the pleckstrin-homology (PH) domain that mediate protein targeting to cellular membranes (Praefcke et al. 2004).

The classical dynamins (for brevity, dynamins) are the better characterised members. They possess the PH domain for membrane targeting and also the C-terminal PRD through which they interact with a wide range of traffic-related proteins. Classical dynamins include three members expressed in several different tissues (Cao et al. 1998):

(i) dynamin 1: enriched in brain and present in eight splice isoforms.

(ii) dynamin 2: ubiquitous and present in four splice isoforms.

(iii) dynamin 3: primarily found in testis and present in thirteen splice isoforms.

Dynamin was first identified as a 100-kDa protein associated to purified calf brain microtubules in a GTP-dependent fashion (Shpetner et al. 1989). The association
of dynamin to microtubules has been further investigated in vitro and it was observed that dynamin decorates the surface of microtubules forming tightly packed helical structures (Maeda et al. 1992). Years after, in vitro evidences were produced that showed how dynamin is able to bind membranes through its PH domain and oligomerise into ring structures. This conformation induces membrane tubulation and then following GTP hydrolysis on dynamin, the membranes fragment into vesicles (Carr et al. 1997; Sweitzer et al. 1998; McNiven et al. 2000; Danino al. 2004; Sever et al. 2000; Kozlov 1999). Implication that the dynamins are involved in membrane traffic first derived from the observation that dynamin is the mammalian homologue of the shibire gene product in Drosophila (van der Bliek et al. 1991). Drosophila flies carrying the temperature sensitive shibire mutation exhibit paralysis due to a block of endocytosis that depletes synaptic vesicles (van der Bliek et al. 1991). Indeed, in mammalian cells the inhibition of dynamin was seen to block the formation of coated vesicles at the plasma membrane (van der Bliek et al. 1993). The GTP-dependence of dynamin-induced membrane fission has been clearly demonstrated in vitro by the use of the non-hydrolysable form of GTP (GTP\textsubscript{y}S; Shpetner et al. 1992; Maeda et al. 1992) and in vivo by the overexpression of a GTPase-defective dynamin mutant (dyn\textsuperscript{K44A}; van der Blik et al. 1993; Damke et al. 1994). Both treatments are inhibitory towards the fission step and result in the accumulation of dynamin-decorated fission intermediates, although in vivo evidences for dynamin spirals around membranes are scarce (Damke et al. 1994; Iversen et al. 2003).
A

Classical dynamins

Dynamin-related proteins

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GTPase domain □ Middle domain □ PH domain □ Predicted transmembrane domain □ Mitochondrial leader sequence

B

Clathrin-mediated endocytosis

Endosome-to-Golgi transport

Actin–membrane dynamics

Fluid-phase endocytosis

Membrane retrieval

Secretory vesicle formation from the TGN

Phagocytosis?

Mitochondrial morphogenesis?

ER–mitochondrial trafficking?

Caveolae

TR/RS
**Fig 1.9. The dynamin protein family.** A. Schematic representation of the domain structure of the most relevant members of the human dynamin superfamily (adapted from Praefcke et al. 2004). B. Comprehensive map of the different intracellular membrane fission events regulated by members of the dynamin family (key: red, dynamin; green, dynamin Like Proteins. From McNiven et al. 2000). C. Models of dynamin activity as membrane fission protein depicting the “pinchase” and “poppase” hypothesis (from Praefcke et al. 2004). D. A model of the interactions between dynamin and the actin cytoskeleton in the formation of secretory and endocytic membranous transport carriers (from Orth et al. 2003).
Deriving from in vitro studies, there are several proposals for the mechanism of membrane fission mediated by dynamin and those that have merited most attention include the “pinchase” and the “poppase” models (Fig. 1.9 C; Praefcke et al. 2004; Sever et al. 2000). The former is based on the observation that following GTP hydrolysis dynamin undergoes a conformational change that shrinks the rings of dynamin oligomers around the necks of the forming membranous carriers thereby inducing fission (Sweitzer et al. 1998). The poppase model is based on the more recent observation that rather than being more compressed, dynamin oligomers expand when they are bound to GDP, inducing a stretching of membranes that might lead to fission (Stowell et al. 1999). Currently it is impossible to define which model fits the real situation best. Nevertheless, it is generally understood that dynamin by itself is not able to induce fission unless its activity is integrated with that of other proteins that induce a local remodelling of membrane lipids to initiate the budding process (section 1.2.1). Interestingly, dynamin interactors include endophilin (Reutens et al. 2002; Modregger et al. 2003) that has LPAAT activity that results in membrane bending through the conversion of LPA to PA.

The demonstration of a functional link between dynamin and actin at the level of podosomes (Ochoa et al. 2000) is of particular interest since it bridges membrane remodelling events with the actin cytoskeleton. Since the first observations in 2000 a growing number of evidences have been published supporting the interaction between dynamin and acting binding proteins (Orth et al. 2002; Baldassarre et al. 2003; Lee et al. 2002; Pelkmans et al. 2002). Current predictions envision dynamin to polymerize on cellular membranes to form a membrane-deforming scaffold; by doing this PRDs on each dynamin monomer remain
exposed and available for the binding of the numerous acting binding protein through their SH3 domains (Fig. 1.9 D; Orth et al. 2003).

At first the dynamins were studied in the context of clathrin-coated pit (CCP) endocytosis at the plasma membrane where dynamin was seen to localise at the neck of clathrin-positive invaginations. The inhibition of dynamin induced a strong reduction of receptor-mediated endocytosis without affecting the number of CCPs at the cell surface (Damke et al. 1994). More recently it has been observed that dynamin also regulates caveolae-dependent endocytosis (Henley et al. 1998) while a role in certain types of fluid-phase endocytosis, although unlikely, is under debate. Whereas inhibition of dynamin is without effect on fluid-phase endocytosis in mammalian cells (Damke et al. 1994; Altschuler et al. 1998), in Drosophila expression of a dominant-negative (DN) mutant (shibire°; Chen et al. 1991) causes a small and inconsistent decrease (Guha et al. 2003). More recently other traffic steps have been identified that require the fissiogenic activity of dynamin. In respect of the retrograde transport of toxins in mammalian cells there is a role for dynamin in endosome-to-Golgi transport. At this stage the cells that overexpress DN mutants of dynamin do internalise ricin but the further transport of the toxin to the Golgi complex is inhibited, as also indicated by a reduction in the toxic effects of ricin under these conditions (Llorente et al. 1998). Similarly, dynamin is present on perinuclear endosomes that are positive for the cation-independent mannose-phosphate receptor (CI-MPR); again, the impairment of dynamin activity (by overexpression of the DN K44A mutant) inhibits recycling of the CI-MPR from endosomes to the TGN (Nicoziani et al. 2000).
Dynamin is also present at the Golgi complex where it partially co-localised with clathrin (Jones et al. 1998), TGN38 (Cao et al. 2000), Giantin and cortactin (Cao et al. 2005) and where it has a role in the formation of both clathrin-coated and uncoated secretory carriers (Jones et al. 1998; Cao et al. 2005; Cao et al. 2000). Here it appears to regulate the release of secretory vesicles that contain the polymeric-immunoglobulin A (pIgA) receptor apical marker (Jones et al. 1998) though there is some evidence in favour of the dynamin-independent transport of this entity (Altschuler et al. 1998). The discrepancies between these two lines of evidence have been addressed by the use of different assay systems so as to decide whether dynamin actually regulates the post-Golgi transport of pIgA or not. This is still an open question.

A role for dynamin has also been discerned in the formation of heparan sulphate proteoglycans (HSPG; Dong et al. 2000) and in hormone secretion in mouse pituitary corticotrope cells (Att20; Yang et al. 2001). Recent evidence indicates an in vivo role for dynamin in apical and basolateral post-Golgi transport. Here, overexpression of a GTPase-defective dynamin inhibits post-Golgi transport of the p75 neurotrophin receptor apical marker in MDCK cells (Kreitzer et al. 2000) and the VSVG basolateral marker in baby hamster kidney (BHK) cells (Cao et al. 2000; Cao et al. 2005). Of particular relevance is the recent observation that dynamin is recruited to the Golgi apparatus together with cortactin via Arf1 (Cao et al. 2005). Although its role in apical secretion is widely accepted there is conflicting evidence (Kasai et al. 1999) regarding the effects of inhibition of dynamin on basolateral protein transport. This will be discussed below.
In yeast, Vps1 (GenBank accession number: M33315) is the only dynamin-like protein (sharing 66% sequence identity) involved in vesicular transport and its role appears to be limited. Here it regulates a clathrin-dependent branch of the secretory pathway from the Golgi complex to an endosomal sorting compartment but does not affect the direct Golgi-to-plasma membrane pathway (Gurunathan et al. 2002; Harsay et al. 2002; Praefcke et al. 2004), or any other exocytic or endocytic trafficking steps (Luo et al. 2000). The protein(s) involved in the regulation of these last transport pathways are still unidentified.

It is clear that dynamin is a widespread fission-inducing protein that acts at several intracellular transport steps that have been precisely mapped (Fig. 1.9 B; McNiven et al. 2000; Praefcke et al. 2004). Nevertheless, if on the one hand it is clear that dynamin is involved in a wide range of fission processes, it is also true that several other dynamin-independent transport steps exist that still await characterisation.

1.2.2.2. BARS

The Brefeldin A-Ribosylated Substrate (BARS) is a 50-kDa protein that shares a high sequence identity with members of the C-terminal-binding proteins (CtBPs; 97% identity with CtBP1 and 72% with CtBP2; Spanò et al. 1999). These proteins were named after their ability to interact with the C-terminal region of the human adenovirus E1A proteins, and CtBP1 and CtBP2 are known to be co-repressors of gene transcription (Chinnadurai 2003). It is thought that they act by forming a bridge between DNA-binding proteins and histone deacetylases, which in turn favours histone condensation and inhibits transcription (Chinnadurai 2002). The
importance of these proteins derives from the observation that their interaction with the N-terminal portion of E1A reduces the oncogenic propensity of this viral protein (Boyd et al. 1993). CtBP1 co-repressor activity of these proteins is regulated by the binding of NAD⁺ and NADH which stabilises them in the dimeric form that is required for the co-repression activity (Fjeld et al. 2003; Balasubramanian et al. 2003). Moreover, the CtBPs are phosphoproteins and p21-activated kinase 1 (PAK1) is able to phosphorylate CtBP1 on Serine 158. This modification has a strong preference for NADH-bound CtBP with respect to the NAD⁺-bound form and it down-regulates the co-repressor activity of CtBP inducing the translocation of the protein from the nucleus to the cytoplasm (Barnes et al. 2003). Thus, it appears that the CtBP co-repressive activity is modulated both by changes in the supra-molecular organisation of the protein and by its translocation between different intracellular compartments.

Other factors can influence the intracellular localisation of CtBP. CtBP binding to neuronal nitric oxide synthase (nNOS) through its PDZ domain (Riefler et al. 2001) induces its translocation from the nucleus. Conversely, a ubiquitin-like modification of CtBP (the SUMOylation induced by the small ubiquitin-like modifier 1, SUMO1), favours nuclear retention (Lin et al. 2003). Interestingly, these two phenomena affect the same domain of CtBP, responsible for the binding to the PDZ domain of nNOS (Lin, Sun et al. 2003).

The third member of the CtBP family, CtBP3/BARS (i.e. BARS), was first described in 1994 in a search for mono-ADP ribosylated substrates of the fungal metabolite BFA (De Matteis et al. 1994). This derived from the observation that
BFA is a co-factor for the enzymatic activity of cholera toxin, a bacterial ADP-ribosyltransferase (Kahn et al. 1984; Tsai et al. 1987; Price et al. 1988). It was seen that BFA-induced ribosylation (where NAD is the ADP-ribose donor) is a key event for the BFA promotion of Golgi disassembly as NAD-depleted cytosol is unable to support the effects of BFA (Mironov et al. 1997).

Together with the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH), BARS is ribosylated upon treatment with BFA (De Matteis et al. 1994). Interestingly, depletion of both NAD and BARS from cytosol recovers the activity of BFA which indicates that the inactivation of BARS by BFA-induced ribosylation is indeed required for Golgi disassembly and Golgi-enzyme redistribution into the ER (Spanò et al. 1999). BARS induces fission of Golgi-derived tubules (Fig 1.10 C; Weigert et al. 1999) and live imaging of BFA-treated cells indicates that the Golgi enzymes redistribute into the ER via long membranous tubules (Sciaky et al. 1997). Since BARS is a fission-inducing protein, it is possible that the preservation of Golgi tubular structures from fission depends on the inhibition of this activity.

It is interesting to note that palmitoyl coenzyme A (pCoA) can replace the cytosol in the in vitro conditions for BARS-driven fission of membranes. pCoA also promotes the BARS-induced production of PA (a fission inducing lipid; section 1.2.1.1) from LPA (Fig 1.10 B; Weigert et al. 1999). It has thus been suggested that BARS induces fragmentation of Golgi membranes by exploiting this slow acyltransferase activity that destabilises membrane bilayers. BARS-induced fission is preceded by the appearance of “pearled” tubules in which highly
constricted pinch-points form at regular intervals (Fig 1.10 C). The diameter of
the tubules at these constrictions decreases from around 50 nm to 10 nm, giving a
curvature that is compatible with the hypothetical presence of type II conical
lipids (Shemesh et al. 2003) such as PA or DAG (that can derive from PA
metabolism). It is likely that the pearling on tubules represents "fission
intermediates", zones where BARS activity has prepared the membranes for
fission (Weigert et al. 1999). However, the LPAAT activity detected \textit{in vitro} is
quite low and its \textit{in vivo} relevance on Golgi membranes remains uncertain.

Although a role for BARS in the regulation of gene transcription has not yet been
investigated, the fact that it shares such a high identity with the other members of
the CtBP family (that are known to be transcriptional co-repressors) is clearly
intriguing. If indeed the members of the CtBP family are endowed with two
radically different functions, it would be interesting to understand the mechanisms
that regulate the switch between each of the activities. In this respect, the recently
reported crystal structure of BARS is relevant (Fig. 1.10 A; Nardini et al. 2003).
As with the other members of the CtBP family, BARS is able to bind NAD(H)
and this stabilises the protein as a dimer. Nevertheless, to exert its acyltransferase
activity BARS requires acyl-CoA and this binding competes with that of
NAD(H), indicating that the two molecules interact with the same binding site
(Nardini et al. 2003). Acyl-CoA binding favours an "open conformation" of the
monomeric form that facilitates its interaction with biological membranes. This
switch between the closed dimeric and the open monomeric forms of BARS might
represent the key to its regulation (and possibly of the other members of the CtBP
family).
Fig 1.10. BARS induces fission of Golgi membranes by acylating lysophosphatidic acid. A. Crystal structure of BARS depicting the Nucleotide-Binding Domain (NBD, with an NADH molecule in purple) and the Substrate Binding Domain (SBD). B. BARS exhibits an in vitro acyltransferase activity stimulating the formation of PA from LPA (from Weigert et al. 1999). C. BARS induces fission of isolated Golgi membranes (visualised by negative staining) when added together with cytosol or with pCoA that replaces the cytosolic component (from Weigert et al. 1999). Scale bars: left and middle panels 100 nm; right panel 40 nm.
1.3. Coat proteins and protein sorting (Fig. 1.11)

In most cases, protein sorting appears to be controlled by the assembly of coat protein complexes. These are also key factors for the budding of membranes which precedes the formation of carriers at the donor compartment (reviewed in Barlowe 2000; Bonifacino et al. 2003). Nevertheless, the formation of a coated bud is insufficient for the induction of membrane fission, a process in which lipids play a fundamental role (Burger 2000).

To date, four classes of coat complexes have been characterized: the clathrin, caveolin, COPI and COPII coats. The assembly of membrane coats is initiated by the recruitment and activation of small GTPases (such as the members of the ARF family of proteins and Sar1; reviewed in Bonifacino et al. 2003), the choice of which varies in relation to the type of coat that is formed. The small GTPase also remains as a part of the final assembled coat.

Clathrin coats which form at the plasma membrane were the first to be described at the EM level. These coats are involved in the formation of the carriers that mediate transport from the cell surface to endosomes and between the TGN and the endosomal/lysosomal compartment. They are formed by the polymerisation of multiple triangular-shaped units, known as triskelions, each of which is made up of three clathrin heavy chains and three clathrin light chains. Clathrin coats form on platforms composed of heterotetrameric adaptor protein complexes (AP1, AP2, AP3 and AP4; reviewed in Kirchhausen 1999; Bonifacino et al. 2003), and the process is typically initiated by the activation of small GTPases of the ARF family.
Caveolae are flask-shaped structures surrounded by the protein caveolin, a cholesterol-binding protein that is able to maintain a “caveolar” shape (Anderson 1993; Stahlhut et al. 2000). They are associated with dynamin-dependent endocytosis (Pelkmans et al. 2003).

The function of COPII appears to be restricted to the sorting and packaging of newly synthesized proteins en route to the Golgi complex (Aridor et al. 1998; Bannykh et al. 1998; Stephens et al. 2001). COPII coats are generated by the assembly of two heterodimers, the Sec23/24 and Sec13/31 complexes, and the small GTPase Sar1 (reviewed in Schekman et al. 1996). The process of assembly is regulated by the activation of the GTPase Sar1, which is in turn controlled by the guanine-nucleotide exchange factor Sec12, a transmembrane protein localized to the ER (Barlowe et al. 1993).

The role of COPI is under greater debate. COPI is a 600-kDa complex composed of seven subunits (α-, β-, β'-, γ-, δ-, ε-, ζ-COP) and is present in its preassembled form in the cytosol (Bonifacino et al. 2003). Its recruitment to membranes was suggested to be the key event for vesicle budding (Orci et al. 1993) and it is mediated by ARF (Serafini et al. 1991; Donaldson et al. 1992). Although its molecular composition and association with the membrane in vitro are well described, the precise role of the COPI coat complex in living cells remains to be fully elucidated. Initially, studies were performed in in vitro transport systems, which indicated a role for COPI in the regulation of anterograde transport from VTCs to the Golgi complex and through the Golgi stacks (Balch et al. 1984). This idea was generated by the observation that GTPγS, a non-hydrolysable analogue
of GTP that strongly inhibited transport, also causes an accumulation of COPI-coated vesicles (Melancon et al. 1987). On the other hand, the idea that COPI mediates the forward intra-Golgi traffic appears to be ruled out because cargo is not enriched in COPI-coated vesicles (Sonnichsen et al. 1996; Martinez-Menarguez et al. 1999). There is general agreement that COPI mediates retrograde transport from the Golgi complex to the ER (Bannykh et al. 1998; Klumperman 2000; Stephens et al. 2001). This conclusion is based on the ability of the coatomer to bind proteins that contain the retrieval motif KKXX (Cosson et al. 1994; Letourneur et al. 1994) and the KDEL receptor, an ER-Golgi recycling protein that is involved in the retrieval of ER proteins containing the KDEL signal sequence (Griffiths et al. 1994). Evidence also exists in favour of a requirement of COPI for anterograde traffic from the ER to the Golgi complex (Scales et al. 1997; Stephens et al. 2000). The importance of COPI in forward traffic to the Golgi complex is probably in the concentration of the anterograde cargo occurring at VTCs by exclusion from COPI-coated buds (Martinez-Menarguez et al. 1999). COPI may also be a negative regulator of tubule formation in the Golgi complex, based on the observations that several experimental conditions that inhibit COPI binding to the membranes, including treatment with BFA, induce the tubulation of the Golgi complex (Klausner et al. 1992; Sciaky et al. 1997).
Clathrin-coat and classical AP2 adaptors

Cop I coat

Cop II coat

Alternative Clathrin Adaptors

Adaptors
AP2
GGA
Hrs

Clathrin
β-propeller domains

COPI
F-subcomplex
COPI
B-subcomplex

Cargo
Lipids with -ve headgroups

Fig 1.11. Coat proteins. Structural and functional homology between COPI, COPII and clathrin coats (from McMahon et al. 2004).
1.4. ARF

Originally identified for its ability to enhance the ADP-ribosylation of the stimulatory G protein of cholera toxin (Kahn et al. 1984; Tsai et al. 1987; Price et al. 1988), ARF is a small GTPase that belongs to the Ras superfamily. Six ARFs have been identified in mammalian cells and three ARFs are expressed in the yeast *Saccharomyces cerevisiae* (reviewed in Roth 1999). The six mammalian ARFs can be subdivided into three classes based on their sequence identity. The class-I ARFs (ARFs 1-3) are the best characterised and appear to be functionally redundant. These proteins regulate not only COPI assembly, but also clathrin-AP1 assembly on the TGN (Stamnes et al. 1993) and clathrin-AP3 assembly on endosomes (Ooi et al. 1998). The class-II ARFs (ARF4 and ARF5) have not been well investigated. Arf6 is the only identified member of the class-III ARFs and is located at the plasma membrane and on endosomes where it controls several types of endocytosis. Yeast ARF1 and ARF2 are required for the 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. Similar to other small GTPases in the cell, the regulatory activity of ARF 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 it mediates the assembly of the coats on membranes. In its GDP-bound state ARF is inactive, present in the cytosol and the coats are 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 et al. 2000 and Jackson et al. 2000).
exchange factor for ARF to be identified was a Golgi-membrane-associated protein that is the BFA-sensitive factor for membrane recruitment of ARF and COPI (Donaldson et al. 1992; Helms et al. 1992). Since then, several proteins with ARF-GEF activity have been identified (reviewed in Jackson et al. 2000), some of which are inhibited by BFA (Peyroche et al. 1996; Morinaga et al. 1997). Others are BFA resistant (Chardin et al. 1996; Meacci et al. 1997). The binding of ARF to membranes which occurs through an N-terminal myristoyl group that interdigitates into the bilayer is 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 a model to be proposed for the mechanism of ARF activation. The conformational change that is responsible for ARF activation appears to be a consequence of the 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, indicating how ARF couples the GDP–GTP conformational switch to membrane binding (Goldberg 1998).
CHAPTER 2

Materials and Methods

2.1. Cell culture

2.1.1. Materials

Normal rat kidney (NRK) and African-green-monkey Cos7 cells were purchased from American Tissue Type Collection (ATTC, USA). Canine-Kidney MDCK cells were kindly provided by Dr. J. Morrow (Yale University, CT, USA), baby hamster kidney (BHK) cells were kindly provided by Dr. J. Donaldson (National Institutes of Health, MA, USA), human fibroblasts (HF cells) were kindly provided by the Istituto Dermatologico dell’Immacolata (IDI, Italy). Dulbecco's modified Eagle's medium (DMEM), foetal calf serum (FCS), penicillin, streptomycin, trypsin-EDTA, and L-glutamine were all from GIBCO (NY, USA). All these reagents were 10x stock solutions. All the plastic materials were from Corning (NY, USA). Filters (0.45 and 0.20 μm) were from Amicon (MA, USA).

2.1.2. Growth conditions

Cos7 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. 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 5% FCS. BHK and MDCK cells were grown in DMEM supplemented with non-essential amino acids, 4.5 g/L glucose, 2 mM L-glutamine, 1 U/ml penicillin and streptomycin, 1 mM sodium pyruvate, and 10% FCS. Complete media were prepared by diluting
concentrated stock solutions with sterile water (DIACO, Italy) and filtering the mixture through 0.2 μm filters.

All of the cell lines were grown under a controlled atmosphere in the presence of 5% CO₂ at 37 °C. The cells were grown in flasks until they reached 90% confluence. For propagation, the medium was removed, the cells were washed in sterile PBS, and 0.25% trypsin solution was added for 2-5 minutes. The medium was then added back to block the protease action and the cells were collected in a plastic tube. After centrifugation for 5 min at 300 xg, the pellet was resuspended in fresh medium.

2.2. cDNA subcloning and amplification

2.2.1. Materials

Restriction enzymes were from Amersham Pharmacia Biotech (NJ, USA). T4 DNA ligase, DNA molecular size standards and the pcDNA3 vector were from Invitrogen (CA, USA). The pEYFP vector was from Clontech (CA, USA). Oligonucleotides were purchased from Gibco-Life technologies (USA) or from Sigma-Genosys (WI, USA). Turbo DNA Polymerase was from Stratagene (CA, USA). The “Wizard Plus Miniprep Kit” was from Promega (WI, USA), and the “QIAGEN Plasmid Maxi Kit” and “QIAEX II extraction kit” were from Qiagen (CA, USA). Tryptone peptone, yeast extract and agar were from Difco, Becton Dickinson (MD, USA). Agarose was from Bio-Rad Laboratories (UK). 3-Morpholino-propane-sulfonic acid (MOPS), RbCl and MnCl₂ were from Sigma Aldrich (WI, USA).
2.2.2. Solutions and media

Luria-Bertani (LB) broth: 1.0% tryptone peptone, 0.5% yeast extract, 1.0% NaCl; autoclaved 15 min at 121 °C. LB-agar: LB plus 1.5% agar; autoclaved 15 min at 121 °C. Tfbl: 30 mM potassium acetate, 100 mM RbCl, 10 mM CaCl₂, 50 mM MnCl₂, 15% glycerol, pH 5.8. Tfb2: 10 mM MOPS, 75 mM CaCl₂, 10 mM RbCl, 15% glycerol, pH 7.0. TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. 50x TAE buffer (1 L): 242 g TRIZMA base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA.

2.2.3. DNA agarose gels

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

2.2.4. PCR amplification of DNA inserts

To amplify specific regions of DNA inserts, PCR was performed by incubating 10 ng DNA plasmid as a template in 50 μl of 20 mM Tris-HCl, pH 8.8, 10 mM KCl, 2 mM MgSO₄, 10 mM (NH₄)SO₄, 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. 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. MA, USA). Melting-, annealing-, and elongation-temperatures were adjusted according to the features of the template.
and the primers. To facilitate the subsequent subcloning of PCR products, the forward and reverse primers were provided with restriction sites at their 5’ ends.

2.2.5. Restriction and ligation

The DNA (vectors and inserts) was cut with 5 U/µg of the appropriate restriction enzymes in the buffer supplied with each enzyme by Amersham Pharmacia Biotech (NJ, USA). After restriction, the enzymes were usually inactivated by incubating at 65 °C to 75 °C for 10-20 min, according to the manufacturer instructions, and loaded onto 1.0% to 1.4% agarose gels. The bands of interest were cut from the gels with sterile scalpels, and the DNA was extracted from the gels with the Qiaex II extraction kit, according to the manufacturer instructions. The DNA was eluted in 10 mM Tris-HCL, pH 8.0. To ligate the vector and to insert, ~100 ng of vector and ~3-fold molar amounts of insert were incubated with 1U T4 DNA ligase in T4-DNA-ligase buffer, for 10 min at room temperature.

