Molecular mechanisms of secretory granule biogenesis in neuroendocrine cells

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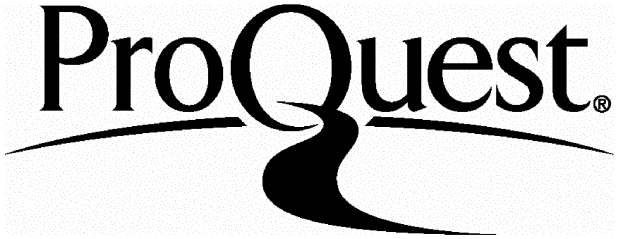

ABSTRACT

Secretory granules are storage organelles that form from the *trans*-Golgi network (TGN) as immature secretory granules (ISG) and subsequently undergo a maturation process characterised by a series of biochemical and morphological modifications.

The first part of this thesis concerns the characterisation of the ISG with respect to the conversion of prohormones to active hormones and the progressive acidification of the granule lumen. Secretogranin II (SgII) was used to study endoproteolytic processing by the endopeptidase PC2 in PC12/PC2 cells, a neuroendocrine cell-line. Processing could be observed in isolated ISGs but not in the TGN and was strongly dependent on the intragranular pH. Using processing of SgII as a pH indicator for the granule interior, an intragranular pH of 6.3+/- 0.1 for ISGs in a physiological buffer in the presence of ATP was determined.

The second part of this thesis describes the reconstitution of homotypic fusion of ISGs in a cell-free assay based on content mixing of two ISG populations isolated from PC12 or PC12/PC2 cells. Fusion was monitored by detecting the cleavage of [^{35}S]-sulphate labelled SgII by PC2. Antibodies were generated and characterised for quantitation of the end product p18. Fusion of ISGs was shown to be temperature, time, ATP and cytosol dependent and was competed by the addition of unlabelled ISGs. Mature secretory granules and TGN derived from PC12/PC2 cells were unable to fuse with ISGs. The time window within which ISGs are fusion competent was characterised and fusogenicity was shown to decrease rapidly after budding from the TGN. Trypsin treatment of the ISGs abolished fusion whilst clostridial neurotoxins had no effect. *N*-ethylmaleimide treatment was inhibitory and fusion could be rescued with untreated cytosol and to some extent with recombinant NSF and α-SNAP.

Table of contents

Chapter 2: Materials and Methods

Chapter 3: PH-dependent processing of secretogranin II by the endopeptidase PC2 in isolated ISGs

Chapter 7: Characterisation of the time window for fusion of immature secretory granules

Chapter 8: Conclusion

List of Figures

List of tables

Abbreviations

Ab	Antibody
AMBA	30% Acrylamide mixed with 0.8% bis-acrylamide
AMP-PNP	5'Adenylylimidodiphosphate
ARF	ADP-ribosylation factor
AP	Alkaline phosphatase
AP1	Adaptor protein 1
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
ATPγS	Adenosine 5'-3-*O*-(thio)triphosphate
BFA	Brefeldin A
BiP	Immunoglobulin heavy chain binding protein
CgA	Chromogranin A
CgB	Chromogranin B
CpE	Carboxypeptidase E
C-terminus	Carboxy-terminus
DAMP	3-(2,4-dinitroanilino)-3'-amino-*N*-methyldipropylamine
DMEM	Dulbecco's modified Eagle medium
DMEM-S	Dulbecco's modified Eagle medium without sulphate
DMF	*N*,*N*-Dimethylformamide
DTT	Dithiotreitol
EDTA	Ethylene diamine tetra-acetic acid
ELH	Egg-laying hormone
ER	Endoplasmic reticulum
FB	Fusion buffer
FSH	Follicle-stimulating hormone
GAP	GTPase activating protein
GDI	GDP-dissociation inhibitor

GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GGTase	Geranyl-geranyl transferase
GlcNac	N-acetylglucosamine
GLUT-4	Glucose transporter 4
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
GTPγS	Guanosine 5'-3-*O*-(thio)triphosphate
HB	Homogenisation buffer
HEPES	4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid
HG	Haemoglobin
HRP	Horse radish peroxidase
hr	hour
hrs	hours
hsp	heat shock protein
hsPG	heparin sulphate proteoglycan
ISG	Immature secretory granules
kDa	Kilodalton
LH	Lutenising hormone
MBS	m-Maleimidobenzoyl-N-hydroxysuccimide ester
MES	(2-[N-Morpholino]ethanesulfonic acid
MSG	Mature secretory granules
min	minute(s)
NEM	*N*-Ethylmaleimide
NSF	NEM-sensitive fusion protein
N-terminus	Amino-terminus
PAGE	Polyacrylamide gel electrophoresis
PAM	peptidyl α-amidating mono-oxygenase
PAPS	3'phosphoadenosine 5'-phosphosulphate

PBS	Phosphate buffered saline
PC	prohormone convertase
PC12	Rat pheochromocytoma cell line
PC2	Prohormone convertase PC2
PDI	Protein disulfide isomerase
PFA	Paraformaldehyde
PLD	Phospholipase D
PMSF	Polymethylsulphonyl fluoride
POMC	Proopiomelanocortin
PNS	Post-nuclear supernatant
PtdIns	Phosphatidylinositiols
REP	Rab reporter protein
rpm	rounds per minute
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SgII	secretogranin II
SNAP	soluble NSF attachment protein
SNAP-25	Synaptosome Associated Protein of 25 kDa
SNARE	SNAP-receptor
SV2	Synaptic vesicle protein 2
TBS	Tris Buffered Saline
TCA	Trichloroacetic acid
TGN	*trans*-Golgi Network
VAMP	Vesicle Associate Membrane Protein
VMAT1	Vesicular monoamine transporter 1
VSV-G	Vesicular Stomatitis Virus Glycoprotein

Dedication

This thesis is dedicated to my father Jean-Pierre Urbé

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Chapter 1: Introduction

1.1 Compartmentalisation of the eukaryotic cell: Concepts and definitions.

1.1.1 Protein targeting and vesicular transport

Eukaryotic cells are characterised by a complex system of membrane bound compartments or organelles (Alberts, *et al.*, 1994). This organisation makes it possible that biochemical reactions can take place in an optimal milieu that is regulated by the flux of ions and small molecules across the delimiting semi-permeable membrane. All proteins (except those encoded by the genome of mitochondria and chloroplasts) are synthesised in the cytosol and have to be transferred to their correct location. Protein targeting is dependent on sorting signals that mediate recognition and uptake into the membrane or the lumen of the organelle (Verner and Schatz, 1988; Walter and Lingappa, 1986) Proteins that have no signal, remain by default in the cytosol. Different sorting signals exist to direct proteins into the nucleus, mitochondria, chloroplasts, peroxisomes and into the ER. Proteins that move from the cytosol into the nucleus are transported through nuclear pores that function as selective gates, while proteins that move into the ER, mitochondria and chloroplasts are transported by protein translocator complexes (Alberts, *et al.*, 1994; Verner and Schatz, 1988; Walter and Lingappa, 1986). Proteins destined for secretion or for other compartments of the endomembrane system first enter the ER (Blobel and Dobberstein, 1975) and are transported onwards to their respective destinations via transport vesicles (Palade, 1975).