2.2.6. BARS YFP construct preparation

The coding sequence of BARS was amplified by PCR with the following primers:
5’-GTGCTCGAGATGTCAGGCGTCCGAC-3’ (forward primer) and 5’-GCGGAATTCGCAACTGGTCAGTCGTATG-3’ (reverse primer). The PCR product was then digested with XhoI and EcoRI and subcloned in XhoI/EcoRI-digested pEYFP vector (Invitrogen, CA, USA), generating the YFP-BARS plasmid.
2.2.7. BARS<sup>D35A</sup> construct preparation

The coding sequence of the BARS<sup>D35A</sup> mutant was amplified by PCR with the following primers: 5'-GCGGAATTCATGTCAGGCCTGACCTC-3' (forward primer) and 5'-GATGCGGCCCTACAACTGGTCAGTCGTATG-3' (reverse primer). The PCR product was then digested with EcoRI and NotI and subcloned in EcoRI/NotI-digested pcDNA3 vector (Invitrogen, CA, USA), generating the pcDNA3-BARS<sup>D35A</sup> plasmid.

2.2.8. Preparation of bacteria competent for transformation

A single colony of XL-1Blue <i>E. Coli</i> bacteria was picked from an LB-agar plate and used to inoculate 10 ml of LB-broth. Bacteria were grown overnight; the culture was diluted in 190 ml of fresh LB-broth and incubated at 37 °C until the optical density reached 0.5 (at 600 nm). The bacteria were harvested by centrifugation at 6,000 rpm in a JA10 rotor for 10 min at 4 °C. The bacterial pellet was resuspended in 40 ml of Tfb1 and left on ice for 2 h. After centrifugation and resuspension of the pellet in 4 ml of Tfb2, the cells were stored at −80 °C in 400 μl aliquots.

2.2.9. Transformation of bacteria

The DNA plasmid of interest (10 ng) was added to 200 μl of competent bacteria that had been previously thawed on ice. After gentle mixing, the cells were left on ice for 30 min, heat shocked for 90 sec at 42 °C, and after the addition of 800 μl LB-broth, 10 g/l NaCl, 5 g/l yeast extract, 10 g/l tryptone peptone, plus 100 μg/ml
ampicillin, the cells were incubated with shaking at 37 °C for 45 min. The bacteria were plated on LB agar containing the appropriate selective antibiotic and incubated overnight at 37 °C. The next day, an isolated bacterial colony was picked and used to inoculate 2 ml of LB-broth containing the appropriate antibiotic. The culture was incubated at 37 °C overnight. Sterile glycerol (200 µl) was added to 500 µl of the bacterial culture and this was then stored at −80 °C.

2.2.10. Small-scale preparations of plasmid DNA (miniprep)

The clones obtained after the 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 overnight growth at 37°C under continuous shaking (200 rpm), 700 µl of the culture was mixed with 300 µl 50% sterile glycerol and stored at −80°C, with the rest of the culture being chilled on ice and centrifuged for 10 min at 4,000 xg. The DNA was extracted using the “Wizard Plus miniprep kit”, according to the manufacturer instructions, and analysed by restriction analysis and separation on agarose gels.

2.2.11. Large-scale preparation of plasmid DNA (maxiprep)

For large-scale DNA preparation, a Maxi Prep Kit from Qiagen (USA) was used. A single colony of XL-1Blue E. Coli bacteria transformed with the plasmid of interest was used to inoculate 500 ml of LB-broth plus selective antibiotics. After 5-20 h of incubation, the bacteria were collected by centrifugation at 6,000 rpm in a JA10 rotor for 10 min at 4 °C and processed according to the maxi-prep purification protocol of the “Qiagen-plasmid-kit”. The DNA obtained was
resuspended in TE buffer to a final concentration of 1 mg/ml and stored at 4 °C, or for long-term at –20 °C.

2.3 Cell transfection for protein overexpression

2.3.1. Materials

Lipofectamine 2000, Lipofectamine-Plus, OptiMEM serum-free reduced culture medium, were from Invitrogen/GIBCO (CA, USA). Lipofectamine 2000 was used for single cDNA transfection because of its higher efficiency of transfection. Lipofectamine-Plus was used when double transfection was required as it gave the best results in terms of co-expression of two cDNAs.

Table 2.1 DNA constructs used in this study

<table>
<thead>
<tr>
<th>Construct Name</th>
<th>Protein</th>
<th>Vector</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>BARS</td>
<td>CtBP3/BARS</td>
<td>pCDA3 (Invitrogen, USA)</td>
<td>Stefania Spanò</td>
</tr>
<tr>
<td>VSVG-GFP</td>
<td>tsO45 vesicular stomatitis virus G-protein</td>
<td>pEGFP (Clontech, USA)</td>
<td>J. Lippincott-Schwartz (NIH, MA, USA)</td>
</tr>
<tr>
<td>BARS-YFP</td>
<td>CtBP3/BARS</td>
<td>pEYFP (Clontech, USA)</td>
<td>Our laboratory</td>
</tr>
<tr>
<td>VSVG-CFP</td>
<td>tsO45 vesicular stomatitis virus G-protein</td>
<td>pECFP (Clontech, USA)</td>
<td>J. Lippincott-Schwartz (NIH, MA, USA)</td>
</tr>
<tr>
<td>Dyn2K44A</td>
<td>A dominant-negative dynamin-2</td>
<td>pCR3.1 (Invitrogen, USA)</td>
<td>M.A. McNiven (Mayo Clinic and Foundation, MN, USA)</td>
</tr>
<tr>
<td>BARSP355A</td>
<td>A dominant-negative CtBP3/BARS</td>
<td>pCDNA3 (Invitrogen, USA)</td>
<td>Our laboratory</td>
</tr>
<tr>
<td>P75-GFP</td>
<td>P75 neurotrophin receptor</td>
<td>pEGFP (Clontech, USA)</td>
<td>E. Rodriguez-Boulan (Cornell University, NY, USA)</td>
</tr>
</tbody>
</table>
2.3.2. Lipofectamine reagent-based cell transfection

*Lipofectamine-Plus*: the cells were plated on glass coverslips or glass-bottomed petri dishes in normal culture medium at a concentration suitable to have 50-70% confluence for transfection. The day after, a transfection mixture was prepared by diluting the DNA in OptiMEM culture medium and adding Plus Reagent in a test tube, according to the manufacturer instructions. The mixture was then shaken and incubated at room temperature for 15 min. In a separate test tube, Lipofectamine Reagent was diluted in the same medium, according to manufacturer instructions, added to the transfection mixture, shaken and kept at room temperature for another 15 min, to allow the DNA-Lipofectamine complex to form. In the meantime, the cells were washed twice with OptiMEM medium. The cells were then incubated with the transfection mixture for 3 h at 37 °C in the presence of 5% CO₂. At the end of the incubation, the transfection mixture was replaced by complete culture medium and the cells incubated for an additional 12-20 h prior to assay. Transfection efficiencies were ranging between 30-40% depending on the constructs used.

*Lipofectamine 2000*: the cells were plated on glass coverslips or glass-bottomed petri dishes in normal culture medium at a concentration suitable to have 50-70% confluence for transfection. The following day, a transfection mixture was prepared by diluting the DNA in OptiMEM medium in a test tube, and Lipofectamine 2000 with the same medium in a second test tube, according to manufacturer instructions. The tubes were gently shaken and incubated for 5 min at room temperature; after this, the diluted DNA was mixed with diluted Lipofectamine 2000 and further incubated for 20 min at room temperature, to
allow the DNA-Lipofectamine complex to form. The transfection mixture was then added to the cells and incubated for 12-24 h at 37 °C in a CO₂ incubator prior to assay. Transfection efficiency was approximately 70% depending on the constructs used.

2.3.3. Electroporation

For electroporation, confluent HF cells were trypsinised by the addition of 0.25% trypsin solution for 2-5 minutes, and then resuspended in 400 µl PBS at 6 x 10⁶ cells/ml. The cells were then transferred to a sterile electroporation cuvette (0.4 cm) (Biorad, UK) and 20 µg of plasmid DNA was added. After 10 min of incubation on ice, the cells were electroporated using the following parameters: voltage: 380 V, capacitance: 850 mF. After the pulse, 300,000 cells were plated on each coverslip. Transfection was performed in a Gene-Pulser Electroporator (Biorad, UK). Transfection efficiency was approximately 60% depending on the constructs used.

2.3.4. cDNA nuclear injection-based transfection

See section 2.6.2. for details.

2.4. Cell infection with Vesicular Stomatitis Virus (VSV)

2.4.1. Materials

The infectious stocks of the temperature-sensitive mutant of VSV were prepared by Alessio Di Pentima (Department of Cell Biology and Oncology, Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy) according to Bergmann et al. (1989).
For each infectious stock, the optimal working concentration was experimentally defined as the lowest causing 100% infection of Cos7 cells, as judged by staining for the viral membrane glycoprotein. Cycloheximide (Sigma Chemicals, WI, USA) was diluted in PBS (pH 7.4) at a concentration of 10 mg/ml, and aliquots were stored at –20 °C.

2.4.2. Procedures

Cells were washed twice in serum-free culture medium and incubated with the diluted VSV infectious stock for 45 min at 32 °C. The virus was removed by replacing infection medium with normal complete growth medium and the cells were kept at 40 °C in a CO₂ incubator for 2 h to allow the VSVG to accumulate in the ER. The temperature was then shifted to 32 °C to allow protein transport out of the ER, in the presence of 100 µg/ml cycloheximide to stop protein synthesis. Alternatively, after the incubation at 40 °C, the cells were kept at 20 °C for 2 h with 100 µg/ml cycloheximide, to accumulate the VSVG protein in the Golgi complex, before shifting the temperature to 32 °C to follow Golgi-to-PM transport of the viral protein.

2.5. Glutathione-S-transferase (GST)-fusion protein purification

2.5.1. Materials

Glutathione, lysozyme, IPTG, sodium azide, Triton X-100, potassium acetate, RbCl, CaCl₂ and MnCl₂ were from Sigma Chemicals (MO, USA). The glutathione sepharose resin and the pGEX 4T vectors were from Pharmacia Bio-Tec, UK. Leupeptine, benzamidine, and PMSF were from Sigma Chemicals (MO, USA).
2.5.2. Expression and purification of GST fusion proteins

A single colony of *E. Coli* transformed by a pGEX vector containing the GST fusion protein of interest was used to inoculate 100 ml of LB-broth. The bacterial culture was grown to an optical density of 0.6 at 600 nm. Expression was induced with 1 mM IPTG for 3 h at 37 °C. The bacteria were harvested by centrifugation (6,000 rpm, JA10 rotor, Beckman, CA, USA) for 10 min at 4 °C. The bacterial pellet was resuspended in 25 ml of 20 mM TRIS-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mg/ml lysozyme in the presence of a cocktail of protease inhibitors (leupeptine, benzamidine and PMSF). TritonX-100 (1%) was added and the suspension agitated continuously for 30 min, and then sonicated for 2 min on ice.

The lysed bacteria were centrifuged for 20 min (1,800 rpm, JA20) and the pellet was discarded. Meanwhile, 2.5 ml of a glutathione-sepharose resin were diluted to 40 ml and centrifuged (2,000x g) for 5 min to remove the preservative. The supernatant was discarded and the resin was resuspended in PBS. This procedure was repeated twice. The cleared bacterial lysate (see above) was then added to the glutathione-sepharose resin and incubated for 30 min at 4 °C with occasional agitation. The beads were washed 3 times with 50 ml PBS and percolated on a column. The resin was allowed to pack for 10 min, and the protein was eluted with 1 ml elution buffer (100 mM Tris-HCl, 20 mM glutathione, 5 mM DTT). The eluate was collected and this operation was repeated an additional five times.

The protein content of each of the six fractions collected was analysed by SDS-PAGE and the fractions containing the higher protein concentrations were pooled, dialysed against microinjection buffer (70 mM KCl, 10 mM NaH₂PO₄, pH 7.2) and stored in aliquots at −80 °C.
2.6. Cell microinjection

The day before injection, the cells were plated on glass coverslips or on glass-bottomed petri dishes, in normal culture medium. For injection of cells grown on coverslips, the coverslips were put into small petri dishes (3-cm diameter) containing normal culture medium buffered with 20 mM HEPES, pH 7.4. The petri dishes were then transferred to the thermo-regulated stage of an inverted Zeiss IM 35 microscope, kept at the temperature required for experimental protocols. Injections were performed using a transjector 5246 controlled by a 5171 micromanipulator (Eppendorf, Germany), in the manual mode. The injection pressure was variable, depending on the conditions of the injection needle or the viscosity of the solution to be injected, ranging from 50 to 500 hPa. The injection time was manually defined by the operator as the shortest necessary to observe the propagation through the cytoplasm or the nucleus of a circular wave originating from the tip of the needle.

2.6.1. Polypeptide microinjection

The injections were made in the perinuclear area where the thickness of the cell is greater than that in the periphery. The needles for injection were prepared from 1.0-mm-internal-diameter glass capillaries (World Precision Instruments, USA), using an automatic capillary puller (Inject+Matic,CH) with the heat controller set to 3. Glass needles were then loaded with 3 μl of injection solution containing the polypeptide to be injected in microinjection buffer (70 mM KCl, 10 mM NaH₂PO₄, pH 7.2). The solution diffused through capillarity action within a few seconds. Where needed, lysine-fixable fluorescent dextran (Molecular Probes, The
Netherlands) was added to the injection mixture, to trace efficient injections. Injected cells were also relocated by means of a grid present on the coverslip (see section 2.12.3 and 2.13.4)

2.6.2. DNA injection

For nuclear DNA injection, the use of commercial needles (Sterile Femtotips, Eppendorf, Germany) was more suitable. Needles were loaded with 3 μl of DNA diluted in microinjection buffer (70 mM KCl, 10 mM NaH₂PO₄, pH 7.2). Depending on the efficiency of expression, the DNA concentrations ranging from 5 to 40 ng/μl were used. After injection, the cells were incubated for 1 h at 37 °C in a CO₂ incubator for efficient protein synthesis prior to the assays.

2.7. BARS depletion by antisense oligonucleotides

2.7.1. Conventional antisense oligonucleotides

The oligonucleotide sequences were from MWG-Biotech (Italy). A 20 bp sequence (5’ -tga TGC CCA AGG TCT CTC cac- 3’) was designed based on the amino-acidic sequence of CtBP3/BARS, in order to inhibit the synthesis of CtBP1 & 3. A second sequence (5’ -gag CCT TTC TCA GCC ACC tag- 3’) that was obtained by randomly scrambling the nucleotides in the former sequence was used as the control oligonucleotide. After designing the sequences, FASTA searches were performed to verify the specificity of the anti-CtBP1/3 sequence designed and the mismatches of the scrambled sequence. For experiments, cells were plated on glass coverslips in normal growth medium. Oligonucleotide sequences were added directly to culture medium at 8 μM for five days prior to assay. To test the
efficiency of the oligonucleotide treatment, after 5 days of treatment the sample buffer (Laemmli 1x) was substituted for the culture medium and the cells were scraped and collected in 1.5 ml Eppendorf tubes. The samples were loaded directly onto polyacrylamide gels. Bands were revealed with the p50-2 anti-BARS antibody, followed by treatment with an ECL kit (Amersham Biosciences, UK) according to the manufacturer instructions. After digital scanning, the intensity of each band was measured with Image J.

2.7.2. RNA interference (RNAi)

2.7.2.1. Materials

Small interfering RNAs (siRNAs; stock 50 μM) were from a smart pool:
duplex1: CCGUCAAGCAGAUGAGACAUU;
duplex2: GGAUAGAGACCACGCCAGUU;
duplex3: GCUCGCACUUGCUCAACAAU;
duplex4: GAGCAGGCAUCCAUCGAGAU
from Dharmacon (CO, USA). OptiMEM culture medium and Oligofectamine were from Invitrogen/GIBCO (USA).

2.7.2.2. Procedures

The RNA smart pool was diluted in OptiMEM according to the manufacturer instructions, mixed gently and incubated for 5 min at room temperature. In a separate vial, Oligofectamine was diluted in OptiMEM, mixed gently, and incubated at room temperature for 5 min. The solutions were then mixed together...
and incubated at room temperature for another 20 min. The cells were washed in serum-free culture medium and then incubated with the transfection mixture for 4 h at 37 °C, in the presence of 5% CO₂. A suitable volume of culture medium containing three times the normal concentration of serum was added to cell cultures to re-establish normal growing conditions. The cells were then incubated at 37 °C in the presence of 5% CO₂ for 48 h, prior to further experimental procedures.

2.8. Transport assays

2.8.1. Mini wave

Cells expressing VSVG (following infection) or VSVG-GFP (following transfection) were incubated at 40 °C in a CO₂ incubator for 2 h or overnight, respectively. The cells were then shifted to 15 °C for 15 min in the presence of 100 μg/ml cycloheximide to accumulate VSVG in the ERGIC. After that, the cells were incubated again at 40 °C (always in the presence of cycloheximide) to chase the VSVG that had accumulated within the ERGIC, while preserving the rest of the glycoprotein synthesised within the ER. The samples were fixed in 4% paraformaldehyde at the indicated times and processed for immunofluorescence as described in section 2.11.

2.8.2. ER-to-PM transport assay

Infected or transfected cells expressing VSVG were incubated at 40 °C in a CO₂ incubator for 2 h or overnight, respectively, and then shifted to 32 °C in the presence of 100 μg/ml cycloheximide. The cells were fixed in paraformaldehyde
after 0 (40 °C), 45 and 60 min of chase and blocked in the Blocking Solution without permeabilisation. To detect the amount of viral glycoprotein on the cell surface, the cells were incubated with an antibody against the luminal domain of the protein and then revealed with a Cy5-conjugated secondary antibody. After this first round of immuno-fluorescence staining, the cells were permeabilised in Blocking Solution with saponin, and the intracellular VSVG was stained with the Cy3-conjugated P3D4 antibody as described in section 2.11. ER-to-Golgi transport was measured both by measuring the VSVG-associated cell surface fluorescence and by counting the number of VSVG-positive intermediates formed during the chase using an LSM510 Zeiss confocal microscope as described in section 2.11.3.

2.8.3. TGN-exit assay in non-polarized cells

For the TGN-exit assay of VSVG, cells were transfected with VSVG-GFP cDNA or infected with VSV, as described in section 2.3 and 2.4 respectively. The cells were then incubated for 2 h at 40 °C, followed by 2 h at 20 °C (with 100 µg/ml cycloheximide) to accumulate VSVG in the Golgi complex. For the TGN-exit assay of p75, the cells were transfected with p75-GFP cDNA, incubated overnight in complete medium, and for 3 h at 20 °C with 100 µg/ml cycloheximide. To monitor VSVG and p75 exit from the TGN, the temperature was then shifted to 37 °C and the samples were fixed with 4% paraformaldehyde at the indicated times. For tannic acid treatment, 0.5% tannic acid was added to the cells just before the release of the 20 °C temperature block (Newman, Tian et al. 1996; Polishchuk, Di Pentima et al. 2004).
2.8.4. TGN-exit assay in polarized MDCK cells

MDCK cells were grown on glass coverslips until they were over-confluent; the degree of polarisation was tested in a parallel sample with an anti-occludin antibody for the visualisation of tight junctions. To test apical protein transport, p75-GFP cDNA was microinjected into the nucleus, either alone or in combination with the cDNAs of the other proteins to be tested. After injection, the cells were incubated for 1 h at 37 °C to allow the expression of the protein, and then for 2 h at 20 °C in the presence of 100 μg/ml cycloheximide to accumulate p75-GFP in the Golgi complex. The temperature was then shifted to 37 °C, and the samples were fixed with 4% paraformaldehyde after 0, 30 or 60 min. Finally, they were labelled without permeabilisation with the lumenal domain anti-p75 antibody. To test basolateral protein transport, over-confluent MDCK cells were infected with VSV, incubated at 40 °C for 1 h, microinjected into the nucleus with the cDNA of the perturbing protein, and incubated at 40 °C for a further 1 h. The cells were then incubated for 2 h at 20 °C in the presence of 100 μg/ml cycloheximide and shifted to 32 °C in the presence of 10 mM EGTA. The samples were fixed after 0, 30 or 60 min, and first labelled without permeabilisation with the lumenal domain anti-VSVG antibody, and then permeabilised and incubated with the P₁D₄ anti-VSVG antibody. For both p75-GFP and VSVG, confocal z-stacks were captured for at least 15 cells for each condition, and the plasma membrane arrival was quantified as the ratio of surface versus total fluorescence in reconstructed three-dimensional images (see section 2.11).
2.8.5. Receptor-mediated endocytosis

Cells grown on glass coverslips were washed in ice-cold serum-free growth medium and incubated on a 50 µl drop of serum-free medium containing 50 µg/ml Cy3-conjugated transferrin (Tf; Sigma WI, USA), 0.1% fatty-acid-free BSA, on ice for one hour. After that cells were extensively washed in ice-cold complete growth medium to remove unbound Tf and the temperature was released to 37 °C to chase the Tf inside the cells. The cells were then fixed in 4% paraformaldehyde after 0, 10 or 20 min of chase, and processed for immuno-fluorescence. Where needed, before fixation cells were subjected to an acid wash (500 mM NaCl, 0.5% acetic acid) for 1 min on ice, to remove PM-associated Tf.

2.8.6. Fluid-phase endocytosis

Cells were grown on glass coverslips in normal growth medium. Before the experiments, the growth medium was replaced with serum-free growth medium and lysine fixable fluorescent dextran (FITC or TRITC, Molecular Probes, The Netherlands) was diluted to 2 mg/ml in a vial with serum-free growth medium. The coverslips were then incubated on a 50 µl drop of dextran-containing medium at 37 °C for the indicated times. Alternatively, the cells were incubated at 19 °C for 1 h to internalise the probe into the early endosomal compartment; the cells were then washed to eliminate non-internalised dextran and the temperature was released to 37 °C, as above. The cells were then washed three times in PBS, fixed in paraformaldehyde, and processed for immunofluorescence where needed.
2.9. Endo-H resistance test

This assay was performed in collaboration with Gabriele Turacchio (Department of Cell Biology and Oncology, Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy). Cells grown in small petri dishes (35-mm diameter) were co-transfected with VSVG and BARS (see section 2.3) and incubated at 37 °C for 48 h to maximize transfection efficiency. After this, the cells were incubated overnight at 40 °C to accumulate VSVG in the ER. The cells were then washed twice in growth medium without methionine and cysteine (always at 40 °C) and then starved in the same medium for 10 min at 40 °C. The cells were then loaded with [35S]-methionine (80-100 μCi/ml in growth medium without methionine and cysteine) for 10 min at 40 °C. The growth medium was then changed for cold methionine-containing medium plus 100 μg/ml cycloheximide, and the cells were incubated for a further 5 min at 40 °C. The temperature was then released to 32 °C, to chase the VSVG to the plasma membrane, for 0, 40 or 60 minutes. At the end of each time step, the cells were blocked with 5 min on ice and washed three times in cold PBS. The PBS was then removed and the cells lysed in 500 μl of RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton, 1mM EDTA) for 15 min on ice under continuous shaking. The cells were then scraped and repeatedly passed through a 0.45 mm needle. Lysates were centrifuged for 10 min at 4 °C at 21000 x g, and the supernatants collected and incubated overnight at 4 °C with the P3D4 anti-VSVG antibody on a spinning wheel. The samples were then subjected to immunoprecipitation of VSVG. After immunoprecipitation, the samples were collected and 20 μl of 2.5 M Na citrate, pH 5.5, was added to each sample. Each sample was divided into two vials, and 400 U/ml of Endo H were added to one
vial, keeping the second vial as a control. The samples were then vortexed and incubated overnight at 37 °C. The Endo H reaction was stopped with 24 µl of 5x Sample Buffer, and the samples were boiled for 5 min and loaded onto a 10% polyacrilamide gel.

2.10. Cell treatments

2.10.1. BFA treatment

BARS<sup>DSSA</sup>-transfected cells were incubated overnight at 37 °C in a CO<sub>2</sub> incubator and then treated with 5 µg/ml BFA (Sigma Chemical, MO, USA) for 15/30 min at 37 °C in normal culture medium buffered by 20 mM HEPES, pH 7.4.

2.10.2. Leptomycin B (LMB) treatment

LMB was from Minoru Yoshida (Department of Biotechnology, Graduate School of Agriculture and Life Sciences, University of Tokyo, Tokyo, Japan). The LMB stock solution was 1 mg/ml in ethanol. Cos7 cells were incubated with 10 ng/ml LMB in normal culture medium buffered by 20 mM HEPES, pH 7.4, for 3 or 6 h at 37 °C. The cells were then fixed and processed for immunofluorescence. Control cells were incubated with complete medium containing 0.1% ethanol.
2.10.3. Lipid modifying treatments

2.10.3.1. Materials
The 1-butanol was from Carlo Erba (Italy). Propranolol was from Calbiochem (Germany), and a stock solution in methanol (100 mM) was aliquoted and stored at -20 °C. DGK inhibitors I and II were from Sigma Chemical (MO, USA), with stock solutions in DMSO at 500 mM; this was aliquoted and stored at -20 °C.

2.10.3.2. Procedures
Cos7 cells were transfected with VSVG-GFP together with either an empty vector or with BARS, as indicated in section 2.3, and incubated overnight at 40 °C in a CO₂ incubator. The following day, the cells were incubated at 20 °C for 2 h in the presence of 100 μg/ml cycloheximide. At the release of the temperature block, the cells were washed and new complete medium was added at 32 °C, supplemented with one of the following:

1.5% 1-butanol
500 μM Propranolol
50 μM DGK inhibitor I (R59022)
50 μM DGK inhibitor II (R59949)

The cells were fixed after 0, 20 or 40 min of chase and assayed for VSVG transport as described in the post-Golgi transport assay (see section 2.8.3).
2.10.4. Cell cycle synchronisation

2.10.4.1 Materials

Aphidicolin and Hoechst 33258 were from Sigma Chemical (MO, USA)

2.10.4.2 Procedures

NRK cells were incubated in complete culture medium containing 2.5 mg/ml aphidicolin O/N at 37 °C. After that cells were washed twice in PBS to remove aphidicolin-containing medium and further incubated in complete medium containing 0.1 mg/ml Hoechst for 18h. Cells were then fixed in 4% paraformaldehyde in PBS and processed for immunofluorescence with anti-H1 and anti-H3 antibodies as described in section 2.11 to quantify the number of cells synchronised in the G2 phase of the cell cycle. Alternatively cells were treated with aphidicolin as above and transfected with VSVG-GFP (see section 2.3) in Hoechst-containing medium. After 14 h cells were subjected to the post-Golgi transport assay (see section 2.8.3) in the presence of 0.1 mg/ml Hoechst in the culture medium.

2.11. Immunofluorescence

2.11.1. Materials

Paraformaldehyde was from Electron Microscopy Science (EMS; PA, USA); this was at 16% in H2O, and was diluted in 0.2 M HEPES (pH 7.4) to the appropriate concentration and stored at 4 °C. The Blocking Solution was prepared as follows: 0.5% BSA, 50 mM NH4Cl in PBS, pH 7.4. Where needed, 0.05% saponin (Serva,
IT) was added to the preparation. Aliquots were stored at -20 °C. Samples on glass coverslips were mounted on glass microscope slides (Carlo Erba, IT) with MOWIOL.

2.11.2. Sample preparation
Cells were fixed in 4% paraformaldehyde for 15-20 min at room temperature, washed three times in PBS, and incubated in Blocking Solution with or without saponin as necessary, for 20 min at room temperature. The cells were subsequently incubated with the specified antibodies diluted in Blocking Solution for 1 h at room temperature, or overnight at 4 °C. After incubation with the primary antibody, the cells were washed six times in PBS and incubated with a fluorescent-probe-conjugated secondary antibody, directed against the constant region of the primary IgG molecule, for 45 min at room temperature. Commonly, Cy3-, Cy5- (Sigma Chemical Co. MO, USA) or Alexa-488 (Molecular probes, OR, USA)-conjugated anti-rabbit or anti-mouse goat antibodies were used at a dilution of 1/500 in Blocking Solution. After immuno-staining, the cells were washed six times in PBS and twice in sterile water, to remove salts. The coverslips were then mounted on glass microscope slides with MOWIOL.
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2.11.3. Light and immuno-fluorescence analyses

Confocal images were acquired using a Zeiss LSM510 inverted confocal microscope system (Carl Zeiss, Gottingen, Germany). Fixed cells were analysed using a 60x oil-immersion objective, maintaining the pinhole of the objective at 1 airy unit. Images were scanned using an Argon 488 laser, a HeNe 543 laser and a HeNe 633 laser accordingly, under non-saturating conditions (pixel fluorescence below 255 arbitrary units).

To monitor protein transport to the cell surface, the acquisition settings were calibrated at non-saturating conditions with control cells at the longest time of protein chase to the plasma membrane, when the protein fluorescence at the cell surface is higher; images were then acquired for all of the samples without altering these settings. After acquisition, the fluorescence was measured using Image J software (NIH, MA, USA). For each cell, the plasma-membrane-associated fluorescence of the monitored protein was normalised for its intracellular fluorescence and the resulting values were monitored over time.

To quantify cargo-positive intermediates within cells, images were acquired at the focal plane closest to the base of the cell, where these structures are more abundant, and structures of the appropriate size (typically ranging between 0.5 and 1 μm) and co-localising with the trans-Golgi marker TGN46 were considered. At least 15 microinjected cells were monitored for each time point and the values were then plotted over time.
To quantify the levels of transferrin and dextran endocytosis images acquired at the confocal microscope were analysed using Image J software (NIH, MA, USA). The fluorescence associated to dextran or transferrin was measured in approximately of 40 cells for each time points and the average values were plotted over time. The possibility that non-intemalised dextran or transferrin affected the measurements was considered negligible since the cells were acid-washed or extensively washed in PBS to remove non-internalised transferrin or dextran respectively (see sections 2.8.5 and 2.8.6).

2.11.4. Co-localisation measurements

To quantify the level of co-localisation, the scatter diagram function of the LSM software was used. Only the spots labelled for cargo were considered. In each cargo-containing pixel, the intensity of the appropriate marker was measured. Co-localisation was considered positive if the intensity of the analysed marker fluorescence was not more than two-fold higher or two-fold lower than the intensity of the cargo fluorescence in the same pixel. Because fluorescence intensities depend on labelling conditions, each experiment condition was calibrated to give a VSVG/marker ratio close to 1.0 in the spots where co-localisation was obviously occurring. Similar levels of laser power (25%) and detector amplification were used for both channels. The percentage of co-localisation-positive pixels was then estimated.

2.11.5. Z-Stack acquisitions and three-dimensional reconstructions

For Z-stacks and three-dimensional (3D) reconstruction, the images were acquired along the z-axis of the cell using the appropriate function of the LSM software.
Acquisition intervals were selected so that the top and bottom images were the first containing no fluorescence, and approximately 50 images were acquired for each cell. Z-stacks were then processed using the 3D projection function of the LSM software, to provide a 3D image of the cell.

2.12. Live imaging

2.12.1. Video microscopy

Time-lapse images were obtained using a Till Photonics (Till Photonics, Gräfelfing, Germany)-equipped IX70 Olympus inverted microscope system (Olympus, Hamburg, Germany). Samples were installed in a thermostatic device on top of the microscope stage, and images were acquired at 5-sec intervals for a total time of approximately 20 min. Where needed, the particle-tracking software of the Till Vision software was used to follow post-Golgi intermediate movements: subsequent images were superimposed one on top of the other and perfectly matching fluorescent pixels were automatically eliminated by the software as non-moving objects. The resulting images showed the tracks followed by all of the moving fluorescent objects in the movie analysed.

2.12.2. Fluorescence recovery after photobleaching (FRAP)

FRAP experiments were performed using a Zeiss LSM510 inverted confocal microscope system (Carl Zeiss, Gottingen, Germany), using a wide-open pinhole in order to have an efficient bleaching throughout the cell thickness. To bleach a defined region within the cell, the appropriate area of interest (ROI) was selected accordingly using the LSM software. Typically, four images were acquired,
applying 25% laser power at 5-sec intervals before bleaching; after that the selected ROI was rapidly hit by 100 iterations of the full-power laser. After bleaching, the cells were monitored for approximately 15 min by scanning images at 5-sec intervals using 25% laser power. At the end of each experiment, the fluorescence of the bleached ROI and of a new ROI from a fluorescent area not hit by the bleaching laser, were measured over time. Values from the normalised bleached ROI fluorescence were then plotted over time as a readout of the fluorescence recovery.

2.12.3. Correlative video immuno fluorescence (CVIF)

After microinjection, cells grown on CELLocate coverslips (Eppendorf, Germany) were subjected to the post-Golgi transport assay, as described in section 2.8.3, and analysed by video microscopy to follow VSVG carrier formation. After the video monitoring, the position of the cell of interest was determined by means of the grid present on the coverslip, and then the cells were fixed in 4% paraformaldehyde for 15-20 min at room temperature. Following this, the cells were normally processed for immuno-fluorescence as described in section 2.11.

2.13. Electron microscopy techniques

2.13.1. Materials

Paraformaldehyde was from EMS (PA, USA). As 16% in H₂O, the paraformaldehyde was diluted in 0.2 M HEPES (pH 7.4) to the appropriate concentration and stored at 4 °C. Glutaraldehyde was from Fluka (Switzerland). The Blocking Solution was prepared as follows: 0.5% BSA, 50 mM NH₄Cl,
0.02% NaN₃ in PBS, pH 7.4. Where needed, 0.05% saponin (Serva, IT) was added to the preparation. Aliquots were stored at -20 °C. Nanogold-conjugated Fab fragments of anti-rabbit IgG and Gold Enhancer were from NanoProbes (NY, USA). EPON was from Fluka (Switzerland).

2.13.2. Immuno-gold labelling

Cells grown in 6-wells plates (Falcon, NJ, USA) were fixed with 0.05% glutaraldehyde, 4% paraformaldehyde in 0.2 M HEPES (pH 7.4) for 5 min, and then with 4% paraformaldehyde in the same buffer for 20 min at room temperature. The cells were then washed three times in PBS and incubated in Blocking Solution with saponin for 20 min at room temperature, and then with a polyclonal anti-BARS antibody in Blocking Solution overnight at 4 °C. The cells were then washed 6 times with PBS and incubated with nanogold-conjugated Fab fragments of anti-rabbit IgG for 60 min at room temperature, extensively washed, and fixed with 1% glutaraldehyde in 0.2 M HEPES (pH 7.3) for 5 min. The formation of larger gold particles was enhanced by Gold Enhancer, according to manufacturer instructions. The cells were then scraped and pelleted in 1.5 ml Eppendorf tubes at 10,000 rpm for 15 min. After 3 washes in PBS, the samples were treated with 1% OsO₄ plus 1.5% potassium ferrocyanide in 0.1 M cacodylate buffer (pH 7.3) for 2 h on ice in the dark, and then processed for EPON embedding.

2.13.3. EPON embedding

Samples were washed once in PBS and dehydrated in alcohol through incubations in increasing ethanol concentrations (50%, 70%, 90% and 100% in distilled water,
3 incubations for each dilution) of 10 min each. The samples were then incubated in EPON+ Ethanol (1:1) for 4 h at room temperature, followed by a further 4-h incubation in pure EPON at room temperature. To allow the EPON to polymerise, the samples were then incubated for 24 h at 60 °C.

2.13.4. Correlative video electron microscopy (CVEM)

After microinjection, cells grown on CELLocate coverslips (Eppendorf, Germany) were subjected to the post-Golgi transport assay as described in section 2.8.3 and analysed by video microscopy to follow VSVG carrier formation. The cells were then fixed with 0.05% glutaraldehyde, 4% paraformaldehyde in 0.2 M HEPES (pH 7.4) for 5 min, and then with 4% paraformaldehyde in the same buffer for 20 min at room temperature. After 3 washes in PBS, the cells were incubated in Blocking Solution containing 0.05% saponin for 20 min at room temperature, and then further incubated with the P5 D4 monoclonal anti-VSVG antibody in Blocking Solution overnight at 4 °C. After washing, the samples were analysed by LSM (the cell of interest was detected by means of FITC-dextran, used as a tracer for microinjection; see section 2.6) and its position was determined by means of the grid present on the coverslip. The sample was further incubated with nanogold-conjugated Fab fragments of anti-mouse IgG diluted in Blocking Solution (1:100), for 60 min at room temperature, extensively washed and fixed with 1% glutaraldehyde in 0.2 M HEPES (pH 7.3) for 5 min. Formation of larger gold particles was enhanced by GoldEnhancer, according to the manufacturer instructions. After washing, samples were treated as above for EPON embedding. After EPON polymerisation, the CELLocate coverslips were dissolved with 40% hydrofluoric acid, and the samples were extensively washed.
with 20 mM HEPES, pH 7.4. The cell of interest was sectioned tangentially by Gabriele Turacchio (Department of Cell Biology and Oncology, Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy). One-hundred-nm serial sections were collected on slot grids covered with formvar-carbon supporting film and examined at 80 kV in a Philips TECNAI electron microscope.

2.13.5 Morphometrical analysis

Morphological measurements were performed analysing five different sections from five different cells for each experimental condition. Quantifications were performed counting points of intersections of intracellular structures with an overlapping grid (250x250 nm). Round profiles with a diameter up to 100 nm present in a single 100 nm section but absent from the preceding or following serial sections were considered vesicles. Elongated structures of variable size present in single 100 nm sections and absent in the preceding or following serial sections were considered tubules.
CHAPTER 3

BARS overexpression alters Golgi morphology and inhibits transport of VSVG to the cell surface

3.1. Background

BARS has been described as a fission-inducing protein that acts on Golgi-derived membranous tubules \textit{in vitro} (Weigert et al. 1999). It can bind acyl-CoA and associate to membranes, where it induces the formation of constricted areas that have been referred to as fission intermediates. This ultimately leads to the fragmentation of the Golgi tubules, an effect that has been indirectly associated with the LPAAT activity of BARS. Although the LPAAT activity of BARS appears to be low as compared to that of other known acyl-transferases, it was proposed that it stimulates the production of PA from LPA to induce changes in the curvature of membranous surfaces (Weigert et al. 1999).

In this first part of my study, the effects of BARS on Golgi membranes were studied \textit{in vivo} by using BARS overexpression as a means of stimulating its activity. The Cos7 cell line was chosen for this analysis as they are an easy-to-handle, non-polarised cell system where the well-characterised protein transport assay with the temperature-sensitive mutant of the viral glycoprotein VSVG can be used to study Golgi function (ts045VSVG; Flamand 1970).
3.2. Effects of BARS overexpression on the morphology of the Golgi complex

BARS-overexpressing Cos7 cells were labelled with the SN1 anti-BARS antibody that was developed in our laboratories. When processed for EM, the anti-BARS antibody was revealed by the gold-enhance technique (Section 2.13.2.) and the samples were embedded in EPON for sectioning (Section 2.13.3.). The resulting sections (80 nm) were analysed under EM and the sub-cellular localisation of BARS and the morphology of the Golgi complex were analysed (Fig. 3.1). BARS labelling was mainly visible in overexpressing cells as endogenous BARS is difficult to detect using the SN1 antibody. In these cells, high levels of cytoplasmic BARS were seen due to the overexpression, although significant levels of BARS were also detected in association with cellular membranes (Fig. 3.1A). BARS was detected at the plasma membrane, on membranes that are probably a part of the endocytic network, on the nuclear envelope, within the nucleus, and in a vast area surrounding the Golgi complex (Fig. 3.1A). Notably, although membranous compartments at both poles of the Golgi stacks were decorated with BARS, the stacks themselves and the intra-stack regions were totally devoid of SN1 labelling (Fig. 3.1B). Morphometric analyses (Section 2.13.2.) of the Golgi complex under these conditions showed that the number of cisternae forming each Golgi stack was not affected by this BARS overexpression, although the presence of BARS was often associated with a “loss of curvature” of each cisternae: control mock-transfected cells often presented stacks formed of curved cisternae, whereas in BARS-overexpressing cells the Golgi membranes were often particularly planar (Fig. 3.1C). Furthermore, the BARS-overexpressing cells showed a strong reduction (50%) in the presence of
“round profiles” surrounding the Golgi area (Fig. 3.1C), although as the analysis was conducted on single sections it was impossible to determine whether these structures represent cross-sections of tubules, vesicles, or parts of pleiomorphic membranous structures. However, the BARS-overexpressing cells often exhibited Golgi stacks that were devoid of surrounding small membranous compartments (Fig. 3.1B).
Fig 3.1. Intracellular localisation of overexpressed BARS and effects on Golgi morphology. A. Cos7 cells were transfected with BARS at steady state and incubated o/n at 37 °C. The following day cells were fixed and processed for immuno EM. Overexpressed BARS was labelled by immuno-nanogold and gold enhance labeling (black irregular dots). White arrows indicate BARS localisation at the Golgi complex; black arrows indicate BARS at the PM. B. Higher magnification of the Golgi area of Cos7 cells treated as in A. Scale bars 100 nm C. Morphometrical analysis of cisternal/tubular profiles and round profiles in control and BARS-overexpressing cells. Values are means ± s.d. of three independent experiments.
To further investigate this issue, immunofluorescence was used under the same conditions, to allow for multiple antibody staining. Here, transfected Cos7 cells were labelled for BARS with the SN1 antibody, and the Golgi complex was labelled with a set of markers that included GM130 for CGN staining (Barr et al. 2003; Short et al. 2003), giantin for general stack labelling (Linstedt et al. 1993; Seelig et al. 1994) and TGN46 for TGN labelling (Ponnambalam et al. 1996; Banting et al. 1997; Polishchuk et al. 2003). The mock-transfected and BARS-overexpressing cells were then analysed under confocal microscopy to allow the acquisition of images along the z-axis of the cells when needed. As seen by EM, there was an even distribution of the overexpressed BARS throughout the cytoplasm, although BARS-devoid structures were clearly detectable in the reticular-like appearance to the antibody staining (Fig. 3.2). Due to the limited resolution of light microscopy, no information could be gained on the morphology of single Golgi stacks, and the structures labelled by giantin in control and transfected cells appeared comparable, as did those labelled by GM130. On the other hand, at the level of the TGN it was possible to observe several differences: control cells showed TGN46 staining that was mainly concentrated within the perinuclear Golgi area, but was also present on smaller structures distributed throughout the cytoplasm and at the level of the plasma membrane, as would be expected for TGN46 (Fig. 3.3; Banting et al. 1997). Moreover, although membranous Golgi tubules are difficult to see in fixed samples, some tubular structures labelled by TGN46 were seen emanating from the Golgi complex (Fig. 3.3). In contrast, the BARS-overexpressing cells showed TGN46 labelling that was mainly concentrated in the perinuclear Golgi area, with TGN46-positive structures within the cytoplasm being rare and no tubular structures seen to be
elongating from the fluorescent mass of the Golgi complex (Figs. 3.2; 3.3). These observations were confirmed when the cells were visualised along their z-axis, further supporting that these effects were not due to misleading aspects of single optical sections (Fig. 3.3).
Fig 3.2. Intracellular localisation of overexpressed BARS. Cos7 cells were transfected with BARS and incubated o/n at 37 °C. The following day cells were fixed and processed for immunofluorescence and the intracellular localisation of BARS (red) was examined together with different markers of the Golgi complex (green). Scale bars, 10 μm.
**Fig 3.3. BARS overexpression alters TGN46 distribution.** Cos7 cells were mock-transfected (control; left panels) or transfected with BARS (BARS; right panels) and incubated overnight at 37 °C. Cells were then fixed and processed for immunofluorescence to investigate the distribution of the TGN marker TGN46. Optical sections along the Z-axis of control and BARS-overexpressing cells were acquired using a confocal microscope with the objective’s pinhole reduced to 1 airy unit. Scale bars, 10 μm.
3.3. BARS overexpression alters Golgi function during protein secretion

Once the effects of BARS overexpression on Golgi morphology had been assessed, the functionality of this organelle was tested under the same conditions. As the Golgi complex is the central station for the secretory pathway, the ER-to-plasma membrane transport of the well-known GFP-tagged marker protein VSVG (VSVG-GFP) was tested in these BARS-overexpressing Cos7 cells. The cells were co-transfected with the BARS cDNA (or an empty DNA vector as control) and the temperature sensitive mutant of VSVG-GFP and incubated overnight at 40 °C in a CO₂ incubator. At this temperature, although the viral glycoprotein is synthesised in the ER, due to its mutation it cannot fold properly and it thus remains trapped within the ER until the temperature is decreased to 32 °C (Flamand 1970).

On the following day, these cells were incubated at 32 °C to release the VSVG-GFP from the ER. The VSVG-GFP transport to the cell surface was followed by fixing samples in 4% paraformaldehyde at different times from 0 min to 180 min (where 0 min represent the cells immediately prior to the removal of the 40 °C block). At the release of the temperature block, both mock-transfected and BARS-overexpressing cells showed VSVG-GFP within the ER (Fig. 3.4A). Upon shifting to the permissive temperature, VSVG-GFP transport from the ER to the Golgi complex appeared not to be perturbed by BARS, with more or less all of the viral protein concentrated in a perinuclear area after 30 min of chase (Fig. 3.4A).
Fig 3.4. BARS overexpression inhibits secretion of VSVG-GFP. A. Cos7 cells were transfected with VSVG-GFP alone or together with BARS and subjected to a 40°-32° C. temperature block protocol to assay protein secretion. Cells were fixed at the indicated times and intracellular localisation of VSVG was followed during the chase. Scale bars 10 µm. B. For each time step of the chase described in A, the ratio of the Golgi fluorescence versus that of the cytoplasm was measured over a random sample of 10 cells and plotted over time. BARS overexpressing cells exhibit a higher and prolonged accumulation of VSVG within the Golgi as further transport to the PM appears inhibited. Values are means ± s.d. of three independent experiments.
At later time points, the control cells showed VSVG-GFP exiting the Golgi complex through the formation of fluorescent carriers of variable sizes and of GFP-positive tubular structures that emanated from the Golgi complex. In contrast, in the BARS-overexpressing cells, the VSVG-GFP transport appeared to be blocked at the level of the Golgi complex, with a very limited number of GFP-positive carriers and tubular structures being detected during the chase (Fig. 3.4A). As a consequence, after 45 min to 60 min of chase, VSVG-GFP was becoming visible at the cell surface of the control cells and the Golgi-associated fluorescence had decreased, whereas in the BARS-overexpressing cells VSVG-GFP was still concentrated within the Golgi complex and the plasma membrane was hardly visible (Fig. 3.4A).

To better characterise these observations, the ratios between the Golgi complex GFP fluorescence (detected as that co-localising with the antibody giantin) and the cell-associated fluorescence were calculated in samples of approximately 10 cells for each time point. The fluorescence intensities were then plotted against time, and it was again clear that in the control cells VSVG-GFP transport was not perturbed within the first 30 min of the chase, where the GFP fluorescence peaked, before decreasing at the later time points as the VSVG fluorescence was redistributed from the Golgi complex to the cytoplasm/plasma membrane (Fig 3.4B; dashed line). Instead, in the BARS-overexpressing cells there was a continuing increase in the accumulation of VSVG within the Golgi complex that was almost 3-fold that of the control cells and that peaked with a 20-min delay as compared to the control cells (Fig. 3.4B; black line). This is probably because in the control cells the further VSVG-GFP accumulation within the Golgi complex is
offset by the amount of VSVG-GFP that is already on its way to the plasma membrane. Thus if this last step is inhibited in the BARS-overexpressing cells, this will ultimately result in a larger amount of VSVG-GFP remaining within the Golgi stacks.

At the later time points, the BARS-overexpressing cells did show a partial redistribution of the fluorescence from the Golgi complex to the cytoplasm/plasma membrane. However, as the Golgi complex maintained its fluorescence in BARS-overexpressing cells even after 180 min of chase, this redistribution was considerably slower and hence severely inhibited. At this time, the Golgi fluorescence in the control cells was reduced to one third of its peak levels (Fig. 3.4B).

These initial observations thus indicated that BARS overexpression has no effects at the level of ER-to-Golgi transport, but it was indeed able to perturb transport at the Golgi complex. At this stage, it was not possible to determine whether this BARS-induced transport block occurred at the level of intra-Golgi transport or the TGN.

3.4. BARS overexpression inhibits VSVG transport at the level of the TGN

To characterise this BARS-induced block of VSVG transport, the Golgi-to-plasma membrane transport was dissected into the intra-Golgi and post-Golgi stages. First, a biochemical approach was used to monitor intra-Golgi transport by the endoglycosidase H (Endo-H) resistance test (Section 2.9.). This assay is based on
the principle that proteins traversing the Golgi complex undergo a series of glycosylations, one of which (sialylation) occurs at the level of the trans-Golgi and confers resistance to the cleaving activity of the enzyme Endo-H (Davidson et al. 1993). When cell lysates from different time points of the chase (as above) were run on polyacrilamide gels and the gels were stained with an anti-VSVG antibody, it was possible to determine when the viral glycoprotein arrived at the trans-Golgi compartment. Typically, protocols for Endo-H resistance tests involve cells infection with VSV so that the overall amount of this viral glycoprotein is detectable over the total cellular proteins. However, here infection was not applicable as only about 30% of the cells were overexpressing BARS after transfection, and the effects of BARS would have been “diluted” by the remaining 70% of cells where transport was not perturbed. On the other hand, co-transfection of BARS and VSVG would have resulted in the production of a low amount of viral glycoprotein, making it difficult to discriminate it from the total pool of cellular proteins. The Endo-H resistance protocol was thus modified as described in Section 2.9., with the co-transfected Cos7 cells being incubated with Endo-H and lysed. The resulting cell lysates were immuno-precipitated to isolate the VSVG from the total protein pool. To monitor VSVG transport, the cells were analysed at three different time points: 0 min, that corresponded to the 40 °C incubation, and after 40 and 60 min of chase, when VSVG should be accumulated within the Golgi complex and on the plasma membrane, respectively. As expected, at 40 °C all of the VSVG was sensitive to Endo-H in both the control and the BARS-overexpressing cells, which was seen as a shift in the VSVG band on the gels (Fig. 3.5A). Later in the chase, both samples showed resistance to Endo-H, which indicated that in both control and BARS-overexpressing cells the
transport of VSVG at the level of the medial/trans-Golgi stack was also not perturbed when BARS was overexpressed (Fig. 3.5A).

The second approach that was used to test intra-Golgi transport was based on the co-localisation between VSVG-GFP and different Golgi markers on immunofluorescence treated samples (Mironov et al. 2001). Here, after the co-transfection of BARS and VSVG-GFP, the Cos7 cells were subjected to a miniwave protocol (Section 2.8.1.) where the temperature was taken from 40 °C to 15 °C for a short time to allow only a small portion of the blocked VSVG to leave the ER and thus to accumulate in the intermediate compartment between the ER and the Golgi complex. In this way it was possible to follow a small pulse of the viral glycoprotein while it was on its way through the Golgi complex, while leaving the major pool of VSVG-GFP trapped within the ER (Mironov et al. 2001).

To monitor this VSVG transport through the Golgi complex, two antibodies were used: an anti-GM130 antibody to label the cis side, and an anti-TGN46 antibody for the trans side. The samples were fixed at the end of the 15 °C incubation and after 5, 10 and 30 min of chase at 40 °C. For each time point, the co-localisation between VSVG-GFP and the Golgi markers (Section 2.11.4) was measured in both the control and the BARS-overexpressing cells. At the release of the 15 °C temperature block, the VSVG-GFP showed very little co-localisation with the cis-Golgi marker GM130 both in control and in BARS-transfected cells. The level of co-localisation then increased with time up to 10 min of chase, and then decreased again at later times as the VSVG-GFP moved through the Golgi stack towards the
TGN (Fig. 3.5B). Overall, the transport of the VSVG-GFP through the cis-side of the stack was unaffected by BARS overexpression (Fig 3.5B). Conversely, the VSVG-GFP co-localisation with TGN46 was negligible within the first 10 min of chase, and then peaked at 15 min both in control and in BARS-transfected cells (Fig. 3.5B). Following this, the control cells exhibited a decrease in VSVG-GFP and TGN46 co-localisation within the Golgi area as the VSVG-GFP moved on to the plasma membrane. In the BARS overexpressing cells this co-localisation remained high until the end of the chase. This thus indicated that the viral protein somehow remained trapped within the TGN (Fig. 3.5B).

This evidence that indicates that VSVG transport from the ER to and through the Golgi complex is not perturbed by BARS overexpression is also in agreement with the previous observations (Section 3.2.). Thus when BARS was overexpressed, it localised on intracellular membranes that included those that surround the Golgi complex, while it was always excluded from the Golgi stacks themselves, suggesting that it should not affect transport events within the Golgi complex. Instead, VSVG transport appears to be inhibited by BARS at the level of the TGN, where this cargo accumulates without entering post-Golgi carriers. Moreover, the observation that in BARS-overexpressing cells the marker TGN46 is concentrated in the Golgi area suggests that the block in VSVG transport is not due to a mistargeting of VSVG-GFP to the forming cargo domains, but rather to an inhibition of the formation of the post-Golgi carriers themselves.
Fig 3.5. Intra-Golgi VSVG transport is not affected by BARS overexpression. A. Endo-H resistance test was performed on lysates from control and BARS-overexpressing Cos7 cells (section 2.9). B. Cos7 cells were mock-transfected (blue line) or transfected with BARS (pink line) and intra-Golgi transport of VSVG was assayed by measuring the colocalisation of VSVG with GM130 and TGN46 (markers of the cis- and trans-Golgi networks respectively) during the chase. Values are means of three independent experiments.
3.5. Overexpression of BARS inhibits VSVG delivery to the cell surface

Once VSVG has been sorted into post-Golgi carriers it is transported to the plasma membrane, where it is exposed to the extracellular milieu before being re-internalised and delivered to lysosomes for degradation. As a consequence, it is possible to observe the time-dependence of VSVG transport to the cell surface by means of a specific antibody against the lumenal domain of the viral protein. In this way, by applying the antibody to non-permeabilised cells and measuring cell surface fluorescence, it is possible to detect only the fraction of VSVG that has reached the cell surface, without contamination from the intracellular VSVG fraction. If BARS does indeed affect post-Golgi transport, then the monitoring of VSVG delivery to the plasma membrane in a post-Golgi transport assay (Section 2.8.3) should make it possible to see a reduction in the amount of VSVG that reaches the cell surface during the chase.