The intracellular synthesis of secretory products and the vesicular nature of the transport intermediates was first studied in pioneering work on pancreatic acinar cells by Caro and Palade (Caro and Palade, 1964) who used autoradiography and electron microscopy to follow the journey of metabolically labelled proteins from the ER to the lumen of the acini where they were secreted. Transport vesicles are loaded with cargo from the lumen of one compartment, pinch off and deliver their content to a second compartment by fusing with its membrane (Rothman, 1994). Vesicular transport does not only move soluble proteins but also lipids and membrane proteins from one compartment to another. The identity of the compartments of the endomembrane system relies on positive selection of the cargo for entry into vesicles and exclusion, retention and retrieval (by vesicles) of other proteins including resident proteins of the compartments (Aridor and Balch, 1996; Nilsson and Warren, 1994). Positive selection involves specific sorting signals, for example the mannose 6-phosphate signal that mediates sorting of lysosomal enzymes into clathrin-coated pits (Hille-Rehfeld, 1995). Retention operates for example at the level of the Golgi-complex: both transmembrane domains and the cytosolic "stalk"-region have been implicated in preventing Golgi enzymes from entering transport vesicles (Nilsson and Warren, 1994). Proteins that are not properly retained, missorted or included into the vesicle as an integral part of the budding, targeting or fusion machinery, are retrieved by retrograde vesicles in a process that also involves sorting signals (Aridor and Balch, 1996; Pelham, 1994; Pelham, 1998). Such a recycling pathway operates for example between the Golgi and the ER and involves a specific C-terminal signal sequence (KDEL for soluble proteins and KKXX for transmembrane proteins) (Munro and Pelham, 1987, Jackson *et al,* 1990).

Vesicular transport of proteins takes place both along the secretory pathway (from the ER to the plasma membrane) and the endocytic pathway (from the plasma membrane to the lysosomes) and provides routes of communication between the interior of the cell and its surroundings (Palade, 1975). A combination of biochemical and genetic approaches has led to the identification of components involved in vesicle

formation and fusion (Hay and Scheller, 1997; Novick, *et al.*, 1980; Rothman, 1994). Transport from the ER to the Golgi and through the Golgi apparatus involves COP-I and COP-II coated vesicles (Schekman and Orci, 1996; Pelham, 1998; Urbé, *et al.*, 1997). Whereas all proteins leaving the ER are thought to be directed to the Golgi complex, the sorting process at the *trans*-side of the Golgi, in the "*trans*-Golgi network"(TGN) is far more complex due to the multitude of intracellular destinations fed from this site (Figure 1/1) (Griffiths and Simons, 1986; Traub and Kornfeld, 1997). In the TGN, proteins destined for secretion are sorted away from proteins that are targeted to the endosomal system. Lysosomal enzymes bind to the mannose-6-phosphate receptor which is recruited via adaptor proteins into clathrin coated pits (Hille-Rehfeld, 1995). These pits form clathrin coated vesicles that bud from the TGN and fuse with an endosomal compartment. In regulated secretory cells, proteins destined for secretion are further sorted into two distinct pathways, one of which is regulated whilst the other operates constitutively (Gumbiner and Kelly, 1982; Halban and Irminger, 1994; Urbé, *et al.*, 1997). In polarised cells, exemplified by MDCK cells and some neuronal cell-types, proteins destined to the apical plasma-membrane (or to the axon) are sorted in the TGN from proteins directed to the basolateral site (to the dendrites) in a process that involves both targeting signal sequences and lipid-mediated sorting events (Dotti and Simons, 1990; Matlin, 1992; Matter and Mellman, 1994; Simons and Wandinger-Ness, 1990).