To achieve this, Cos7 cells were again co-transfected with BARS (or the empty DNA vector as control) and VSVG-GFP and then incubated overnight at 40 °C. The next day, the cells were incubated for 2 h at 20 °C to allow the VSVG-GFP to accumulate in the Golgi complex (Griffiths et al. 1985). The temperature block was then released to 32 °C to chase the VSVG-GFP to the cell surface. Samples were fixed at the end of the 20 °C block and after 45, 60 and 90 min of chase, when substantial amounts of VSVG-GFP should be detectable at the plasma membrane. After fixation, the cells were immediately labelled with the anti-VSVG antibody and only then permeabilised, prior to their labelling for BARS, with the intracellular VSVG being detected through the GFP tag (Fig. 3.6A). For
each sample, the plasma membrane-associated VSVG-GFP fluorescence was measured in control and BARS-overexpressing cells, and after these values had been normalised with respect to the intracellular levels of VSVG-GFP, the resulting fluorescence intensities were plotted against time (Fig. 3.6B). As expected, at the release of the temperature block no VSVG was detectable on the cell surface of either control or BARS-overexpressing cells. After 45 min of chase, a significant amount of VSVG had reached the cell surface in the control cells, whereas in the BARS-overexpressing cells there was very little VSVG visible at the plasma membrane (Fig. 3.6B). After 60 min of chase, the control cells had doubled their amount of VSVG at the plasma membrane and the Golgi was nearly devoid of VSVG (Fig. 3.6A). In contrast, the BARS-overexpressing cells accumulated only low levels of the viral glycoprotein on the cell surface (Fig. 3.6B) while retaining the majority of the VSVG in the Golgi complex (Fig. 3.6A). Eventually, by the end of the chase (90 min), both the control and the BARS-transfected cells showed reductions in the amounts of plasma membrane VSVG, possibly due to re-internalisation of the viral protein.
Fig 3.6. BARS overexpression inhibits post-Golgi transport of VSVG.

A. Cos7 cells were transfected with VSVG-GFP alone (Control) or in combination with BARS (BARS) and subjected to the post-Golgi transport assay (section 2.8.3). Cells were fixed and processed for immunofluorescence and the fraction of VSVG that had reached the PM was revealed by an antibody against the extracellular domain of the viral glycoprotein in non permeabilised cells. Scale bar 10 μm

B. Cos7 cells treated as in A were fixed and processed for immunofluorescence at the indicated time and VSVG fluorescence at the PM was measured over a sample of at least 10 cells/time point. Values are means ± s.d. of three independent experiments.
Overall, this BARS overexpression appeared to induce both a delay and a reduction in the amount of VSVG-GFP delivered from the Golgi complex to the cell surface, i.e. the control cells exhibited a significant amount of VSVG-GFP at the plasma membrane after 45 min of chase, whereas similar levels of VSVG-associated fluorescence were detectable in BARS-overexpressing cells only after 60 min of chase (Fig. 3.6B). Moreover, the control cells continued to accumulate VSVG at the cell surface until re-internalisation of the viral protein prevailed 90 min after the release of the temperature block. The total amount of VSVG accumulated at the plasma membrane of BARS-overexpressing cells never reached the control levels, with the amount of VSVG accumulated in this case being around 40% of that of the control cells.

3.6. Overexpression of BARS blocks VSVG transport by inhibiting post-Golgi-carrier formation at the level of the TGN

To further characterise this inhibition of post-Golgi transport observed in the BARS-overexpressing cells, VSVG transport from the TGN was analysed in living cells by confocal imaging. To achieve this, YFP-tagged BARS was produced by sub-cloning BARS cDNA into a pEYFP vector (Section 2.2.6.). When the construct was tested for its intracellular localisation and effects on post-Golgi transport of VSVG, it exhibited the same features as the BARS construct used in the previous assays. Thus in the BARS-YFP-overexpressing cells it localised within the cytoplasm, with local enrichments at the plasma membrane and the Golgi complex (Fig. 3.7A). It also inhibited the post-Golgi transport of VSVG. To monitor this post-Golgi transport of VSVG in BARS-overexpressing
cells, the Cos7 cells were co-transfected with BARS-YFP (or the pEYFP vector alone as control) and VSVG-CFP and subjected to the post-Golgi transport assay using a 20 °C to 32 °C temperature block. At the end of the 20 °C incubation, the cells were moved to a thermostatic chamber installed on a Zeiss confocal microscope and temperature was released to 32 °C. Images were acquired with an open pinhole every 10 s for 20 min (Fig. 3.7B). In the control cells, the VSVG-CFP was normally transported from the Golgi complex to the plasma membrane through the formation of CFP-positive carriers that mainly arose from tubular structures that emanated from the fluorescent mass of the Golgi complex (Fig. 3.7B). During the chase, the cytoplasm had an increasing number of CFP-positive structures and the plasma membrane gradually became visible (movie 1). On the other hand, in BARS-YFP-overexpressing cells there were only a very limited number of CFP-positive structures that left the Golgi complex, with the vast majority of the VSVG-CFP remaining trapped within the Golgi complex, as would be expected (Fig. 3.7B). Interestingly, although the Golgi mass appeared dynamic during the chase, no tubular structures were seen to emanate from the Golgi complex, thus indicating that overexpression of BARS can inhibit the step of carrier precursor formation (movie 2).
Fig 3.7. A. BARS-YFP overexpression. Cos7 cells were transfected with the BARS-YFP construct; after an o/n incubation at 37 °C cells were fixed and processed for immunofluorescence using an anti BARS antibody to investigate the intracellular localisation of the YFP construct. B. Time-lapse imaging of VSVG-CFP post-Golgi transport. Cos7 cells were transfected with VSVG-CFP alone (left panel set) or together with BARS-YFP (right panel set) and treated for the post-Golgi transport assay (section 2.8.3). At the release of the temperature to 37 °C., cells were imaged with a confocal microscope, using a wide open pinhole and acquiring single images every 10 sec (movie 1; 2). Scale bars, 10 μm.
3.7. Discussion

This first information as to the intracellular localisation and effect of overexpressed BARS in Cos7 cells clearly suggest that BARS has a role in intracellular transport at the level of the Golgi complex. Moreover, it appears that its major effect is on the tubular compartments of the TGN, as was also suggested by previous in vitro studies (Weigert et al. 1999). However, this does not clearly fit with the previous observation whereby BARS stimulates the fission of Golgi tubules on isolated Golgi membranes. A stimulation of the fission step should lead to an increase in the formation of transport carriers and ultimately to an increase in transport from the Golgi complex. The present observations from BARS-overexpressing cells indicate that it actually inhibits post-Golgi transport by inhibiting the formation of VSVG-positive transport carriers. Moreover, the observation that the TGN marker TGN46 is also mainly localised within the Golgi complex and is scarcely visible within the cytoplasm of BARS-overexpressing cells suggests that the block in post-Golgi transport is not dependent on a defect in cargo sorting into carrier precursors at the level of the TGN, but rather to an impairment of the formation of the transport carriers themselves.

With its relevance still being uncertain, the LPAAT activity of BARS would imply that high concentrations of BARS can lead to an accumulation of lipids like PA. Although considered to be a fission-inducing lipid, as PA is a cylindrical lipid at μM calcium concentrations it could also infer rigidity to biological membranes at high concentrations. In transfected cells expressing BARS during an overnight incubation, it is possible that the intracellular levels of BARS could reach a
critical concentration that indeed stimulates post-Golgi transport. However, with further continued accumulate of BARS, the end result could be its inhibitory activity on protein secretion at the TGN. As the levels of PA at the Golgi complex are almost impossible to measure in an *in vivo* system, there are two possible ways of further investigating this possibility: one would be to treat BARS-overexpressing cells with compounds that are able to interfere with lipid metabolism such that this potential accumulation of PA would be removed or overcome, to see if transport is recovered; and the other would be to induce an acute increase in the intracellular concentration of BARS (i.e. by microinjection) and observe its effects on VSVG transport from the TGN. These two approaches were thus applied to this system, and are described in the following Chapters.
CHAPTER 4

Modifications of the membrane lipid composition alter post-Golgi transport

4.1. Background

Membrane lipid composition is a fundamental factor in the regulation of membrane curvature and fission along the endocytic and secretory pathways (Corda et al. 2002). Lipids like PA and DAG can serve both as recruiting platforms for fission-inducing factors, such as protein kinase D (PKD), phospholipase D (PLD) and ARFGAP (Maeda et al. 2001; Manifava et al. 2001; Baron et al. 2002; Ktistakis et al. 2003; Antonny et al. 1997), and as membrane destabilising elements that are able per se to drive membrane fission (Kooijman et al. 2003). There is also evidence in favour of a role for these lipids in both the stimulation and the inhibition of protein secretion at the Golgi complex (Chen et al. 1997; Siddhanta et al. 2000).

Although often of indefinite specificities, a wide range of compounds have been used to modulate the concentrations of PA and DAG within cellular membranes (de Chaffoy de Courcelles et al. 1985; de Chaffoy de Courcelles et al. 1989; Flores et al. 1999; Siddhanta et al. 2000; Baron et al. 2002; Skippen et al. 2002; Sweeney et al. 2002; Deretic et al. 2004). Given the technical difficulties associated with the measurements of lipid concentrations within a single organelle
like the Golgi complex in an \textit{in vivo} system, and particularly where only BARS-overexpressing cells should be considered, the aim of this part of the study was to use a more indirect approach to determine whether the block in VSVG transport induced by BARS overexpression is associated with an increase in PA that could be generated by the LPAAT activity of BARS. As indicated in the scheme in Figure 4.1, primary alcohols like 1-butanol can reduce the intracellular levels of PA through their inhibition of PLD activity (Siddhanta et al. 2000; Skippen et al. 2002; Sweeney et al. 2002), and DAG kinase inhibitors can block the conversion of DAG to PA, thereby also inducing a reduction in intracellular PA concentrations and a concomitant increase in DAG (de Chaffoy de Courcelles et al. 1985; de Chaffoy de Courcelles et al. 1989; Flores et al. 1999). The opposite effects can be achieved by inhibition of PA phosphatases with propranolol (Baron et al. 2002; Deretic et al. 2004).

To verify their efficacies and determine any possible toxic effects, all of these compounds were tested in the \textit{in vivo} transport assay for VSVG that was described and used in the previous Chapter. A morphological approach was used for the characterisation of the effects of different treatments through the quantification of the variations in the numbers of VSVG-positive intermediates and VSVG-positive tubules, as a measure of the stimulation or inhibition of transport, respectively. In parallel, and as before, the levels of cell surface VSVG were assessed to provide confirmation of the morphological evidence.
Fig 4.1. Lipid metabolic pathways for the formation of phosphatidic acid (PA). Schematic representation of the main metabolic pathways that modulate the intracellular concentrations of phosphatidic acid. Key: phospholipase D (PLD); phosphatidyl choline (PC); lysophosphatidic acid (LPA); diacylglycerol kinase (DGK); diacylglycerol (DAG).
4.2. While blocking anterograde transport, 1-butanol also disrupts the Golgi complex

As mentioned above, treatment with 1-butanol inhibits the formation of PLD-derived PA due to the synthesis of metabolically inactive phosphatidylbutanol. This has a large effect on cellular membranes that leads to the disruption of the intracellular organelles, including the Golgi complex (Siddhanta et al. 2000). One means of determining whether BARS-overexpression can indeed lead to the stimulation of PA is to see if BARS is able to revert these effects of primary alcohol treatment, and thus to restore the secretory transport of VSVG.

To achieve this, Cos7 cells were co-transfected with VSVG-GFP and BARS (or an empty vector as the transfection control) and incubated overnight at 40 °C to accumulate VSVG-GFP in the ER. To chase the VSVG-GFP from the ER to the cell surface and to determine the effects of PLD activation, the temperature was shifted to 32 °C and 1.5% 1-butanol was added to the culture medium, as needed (Siddhanta et al. 2000; the 2-butanol control was not included in the protocol in the first round of experiments). The cells were fixed after 0, 20, 40 and 60 min of chase and processed for immunofluorescence. For each time point, VSVG transport was assessed by staining the cell-surface-associated VSVG in non-permeabilised cells with the antibody against the lumenal domain of the protein. The conditions included control cells that were not overexpressing BARS and cells overexpressing BARS without and with the addition of 1-butanol. In the control cells the VSVG-GFP was transported from the ER to the Golgi complex and on to the plasma membrane at normal rates, whereas in the BARS-
overexpressing cells there was a marked inhibition of post-Golgi transport of VSV-GFP, as expected (Fig. 4.2A). Unfortunately, all of the cells treated with 1-butanol, whether mock transfected or BARS overexpressing, presented a disrupted Golgi complex, as judged from the lack of perinuclear accumulation of VSV-GFP during the chase. VSV-GFP remained trapped within the ER (Fig. 4.3) and there was no VSVG-associated fluorescence at the plasma membrane (Figs. 4.2A; 4.3). These results would indicate that where present, BARS-induced PA synthesis is not sufficient to recover the overall effects of this 1-butanol treatment on PA depletion. Of note, such primary alcohol treatment is known to be relatively non-specific and to produce more general effects on cellular membranes, as also seen here by the toxicity of prolonged 1-butanol treatment (which induced apoptosis). In terms of the cells overexpressing BARS, these would normally be viable for at least 48 h after transfection, indicating that although the increased levels of BARS are inhibitory on post-Golgi secretion, they are still compatible with cell growth. Thus in terms of the 1-butanol treatment, at this level all that can be specifically said is that the overexpression of BARS is not sufficient to compensate for the 1-butanol effects on the cells, which should have included depletion of PA. Because of the toxicity of the 1-butanol treatment, this experimental procedure was not carried on any further.

4.3. Propranolol and DAG kinase inhibitors modulate membrane traffic

The metabolic interchange between PA and DAG is very rapid and is mediated by two classes of enzymes: the DAG kinases and the PA phosphatases. The balance between these two lipids can be modified by applying an inhibitor of one of the
two reactions, thereby increasing the concentration of one lipid by blocking its conversion to the other. Propranolol is an inhibitor of PA phosphatases (Baron et al. 2002; Deretic et al. 2004), and the resulting increases in the intracellular levels of PA have clear consequences on membrane traffic (Chen et al. 1997). DAG kinases, on the other hand, preserve DAG from being converted to PA. Here, there are several classes of DAG kinases (Topham et al. 1999) and two main inhibitors: R59022 (de Chaffoy de Courcelles et al. 1985) and R59949 (de Chaffoy de Courcelles et al. 1989). These are generally just referred to as DAG kinase inhibitors I and II, respectively, and they have different specificities for the various DAG kinases.

To verify the suitability of these tools, propranolol and these DAG kinase inhibitors were tested in the VSVG transport assay before being combined with BARS overexpression. As above, Cos7 cells were transfected with VSVG-GFP and incubated overnight at 40 °C to accumulate the viral glycoprotein within the ER. On the following day, the further temperature block of 2 h at 20 °C was used to accumulate the VSVG-GFP within the Golgi complex in order focus on the segment of traffic that is affected by BARS overexpression, namely post-Golgi transport. At the release of the temperature block, 500 μM propranolol or 50 μM of the DAG kinase inhibitors were added to the culture medium (Section 2.10.3.2.) and the formation of GFP-positive carriers (Figs. 4.2B; 4.3) and the arrival of VSVG at the cell surface (Figs. 4.2A; 4.3) were followed.

The treatment with propranolol stimulated the formation of post-Golgi transport carriers (Figs. 4.2B; 4.3) and almost totally inhibited the formation of VSVG-
GFP-positive tubules (Fig. 4.2C). On the basis of inhibition of the PA phosphatases, the combination of these two results suggests that an increase in intracellular PA does not inhibit tubule formation per se, but rather increases the fission of these tubules, as judged by the increase in post-Golgi carriers. Interestingly, although transport from the Golgi complex appeared to be stimulated by propranolol treatment, the plasma-membrane-associated VSVG was decreased when compared to untreated control cells (Figs. 4.2A; 4.3). However, this is perhaps not totally surprising as Deretic et al. (Deretic et al. 2004) have reported that propranolol can also have effects on other cellular membranes. As these can include the plasma membrane, propranolol might interfere with the tethering complexes formed between the post-Golgi carriers and the plasma membrane. Of note, this propranolol treatment also had a detectable toxicity in these Cos7 cells, and therefore post-Golgi transport could not be followed for more than 40 to 60 min.

In contrast, the addition of DAG kinase inhibitors (both type I and II) resulted in a reduction in post-Golgi carrier formation to approximately 1/3 of those formed in the untreated control cells (Figs. 4.2B; 4.3). At the same time, the numbers of VSVG-GFP-positive tubular structures emanating from the Golgi complex were slightly increased (Fig. 4.2B), in agreement with the concept of an increase in DAG and a concomitant decrease in PA. As a consequence, the levels of VSVG at the cell surface were also significantly reduced (Figs. 4.2A; 4.3).

When taken together with previously published biochemical characterisations that have shown variations in the levels of DAG and PA after propranolol and DAG kinase inhibitor treatments (Baron et al. 2002; Deretic et al. 2004; de Chaffoy de Courcelles et al. 1985; de Chaffoy de Courcelles et al. 1989; Flores et al. 1999),
these results indicate that these compounds may indeed be useful to provide further information as to whether BARS overexpression results in effects on membrane lipid composition.

4.4. Increasing the levels of intracellular DAG can restore VSVG transport in BARS overexpressing cells

Once the effects of the inhibitors of the lipid modifying enzymes acting on the DAG/PA cycle had been characterised in the post-Golgi transport assay for VSVG, the same compounds were tested on BARS-overexpressing Cos7 cells. The cells were co-transfected with BARS (or an empty plasmid as control) and VSVG-GFP, incubated overnight at 40 °C and then for 2 h at 20 °C to accumulate the VSVG within the Golgi complex. At the release of the temperature block, the cells were treated with the individual compounds (Section 2.10.3.2.) and the VSVG-GFP transport was followed. The propanolol treatment was unable to restore VSVG transport (Fig. 4.3), in agreement with a putative increase in PA as being the cause of the membrane traffic inhibition. Moreover, when applied to BARS-overexpressing cells, propanolol was unable to stimulate the formation of the post-Golgi transport carriers that were seen when it was applied to control cells (Fig 4.2B). Instead, the combined BARS and propanolol treatment resulted in a limited number of intermediates and in the complete inhibition of the formation of Golgi-derived tubules (Fig. 4.2B; C). As a result, VSVG transport to the cell surface remained inhibited, as seen for each of the two treatments (BARS and propanolol) when applied separately (Fig. 4.2A; 4.3).
Fig 4.2. Lipid metabolism regulates VSVG transport. A. Cos7 cells transfected with VSVG-GFP or together with BARS were assayed for ER-to-PM VSVG transport (section 2.8.2) in the presence of specific inhibitors of lipid metabolism. Cells were fixed after 40 min of chase and VSVG surface fluorescence was measured through antibody staining on non-permeabilised cells. In parallel VSVG-positive intermediates (B) and tubules (C) were quantified for each sample. Values are means ± s.d. of three independent experiments. Key: Ppl (propranolol); DAGKin (DAG kinase inhibitor); ButOH (Butanol).
Fig 4.3. Lipid metabolism regulates VSVG transport. Cos7 cells transfected with VSVG-GFP were assayed for ER-to-PM VSVG transport (section 2.8.2) in the presence of specific inhibitors of lipid metabolism. Treatments were applied at the release of the 40 °C temperature block; cells were fixed after 40 minutes of chase and treated for immunofluorescence. Insets show the amount of VSVG at the cell surface as revealed by antibody staining on non-permeabilised cells. Scale bar, 10 μm.
In contrast, when the DAG kinase inhibitors were applied to BARS-overexpressing cells under the same conditions as above, the transport of VSVG to the cell surface was restored to the control levels (Figs. 4.2A 4.3). This correlated with the full reconstitution of the formation of VSVG-positive carriers, which was even slightly increased over the control levels (Fig. 4.2B). There was also the re-appearance of tubular structures, although their frequency was lower than that in control cells and their number was difficult to assess because of the high variability within the randomly chosen cell sample (Fig. 4.2C). Although indirect, this suggests that BARS-overexpression can indeed induce alterations in membrane lipid composition that can exert inhibitory effects on post-Golgi transport.

4.5. Discussion

Given the technical difficulties in the detection of variations in the lipid composition of membranes of a sub-compartment of an intracellular organelle like the TGN, alterations in lipid metabolism were obtained *in vivo* using a set of different modifying compounds. The biochemical evidence regarding BARS indicates the presence of an acyl transferase activity, which would stimulate the production of PA by acylating LPA (Weigert et al. 1999). *In vitro* studies on the role of BARS on isolated Golgi membranes demonstrated a fissioning activity of BARS on Golgi-derived tubules (Weigert et al. 1999), whereas the *in vivo* evidence has indicated that BARS overexpression results in an inhibition of the formation of post-Golgi carriers, an event that requires membrane fission. The main differences in these two approaches can be found in the concentrations of
BARS applied to the cellular membranes and the prolonged exposure of these membranes to high levels of the overexpressed protein. *In vitro*, the BARS concentrations can be easily controlled and the incubation time is around 20 min, whereas the *in vivo* cDNA expression often leads to high intracellular BARS concentrations and protein overexpression typically requires an overnight incubation. In the latter case, it is thus possible that the resulting overnight exposure to increasing concentrations of BARS stimulates the local synthesis of PA that can be “toxic” in terms of protein transport when VSVG secretion is investigated. To test this possibility without directly measuring the lipid content at the level of the Golgi complex, the cellular lipid metabolism was altered to further stimulate PA synthesis or, on the other hand, to reduce the intracellular PA content in favour of DAG. In line with the hypothesis of an “over production” of PA induced by BARS overexpression, the use of propranolol alone gave a phenotype that was highly similar to that observed in BARS-overexpressing cells, and the combination of BARS and propranolol was inhibitory on VSVG secretion (Figs. 4.2A; 4.3). Interestingly, on the other hand, when BARS overexpression was coupled with DAG kinase inhibition, VSVG secretion was restored and the formation of post-Golgi carriers was as efficient as in the control cells, if not even slightly stimulated (Figs. 4.2A; 4.3). DAG kinase inhibitors will reduce the pool of PA by preserving DAG from being converted to PA itself, thereby shifting the lipid balance in favour of DAG (Fig. 4.1). Accordingly, and as described above, the incubation of cells with DAG kinase inhibitors alone actually reduces fission (Fig. 4.2B; C), in agreement with the prediction of PA as a fission-inducing lipid. Of note, although treatments with chemical inhibitors are not always considered “specific” due to effects on metabolic processes in a wide range of intracellular
compartments, the DAG kinase inhibitors used in this study appear to have a degree of specificity for Golgi-associated kinases such as DGKα (Jiang et al. 2000; Shirai et al. 2000), supporting the reliability of this assay. This evidence, on the one hand, is in favour of a role for BARS in the modulation of the composition of the membrane lipids, but on the other hand, it also indicates that transport inhibition could be the result of a secondary effect that is due to this overexpression. To further dissect the role for BARS in intracellular transport, a different assay is needed, where the intracellular concentrations of BARS can be modulated with more precision and more acutely.
CHAPTER 5

Acute increases in cytosolic BARS stimulate the formation of post-Golgi transport carriers

5.1. Background

In the first part of this study, the intracellular localisation and the role of BARS in intracellular transport were assessed by means of its overexpression in Cos7 cells. If on the one hand the high levels of intracellular BARS were helpful in determining its intracellular localisation, on the other hand BARS overexpression induced a block in post-Golgi transport of VSVG that was potentially due to secondary effects induced by the prolonged exposure to high intracellular levels of BARS. As it appeared clear anyway that BARS has a relevant role in the formation of carriers from the TGN, in the second part of this study a different approach was used to acutely increase the intracellular levels of BARS. This was achieved by the microinjection of the purified BARS protein. In this way, it was possible to better synchronise the system used and to apply a sharp and regulated pulse of injected BARS when the temperature-sensitive mutant of VSVG (used as the cargo protein) was trapped within the TGN (using again the 20 °C temperature block; Section 2.8.3.).
5.2. Microinjection of BARS stimulates the formation of VSVG-positive carriers

Cos7 cells were infected with VSV and subjected to the post-Golgi transport assay (Section 2.8.3). After the first hour at 20 °C, the cells were moved to a refrigerated (20 °C) microinjection stage and 2 mg/ml of GST-tagged BARS (BARS-GST) in microinjection buffer was injected into the cytoplasm of approximately 100 cells per coverslip (Section 2.6.1.), with the control cells being injected with purified GST. After injection, the cells were incubated further at 20 °C to recover from the stress of microinjection and then assayed for post-Golgi transport of VSVG. The GST-injected cells showed no alteration in VSVG transport from the TGN, with the viral glycoprotein exiting the Golgi complex at normal rates as compared to non-injected cells (Fig. 5.1A). Thus, at the release of the 20 °C temperature block, the VSVG was concentrated within the Golgi complex in both GST-injected and non-injected cells, and a very low number of VSVG-positive carriers were visible throughout the cytoplasm (5 intermediates/cell, on average; Fig. 5.1A, B). During the chase at 32 °C, the control mock-injected cells produced an increasing number of VSVG-positive carriers that reached a plateau after approximately 40 min of chase (Fig. 5.1A, B). The BARS-injected cells had already produced an explosive formation of VSVG-positive structures scattered throughout the cytoplasm at 20 °C (70 intermediates/cell, on average), which is normally considered a non-permissive temperature for the formation of post-Golgi carriers (Fig. 5.1A; B; Griffiths et al. 1985). During the following chase, the formation of VSVG-positive structures gradually returned to the normal level, reaching that of the mock-injected cells.
after 40 min of chase (Fig. 5.1A; B). In parallel, when the cells were immuno-
labelled for VSVG without permeabilisation, a larger fraction of VSVG had
reached the plasma membrane in BARS-injected cells at the end of the 40 min
chase, as compared to the control cells (Fig. 5.1A; B). However, at the beginning
of the chase (20 °C, time 0) the plasma membrane of both BARS- and mock-
injected cells was devoid of fluorescence (Fig. 5.1A), thus indicating that although
BARS-induced intermediates were forming, they were unable to deliver their
cargo to the cell surface. Given the unusual phenotype of VSVG-positive carrier
formation at the restrictive temperature of 20 °C, BARS was also injected into
Cos7 cells 10 min after the release of the 32 °C temperature block. Interestingly,
although VSVG carriers were already forming following the release of the
temperature block, the BARS injection induced a rapid burst in their formation
that was not observed in GST-injected cells under the same conditions. This
enhanced formation of intermediates was limited in time, as was that observed
when BARS was injected at 20 °C, and it was exhausted after approximately 30
min (Fig. 5.1B).
Fig 5.1. Effects of BARS microinjection on post-Golgi transport of VSVG. A. VSV-infected cells were incubated at 20 °C for 1 h, injected with GST (control, upper panels) or BARS (lower panels), and assayed for post-Golgi transport as described in methods. Inserts show the surface staining of VSVG assessed by immunofluorescence. Scale bars, 10 μm. B. Cos7 cells treated as above were shifted to 32 °C, fixed at the indicated times, and treated for immunofluorescence. Alternatively, Cos7 were injected 10 min. after the release of the temperature block (green line). VSVG-positive carriers were counted (left chart) and VSVG fluorescence at the cell surface (right chart) measured at the respective time points. All values are means ± s.d. from three independent experiments. Key: blue line, GST-injected cells; pink line, BARS-injected cells.
5.3. BARS-induced VSVG-positive carriers are *bona fide* post-Golgi transport intermediates

Considering that, as indicated above, the 20 °C temperature block is usually considered to be non-permissive for the formation of post-Golgi transport carriers (Griffiths et al. 1985), a morpho-functional analysis was carried out on the BARS-induced VSVG-positive structures to determine their characteristics.

5.3.1. BARS-induced carriers are compositionally and morphologically the same as post-Golgi transport carriers

Recent studies from Polishchuk and colleagues (Polishchuk et al. 2003) have characterised the protein composition of post-Golgi carriers. This included a listing of the proteins that are normally retained within the Golgi complex and those that are instead sorted into constitutive post-Golgi carriers. Based on this characterisation, an immunofluorescence analysis was carried out on VSVG-positive structures in BARS-injected cells before the release of the 20 °C temperature block, when the presence of these structures is more pronounced (Fig. 5.1A; B).

First, to exclude the possibility that these structures were pre-Golgi carriers on the way from the ER to the Golgi complex, they were tested for the presence of GM130 and COPI coatomer. Neither of these two proteins co-localised with these VSVG-positive structures in BARS-injected cells, and co-localisation was only
detectable at the level of the Golgi complex, where a large fraction of VSVG was still present (Fig. 5.2A).

The transmembrane protein TGN46 is a typical marker of post-Golgi transport carriers and it is present in the large majority of constitutive post-Golgi carriers (Polishchuk, Di Pentima et al. 2003); (Ponnambalam et al. 1996; Banting et al. 1997). When the BARS-injected cells were tested for TGN46, this marker was present on approximately 80% of the VSVG-positive structures induced by BARS microinjection (Fig. 5.2A).

Finally, to rule out the possibility that these structures were induced by a non-specific fragmentation of the Golgi complex rather than by carrier formation from pre-formed exit sites, the presence of a typical Golgi-resident protein that is normally excluded from post-Golgi carriers, sialyl-transferase (Polishchuk et al. 2003), was tested for. Again, sialyl-transferase could only be detected at the level of the Golgi complex and it was excluded from all of the VSVG-positive structures (Fig. 5.2A).

The VSVG carriers were also analysed by correlative-video electron microscopy (CVEM; Polishchuk et al. 2003). Briefly, the injected cells were localised on a CELLocate grid by immunofluorescence and then processed for immuno EM (Section 2.13.4.). At the ultrastructural level, the sizes and shapes of these BARS-induced VSVG carriers were the same as those of control cells, as they were pleiomorphic structures of an average size of 100 nm (Fig. 5.2C). Furthermore, the structure of the whole Golgi complex was analysed under EM before the
release of the temperature block (20 °C). Here, the control cells presented a
typical Golgi complex that was organised into stacked cisternae. The large
tubulated TGN that was at one pole of the stack contained a large fraction of the
VSVG that was trapped by the temperature block (Fig. 5.2B). The BARS-injected
cells also preserved a stacked organisation of their cisternae, the morphology of
which appeared unaffected by the BARS injection (Fig. 5.2B). The more evident
phenotype was visible at the level of the TGN, which was still largely labelled by
VSVG, but which appeared to be fragmented into smaller structures (Fig. 5.2B).
Interestingly, it was also possible to observe the presence of tubular structures
with constrictions, similar to the fission intermediates observed in in vitro
preparations of Golgi membranes that were incubated with purified BARS (Fig.
5.2B; Weigert et al. 1999).
Fig 5.2. Characterization of the VSVG-positive intermediates formed during the 20 °C block. A. immuno-fluorescence staining of control (upper panels) and BARS-injected (lower panels) cells. The majority of BARS-induced intermediates co-localized with a TGN marker (TGN46; green), but did not co-localize with cis-Golgi markers (GM130, COP; green). Scale bars 10 μm. B. Correlative light-electron microscopy of GST-injected (control) and BARS-injected (BARS) cells fixed during the 20 °C temperature block, and stained and revealed for VSVG by immuno-nanogold and gold enhance labeling (black irregular dots). The first two panels show a Golgi stack (S) and the TGN (T); the TGN is preserved in control cells but fragmented in BARS-injected cells (arrows in centre panel). The right-hand panel shows higher magnification of a tubular structure that is reminiscent of the ‘fission intermediates’ (arrows). C. higher magnifications of transport intermediates formed in control and BARS-injected cells (BARS). Scale bars, 150 nm.
5.3.2. The VSVG-GFP-carrier dynamics are similar in control and BARS-injected cells

From the functional point of view, post-Golgi carriers move across the cytoplasm in a “stop-and-go” fashion as a result of their anchoring to microtubules. In this way, they eventually deliver their cargo to the cell surface (Hirschberg et al. 1998). To determine if this was also the case for BARS-induced carriers, VSVG-GFP was transfected into Cos7 cells, and after the BARS injection, the cells were analysed at the video microscope before being fixed (2.12.1.). At the release of the temperature block, the mock-injected cells started forming VSVG-GFP-positive carriers, mainly from tubular precursors emanating from the fluorescent Golgi mass, as previously described (Hirschberg et al. 1998). Once formed, these structures moved across the cytosol in a saltatory manner (movie 3). With the help of particle tracking software (Section 2.12.1.), it was possible to record the overall net movements of these carriers and to demonstrate that they were oriented towards the plasma membrane (Fig. 5.3A). The VSVG-GFP transport in BARS-injected cells appeared the same as that of mock-injected cells, with the obvious exception that with BARS-injection the VSVG-GFP-positive carriers were more abundant at the release of the temperature block (movie 4). The major differences were observed in carrier formation at the level of the Golgi complex. Here, the carriers did not derive from the fission of tubular structures, as these were never visible. Rather, the VSVG-positive carriers appeared to “pop out” of the fluorescent Golgi mass as already formed structures (movie 4), suggesting that the fission event occurred early after carrier precursor formation, thus prior to the formation of tubules that extend out of the Golgi mass.
Fig 5.3. BARS-induced post-Golgi carries move toward the cell periphery. Cos7 cells were transfected with VSVG-GFP and incubated overnight at 40 °C. The cells were then incubated at 20 °C for 1 h, mock injected (A) or injected with recombinant BARS (B), and shifted to 32 °C 1 h after the injection. Images were captured every 4 s by video microscopy (movie 3; 4), and 40 inverted images were overlaid using particle analysis software. The left-hand panels show the distribution of VSVG-GFP at the time of the temperature shift to 32 °C, and the right-hand panels track the routes of single post-Golgi carriers towards the cell periphery. The arrows indicate examples of the directions of motion of individual carriers. Scale bars 5 μm.
Also in this case, the particle tracking analysis indicated that once they were formed, the VSVG-positive carriers moved in an overall centrifugal direction (Fig. 5.3B).

5.3.3. BARS-induced VSVG carriers deliver their cargo to the cell surface

Although a correlation was observed between an increase in VSVG-containing carriers and a larger fraction of VSVG at the cell surface (Fig. 5.1B), the final destination of the BARS-induced carriers was further investigated using two independent approaches. First, VSV-infected Cos7 cells were subjected to the post-Golgi transport assay and injected with BARS, as previously described (Section 2.8.3.). At the release of the temperature block, 0.5% tannic acid was added to the incubation medium to inhibit carrier fusion with the plasma membrane (Newman et al. 1996; Polishchuk et al. 2004). In control cells, the VSVG-positive carriers formed shortly after the release of the temperature block and moved towards the plasma membrane (Fig. 5.4, top panels). Once they had reached their destination, as they were unable to fuse with the plasma membrane and deliver their content due to the inclusion of the tannic acid, they accumulated as small punctate structures all around the edge of the cell (Fig. 5.4, top panels). Other carriers were still visible throughout the cytoplasm, which were representative of intermediates that were still forming at the level of the Golgi complex, and also of intermediates that had already docked at the plasma membrane before reaching the edge of the cell (Fig. 5.4, top panels). As expected, the BARS-injected cells already presented a large number of intermediates at the release of the temperature block (Fig. 5.4, bottom panels).
Fig 5.4. BARS-induced post-Golgi carriers deliver their cargo to the PM. VSV-infected Cos7 cells were incubated for 2 h at 40 °C. The cells were then incubated at 20 °C for 1 h, injected with GST (Control; upper panels) or recombinant BARS (BARS-injected; lower panels), and shifted to 32 °C in the presence of 0.5% tannic acid 1 h later. The cells were then fixed either at the end of the 20 °C block (t0), or 20 min (t20) or 40 min (t40) after the shift to 32 °C, and analyzed by immuno-fluorescence microscopy. Scale bars 10 μm.
Further on during the chase, and again with fusion with the plasma membrane being inhibited by tannic acid, the number of intermediates increased until the end of the 40-min chase, where the large majority of the intermediates were seen to be decorating the edge of the cell (Fig. 5.4, bottom panels). This observation is in agreement with the evidence collected from the particle tracking analysis of live imaging of BARS-injected cells, and also confirms the observation that after microinjection of BARS, not only the formation of VSVG transport carriers is stimulated, but also the delivery of the protein to the cell surface, as assessed by the antibody staining for VSVG in non-permeabilised cells (Fig. 5.1B).

In a second approach, the Cos7 cells were transfected with VSVG-GFP and subjected to the post-Golgi transport assay (Section 2.8.3). As before, after the first hour at 20 °C the cells were injected with BARS-GST, and then further incubated at 20 °C. Before the release of the temperature block, the cells were moved to a thermostatic stage at the confocal microscope and the Golgi area was bleached (Section 2.12.2.), leaving the VSVG-GFP-positive intermediates induced by the BARS microinjection unperturbed (Fig. 5.5A). In this way, at the release of the temperature block only the intermediates formed before the bleaching remained, and it was therefore easier to study their dynamics by fluorescence recovery after bleaching (FRAP; Section 2.12.2.). For instance, pre-Golgi carriers would partially restore the Golgi fluorescence during the 32 °C chase by moving into the bleached area, whereas post-Golgi carriers would gradually disappear as they deliver their fluorescence to the cell surface, thereby leaving the overall cell fluorescence unperturbed. Indeed, during approximately 12 min of chase, there was a gradual disappearance of VSVG-GFP-positive
carriers (movie 5, Fig. 5.5A; B), in that they decreased from an average of 50 per cell to 10 per cell, and in some cases it was also possible to see single fusion events close to the edge of the cell (movie 5). When monitoring fluorescence recovery, it was possible to see a minimal recovery of fluorescence that remained diffuse and was not concentrated in the Golgi area, whereas the rest of the cell maintained the same level of fluorescence during the chase, although the highly fluorescent intermediates had almost totally disappeared by the end of the chase (Fig. 5.5A; B). This indicates that: a) cell-surface-associated VSVG diffused within the bleached area; and most importantly, b) the fluorescence contained within the VSVG-GFP-positive intermediates was “transmitted” to the plasma membrane. Altogether, the FRAP experiments indicated that VSVG-GFP-positive carriers were indeed not moving towards the Golgi complex, but rather to the plasma membrane, where they eventually delivered their cargo, thus increasing the fluorescence of the plasma membrane itself.
Fig 5.5. BARS-induced post-Golgi carriers deliver their cargo to the PM. A. Cos7 cells were transfected with VSVG-GFP and incubated overnight at 40 °C. The cells were then incubated at 20 °C for 1 h, injected with recombinant BARS, and shifted to 32 °C 1 h later. Before the release of the temperature block, the Golgi area was photobleached (central circle), and the dynamics of VSVG-containing carriers were analyzed under confocal video microscopy (movie 5). The sample images, from those captured every 4 s for a total time of 13 min (with the pinhole fully open), show: the distribution of BARS-induced post-Golgi carriers before bleaching of the Golgi (Pre-bleach), the same cell just after the bleach (Bleach) and at the end of the acquisition (End). Scale bar, 10 μm. B. Quantification of Golgi-associated fluorescence (blue), total cell fluorescence after subtraction of the total Golgi fluorescence (red), and total number of post-Golgi carriers observed during the acquisition described in D. (green triangles).
5.4 The formation of BARS-induced VSVG-positive carriers is concentration dependent

To further characterise the effects of BARS injection on transport, several BARS dilutions were injected and tested for their ability to stimulate carrier formation. Starting from the maximum concentration obtained from the protein purification of 2 mg/ml, various dilutions were injected, and the cells were subjected to the post-Golgi transport assay, with measurements taken of the number of VSVG-positive transport carriers formed at 20 °C (Fig. 5.6). Notably, BARS was still fully active when injected at 1 mg/ml and its activity was only slightly reduced when injected at 0.5 mg/ml. At this latter concentration, BARS was able to induce the formation of an average of 48 intermediates/cell, as compared to 58 induced at 2 mg/ml (Fig. 5.6). Below the concentration of 0.5 mg/ml, there was a continued concentration-dependence, although BARS was still able to induce the formation of 36 intermediates/cell at 0.1 mg/ml, with this activity was not being detectable at 0.05 mg/ml (Fig. 5.6). This evidence is relevant in any discussions around the specificity of this BARS effects on the fissioning of membranes, since it demonstrates the efficiency of the protein at concentrations that are actually only a few-fold higher than those estimated for endogenous BARS.
Fig 5.6. Concentration-dependence of BARS effects on post-Golgi transport carriers formation. Cos7 cells were infected with VSV and subjected to the post-Golgi transport assay. After 1 h at 20 °C, cells were microinjected with increasing concentrations of BARS or with a BARS-BARS$^{D355A}$ mix at the indicated concentrations and further incubated at 20 °C for another hour. Cells were then fixed and prepared for immunofluorescence and VSVG-positive carriers were quantified. Values are means ±s.d. of three independent experiments.
5.5. Discussion

Given the presumed secondary effects induced by BARS overexpression, the approach for the study of the role of BARS on Golgi membrane fission was modified to analyse the acute effects of a more regulated increase in the intracellular concentration of BARS. By exploiting the GST-tagged purified protein, VSV-infected cells were microinjected at 20 °C when VSVG was trapped in the Golgi complex, before releasing the temperature block. Strikingly, after only a short incubation, BARS-GST was able to stimulate an explosive formation of VSVG-positive carriers when cells were still being incubated at 20 °C, whereas the GST control injections had no effects (Fig. 5.1). In a very detailed study in 1985, Griffiths and colleagues (Griffiths et al. 1985) showed that reducing the temperature to 20 °C is sufficient to reversibly arrest VSVG within the TGN. This observation, together with previous studies from Matlin and Simons in 1983 (Matlin et al. 1983) relating to the effects of the same temperature block on the secretion of the influenza virus hemagglutinin, have suggested that reducing the temperature to 20 °C should be considered as a universal tool for the study of protein secretion. However, the molecular mechanisms underlying this temperature block have never been investigated, and since the only variable parameter is temperature, it has been postulated that low temperatures reduce membrane plasticity, thereby inhibiting transport carrier formation. Nevertheless, at 20 °C, other fission events still take place in other cellular compartments, such as the formation of endocytic vesicles at the plasma membrane (Griffiths et al. 1985; van Deurs et al. 1988), and the enrichment of tubular structures at the TGN. This would thus suggest that perhaps the block in secretion has something to do
with some protein machinery problems rather than with membrane plasticity. It is therefore difficult to imagine the precise scenario that BARS encounters when microinjected at 20 °C. It has been shown that a block of VSVG transport at the TGN results in a marked enlargement of this compartment (Griffiths et al. 1989). It is possible that under these conditions, BARS can act on the wide membranous surfaces that are already arranged into tubular structures, which would facilitate the fission process. However, microinjection of BARS at the permissive temperature of 32 °C also indicates that the stimulation of carrier formation is not dependent on the temperature block.

When analysed for their morphology, composition and dynamics, these intermediates were apparently bona fide post-Golgi transport carriers (Fig. 5.2). Moreover, although judging from the protein composition of these carriers the sorting at the level of the TGN was as efficient as that in control cells, there was a total absence of tubular structures emanating from the Golgi complex in BARS-injected cells (movie 4). Post-Golgi carriers exited the fluorescent Golgi mass as already formed structures, suggesting that the increase in the levels of cytosolic BARS induced an acceleration of the fission process, thereby inhibiting tubule growth. Besides the relevance of this stimulation of post-Golgi transport induced by BARS microinjection, this also supports the hypothesis that the overexpression of BARS induces lipid modifications on Golgi membranes, which alter its actual effects on membrane fission. To determine if this is indeed the case, BARS-injected cells were incubated overnight at 37 °C prior to the VSV infection and post-Golgi transport assay. As expected, VSVG transport to the cell surface was inhibited at the level of the Golgi complex in BARS-microinjected cells, whereas it was unperturbed in control GST-injected cells (Fig. 5.7).
Fig 5.7. Prolonged exposure to microinjected BARS inhibits post-Golgi transport. Cos7 cells were microinjected with GST (upper panels) or BARS (lower panels) and incubated overnight at 37 °C. The following day cells were infected with VSV and subjected to the post-Golgi transport assay. After the release of the temperature block to 32 °C cells were fixed at the indicated times and processed for immunofluorescence to follow post-Golgi transport of VSVG.
CHAPTER 6

Inhibition of BARS activity blocks post-Golgi transport of VSVG at the level of the TGN

In a second approach to investigate the effects of BARS on VSVG transport, microinjection techniques were exploited to microinject VSV-infected cells with a set of BARS mutants. These mutants were prepared based on the recently reported BARS crystal structure (Nardini et al. 2003). Structurally, BARS is similar to a family of dehydrogenases that are comprised of two regions, the nucleotide-binding domain (NBD; aa113-308), which is involved in the binding of NAD(H) and acyl-CoAs, and the “substrate”-binding domain (SBD; containing both the C- and N-termini, aa1-112 and 309-430, respectively), which is required for the binding of E1A (Fig. 6.1; Nardini et al. 2003). For this part of the study, two point mutants and three deletion mutants were tested to screen for their potential inhibitory activities: (i) a G172E mutant of the NBD that has a point mutation that totally removes the ability of NBD to bind to NAD (Nardini et al. 2003); (ii) a D355A BARS mutant (BARS<sup>D355A</sup>) that has a point mutation in the C-terminus that could be critical for fission; and (iii) the NDB and SBD themselves and a truncated form of BARS (BARS-1-350) that lacks the last 80 aa (Fig. 6.1B). First, the intracellular localisation of these mutants was determined by protein microinjection into non-infected cells at steady-state (Fig. 6.2), and then the effects of each mutant on the intracellular transport of VSVG was determined (Fig. 6.4).
Fig 6.1. A. Schematic representation of the three-dimensional structure of the BARS 1-350 deletion mutant (PDB code: 1HKU; GenPept accession number: AAC79427.2), depicting SBD and NBD. B. Schematic representations of wtBARS and the mutants used in the present study.
6.1. Intracellular localisation of the BARS mutants

The various mutant proteins were microinjected into Cos7 cells at 37 °C, without any accumulation of cargo proteins (Section 2.6.1.). One hour after microinjection, the cells were fixed and labelled with the SN1 anti-BARS antibody. Due to the low sensitivity of the SN1 antibody, the endogenous levels of BARS could not be detected, and thus the fluorescent signal detected indicated the localisation of the microinjected proteins only.

As mentioned above, the G172E mutant has a point mutation in the NBD where glycine is substituted with glutamate. A biochemical characterisation of the mutant (Nardini et al. 2003) has shown that this mutation is sufficient to impair the binding of NAD, due to the amino acidic change that blocks access to the cleft where NAD should localise. Interestingly, the intracellular localisation of this mutant protein showed why BARS-G172 itself is not present at the Golgi complex and remains completely cytoplasmic (Fig. 6.2).

The BARS\textsuperscript{D355A} mutant has a point mutation in the C-terminus, where an aspartate has been changed into an alanine. A precise biochemical characterisation of this mutant is not yet available because this part of the protein was not present in the published BARS structure, as the crystal was obtained from a truncated form of BARS (Nardini et al. 2003). The D355 residue was chosen because it belongs to a cluster of charged aminoacids towards the C-terminus that are supposed to be critical for fission. When microinjected, BARS\textsuperscript{D355A} forms very peculiar and
convoluted ribbons (Figs. 6.2; 6.3) that originate from a centrosomal/Golgi area and extend throughout the cytoplasm. Of note, similar structures were observed when CtBP1 was overexpressed in Cos7 cells, and in some cases, when BARS was overexpressed and the cells were fixed after an overnight incubation at 40 °C. So far, even with the use of several antibodies against the most common Golgi complex markers and other proteins involved in the secretory pathway, it has remained difficult to interpret this pattern (Fig. 6.3). When used to visualise these structures, the SN1 polyclonal anti-BARS antibody cross-reacts with every other antibody used, probably because of the abundance of protein contained in these ribbons. Better results were obtained when a monoclonal anti-BARS antibody (known as BC3; Section 2.11. and table 2.2) was used. Here, the BARS labelling was totally excluded from the Golgi complex, although the ribbons appeared to originate from the Golgi area, sometimes to be wrapped around the whole of the Golgi complex, as seen by immunofluorescence (Fig. 6.3). The presence of VSVG within these structures can be excluded, as when VSVG transport was followed in BARS^{D155A}-microinjected cells the viral protein was never observed to form similar structures (Fig. 6.3). Among the other proteins tested, the presence of GM130 (Golgi marker), kinesin (molecular motor marker) and tubulin can be excluded, as none of these proteins co-localised with these BARS^{D155A}-induced ribbons (Fig. 6.3). The presence of giantin and/or TGN46 remains unclear. For giantin, the co-staining was detected using a different combination of antibodies and the association of this Golgi marker with the ribbons is also preserved after BFA treatment, although the Golgi complex staining is affected (not shown; Section 2.10.1.).
**Fig 6.2. Intracellular localisation of BARS mutants.** Cos7 cells were microinjected at steady state with BARS mutants and incubated 1 h at 37 °C. Cells were then fixed and processed for immunofluorescence to study intracellular localisation of each mutant. The Golgi complex was labelled with the antibody against the protein Giantin. Scale bars 10 μm.
For TGN 46, co-staining was detected using a secondary antibody for BARS that was conjugated with biotin and followed using fluorescent streptavidin to reduce the cross-reactivity for the labelling of the \( \text{BARSD}^{355A} \)-induced ribbons (Fig. 6.3). The nature of these structures is therefore controversial, and it remains unclear whether they contain membranes or if they are solely made up of the polymerised mutant protein. The only information about the dynamics of these structures comes from the observation that the ribbons are temperature sensitive, as they are not present in \( \text{BARSD}^{355A} \)-injected cells that are incubated at 20 °C, whereas they are detectable in these cells when they are incubated at temperatures ranging from 32 °C to 40 °C. Also, when studying the effects of \( \text{BARSD}^{355A} \) on VSVG transport using the 20 °C to 32 °C temperature block, there was an increasing number of cells presenting these ribbons when the temperature was raised from 20 °C to 32 °C. Unfortunately, when overexpressed in Cos7 cells at steady-state the YFP-tagged version of \( \text{BARSD}^{355A} \) did not induce the formation of these convoluted ribbons, so that the dynamics of these structures could not be studied in these living cells.

NBD is the portion of BARS involved in the binding of NAD and acyl-CoA (Nardini et al. 2003). When microinjected, NBD shows a predominant Golgi complex localisation, with reduced cytoplasmic staining (Fig. 6.2). This correlates well with the localisation of the G172E mutant, which is non-functional. There are several ways to interpret this particular localisation, as on the one hand it could be that the NBD contains a Golgi-localisation signal that is able to target the protein to the membranes of the Golgi network. The lack of a large portion of the BARS protein (the SBD) would also make NBD non-functional, and thus unable to cycle.
from the Golgi complex to the cytoplasm, thus inducing its accumulation in the Golgi area. On the other hand, there is the possibility that endogenous BARS is present on Golgi membranes in a tight complex with other interacting proteins. These proteins could “cover” the epitopes required for the binding between BARS and its specific antibodies, thus affecting the detection of a clear Golgi complex localisation. With NBD, this protein complex might be disrupted, giving a better access for the antibodies.

The SBD is the remaining part of BARS when NBD is deleted and it contains a portion of the N-terminus and all of the C-terminus fused together. The microinjection of this deletion mutant reveals its cytoplasmic localisation with no specific enrichment in the Golgi area, which is similar to what was observed when BARS itself was microinjected (Fig. 6.2). This localisation is also in agreement with the above hypothesis: in this case, the protein does not contain the NBD and its localisation at the Golgi complex is very poor, suggesting a role for the NBD in the Golgi complex localisation of BARS.

The last of the deletion mutants tested, BARS-1-350 (truncated BARS) is missing the last 80 aa at the C-terminus, and it also showed a localisation that is very similar to that observed in BARS-injected cells. Thus it shows a predominant cytoplasmic localisation, with no particular enrichment within the Golgi area (Fig. 6.2).
Fig 6.3. Intracellular localisation of injected BARS<sup>D355A</sup>. Cos7 cells were injected with BARS<sup>D355A</sup> at steady state, incubated for 1 hour at 37 °C and processed for immunofluorescence. A set of markers for intracellular membrane transport were tested for their co-localisation with the BARS<sup>D355A</sup>-induced structures. Scale bar, 10 μm.
6.2. The effects of microinjection of the BARS mutants on VSVG transport

Once the intracellular localisation of the different mutants had been characterised, their role in the transport of VSVG was assessed by microinjecting them into VSV-infected Cos7 cells. These cells were then subjected to the post-Golgi transport assay (Section 2.8.3.). The effects of each mutant were characterised in terms of VSVG-positive carrier formation and VSVG delivery to the cell surface, in comparison with that described above for BARS itself (Chapter 5).