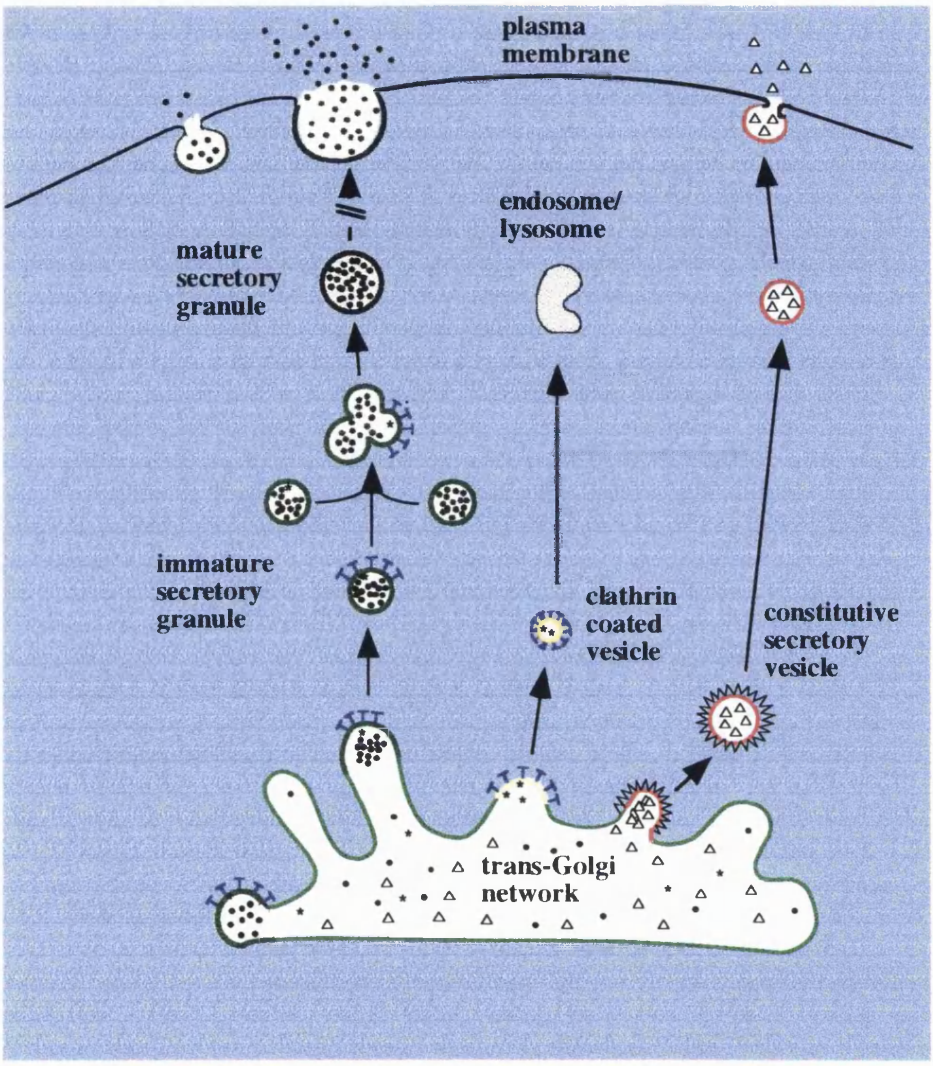


Figure 1/1: *Sorting of proteins in the TGN and formation of secretory granules in neuroendocrine cells.*

Legend:
- ⊤ clathrin triskelion
- ⋆ lysosomal enzyme
- △ constitutive secretory protein
- • regulated secretory protein

1.1.2 Definitions of compartments

In an effort to analyse the structure and function both of the biosynthetic and the endocytic pathways in the eukaryotic cell, the boundaries of the compartments that constitute this endomembrane system have to be clearly defined. The identity of a compartment is generally assessed by the following criteria:

Morphological appearance

Some compartments have a very unique characteristic structure that gives them an unmistakable morphological appearance. The stacked cisternae of the Golgi apparatus illustrate this point (Farquhar and Palade, 1981). However it has to be noted that these structures characterise a steady state equilibrium of ongoing membrane traffic and can change dramatically for example during mitosis or as a result of various treatments leading to the disruption of the cytoskeleton (Lowe, *et al.*, 1998). An other example is the regulated secretory granule of neuroendocrine cells which is characterised by an electron dense core (Farquhar and Palade, 1981) consisting of densely packed, aggregated proteins and catecholamines in a complex with smaller molecules including ATP and calcium (Winkler, *et al.*, 1986). This dense core gives the secretory granules a unique appearance on electron micrographs.

Enzymatic content

Enzymatic reactions can be used to identify the location of a specific compartment if the enzyme is a resident protein of this compartment. This approach is particularly useful when a specific compartment is purified by subcellular fractionation and the purification can be followed by assaying the enzymatic activity of the subcellular fractions. For example the purification of Golgi apparatus from rat liver can be followed by measuring the galactosyltransferase activity (Slusarewicz, *et al.*, 1994). The enzymatic activity of sialyltransferase has been used to determine the position of the TGN in relation to post-TGN vesicles after subcellular fractionation of cell

homogenates by gradient centrifugation (Tooze and Huttner, 1990). In the same way lysosomal enzymes can be used to identify lysosomes both in subcellular fractions and on microscopical sections by enzyme cytochemistry (de Duve, 1975; Holtzman, 1989; and references therein).