6.2.1. SBD microinjection

To assess the in vivo relevance of the BARS LPAAT activity in the regulation of intracellular transport that was described in previous in vitro studies (Weigert et al. 1999), the role of the SBD deletion mutant was tested. Of note, SBD totally lacks the portion of BARS that is required for its binding with acyl-CoA. Interestingly, when microinjected into the cells at 6 mg/ml, SBD was able to increase the post-Golgi transport of VSVG, albeit less potently than BARS itself. As described in the previous chapter, at 20 °C BARS-injected cells showed an average of 70 intermediates/cell as compared to 5 intermediates/cell in GST-microinjected cells. With SBD microinjection, the formation of an average of 46 intermediates/cell was induced (Fig. 6.4A). During the chase, the average number of intermediates in the SBD-injected cells followed the same dynamics as with BARS injection, in that the number of intermediates decreased to normal levels (31 after 20 min of chase, and 20 after 40 min; Fig. 6.4). Accordingly, 40 min
from the release of the 20 °C temperature block, an increase of approximately 20% was observed in the viral glycoprotein at the surface of the injected cells (Fig. 6.4B). This suggests that the LPAAT activity of BARS may have a facilitating (but not necessary) role in fission in vivo. Of note, there is a significant difference between the working concentrations of SBD and BARS here (6 mg/ml compared to 2 mg/ml, respectively). Moreover, dose-response experiments with BARS (as described above) have shown that concentrations as low as 0.1 mg/ml are sufficient to stimulate the formation of post-Golgi intermediates. Altogether, this suggests that although it is able to stimulate the formation of VSVG-positive carriers, the SBD mutant is much less potent than BARS itself. Whether this is due to the loss of the acyltransferase activity or to the fact that a large portion of the protein is missing is difficult to ascertain at this stage.

6.2.2. BARS$^{G172E}$ microinjection

To further evaluate the requirements for the LPAAT activity of BARS in the regulation of post-Golgi transport, the effects on transport of this point mutant were tested, noting that this point mutation is sufficient to inhibit the binding of BARS with acyl-CoA (Nardini et al. 2003). Unexpectedly, the microinjection of this mutant at a concentration of 5 mg/ml had no effects on VSVG transport from the Golgi complex to the plasma membrane, in that the number of VSVG-positive intermediates and the amount of VSVG that reached the plasma membrane during the 32 °C chase were the same as for mock-injected cells (Fig. 6.4). Although the mutant should theoretically have the same ability to act on VSVG transport as SBD, it is important to stress that in contrast to the deletion mutant, BARS$^{G172E}$
does not localise at the Golgi complex (Fig. 6.2), and this may be relevant in terms of regulation of the Golgi-localised fission event.

6.2.3. BARS^{D355A} microinjection

As mentioned above, the microinjection of this mutant induced the formation of convoluted ribbons that contained high levels of the mutant protein (Fig. 6.3). When tested in the post-Golgi transport assay, the microinjection of BARS^{D355A} at a concentration of 3.5 mg/ml interfered with VSVG transport. The number of VSVG-positive carriers was severely reduced to an average of 12 per cell throughout the chase, whereas the control cells showed an average of 40 intermediates per cell after 20 min of chase, and 30 after 40 min (Fig. 6.4A). Accordingly, after 40 min of chase, the VSVG-GFP appeared to be trapped in the Golgi complex and only a weak labelling of the plasma membrane was detected (50% of the control labelling of the cell surface; Fig 6.5B). To better characterise this effect, VSVG-GFP-transfected cells were microinjected with BARS^{D355A} during the 20 °C temperature block. At the release of the temperature to 32 °C, it was possible to observe the formation of long dynamic tubules containing VSVG that emanated from the fluorescent Golgi mass in approximately 70% of the injected cells (Fig. 6.5; movie 6). These tubules formed and then extended from the Golgi complex, but they were essentially unable to undergo fission, as they were seen to move back and forth from their site of origin (Fig. 6.5A; movie 6). Once fixed, the cells were immuno-labelled with TGN46 and the majority of these tubules were positive for this trans-Golgi marker (Fig. 6.5B), thus indicating that
the transport step that is inhibited at the level of the TGN is that of fission, and not of formation, of the carrier precursors. It is important to stress that in no cases did VSVG form structures that resembled the ribbons induced by this BARS mutant, supporting the hypothesis that BARS\textsuperscript{D355A}-induced ribbons are not membranous.

6.2.4. NBD microinjection

The microinjection of NBD at 3.6 mg/ml strongly inhibited Golgi-to-plasma membrane transport, to a similar extent to the inhibition induced by BARS\textsuperscript{D355A} microinjection. At the release of the temperature block, VSVG appeared concentrated in the Golgi area and the number of intermediates labelled by the viral glycoprotein was limited to an average of 14 per cell throughout the chase (Fig. 6.4A). Forty min from the release of the temperature block, only 50% of the control amount of VSVG was detected at the cell surface of these injected cells (Fig. 6.4B). Interestingly, when the transport in living cells expressing VSVG-GFP and injected with NBD was monitored, the microinjection of NBD severely interfered with the movements of the whole Golgi mass, and in contrast to the effects of BARS\textsuperscript{D355A} microinjection, no forming tubules were detected.
Fig 6.4. Effects of BARS mutants microinjection on post-Golgi transport of VSVG. A. Cos7 cells were infected with VSV, incubated at 20 °C for 1 h, injected with GST or BARS mutants (BARS^{G172E}, NBD, SBD), and incubated at 20 °C for a further 1 h. The cells were then shifted to 32 °C, fixed at the indicated times, and treated for immunofluorescence. VSVG-positive carriers were counted for at least 10 cells for each time point. All values are means ± s.d. from three independent experiments. B. Quantification of PM-associated VSVG fluorescence in Cos7 cells treated as described in A, cells were fixed and stained without permeabilisation, using the luminal domain anti-VSVG antibody. All values are means ± s.d. from three independent experiments.
**Fig 6.5. BARS^{D355A} microinjection blocks VSVG transport by inhibiting fission.**

**A.** Representative frames of time-lapse imaging of VSVG-GFP-transfected Cos7 cells injected with BARS^{D355A} during the 20 °C temperature block as described in methods. The images were acquired 20 s (20''), 40 s (40''), 5 min (5'), 10 min (10') and 15 min (15') from 20 min after the shift to 32 °C (0''), and show the formation at and elongation from the Golgi of a VSVG-containing tubule. Even after 15 min of observation, the tubule did not undergo fission (movie 6). Scale bar, 5 μm.

**B.** Correlative Video Immuno Fluorescence (CVIF) indicating the same cell as in A, processed for immunofluorescence after time lapse acquisition and labelled for the trans-Golgi marker TGN46. Arrows indicate two tubular elements co-localising with TGN46.
6.2.5. BARS-1-350 microinjection

Similarly to the effects of the BARS$^{G172E}$, microinjection of this truncated form of BARS at 3.5 mg/ml was also unable to perturb VSVG transport from the Golgi complex. Thus, both the formation of VSVG-positive carriers and the delivery of the viral glycoprotein to the cell surface were not distinguishable from the GST-injected control cells (Fig. 6.4). Interestingly, as with the BARS$^{G172E}$ mutant, BARS-1-350 exhibited a prevalently cytoplasmic localisation (Fig. 6.2), also suggesting that in this case the inability to localise at the level of the Golgi complex impairs the regulation of post-Golgi carrier formation.

6.3. BARS$^{D355A}$ and NBD behave like dominant negatives

Once the role of the different mutants on VSVG transport had been assessed, the two inhibitory mutants, BARS$^{D355A}$ and NBD, were tested in combination with BARS itself to determine whether they were behaving like dominant negatives. Hence, using microinjection, their ability to inhibit the stimulation of BARS-induced post-Golgi carrier formation was investigated. These effects of the single mutants were thus monitored in the post-Golgi transport assay (Section 2.8.3.) after the cells had been injected with a mixture of BARS and increasing concentrations of either BARS$^{D355A}$ or NBD. VSVG-positive carrier formation at 20 °C was used as the read-out.
Starting from the microinjection of BARS alone at 2 mg/ml, increasing concentrations of each mutant inhibited the formation of post-Golgi transport carriers in a BARS-to-mutant concentration-ratio-dependent manner (Fig. 5.6). At an initial 1:1 concentration ratio, the formation of post-Golgi carriers was reduced by 50%, whereas a 1:2 concentration ratio in favour of the mutants reduced carrier formation by 70% (Fig. 5.6). This is in favour of specific and competitive inhibitory effects of both of the two mutants that were selected here, and also indicates the possibility of exploiting their characteristics as true dominant negatives in further investigations. Although efficient in inhibiting post-Golgi transport, NBD appears to have a broad range of effects that eventually immobilise the whole Golgi complex. BARS^{D355A}, on the other hand, appears to be the better dominant negative as it exerts its inhibition at a very specific step, which is the fission of cargo-loaded membranous tubules, with the other transport steps apparently not being perturbed.

6.4. Microinjection of the p50-2 anti-BARS antibody impairs post-Golgi transport

By an independent approach, endogenous BARS activity was inhibited by microinjecting 4 mg/ml of the p50-2 affinity-purified anti-BARS antibody (Section 2.6.1.). As the antibody requires a longer incubation time, the transport assay was slightly modified in that the antibody was microinjected right after VSV infection, and hence during the 40 °C temperature block that causes the accumulate VSVG in the ER. After microinjection, the cells were infected with
VSV and subjected to the post-Golgi transport assay. After 3 h of incubation, the antibody was potently inhibitory on the formation of post-Golgi transport carriers. An average of 2 VSVG-positive intermediates were produced per cell after 20 min of chase, and their number increased to approximately 4 after 40 min, compared to 24 and 27 intermediates/cell at 20 and 40 min of chase, respectively, in control IgG-injected cells (Fig. 6.6A). When monitoring VSVG delivery to the cell surface in non-permeabilised cells, the antibody microinjection inhibited VSVG transport by more than 90% (Fig. 6.6B). Of note, when it was revealed using a secondary antibody that was tagged with a fluorophore, p50-2 was mainly localised at the level of the Golgi complex and the cytoplasmic signal was strongly reduced, as compared to the signal obtained by conventional immunofluorescence on fixed samples (Fig. 6.6C). This could be because cell fixation induces a re-location of BARS away from the Golgi complex, thus enriching the cytoplasmic fraction.
Fig 6.6. Effects of BARS antibody microinjection on post-Golgi transport of VSVG. A. Cos7 cells were infected with VSV, incubated at 40 °C for 1 h, injected with IgG or the anti-BARS antibody p50-2, and incubated at 40 °C for a further hour. The cells were then treated for the post-Golgi transport assay as described in methods, fixed at the indicated times, and treated for immunofluorescence. VSVG-positive carriers were counted for at least 10 cells for each time point. All values are means ±s.d. from three independent experiments. B. Quantification of PM-associated VSVG fluorescence in Cos7 cells treated as described in A, cells were fixed and stained without permeabilisation, using the luminal domain anti-VSVG antibody. All values are means ±s.d. from three independent experiments. Key: dashed line, IgG-injected cells; black line, p50-2-injected cells. C. Cos7 cells treated as in A and processed for immunofluorescence. BARS localization is mainly concentrated in a perinuclear region corresponding to the Golgi apparatus and in the nucleus. Scale bar, 10 μm.
6.5. Discussion

The evidence collected from the inhibition of endogenous BARS is in agreement with an important role for BARS in the formation of constitutive post-Golgi transport carriers originating at the level of the TGN. This further strengthens the hypothesis of the presence of a specific BARS-regulated machinery operating at the level of the Golgi complex that is able to modulate transport to the plasma membrane. Furthermore, the data collected from BARS\textsuperscript{D355A}-injected cells indicate that BARS is specifically required for the fission of membranous carrier precursors, whereas the earlier steps of cargo transport, such as protein segregation into carrier precursors and the formation of membranous tubules (Polishchuk et al. 2003), are BARS independent. Moreover, the observation that BARS\textsuperscript{D355A} behaves like a dominant negative will be a great help for the further understanding of the role for BARS in intracellular transport.

Of note, although both BARS overexpression and BARS\textsuperscript{D355A} microinjection are inhibitory on the post-Golgi transport of VSVG, the phenotypes observed are different. BARS overexpression appears to affect post-Golgi transport at an early step; namely, the formation and protrusion of membranous tubules that contain cargo proteins. In contrast, BARS\textsuperscript{D355A} microinjection does not affect the formation of tubules (Fig. 6.5), but inhibits their fission instead. Judging from the previous characterisation of BARS effects on Golgi membranes performed in in vitro systems (Weigert et al. 1999), it appears that overall microinjection is the best tool to study the role of BARS in intracellular membrane transport.
Moreover, \( \text{BARS}^{\text{D355A}} \) can be used here as a specific inhibitor, in that it selectively blocks the BARS-dependent step in the formation of transport carriers. This combined evidence also suggests that BARS overexpression is indeed not a suitable tool for the investigation of its role in membrane transport, since not only does it not induce the same phenotype as the microinjected purified protein, but the inhibition of VSVG transport also does not reflect that induced by the microinjection of the dominant-negative mutant \( \text{BARS}^{\text{D355A}} \).

Two of the mutants tested (\( \text{BARS}^{\text{G172E}} \) and \( \text{BARS-1-350} \)) that showed no specific enrichment on the Golgi membranes were inactive in the regulation of post-Golgi transport of VSVG. This suggests that the interaction of BARS with membranes is required for its activity, and possibly that there are different sites that are important for these interactions within the protein. Indeed, a point mutation within the NBD of BARS is sufficient to abolish both its activity and Golgi localisation (the G172E mutant). Similarly, there also appears to be a domain relevant for membrane targeting within the C-terminus, judging by the cytoplasmic localisation of the \( \text{BARS-1-350} \) deletion mutant.
CHAPTER 7

Depletion of BARS by oligonucleotide treatment and RNAi

7.1. Oligonucleotide treatment

To assess the effects of long-term inhibition of endogenous BARS activity, two antisense oligonucleotide sequences were designed that were able to suppress the synthesis of either CtBP1 or BARS (CtBP3) in NRK cells (Section 2.7.1.). The synthesis of CtBP2 was not affected by these antisense oligonucleotides, and the control cells were treated with the same nucleotide bases arranged in a scrambled order. FASTA searches for both of the oligonucleotide sequences indicated that these anti-CtBP1 and anti-BARS oligonucleotides were specific for the selected proteins, whereas the scrambled control oligonucleotides had no specific targets. Time-course experiments with different concentrations of the oligonucleotides added to the cell culture medium (Section 2.7.1.) showed that a 5-day treatment of NRK cells with 8 μM oligonucleotides reduced the synthesis of their target protein to 30% of the control cells, whereas the same treatment with the scrambled sequences had no effects (Fig. 7.1).
Fig 7.1. Antisense oligonucleotide treatment reduces BARS concentration. A. NRK cells treated for 5 days with anti-BARS antisense oligonucleotides (Antisense), a scrambled oligonucleotide sequence (Scrambled) or left untreated (Control) as described in methods, were scraped and processed to measure intracellular BARS content. Tubulin concentration was also monitored for reference. B. NRK cells treated as in A were processed for immunofluorescence to monitor intracellular BARS Scale bars 20 μm.
7.1.1. Morphological effects of BARS depletion

From a morphological point of view, the oligonucleotide-treated cells appeared much bigger in size, although both microtubules and actin filaments were not affected (Figs. 7.1B; 7.2C). Interestingly, when the cells were labelled with the p50-2 anti-BARS antibody (which recognises all three members of the CtBP protein family at endogenous levels), although the cytoplasmic fluorescence was reduced, it was still clearly detectable. The nuclear fluorescence, which should derive from CtBP2, was instead completely lacking (Fig. 7.1B). As the antisense sequences had no similarity with CtBP2, the intracellular levels of CtBP2 should have been conserved. Thus there was the possibility that CtBP2 was exiting the nucleus to relocate to the cytoplasm in order to supplement the reductions in the levels of CtBP1 and BARS. Indeed, a 7-h treatment of BARS-depleted NRK cells with leptomycin B, which blocks protein export from the nucleus (Wolff et al. 1997), significantly reconstituted the nuclear fluorescence, indicating that CtBP2 does indeed cycle from the nucleus to the cytoplasm.

The morphology of the Golgi complex was then examined both at the immunofluorescence and at the ultrastructural level (Fig. 7.2A; B). Through the revealing of the Golgi structures using anti-mannosidase II and anti-TGN38 antibodies, at the immunofluorescence level the BARS-depleted cells presented an extended network of anastomotic tubules that extended from a perinuclear area, instead of the compact Golgi structures observed in mock-treated cells (Fig. 7.2A). Interestingly, at the ultrastructural level BARS-depleted cells showed a
meshed network of membranes that were difficult to distinguish, whereas control and mock-treated cells presented organised Golgi structures where the cisternae were clearly distinguishable, as well as the cis- and trans-Golgi networks (Fig. 7.2B). To further investigate the reasons for the unusual size of the treated cells, they were labelled with antibodies to the H1 and H3 phosphorylated histones, a treatment that allows the identification of the cell-cycle phase (Hidalgo Carcedo et al. 2004). This was on the basis that the unusual size could reflect a block of entry into mitosis, thus arresting the cells at the end of their pre-mitotic growth. This was also based on the sub-confluency of the oligonucleotide-treated cells after 5 days of treatment. Indeed, approximately 90% of the oligonucleotide-treated cells were synchronised in the G2 phase of the cell cycle (Fig. 7.3A).
Fig 7.2. BARS depletion by oligonucleotide treatment induces severe changes on Golgi morphology. NRK cells treated for 5 days with anti-BARS antisense oligonucleotides (Antisense) or with a scrambled oligonucleotide sequence (Control) and processed for immunofluorescence (A) or immuno-nanogold and gold enhance labeling (B, black irregular dots). Golgi membranes labeled with an anti ManII antibody appeared as a large network of tubulated membranes both at the immunofluorescence level and at the ultra structural-level. Despite the larger size of BARS-depleted cells the cytoskeleton appeared unperturbed when visualised by means of an anti-tubulin antibody (C). Scale bars 10 μm A, C; 300 nm B.
7.1.2. BARS depletion inhibits the post-Golgi transport of VSVG

In light of the morphological effects of BARS depletion on the Golgi complex, the oligonucleotide-treated cells were tested for VSVG transport. After 5 days of treatment, the cells were infected with VSV and subjected to the post-Golgi transport assay (Section 2.8.3.), with VSVG arrival at the cell surface monitored using the antibody against the lumenal domain of VSVG.

Control cells fixed at 20 °C showed no VSVG at the cell surface, as expected. However, cells fixed at later times during the chase (20 and 40 min.) showed increasing amounts of VSVG on the plasma membrane (Fig. 7.3B; C). By comparing non-treated, mock-treated and oligonucleotide-treated cells, it was possible to observe how the VSVG that was detectable at the plasma membrane was significantly reduced in the BARS-depleted cells (by around 50%; Fig. 7.3C).

Considering the documented block of the cell cycle that is induced by BARS depletion, VSVG transport was also tested in cultured NRK cells that had been synchronised in G2 by means of combined treatments with aphidicolin and bisbenzimide (Section 2.10.4.). This approach was used to verify that the inhibition of VSVG transport was not a secondary effect of the block in the cell cycle. The synchronised cells were then transfected with VSVG-GFP and subjected to the post-Golgi transport assay, as above. Here there was no detectable delay in VSVG transport to the cell surface in cells synchronised in G2, as compared to control cells.
Fig 7.3. BARS depletion by oligonucleotide treatment arrests the cell cycle in G2 and inhibits secretion of VSVG to the PM. A. NRK cells treated with anti-BARS oligonucleotides as described in methods were fixed and processed for immunofluorescence using a combination of antibodies against histones to reveal the different phases of the cell cycle. B. NRK cells treated as in A were infected with VSV and assayed for post-Golgi transport as described in methods. After 40 minutes of chase cells were fixed and stained without permeabilisation, using the luminal domain anti-VSVG antibody. Scale bars 10 µm. C. Quantification of PM-associated VSVG fluorescence in NRK cells treated as described in B. Values are means ± s.d. of three independent experiments. Key: white bars, untreated cells; gray bars, scramble oligonucleotide-treated cells; black bars, anti CtBP1&3 oligonucleotides-treated cells.
Since this conventional oligonucleotide treatment was effective only after 5 days of incubation and resulted in a perturbation of the progression through the cell cycle, BARS was also depleted by an independent approach: using RNA interference (RNAi) in Cos7 cells. For these experiments a “smart pool” of 4 different siRNA sequences was used that was designed to inhibit the synthesis of CtBP1 and BARS (CtBP3). The suppression of both proteins was chosen because: (i) the sequence identity between CtBP1 and 3 is too high to select the inhibition of only one protein; (ii) the sequence identity raises the possibility of redundant functions and (iii) only the rat sequence of BARS has been deposited, it is therefore impossible to design a specific anti-BARS siRNA sequence to be used in higher mammals. To set up the conditions for the suitable and specific protein depletion, the cells were treated for 48, 72 and 96 h with increasing concentrations of the siRNAs (ranging from 20 nM to 100 nM; Section 2.7.2.). After each incubation, the cell lysates were loaded onto polyacrilamide gels to determine protein expression. After 48 h of treatment with 50 nM siRNAs, the CtBP1 and BARS levels were efficiently decreased by some 90%. At the same time, QBP2 expression was not affected, as seen for the GAPDH expression that was monitored as the marker for protein synthesis (Fig. 7.4A). Increasing the concentrations of the siRNAs did not significantly improve this CtBP1 and BARS depletion, while longer exposures to the siRNAs (72 and 96 h) were toxic and induced apoptosis in the treated cells. Under all of the conditions tested, the mock transfections were ineffective (Fig. 7.4A).
Fig 7.4. siRNA treatment reduces BARS concentration and inhibits post-Golgi transport of VSVG. A. Cos7 cells were transfected as indicated and incubated for 48h at 37 °C. Cell lysates were run on a polyacilamide gel to assay RNAi effects. GAPDH concentration was measured to monitor overall protein synthesis. B. Cos7 cells treated as in A were transfected with VSVG-GFP or P75-GFP and subjected to the post-Golgi transport assay. VSVG or P75 fluorescence at the cell surface (see methods) was measured and plotted over time to monitor transport efficiency. Values are means ±s.d. of three independent experiments C. Cos7 cells treated as in A were fixed at the end of the 20 °C block and immunolabelled for the trans-Golgi marker TGN46.
7.2.1. BARS depletion by RNAi enriches the number of cells in the G2 phase of the cell cycle

Since BARS depletion by oligonucleotide treatment caused the synchronisation of the treated cells in the G2 phase of the cell cycle, the siRNAs-treated cells were assayed after 48 h of incubation for their progression through the cell cycle. This was achieved using the same combination of antibodies (Chapter 7.1.1.) that were used to monitor the oligonucleotide treated cells. Indeed, after the RNAi treatment, the population of cells in G2 was enriched by approximately 25% as compared to the control cells. This enrichment, compared to the almost total synchronisation induced by oligonucleotide treatment, was not unexpected since although it is equally efficient, RNAi requires significantly shorter incubations that were possibly insufficient for total synchronisation. Unfortunately, as longer exposures to the siRNAs were toxic to the cells, it was not possible to directly test this hypothesis. Moreover, the cell size and the Golgi complex morphology were not altered by this RNAi.

7.2.2. BARS depletion by RNAi inhibits the post-Golgi transport of VSVG-GFP

Cos7 cells were then co-transfected with the siRNAs and VSVG-GFP (Section 2.7.2.). After 44 h, the cells were shifted to 40 °C for 4 h and then subjected to the post-Golgi transport assay (Section 2.8.3.). At the release of the temperature block from 20 °C to 32 °C, the VSVG-GFP was concentrated within the TGN (as indicated by co-localisation between VSVG-GFP and TGN46 measured by immunofluorescence staining; Fig. 7.4C) both in the control and the siRNAs-
treated cells, suggesting that BARS depletion does not affect ER-to-Golgi transport or intra-Golgi transport. However, during the chase, VSVG-GFP was seen to be efficiently transported to the plasma membrane in the control cells (by measuring the fluorescence of plasma-membrane-associated VSVG detected using the anti-VSVG antibody staining on non-permeabilised cells), whereas it was strongly inhibited (by around 85%) in the siRNAs-treated cells (Fig. 7.4B). As a control, siRNA treated cells were monitored for post-Golgi transport of the apical transport marker p75 (Section 2.8.3). BARS depletion was ineffective on such transport step (Fig. 7.4B).

7.3. Discussion

As well as using the expression or the microinjection of dominant-negative mutants, one of the best ways to investigate the role of a protein in cellular systems is through its depletion by means of antisense oligonucleotides. To reduce endogenous BARS, both traditional antisense oligonucleotide treatments in NRK cells and RNAi in Cos7 cells were used.

The depletion of the endogenous BARS using antisense oligonucleotide treatment induces severe changes on the overall cell morphology, and more specifically on the organisation of the Golgi complex. Under such treatment, the Golgi complex appears as a very large network of anastomotic tubules and at an ultrastructural level it is no longer possible to recognise a classical cisterna organisation (Fig. 7.2B). Moreover, BARS depletion inhibited the post-Golgi transport of VSVG by
50% (Fig. 7.3B; C), indicating again a relevant role for endogenous BARS activity in the formation of post-Golgi carriers. More interestingly, the unusual size of the treated cells, together with the low confluency after 5 days of treatment (as compared to control or mock-treated cells), suggested some effects on the cell cycle. This is also in agreement with recent evidence on the role of Golgi fragmentation as a sensor for the entry into the late phases of mitosis (Sutterlin et al. 2002). When the cell-cycle phase of these BARS-depleted cells was monitored, they were found to be synchronised in the G2 phase. This evidence is of great relevance, as besides confirming a role for BARS in membrane fission, it indicates that BARS has effects not only on membrane traffic, but also on the regulation of other relevant cellular events, such as the cell cycle (Hidalgo Carcedo et al. 2004).

The effects of BARS depletion were also observed by treating Cos7 cells with siRNAs, which reduced the effects on the morphology of the Golgi complex as they were effective after shorter incubations. They therefore also had more limited effects on the cell cycle. This evidence suggests that the phenotype on intracellular transport induced by BARS depletion is indeed specific.
CHAPTER 8

BARS and dynamin operate independently and in different transport pathways

8.1. Background

The molecular mechanisms of fission have been studied extensively over the last decade or so, and it is now clear that many membrane fission events are controlled by the dynamins in vivo (Song et al. 2003). The dynamins are a versatile and structurally diverse family of large GTPases that can be broadly subdivided into ‘classical’ dynamins and dynamin-related proteins (Praefcke et al. 2004). In addition to fission, the dynamins regulate several other cellular processes (McNiven et al. 2000; Orth et al. 2003; McNiven et al. 2004).