Non-enzymatic marker proteins

Resident proteins can be used to identify compartments. For example the hsp70 homologue BiP is used as a marker protein of the ER where it binds to incorrectly folded proteins in the ER and is involved in their refolding (Pelham, 1989). Proteins of the granin family can be used as marker proteins of the secretory granules where they constitute in many cases the majority of the protein content (Fischer-Colbrie, *et al.*, 1987, Huttner, *et al.*, 1991).

Apart from these luminal markers, some proteins that are associated with the cytoplasmic face of compartments are also used as markers. Amongst these is the clathrin coat adaptor protein γ-adaptin, a subunit of the AP1 complex, that is present in a cytosolic and a membrane bound pool in the cell (Pearse and Robinson, 1990). Antibodies against γ-adaptin label a region of the TGN by immunofluorescence microscopy (Robinson, 1997). The same protein is however also localised to an early endosomal compartment (Le Borgne, *et al.*, 1996) and, in endocrine cells, to immature secretory granules (Dittie, *et al.*, 1996). This observation illustrates the difficulty in assigning an identity to a compartment based on proteins that are cycling between compartments. Rab proteins, another family of proteins that cycle between a membrane bound and a cytosolic pool, are also used as markers for subcellular compartments (Zerial, *et al.*, 1992). While some rab proteins have a very restricted distribution (for example rab5 localises to early endosomes; Chavrier, *et al.*, 1991; Zerial, *et al.*, 1992), others are found on a variety of compartments (for example rab11 is localised to TGN, post-TGN vesicles and recycling endosomes; Ullrich, *et al.*, 1996; Urbé, *et al.*, 1993).

Operational definitions

Compartments can be operationally defined using a pulse-chase approach. Compartments of the biosynthetic pathway can be labelled by a pulse of radioactively labelled amino acids, for example [^{35}S]-methionine, [^{3}H]-leucine or [^{3}H]-tyrosine. Detection usually involves subcellular fractionation followed by scintillation counting or separation of the proteins by SDS-PAGE and autoradiography. In addition, [^{3}H]-labelled proteins can also be used for autoradiography of ultrathin sections for electron microscopy (Palade, 1975). While the length of the pulse time determines the spatial resolution of the analysis, the length of the chase time with non-radioactive amino acids determines the position of the label within the biosynthetic pathway. Proteins in the TGN can be labelled with [^{35}S]-sulphate on tyrosines and carbohydrate chains. Tyrosine sulphation (Baeuerle and Huttner, 1987) as well as the sulphation of proteoglycans (Kimura, *et al.*, 1984) is restricted to the TGN. This method can be applied to identify the TGN and immature and mature secretory granules using [^{35}S]-sulphate labelled secretogranin II as a marker (Tooze and Huttner, 1992). Another example for operational definitions is the pulse-chase internalisation of a variety of endocytic tracers, e.g. horse radish peroxidase or colloidal gold, to identify compartments of the endocytic pathway (Griffiths, *et al.*, 1989, Holtzman, 1989)

Temperature blocks

Some compartments have initially been identified on the basis of treatments that perturb membrane traffic. This does not mean that these compartments do not exist under steady state conditions however their morphological and sedimentation characteristics may be more pronounced and their identity is more apparent under these conditions. Temperature blocks have been used to accumulate secretory proteins and viral glycoproteins at different stages of the secretory pathway (De Curtis and Simons, 1988; Saraste and Svensson, 1991; Xu and Shields, 1993; Kuismanen and Saraste, 1989). Incubation at 15°C accumulates proteins in the intermediate compartment (IC or 15°C block compartment (Hauri and Schweizer, 1992; Saraste and Svensson, 1991)

and incubation at 20°C blocks exit from the *trans*-Golgi network (TGN or 20°C block compartment) (Matlin and Simons, 1983).