It has become apparent over these years, however, that not all of the cellular fission events are driven by the dynamins, or at least by the ‘classical’ dynamin subgroup (for brevity, dynamin). Within the secretory pathway, dynamin-2 is essential in the transport of p75 from the TGN to the apical membrane surface (Kreitzer et al. 2000), in the formation of secretory granules in pituitary cells (Kreitzer et al. 2000; Yang al. 2001), and in the in vitro release of CCV and of polymeric-IgA-receptor-containing carriers from liver Golgi membranes (Jones et al. 1998). In contrast, export from the TGN of the transferrin receptor and the polymeric IgA receptor (in vivo) are not controlled by dynamin-2 (Altschuler et al. 1998). In the case of the dynamin-dependence of the export of VSVG (which is an
extensively characterized basolateral traffic marker) from the TGN, this remains controversial (Kasai et al. 1999; Cao et al. 2000).

In this part of the study, BARS and dynamin-2 were tested for their abilities to regulate the different post-Golgi transport steps, to determine whether they cooperate in the same machinery(ies) or if they act exclusively in different protein export pathways from the Golgi complex. So far, the documented roles for dynamin-2 in different intracellular transport steps have been assessed by overexpressing a mutant form of dynamin (Dyn2K44A) that bears a point mutation that impairs GTP binding. The overexpression of dynamin-2 itself has been seen to have no effects. To compare the effects of the inhibition of BARS and dynamin-2 in a comparable assay, the dominant-negative BARS^{D355A} was subcloned into a PcDNA3 vector (Section 2.2.7.) and overexpressed in cultured cells. In an independent approach, BARS and dynamin-2 were also inhibited by micrionjection of their respective blocking antibodies, p50-2 and DYN2.

**8.2. VSVG transport is selectively regulated by BARS and not by dynamin-2**

To assess the roles of BARS and dynamin-2 at the level of the TGN, both proteins were tested in the post-Golgi transport assay for VSVG. Cos7 cells were co-transfected with VSVG-GFP and either BARS^{D355A} or Dyn2^K44A, and after an overnight incubation at 40 °C the VSVG was accumulated in the Golgi complex by incubating the cells for 2 h at 20 °C. The post-Golgi transport was monitored by counting VSVG-GFP-positive intermediates that formed during the chase and by measuring the corresponding fluorescence of surface-associated VSVG.
(Section 2.11.3.). The overexpression of BARS\textsuperscript{D355A} was strongly inhibitory, as had been seen when this mutant was microinjected (Section 2.6.1.), and induced a reduction both in the formation of post-Golgi carriers and in the delivery of VSVG to the cell surface (Fig. 8.1). Under the same conditions, the overexpression of Dyn2\textsuperscript{K44A} had no effects on the post-Golgi transport of VSVG-GFP (Fig. 8.1). The same results were obtained when the two proteins were inhibited by means of antibody microinjection. Here, p50-2 had strong inhibitory effects, as previously described (Chapter 6.4.), while the microinjection of the well characterised anti-dynamin-2 blocking antibody DYN2 (Jones et al. 1998) was not effective (Fig. 8.2).

Although this is in agreement with the previously described role of BARS in post-Golgi transport of VSVG and excludes the possibility that dynamin cooperates at this transport step, it does not resolve the discrepancy with the published evidence from other groups on the role of dynamin-2 in the regulation of post-Golgi transport of VSVG (Cao et al. 2000; Kasai et al. 1999). To resolve this issue, the same post-Golgi transport assay was performed in BHK cells as that described by Cao et al. (Cao et al. 2000). In this cell line the published evidence was confirmed, in that the inhibition of dynamin-2, but not that of BARS, was able to block VSVG transport to the cell surface (Fig. 8.3A; C).
Fig 8.1. The roles of BARS and dynamin in post-Golgi carrier formation. A. Cos7 cells were transfected with VSVG-GFP either with mock transfection (mock) or with co-transfection of Dyn2<sup>K44A</sup> (K44A) or BARS<sup>D355A</sup> (D355A). Sixteen h later, the cells were processed for the TGN-exit assay (see methods), and then fixed and analyzed by confocal microscopy 40 min after the shift to 32 °C both for the formation of VSVG-positive carriers (upper panels) and for the delivery of VSVG to the PM (lower panels). Scale bars, 10 μm. B. Quantification of VSVG-positive post-Golgi carriers and arrival of VSVG at the PM at the indicated times after the shift to 32 °C applying the same conditions as described for A. All values are means ±s.d. of three independent experiments.
Fig 8.2. The roles of BARS and dynamin in post-Golgi carrier formation. Cos7 cells were infected with VSV, incubated at 40 °C for 1 h, injected with IgG, the anti-BARS antibody p50-2, or the anti dynamin antibody DYN2 and incubated at 40 °C for a further hour. The cells were then treated for the post-Golgi transport assay as described in methods, fixed at the indicated times, and treated for immunofluorescence. VSVG-positive carriers were counted for at least 10 cells for each time point. All values are means ± s.d. from three independent experiments.
The main difference between these two cell lines (Cos7 and BHK) is that the BHK cells are collagen-secreting fibroblasts (Schalk et al. 1992), whereas the Cos7 cells, although morphologically fibroblastic, do not produce procollagen-I (PC-I; Gao et al. 1999). In 2001, Mironov et al. (Mironov et al. 2001) demonstrated that in collagen-secreting cells the small cargo proteins enter the same compartments as collagen, and that these cargoes are all transported together to the cell surface in large membranous compartments that are different from those of non-collagen-secreting cells (Volchuk et al. 2000). Thus, in specialised cells the normal cargo is hijacked by other transport routes that are regulated by specialised machineries. To test this possibility, BARS and dynamin-2 were also investigated in another PC-I-secreting cell line, human fibroblasts (HF cells), where transport of VSVG and PC-I were first characterised (Mironov et al. 2001). The HF cells were electroporated (Section 2.3.3.) to overexpress VSVG-GFP together with either BARS\textsuperscript{D355A} or Dyn2\textsuperscript{K44A}, and then they were subjected to the post-Golgi transport assay, as above. As expected, also in this case the inhibition of dynamin-2, but not that of BARS, was able to suppress VSVG transport from the Golgi complex to the cell surface (Fig. 8.3B).
Fig 8.3. The roles of BARS and dynamin in post-Golgi carrier formation in fibroblastic cell lines. A. BHK cells were transfected with VSVG-GFP either with mock transfection (mock) or with co-transfection of Dyn2K44A (K44A) or BARSD355A (D355A). Sixteen h later, the cells were processed for the TGN-exit assay (see methods), and then fixed and analyzed by confocal microscopy 40 min after the shift to 32 °C for the delivery of VSVG to the PM. B. HF cells were treated as BHK in A and analyzed for the formation of VSVG-positive carriers and for the secretion of endogenous PC-I. Scale bars, 10 μm. C. Quantification of VSVG-positive and PC-I-positive post-Golgi carriers and arrival of VSVG at the PM at the indicated times after the shift to 32 °C, applying the same conditions as described for A. All values are means ±s.d. of three independent experiments.
In collaboration with the laboratory of Dr. Roman Polishchuk, the regulation of PC-I transport from the Golgi complex to the cell surface was examined by overexpressing either the BARS or the dynamin-2 dominant negatives. Also in this case the formation of PC-I-containing granules was dynamin-2-dependent, but not BARS-dependent (Fig. 8.3B). This again supports the possibility that in PC-I-secreting cells a specialised dynamin-dependent transport machinery has evolved, which is able to recruit other small secreted proteins that would normally follow a more conventional BARS-dependent pathway, as seen for VSVG in Cos7 and NRK cells.

8.3. Post-Golgi transport of p75 is dynamin-2-dependent, and not BARS-dependent

Transport from the Golgi complex of the p75 neurotrophin receptor was demonstrated to be dynamin dependent by Kreitzer et al. (Kreitzer et al. 2000) in non-polarised MDCK cells. Transport of this marker (linked to GFP) was thus tested here in Cos7 cells as a further means of demonstrating the exclusivity of the dynamin- and BARS-dependent pathways. As p75 is not synchronisable in the ER at 40 °C, a slightly modified post-Golgi transport assay was used. The Cos7 cells were transfected with p75-GFP together with either an empty vector as control, or with BARS<sup>D355A</sup> or Dyn2<sup>K44A</sup>, and then incubated overnight at 37 °C (Section 2.3.2.). The following day, the cells were incubated at 20 °C for 3 h to accumulate p75-GFP within the Golgi complex. At the release of the temperature block to 37 °C, transport of p75-GFP was monitored by measuring the number of p75-GFP-positive carriers that were formed during the chase, and by revealing the surface-
associated fluorescence of the receptor protein in non-permeabilised cells with an antibody against its extracellular domain (Weskamp et al. 1991). BARS inhibition was ineffective in the regulation of p75 transport, as expected (Fig. 8.4), whereas it was possible to confirm the dependency on dynamin-2 of this transport step also in Cos7 cells: in Dyn2<sup>K44A</sup>-overexpressing cells the p75-positive intermediate formation was inhibited by more than 70%, as was the plasma-membrane-associated fluorescence of p75 during the 37 °C chase (Fig. 8.4). This further strengthens the hypothesis that at the level of the TGN there are at least two different pathways for secretion, each of which is regulated by separate and different machineries. Each of the two pathways requires a membrane fission step to form constitutive transport intermediates, and each fission step is mediated by the fission-inducing protein that is specific for its own pathway and that is independent of the other.
Fig 8.4. The role of BARS and dynamin in post-Golgi carrier formation A. Cos7 cells were transfected with P75-GFP either with mock transfection (mock) or with co-transfection of Dyn2^{K44A} (K44A) or BARS^{D355A} (D355A). Sixteen h later, the cells were processed for the TGN-exit assay (see methods), and then fixed and analyzed by confocal microscopy 40 min after the shift to 32 °C both for the formation of P75-positive carriers (upper panels) and for the delivery of P75 to the PM (lower panels). Scale bars, 10 μm. B. Quantification of P75-positive post-Golgi carriers and arrival of P75 at the PM at the indicated times after the shift to 32 °C applying the same conditions as described for A. All values are means ±s.d. of three independent experiments.
8.4. Differential regulation of the basolateral and apical pathways by BARS and dynamin, respectively, in polarised MDCK cells

VSVG and p75 are sorted to the basolateral and apical plasma membrane, respectively, in polarised cells (Kroschewski et al. 1999; Kreitzer et al. 2000). A derivation from the concept that BARS and dynamin-2 selectively regulate post-Golgi transport of the two cargoes selected here would suggest that more generally BARS regulates post-Golgi basolateral sorting while dynamin-2 regulates that apical. Although they are non-polarised, Cos7 cells maintain the separation of these two pathways, as has also been suggested by previous studies on other non-polarised cell lines (Keller et al. 1997). However, to more specifically verify the hypothesis of a differentiation between apical and basolateral sorting in terms of fission machineries, VSVG-GFP and p75-GFP transport were analysed in polarised MDCK cells. For a better synchronisation of the marker proteins within the Golgi complex and also for a better efficiency of transfection of a confluent cell layer (required for complete cell polarisation), different combinations of nuclear microinjections of cDNAs were used (Section 2.6.2.) and the cells were incubated directly at 20 °C 45 min after the microinjection.

After 2 h of incubation at 20 °C, the cells were shifted to either 32 °C or 37 °C to chase the VSVG-GFP or p75-GFP, respectively, out of the Golgi complex. To monitor Golgi-to-plasma-membrane transport, images were acquired along the Z-axis of overexpressing cells and 3D reconstructions were performed using an LSM510 Zeiss confocal microscope (Section 2.11.5.; Figs. 8.5; 8.6; movies 7-12).
Due to the low resolution of the cells along the Z-axis, post-Golgi transport was solely measured as the amount of cargo protein detected at the cell surface during the chase, using the respective antibodies against the protein extracellular domains (Section 2.11.3.). At the release of the temperature block, VSVG-GFP exited the Golgi complex and reached the basolateral plasma membrane (Fig. 8.5B), where increasing amounts of this viral glycoprotein were measured with time (Fig. 8.5C). The overexpression of the dynamin-2 dominant-negative Dyn2<sup>K44A</sup> did not influence this transport step; rather, it was possible to observe a slight increase in protein transport to the basolateral plasma membrane (Fig. 8.5). On the other hand, the overexpression of BARS<sup>D355A</sup> severely inhibited VSVG-GFP sorting out of the Golgi complex by more than 85% as compared to the control cells (Fig. 8.5). Interestingly, this inhibition was even stronger than that observed in non-polarised cell systems, suggesting perhaps a more specialised pathway.

Similarly, the apical transport dependency on dynamin-2 was also confirmed in MDCK cells (Fig. 8.6). In control cells, at the release of the temperature block the p75-GFP exited the Golgi complex and reached the apical plasma membrane, where it became accessible to the specific antibody (Fig. 8.6A). In this case, the overexpression of BARS<sup>D355A</sup> was ineffective and transport of p75-GFP continued unperturbed and at the same rate as in the control cells (Fig. 8.6B; C). Conversely, overexpression of Dyn2<sup>K44A</sup> resulted in a potent inhibition of apical transport where p75-GFP delivery to the apical plasma membrane was reduced by more than 70% (Fig. 8.6B; C), confirming the previously published evidence (Kreitzer et al. 2000).
8.5. Discussion

The relevance of these results are of great interest as apart from further demonstrating the role of BARS in membrane fission in another cell line (MDCK cells), they indicate the presence of two different fission machineries that regulate post-Golgi protein transport and that operate at the level of the TGN. BARS specifically regulates membrane fission in Golgi-to-basolateral plasma membrane transport, and is not effective on apical transport, which is instead regulated by dynamin-2. Vice versa, dynamin-2 is ineffective in the regulation of basolateral-destined cargo transport, as is seen with VSVG, although this might not be the case in specialised cell lines like PC-I-secreting fibroblasts, where a more differentiated pathway appears to have evolved over time. This last point is also of great help in solving the existing discrepancies in the role of the dynamins in protein export from the Golgi complex (Cao et al. 2000; Kasai et al. 1999). The characterisation of the BARS-dependent fission step is also important in light of the ongoing characterisation of the diverse intracellular fission steps and their respective machineries that regulate protein transport. As indicated above, the dynamin family of proteins have been implicated in several transport steps, although in many others the fission-requiring events along the membrane traffic pathways were still awaiting a precise characterisation (McNiven et al. 2000; Praefcke et al. 2004). BARS can now be included among the fission-inducing proteins along with the dynamins, although a precise characterisation of the molecular machinery that cooperates with BARS to induce membrane fission is still under examination.
Fig 8.5. Differential roles of BARS and dynamin in export of VSVG from the TGN in polarized cells. A. Schematic representation of basolateral transport in polarized cells. Proteins accumulated within the Golgi (green) are sorted to the basolateral PM where they are revealed by specific antibody (Ab) without permeabilisation (red line). B. Polarized MDCK cells were infected with VSV either alone (mock) or with the injection of Dyn2^K44A (K44A) or BARS^D355A (D355A) cDNAs during the incubation at 40 °C. One h later, they were processed for the TGN-exit assay and for immunofluorescence. Scale bars 10 μm C. Quantification of the arrival of VSVG at the PM at the indicated times after the shift to 32 °C. Values are means ±s.d. of three independent experiments.
Fig 8.6. Differential roles of BARS and dynamin in export of p75 from the TGN in polarized cells. A. Schematic representation of apical transport in polarized cells. Proteins accumulated within the Golgi (green) are sorted to the apical PM where they are revealed by specific antibody (Ab) without permeabilisation (red line). B. Polarized MDCK cells were injected with p75-GFP cDNA either alone (mock) or with the injection of Dyn2\textsuperscript{K44A} (K44A) or BARS\textsuperscript{D355A} (D355A) cDNAs. One h later, they were processed for the TGN-exit assay and for immunofluorescence. Scale bars 10 μm C. Quantification of the arrival of VSVG at the PM at the indicated times after the shift to 32 °C. Values are means ±s.d. of three independent experiments.
CHAPTER 9

BARS and dynamin behave as alternative fission machineries also in endocytic traffic pathways

9.1. Background

To test the generality of the concept that BARS and dynamin behave as alternative fission machineries, the role of these proteins was also analysed in two endocytic steps: a) the receptor-mediated uptake of transferrin (Tf); and b) the uptake of the fluid-phase marker dextran. The former is known to require dynamin and the coat protein clathrin (Altschuler et al. 1998; Conner et al. 2003; Damke et al. 1994), while the latter is dynamin independent (Guha et al. 2003; Sabharanjak et al. 2002).

Although not much is known on the molecular mechanisms that regulate the internalisation of fluid-phase solutes, it has been shown that it is strongly dependent on the actin cytoskeleton organisation. This pathway involves proteins such as Cdc42, a small GTPase regulator of actin polymerization (Sabharanjak et al. 2002), PAK1 (Dharmawardhane et al. 2000), and members of the protein kinase C (PKC) family (Song et al. 2002). BARS and dynamin-2 were therefore inhibited in Cos7 cells by the overexpression of their respective dominant-negative forms (BARSD355A and Dyn2K44A) or by microinjection of their blocking
antibodies (p50-2 and DYN2, respectively), and the effects of these treatments were then tested on receptor-mediated and fluid-phase endocytosis.

9.2. Receptor-mediated endocytosis of transferrin is dynamin, and not BARS, dependent

To investigate receptor-mediated endocytosis, Alexa-546-labelled Tf was bound to Cos7 cells on ice and allowed to internalize at 37 °C for increasing lengths of time (Section 2.8.5.). The cells were acid-washed and Tf endocytosis was assessed by measuring the internalized Alexa-546 fluorescence (Section 2.11.3; Damke et al. 1994). Both Dyn2K44A overexpression and microinjection of the DYN2 antibody markedly inhibited Tf endocytosis (by approximately 80 %), as has been previously described (Fig. 9.1; Altschuler et al. 1998; Damke et al. 1994; Kasai et al. 1999). In contrast, parallel treatments for the inhibition of BARS (BARSΔ355A overexpression and microinjection of the p50-2 anti-BARS antibody) were completely ineffective under the same conditions that blocked VSVG exit from the TGN (see above; Fig. 9.1). Thus, Tf endocytosis requires dynamin, as expected, and is BARS-independent.
Fig 9.1. Differential requirements for BARS and dynamin in transferrin endocytosis. The requirement for BARS was tested in Cos7 cells by microinjecting or transfecting BARS\textsuperscript{D355A}, and microinjecting the p50-2 Ab, as indicated; that of dynamin by transfecting Dyn\textsuperscript{K44A} or microinjecting the DYN2 Ab, as also indicated. A. After transfection or microinjection, the cells were incubated for 1 h on ice in the presence of Alexa-546-labeled transferrin, acid washed to remove unbound transferrin, incubated at 37 °C for 5 min, and then fixed and analysed for Alexa-546-transferrin incorporation by wide-field microscopy. Dotted lines indicate cell borders. Scale bars 10 μm. B. Quantification of incorporated Alexa-546-transferrin under the conditions in A. Values are means ±s.d. normalized versus their own controls (and expressed as percentages) of three independent experiments.
9.3. Fluid-phase endocytosis of dextran is BARS-dependent and dynamin-2 independent

To test fluid-phase endocytosis, Cos7 cells were treated for the inhibition of either BARS or dynamin as above, and allowed to internalize FITC-dextran at 37 °C for 10 min (Section 2.8.6; Damke et al. 1994). The cells were then fixed and washed, and the intracellular TRITC fluorescence was used as a measure of the rate of endocytosis. In agreement with previous reports (Guha et al. 2003; Sabharanjak et al. 2002), in cells where dynamin-2 was inhibited, the FITC-dextran was internalized with the normal efficiency (Fig. 9.2). In contrast, in the cells overexpressing BARS\textsuperscript{D355A} or that were injected with the p50-2 antibody there was an impairment of the incorporation of the FITC-dextran fluid-phase marker of approximately 60% (Fig. 9.2). To exclude the possibility that the inhibition of fluid-phase endocytosis by BARS inhibitors that was observed might be secondary to the block of TGN export described above (see Chapter 6), which could result from potential links between endocytosis and exocytosis, the FITC-dextran endocytosis was also monitored at 20 °C. At this temperature, endocytosis can proceed up to the early endosomes, but TGN export \textit{per se} is inhibited, ruling out any possible influence of TGN-to-plasma membrane traffic on the assay (Griffiths et al. 1985). Also under these conditions, the internalization of FITC-dextran into the early endosomes (as assessed by immunofluorescence staining of the early endosomal marker EEA1; Section 2.11) was profoundly blocked by BARS\textsuperscript{D355A} (Fig. 9.3). This thus further confirms a direct effect of BARS on fluid-phase endocytosis.
Finally, since VSVG export from the TGN is BARS-independent in BHK cells (see Chapter 8), the experiments to test whether fluid-phase endocytosis was inhibited by the BARS dominant-negative mutant BARS_355_ were repeated in this cell line. The rationale here aims to rule out the possibility that the entire BARS-dependent fission machinery is lacking in PC-I-secreting cell lines, not only at the level of the TGN, but also in other fission-requiring transport steps. Of note, overexpression of this BARS dominant negative was also able to suppress fluid-phase endocytosis in BHK cells, whereas dynamin-2 inhibition induced by overexpression of its dominant negative was still ineffective.

**9.4. BARS depletion by RNAi inhibits fluid-phase uptake of FITC-dextran**

By an independent approach, the efficiency of fluid-phase endocytosis was also tested in Cos7 cells by using RNAi of CtBP1 and BARS (Section 2.7.2.). After 48 h of incubation, the control and siRNAs-treated Cos7 cells were subjected to the fluid-phase endocytosis assay, as follows: the cells were loaded with 2 mg/ml FITC-dextran at 37 °C for 10 min and 20 min, and then extensively washed and fixed in 4% paraformaldehyde. The intracellular FITC fluorescence was then used as a measure of fluid-phase uptake. The siRNAs-treated cells exhibited an inhibition of FITC-dextran uptake of approximately 60%, as compared to the control, mock-treated cells (Fig. 9.2B).
Fig 9.2. Differential requirements for BARS and dynamin in fluid phase dextran endocytosis. The requirement for BARS was tested in Cos7 cells by microinjecting or transfecting BARS\textsuperscript{D355A}, by microinjecting the p50-2 Ab, and by RNA interference; that of dynamin by transfecting Dyn2\textsuperscript{K44A} or microinjecting the DYN2 Ab. A. After transfection or microinjection, the cells were incubated with TRITC-labeled dextran for 20 min at 37 °C, washed, fixed, and analysed for dextran incorporation by confocal microscopy. Dotted lines indicate cell borders. Scale bars 10 μm. B. Quantification of incorporated FITC-dextran under the conditions in A and in RNAi-treated cells. Values are means ±s.d. normalized versus their own controls (and expressed as percentages) of three independent experiments.
Fig 9.3. Inhibition of fluid phase uptake is not dependent on BARS^{D355A}-mediated post-Golgi transport inhibition. After mock or BARS^{D355A} (D355A) transfection, Cos7 cells were incubated with FITC-labeled dextran for 60 min at 20 °C, washed, fixed, and analyzed for dextran incorporation by confocal microscopy. The merged panels show co-localization with the early endosomal marker EE1A. Scale bars 10 μm.
9.5. Discussion

In the previous Chapters of this study, endogenous BARS was demonstrated to be a relevant protein that regulates a new fission machinery at the level of the Golgi complex. Moreover, BARS is not involved in the previously described fission mechanisms that are regulated by the other well-known fission-inducing protein, dynamin (McNiven et al. 2000; Praefcke et al. 2004). Instead, BARS and dynamin regulate two separate machineries for protein sorting at the TGN. In polarised cells, these BARS- and dynamin-dependent machineries drive transport to the basolateral and the apical plasma membrane, respectively.

To test for the presence of BARS-driven fission events in other intracellular transport processes, a dynamin-independent endocytic process, namely fluid-phase endocytosis (Guha et al. 2003; Sabharanjak et al. 2002), was studied.

Interestingly, and also at the level of the plasma membrane, another dynamin-independent process was found to be BARS-dependent, indicating that possibly many other fission processes that do not require dynamin could be regulated by BARS. The inhibition of BARS activity markedly inhibited the fluid-phase uptake of FITC-dextran in a manner that was independent from the previously described (see above) inhibition of Golgi export. This was achieved by overexpression of a dominant negative (BARS^D355A), by microinjection of the p50-2 anti-BARS antibody, and by RNAi. This thus indicates the presence of a BARS-regulated fission machinery at the level of the plasma membrane, as also suggested by the presence of BARS at the plasma membrane when it was overexpressed into Cos7
cells (Fig. 3.1A). Ultrastructural analyses of BARS<sup>D355A</sup>-overexpressing cells have so far failed to show any elongated tubular structures at the level of the plasma membrane, as is seen at the TGN in the case of VSVG export. It is true, however, that the ultrastructural organisation of the TGN is radically different from that of the plasma membrane, and the presence of tubular structures as precursors of endocytic carriers has been demonstrated only for dynamin-dependent processes (Damke et al. 1994; Takei et al. 1995). Nevertheless, the relevance of BARS in the regulation of fluid-phase endocytosis is clear, and this indicates that the BARS-regulated fission machinery is not limited to the Golgi complex, but can be expected to be as widespread as the dynamin-dependent fission machinery.
Intracellular membrane transport is mediated by a series of discrete events that control the formation of constitutive transport carriers. Unlike membrane fusion, which is probably regulated by a process common to all intracellular transport events and mediated by a single group of proteins (the SNARES; Ungar et al. 2003), fission is highly differentiated in terms of the steps that precede the severing of the transport carrier from the donor compartment and in the multiple machineries that drive it (Corda et al. 2002). Over the last decade, the molecular processes controlling membrane fission have been studied intensively and they have indicated a pivotal role of the dynamin family of proteins (Song et al. 2003). Interestingly, although the dynamins are relevant for many fission events there are others that appear to be dynamin independent. This raises the possibility that parallel and independent machineries exist that are regulated by other yet-uncharacterized proteins or protein complexes.

In the course of this study the role of the protein BARS has been characterized in terms of its ability to regulate those fission events that have previously been determined to be dynamin independent. It is understood that these fissions are regulated by a separate machinery and that BARS may represent the counterpart to dynamin in these processes. BARS was identified and cloned during a search for cytosolic factors controlling Golgi complex tubulation (Spanò et al. 1999) and later shown to act as a component of a Golgi tubule-fissioning machinery in vitro (Weigert et al. 1999). From a structural point of view, BARS is a close
homologue (and most likely a splice variant) of CtBP1 (Nardini et al. 2003; Kumar et al. 2002; Chinnadurai 2002).

In agreement with the in vitro studies, overexpression of BARS indicates the presence of the protein at the plasma membrane and at the Golgi complex (cis and trans-poles of the stacks; Fig 3.1). Moreover, the overexpression alters the morphology of the Golgi membranes and this eventually results in blockade of secretory transport at the level of the TGN (see chapter 3). When the intracellular concentration of BARS is raised more mildly by means of the microinjection of the purified protein, the effects on post-Golgi transport are the opposite to those that occur after overexpression. Cargo secretion which is blocked in cells overexpressing BARS is actually enhanced after microinjection (see chapter 4). The reason for this discrepancy remains unclear though there are a number of clues that might aid understanding. First, BARS is associated with a low, but still detectable LPAAT activity in vitro (Weigert et al. 1999). This activity might not be essential for the formation of post-Golgi transport carriers as microinjection of a LPAAT-inactive SBD mutant is still effective (Fig. 6.5). Nevertheless, when the protein is overexpressed this probably alters the lipid composition of the Golgi membranes, as suggested by the finding that addition of DG kinase inhibitors fully reconstitutes protein secretion (Fig. 4.2). It is possible therefore, that when cells are incubated with high concentrations of BARS for a long time, lipid metabolism is sufficiently perturbed to cause severe defects in membrane transport. Of note, when BARS-microinjected cells were incubated overnight instead of for 1 h prior to the assay, the post-Golgi transport of VSVG was again inhibited (Fig. 5.8). This reinforces the possibility that long incubations with high
levels of BARS are toxic to the cells in the same way as overexpression, ultimately blocking intracellular transport rather than stimulating it.

Microinjection of dominant negative forms of BARS indicates that it exerts its function in the fission of membranous cargo from a tubular precursor that has already originated from a donor membrane (Fig. 6.6). Thus the processes of membrane budding and elongation themselves are likely to be BARS-independent.

Microinjection of purified BARS reveals a pivotal role in the formation of constitutive post-Golgi transport carriers destined for the basolateral plasma membrane. Conversely formation of apical-destined carriers which is a well-characterized dynamin dependent process is unaffected (see chapter 8). This difference raises the possibility that BARS operates in those dynamin-independent transport steps that require a fission event, not only at the level of the Golgi complex but in other membrane transport events.

BARS activity was therefore tested on a transport process that does not originate from the Golgi complex. The dependence of receptor-mediated endocytosis on dynamin is well accepted. On the other hand, endocytosis of fluid-phase solutes is dynamin-independent and is driven mainly by cytoskeletal rearrangements (see chapter 1.1.3). In the present study, the dependence of receptor-mediated endocytosis (monitored by the uptake of transferrin) on dynamin has been confirmed. BARS is not implicated in this type of endocytosis. On the other hand, fluid-phase endocytosis (monitored as uptake of fluorescent dextran) is blocked.
by dominant-negative mutants of BARS (see chapter 9). This evidence further supports the existence of a novel fission machinery that operates in a complementary fashion to regulate those transport steps that have been found to be dynamin-independent.

Other relevant evidence indicates that BARS is involved in membrane fission not only along the transport pathways. When BARS activity was depleted by oligonucleotide treatment the cells were arrested in the G2 phase of mitosis (Fig. 7.3). This indicates that BARS is also involved in the regulation of the cell cycle. Work by others has demonstrated that the G2 arrest arises from inhibition of Golgi membrane fission (Hidalgo Carcedo et al. 2004). Thus, BARS inhibition preserves the Golgi apparatus from fragmentation which represents a key checkpoint in the cell cycle, and thereby arrests the cells in G2. (Hidalgo Carcedo et al. 2004; Sutterlin et al. 2002).

10.1. Towards a comprehensive map of the machineries that regulate intracellular fission events

Intracellular membrane transport can be classified into three main pathways:

- The secretory pathway
- The endocytic pathway
- Retrograde transport
Each of these has been widely tested for dependence on dynamin (McNiven et al. 2000; Praefcke et al. 2004) and as mentioned above, although the dynamins and dynamin-related proteins are involved in sections of all three, several branches within each rely on fission events that are independent of dynamin.

10.1.1. The secretory pathway

Along the secretory pathway, proteins leave the ER and are transported first to the Golgi complex. Although no clear picture of this section of the transport process has been established and there are even questions regarding a fission event at this stage (as opposed to some sort of continuity between the ER and the Golgi complex; Griffiths 2000), a role for dynamin has never emerged. Inhibition of dynamin is without effect on the transport of marker proteins from the ER to the plasma membrane (Cao et al. 2000; Kasai et al. 1999; Kreitzer et al. 2000). A dynamin-like protein, DLP1, is associated with ER-derived membranous tubules but whether it is a regulator of ER-to-Golgi transport unknown (Yoon et al. 1998). In the present study, neither overexpression nor microinjection of BARS interfered with VSVG transport from the ER, suggesting either that this step does not involve a fission event, or that a third, still uncharacterized fission machinery regulates protein export from the ER.

Once they have reached the Golgi complex, proteins are transported from the cis- to the trans- side of the stack. This portion of the secretory pathway has been the subject of extensive studies in recent years and the earlier dogma of vesicular transport encompassing many fission and fusion events to transfer cargo from one Golgi cisterna to the other, is no longer universally accepted (Griffiths 2000;
Storrie et al. 2002). Rather, intra-Golgi transport occurs via other mechanisms that could even exclude fission and fusion although a precise and conclusive model is still far from being established. Anyway, as for ER-to-Golgi, neither BARS nor dynamin are able to stimulate or inhibit this transport step. In both cases, when the two proteins are inhibited the cargo proteins localize normally at the level of the Golgi complex and then continue to traverse the stack to reach the TGN (Cao et al. 2000; Kasai et al. 1999; Kreitzer et al. 2000). BARS is enriched at both poles of each stack but is absent from the cisternae. It is thus unlikely that BARS regulates intra-Golgi transport.

At the TGN the secretory pathway branches into four different pathways. Two of these deliver cargo to the apical and the basolateral plasma membranes, one leads to the formation of secretory granules in specialized cells, and the last links the TGN to the lysosomal system.

Apical transport of the p75 neurotrophin receptor is dependent on dynamin (Kreitzer et al. 2000) though for other apical cargo markers (such as the pIgA receptor) some discrepancies still persist. These problems may depend on the methods of assay arising from experiments carried out using cell-free systems (which indicate a dependence on dynamin) as opposed to intact systems (indicating no involvement at this step).

For the basolateral-membrane-directed transport, the situation remains under debate and the role for dynamin in post-Golgi transport appears to vary with cell type. In BHK cells overexpression of the Dyn2K44A dominant-negative dynamin inhibits the delivery of VSVG to the plasma membrane with a concomitant
accumulation of VSVG within the Golgi complex (Cao et al. 2000). However, under similar conditions of Dyn2^K44A overexpression, VSVG transport in Clone 9 cells continues unperturbed (Kasai et al. 1999). In agreement with results obtained with Clone 9 cells, the evidence presented in this thesis indicates that inhibition of dynamin is without effect on post-Golgi transport of VSVG in Cos7 and MDCK cells; instead, this transport step is inhibited by the overexpression of the BARSD355A dominant-negative BARS mutant. As this looked like it could also represent a case of cell-specificity, the same treatment was analyzed in the same cell line used by McNiven and colleagues, namely BHK fibroblasts. In this case, these cells are indeed insensitive to BARS inhibition at the level of the Golgi complex, and VSVG is efficiently transported to the cell surface, and the evidence documented for a dynamin dependence of this transport steps were confirmed. The reason for this specificity could be due to the fact that some cell lines may have evolved a specialized form of secretion in order to transport macromolecules such as procollagen, that assembles into rigid tetramers of 300 nm. In procollagen-secreting cells macromolecules traverse the Golgi apparatus and are transported to the cell surface in particularly large specialized carriers referred to as mega vesicles (Volchuk et al. 2000), along with smaller cargo such as VSVG (Mironov et al. 2001). Interestingly, in HF cells which also secrete collagen, the transport of both procollagen and VSVG again share the same transport carriers is dependent on dynamin and not BARS (Fig. 8.3).

In the regulated secretory pathway the proteins involved are selectively sorted into immature granules, which eventually mature into secretory granules and, on a signal, deliver their contents to the surrounding environment. There is some
evidence for a role of dynamin in the fission step involved in the formation of secretory granule precursors from the TGN, at least as it occurs in mouse pituitary corticotrope cells (AtT20). Here, overexpression of the $\text{Dyn2}^{\text{K44A}}$ dominant-negative mutant, suppression of dynamin-2 synthesis by means of antisense oligonucleotides and the delocalization of dynamin from the plasma membrane by overexpression of a mutant that lacks the SH3 domain all inhibit hormone secretion (Yang et al. 2001). Although a role for BARS in this system has not yet been tested, it seems unlikely that this well established regulated pathway involves multiple overlapping machineries.

Proteins such as cathepsin D that are destined for the late endosomal/lysosomal system also exit the TGN in specialized membranous containers. This pathway is characterized by receptors such as the MPR that can be exploited as tools to label this transport pathway. Within the TGN pro-cathepsin D binds to the MPR and is delivered to lysosomes where it is processed into its mature form. TGN-to-lysosome transport is modulated but not fully dependent on the dynamins. The effect of overexpression of $\text{Dyn2}^{\text{K44A}}$ is to mislocalise the MPR from the TGN to lysosomes but only has a small effect on cathepsin secretion, (Altschuler et al. 1998; Nicoziani et al. 2000). This evidence suggests a role for dynamin in the retrograde transport from lysosomes to the TGN to allow the recycling of the receptors. By preventing the return of MPR from the lysosomes to the TGN this receptor accumulates within the lysosomal compartment. A slight delay that occurs in the processing of cathepsin D cells overexpressing $\text{Dyn2}^{\text{K44A}}$ (Altschuler et al. 1998; Nicoziani et al. 2000) has been ascribed to the decrease in the number of receptors available at the level of the TGN.
Yeast cells have also evolved two different secretory pathways that branch at the level of the TGN leading to the formation of low density secretory vesicles (LDSV) high density secretory vesicles (HDSV). Of these, only the HDSV pathway is suppressed when the single yeast homologue of dynamin (VSP1) is inhibited (Gurunathan et al. 2002). Under these conditions the secretion of the enzyme invertase, which normally follows the HDSV pathway, is re-routed and emerges through the LDSV pathway. Other regulators of the LDSV pathway have not so far been identified the presence of two putative BARS homologues in yeast, the proteins Ser3 and Ser33 (our unpublished observations), is suggestive.

10.1.2. The endocytic pathway

The endocytic pathways are the best understood and most studied in terms of dependence on dynamin. The pathways rely on five different machineries only three of which, namely those mediated by clathrin, caveolae and (some) lipid rafts, are dependent on dynamin (Pelkmans et al. 2003). Although less well characterized, the remaining pathways, which mainly mediate fluid-phase endocytosis, are known to be dynamin-independent (Pelkmans et al. 2003; Damke et al. 1995; Damke et al. 1994), being controlled by filamentous actin-based membrane motility. Proteins such as the PKC enzymes (Song et al. 2002) and Cdc42 (Sabharanjak et al. 2002), which are all regulators of actin polymerization/depolymerization, are involved in the endocytosis of fluid-phase solutes. As for the secretory pathway, those fission steps that do not require dynamin are sensitive to BARS, yet again confirming the existence of at least two different non-overlapping fission machineries. A possible interaction of BARS with other proteins of the fluid-phase endocytosis machinery has yet to be
established but interestingly, p21-activated kinase 1 (PAK1) that is among the regulators of macropinocytosis (a particular kind of fluid-phase, dynamin-independent endocytosis; (Dharmawardhane et al. 2000) also phosphorylates CtBP1 in a region that is conserved in BARS. This triggers its translocation from the nucleus to the cytoplasm (Barnes et al. 2003) and suggests a link between BARS and the other known regulators of fluid phase endocytosis. In contrast to what occurs at the level of the TGN, the increase in the intracellular concentration of BARS does not induce an increase in fluid-phase uptake. This is not surprising, in that the mechanisms of carrier formation at the level of the TGN and at the level of the plasma membrane represent two very different and unsynchronized processes. Overexpression of dynamin does not stimulate those endocytic processes that depend on it (Damke et al. 1994).

A precise description of all the possible branches of the endocytic network has not yet been achieved though some of these steps are certainly dependent on dynamin (Llorente et al. 1998). It is very likely that BARS or other as yet unknown fission-inducing proteins will be found to be involved in other branches.

10.1.3. Retrograde transport

This transport pathway is mainly exploited by bacterial toxins from the plasma membrane via the Golgi en-route to the ER. It doesn’t appear to represent a single trafficking route. Rather, there is a high variability in the paths followed individual toxins (Lord et al. 1998; Sannerud et al. 2003; Cossart et al. 2004). The role of dynamin has so far been tested by considering the ability of certain toxins to exert their effects even under conditions of overexpression of dominant-
negative mutants. Thus, inhibition of dynamin arrests the uptake of ricin at the level of the endosomal/lysosomal system, inhibiting its further transport to the Golgi complex and thereby blocking its toxic activity (Llorente et al. 1998). This indicates that dynamin is involved in the retrograde transport from lysosomes to the Golgi complex (as also suggested by studies on the MPR and cathepsin transport, see above). It becomes evident, anyway for the route followed by ricin, that no steps preceding the delivery of the toxin to lysosomes, requires dynamin. Unfortunately, because ricin transport is arrested at the level of the lysosomal system, there is no possibility of examining the retrograde pathway beyond this point though there is some evidence that indicates that only the TGN is involved in retrograde transport of toxins to the ER. In this case, the Golgi stack is totally avoided (Massol et al. 2004). From this, it can be seen that the monitoring of toxin transport by itself may not be a suitable method for the exploration of retrograde transport at the level of the Golgi complex. In contrast with the endocytosis of ricin there is no involvement of dynamin at any of the steps in the pathway leading to the uptake of cholera toxin. This suggests once again that retrograde transport of toxins involves multiple pathways, some of which are sensitive to dynamin, others of which are not. The role of BARS in retrograde transport of toxins is still under investigation though it has to have a role in the formation of COPI coated vesicles from \textit{in vitro} Golgi membranes. It appears then that BARS is able to modulate at least the final steps of retrograde transport but this possibility has to be investigated further as so far it has been impossible to reproduce the same effects \textit{in vivo}. 

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All the evidence accrued so far indicates that intracellular membrane transport is mediated by at least two non-overlapping fission-inducing machineries. The pathways tested have revealed that BARS acts as a regulator those fission events that are independent of dynamin. Clearly, more work is needed to characterise all of the intracellular membrane fission events fully.

10.2. Regulation of BARS activity

One may anticipate that the mechanisms that regulate BARS activity will provide insights into the principles underlying its involvement (and that of dynamin) in specific transport pathways. Useful mechanistic clues have been provided by the recently resolved crystal structure of BARS (and of its homolog CtBP1). This shows a marked similarity with that of the D-stereoisomer-specific NAD(H)-dependent dehydrogenases (Nardini et al. 2003; Kumar et al. 2002). Consistent with this, BARS (and its CtBP homologues) binds NAD(H) tightly (Nardini et al. 2003; Kumar et al. 2002; Zhang et al. 2002). Moreover, BARS can also bind pCoA and other acyl-CoAs (most likely through the common ADP molecular framework they share with NADH), these different ligands competing for the same binding site (Nardini et al. 2003). This competition can have major structural effects on BARS, with NADH promoting a closed dimeric conformation and pCoA inducing a substantial transition leading to an open monomeric structure. Since pCoA is an essential cofactor in BARS-induced membrane fission (Weigert et al. 1999), these effects might be of functional significance. For instance, this conformational transition could play a direct or regulatory role in the formation of a protein scaffold that could drive constriction.
and cleavage at the neck of fission intermediates. In addition, the ‘opening’ and monomerization of BARS, by facilitating the access of LPA to the bound pCoA may be permissive of its lysophosphatidic-acid-specific acyltransferase activity (Weigert et al. 1999, Nardini et al. 2003). If correct, then BARS-dependent fission could be regulated by lipid metabolic pathways quite separate from those of phosphoinositide metabolism (Godi et al. 2004). Thus, the BARS- or dynamin-dependence of a traffic step might be linked in part to the prevalence of a specific metabolic pathway at that stage.

Other potential means of regulating BARS activity can be derived from published evidence on CtBP1, the other member of the CtBP family that shares the highest sequence identity with BARS. Mostly, this indicates that CtBP1 activity is modulated by a series of interactions and post-translational modifications that in turn result in a change in the intracellular localization of CtBP1 itself. As mentioned above, there is evidence for phosphorylation of CtBP1 by PAK1 that could alter its intracellular localization from the nucleus to the cytoplasm, thereby preventing its activity as a co-repressor of gene transcription (Barnes et al. 2003). BARS is also a substrate for PAK1 but unfortunately, due to difficulties in determining its precise intracellular localization by immunofluorescence, it is difficult to ascertain whether phosphorylation causes a change in its localisation. Also unexplored is the effect of PAK1 activity on BARS in respect of the regulation of macropinocytosis, a pathway exploited by viruses and bacteria to infect cells.
Similar effects on intracellular localization are induced by the interaction of the PDZ domain of the neuronal nitric oxide synthase (nNOS) with CtBP that causes it to exit the nucleus into the cytoplasm, thereby preventing its co-repressor activity (Riefler et al. 2001). So far it is not known which members of the CtBP family bind the PDZ domain of nNOS, but considering the implications of nitric oxide (NO) in the regulation of phagocytosis in cell lines such as murine microglia (Paolucci et al. 2000), it would be interesting to investigate the possibility of an implication of BARS in this process. Moreover, on the basis that BARS also plays a role in Golgi complex partitioning during mitosis (Hidalgo Carcedo et al. 2004) it will be interesting to examine the possibility of interactions with other PDZ-containing proteins.

CtBPs (Lin et al. 2003) and the dynamins (Mishra et al. 2004) are also substrates for sumoylation. This ubiquitin-like modification mediated by SUMO1 increases their retention in the nucleus (Lin et al. 2003). This same modification increases the activity of dynamin. If BARS were retained within the nucleus its fissioning activity would clearly be prevented, whereas that of dynamin would be increased suggesting an interesting double regulation for two distinct intracellular transport pathways.

The observation that CtBP1 and CtBP2 are transcriptional co-repressors with multiple roles in development and oncogenesis (Chinnadurai 2002) may also be related to the trafficking role of BARS and raises the possibility that in addition to controlling fission, BARS itself is involved in transcriptional regulation. Injection of CtBP1 induces a BARS-like phenotype on post-Golgi VSVG transport while CtBP2 is inactive in this process. It appears that the CtBP family (including
BARS) will join the large group of proteins known to be endowed with dual functions, one in the nucleus, the other in the cytoplasm (Woodman 2003; Gillingham et al. 2002; Hyman et al. 2000). Questions then arise as to whether and how the CtBP function in specific traffic segments and the CtBP-dependent transcription are linked. A possible clue comes from the finding that expression of E-cadherin is regulated by the repressor activity of CtBP1 (Grooteclaes et al. 2003). E-cadherin is transported, possibly in a BARS-dependent pathway, from the Golgi to the basolateral plasma membrane and is fundamental in the establishment of cell polarity. The combined activity of CtBP1 and BARS (or possibly even BARS alone) on the synthesis and further transport of E-cadherin might represent a regulatory machinery for the establishment of cell polarity.

10.3. Physiological relevance of BARS activity

This study has documented a role for BARS in the fission of biological membranes in a number of intracellular transport steps involved in basolateral post-Golgi transport and fluid-phase endocytosis. These two membrane transport events are regulated by the same protein, therefore by means different from those regulating other fission steps depending on dynamin and this raises questions regarding the physiological basis underlying these differentiated regulatory machineries. We might ask, what are basolateral transport and fluid-phase endocytosis really for?
Both polarized and non-polarized cells have differentiated domains at their plasma membrane surfaces. These are randomly distributed in non-confluent cells but become spatially organized under contact conditions or in actively migrating cells. Spatial organization of the cell surface into basolateral and apical domains is mainly under the control of the regulated secretion of specialized proteins from the Golgi complex. In epithelial cells (e.g. MDCK), the delivery of specific proteins to the basolateral surface is a prerequisite for polarization as indicated by the loss of polarity induced by inhibition of basolateral transport (Kroschewski et al. 1999; Cohen et al. 2001). Thus we can propose that the regulation of basolateral transport regulates the formation of a basolateral domain itself. No such evidence exists for a similar role for the dynamin-mediated transport of apical markers (inhibition of dynamin does not result in depolarization of the cell). Since all primordial organisms were unicellular and without surface polarity it seems likely that the original transport machinery would have been controlled by dynamin-like proteins. Basolateral transport, and therefore the basolateral fission machinery, evolved later in time. Interestingly, none of these polarization events are definitive: the formation of the leading edge of a migrating cell stops as soon as it comes to rest just as epithelial cells lose their polarity in response to specific signals for organogenesis and wound healing. These processes, generally known as epithelial-mesenchymal transitions (EMTs; Thiery 2003), are mediated both by transcriptional regulation (that involves members of the CtBP family) and by intracellular transport activity. Clearly, the possibility that BARS might be involved in these processes must be worth investigating, especially in light of the implications of EMTs in fibrosis and carcinoma (Thiery 2003).
For fluid-phase endocytosis the situation is more complicated, particularly as no clear physiological role has been assigned to this process. Current views envisage fluid-phase endocytosis as a means of internalizing whole membrane domains and thereby modulating cell growth, polarization and migration.

10.4. BARS as a “moonlighting” protein

As previously indicated, BARS shares high sequence identity with CtBP1 and CtBP2. Although lacking the nuclear localisation signal that characterises CtBP2 it is a splice variant of CtBP1, from which it differs only in the N-terminal segment (Chinnadurai 2002).

Curiously, the first recognised members of the CtBP family have never been implicated in intracellular transport processes; rather, they act exclusively as co-repressors of gene transcription processes involved in suppression of the oncogenic potential of adenoviral infections (Chinnadurai 2002). These properties are very different from those characterised for BARS which is normally located within the cytoplasm and where it regulates membrane fission (Weigert et al. 1999; Hidalgo Carcedo et al. 2004). This raises obvious questions. Although activity of BARS as a co-repressor of gene transcription has yet to be tested, we take this as serious possibility, especially in view of its high degree of sequence identity (97%) with the other members of the CtBP family and its ability to bind E1A (Spanò et al. 1999). Our preliminary observations indicate that like BARS, CtBP1 is also effective in stimulating post-Golgi transport when microinjected,
strengthening the hypothesis that both proteins are regulators of two radically different processes. This is a defining feature of the so-called “moonlighting” proteins (Jeffery 2003).

Many proteins that were first identified and characterised through their possession of a specific activity now turn out also to be involved in totally different pathways. There are numerous examples. The lens crystallins also bind to DNA and RNA in the regulation of transcription and translation (Jeffery 2003; Piatigorsky 1992). The glycolytic enzyme phosphoglucoisomerase has been endowed with at least four different functions when secreted outside the cell (Jeffery 2003). Monomeric GAPDH is a glycolytic enzyme as a monomer, but a nuclear uracil-DNA glycosylase as a tetramer. And many more. Moreover, there are seemingly no rules, no particular common grounds determining their various activities. The state of activation or inactivation of the various activities of such moonlighting proteins can depend on several mechanisms such as their localisation (cytoplasmic/nuclear, intracellular/extracellular), the cell type, their oligomerisation state and interactions with other factors (Jeffery 2003).

In this context, it is interesting to note how several regulatory mechanisms of both BARS and CtBP1 affect their structure and their intracellular localisation. PAK1 mediated phosphorylation of CtBP1 induces its translocation from the nucleus to the cytoplasm (Barnes et al. 2003) thereby inhibiting CtBP activity as a co-repressor of transcription. On the other hand sumoylation of CtBP1 or interaction with the PDZ domain of neuronal nitric oxide synthase enhances its nuclear retention (Lin et al. 2003; Riefler et al. 2001). If translation from the nucleus
appears to suppress a nuclear activity, the likelihood is that the same protein takes on another activity in its new location. Notably, all the regulatory mechanisms mentioned involve sequences that are conserved between CtBP1 and BARS so it is possible that what has been documented for CtBP1 will be found to apply to BARS as well. Indeed, although BARS is mainly cytosolic, there is some nuclear enrichment for in the G2 phase of the cell cycle (our unpublished observation). The binding of NAD and NADH has been exploited as a metabolic sensor that regulates CtBP1 activity (Zhang et al. 2002; Fjeld et al. 2003) and stimulates its DNA-binding activity associated to the dimeric conformation. In parallel, studies on the crystal structure of BARS (Nardini et al. 2003) have indicated that it can also bind NADH, and similar to CtBP1, this interaction stabilises the dimeric conformation. Interestingly, NADH binds at the same site as acyl-CoA but when BARS is bound to lipids it acquires a more “open” conformation that disrupts the dimer and stabilises the monomeric form. This binding of different interactors could therefore serve as a mechanism to switch between the two putative functions of BARS and CtBP1.

Whatever the regulatory mechanisms, BARS and CtBP1 share several commonalities with other moonlighting proteins: translocation within different intracellular compartments, as for the E.Coli PutA protein and GAPDH; conformational changes, as in the case of GAPDH with one of the two activities also related to transcription, the latter being a feature of many moonlighting proteins (Jeffery 1999).
Although not strictly relevant for classification as a moonlighter, some proteins have radically different functions that are still related to each other in different steps of the same pathway (Jeffery 1999). Similarly, the expression of E-cadherin, relevant for the establishment of cell polarity, is regulated by CtBP1 (Groteclaes et al. 2003) and it is transported from the Golgi complex to the basolateral plasma membrane under the regulation of BARS. Possibly, this could represent another example of a single process (cell polarisation) that can be controlled at different levels by the same moonlighting protein.

The reasons for the development of proteins with multiple functions are not clear, and at the moment we can merely speculate that this represents an economic way to serve different pathways with a minimal number of enzymes. Of course, one would like to know which function came first and how the alternative function(s) evolved. Information should be forthcoming by perusing the phylogenetic tree of these moonlighters but so far there are no clear indications. It has been also proposed that moonlight functions evolve from enzymes that that have rather low substrate specificities. On the other hand, an ancestral enzyme might have failed to restrict its specificity thereby maintaining the ability to bind multiple ligands (Jeffery 1999). In the case of BARS, there are preliminary observations of putative homologues in yeast (our unpublished observations) and in C. elegans that could be of help in understanding the evolution of multiple functions.

In conclusion, BARS drives a fission machinery that operates in multiple in vivo pathways that are distinct from those controlled by dynamin. While the mechanisms of action of dynamin have been studied extensively and models have
been proposed where dynamin acts either as a mechano-enzyme or as a regulatory GTPase (Hinshaw 2000; Conner et al. 2003; Lee et al. 2002; Farsad et al. 2001; Gad et al. 2000; Huttner et al. 2002), BARS has been less well studied. Probing into the mechanisms of action of BARS will require the analysis of BARS-interacting proteins (Spanò et al. 1999) and of the yeast and C. elegans homologues of BARS (our unpublished results). Important new tools for the study of BARS will be provided by a CtBP1/BARS and CtBP2 double knock-out mice that have been generated and is embryonically lethal. Cultured cells from these embryos can be viable (Hildebrand et al. 2002). Similarly, cells depleted of dynamin remain viable (Huang et al. 2004; Guha et al. 2003). Work on the redundancies, commonalities and differences between the actions of BARS and dynamin should lead to a precise definition of the principles of the fission process.
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