1.1.3 Secretory granules and regulated secretion.

All cells undergo some form of constitutive secretion whilst regulated secretion is generally understood as a specialised function of exocrine, endocrine, neuronal, neuroendocrine cells and of some cell-types of the blood cell lineage (Burgess and Kelly, 1987). Calcium dependent and phorbol-ester regulated secretion of glycosaminoglycans has recently been observed in fibroblasts (Chavez, *et al.*, 1996). In this respect it should also be noted that lysosomes have been found to undergo regulated fusion with the plasma membrane under certain conditions (Rodriguez, *et al.*, 1997). Lytic granules in cytotoxic T-cells undergo regulated secretion upon antigen stimulation and combine aspects of secretory granules and lysosomes (Griffiths, 1996). Other regulated fusion events with the plasma membrane include the insulin-stimulated fusion of GLUT-4 (Glucose-transporter 4) containing vesicles in adipocytes (Slot, *et al.*, 1991; Smith, *et al.*, 1991) and the histamine-induced fusion of tubulo-vesicular membranes leading to the insertion of the gastric H^+/K^+-ATPase into the apical membrane of parietal cells (Forte, *et al.*, 1989). These vesicles derive from an endocytic compartment but are distinct from synaptic-like vesicles in neuroendocrine cells (Herman, *et al.*, 1994).

Secretory granules of neuroendocrine cells are organelles that are specialised in the storage and release of proteins including bioactive peptides and neurotransmitter molecules (Johnson, 1988). The biogenesis of the secretory granule is characterised by multiple budding and fusion events that will be discussed in detail further below (Tooze and Stinchcombe, 1992; Urbé, *et al.*, 1997). Secretory granules are formed at the TGN and the nascent secretory granules are referred to as immature secretory granules (ISGs) (Tooze, *et al.*, 1991; Urbé, *et al.*, 1997). The ISGs bud from the TGN and

move from the perinuclear region to the cell periphery while undergoing a series of maturation steps (Tooze and Stinchcombe, 1992; Urbé, *et al.*, 1997). Whilst most vesicles in the cell function solely as transport intermediates, the secretory granule both functions as a transport vesicle en route from the TGN to the plasma membrane and a storage organelle. The mature secretory granule (MSG) can be seen as a stable compartment that is only consumed by fusion with the plasma membrane after stimulation of the cell with a specific signal. This signal triggers a change of the levels of intracellular second messengers and often involves an elevation in the calcium concentration (Burgoyne and Morgan, 1995). Secretion is thus coupled to a stimulus and in the absence of a stimulus the turnover of the secretory granules, which is referred to as basal release, is very low.

Both ISGs and MSGs can undergo exocytosis but the extent to which and the conditions under which ISGs fuse with the plasma membrane have not yet been well characterised (Rhodes and Halban, 1987; Tooze, *et al.*, 1991). The mechanisms of regulated exocytosis have been studied in detail and implicate both components of a general fusion machinery and special regulatory molecules that couple fusion to a specific signal (Burgoyne and Morgan, 1993). From the electrophysiological point of view, a very fast component (exocytotic burst), most likely corresponding to already docked secretory granules (Parsons, *et al.*, 1995), and a slow component which involves the recruitment of the so-called latent pool of granules further away from the plasma membrane (Heinemann, *et al.*, 1994) can be observed (Gillis and Chow, 1997). In general, exocytosis seems to involve a docking-step and several ATP-dependent priming steps, however the relationship between docking and priming is still unclear (Martin, 1997).

A large number of proteins involved in both priming/docking and fusion of secretory granules with the plasma membrane have been identified. Amongst these are proteins that are implicated in the disruption of the cortical actin network, recruitment of granules to the plasma membrane, modification of lipids both in the secretory granule

membrane and the plasma membrane, and molecules involved in the specific interaction and fusion of the two membranes with each other (for reviews see Burgoyne and Morgan, 1993; Martin, 1997). While many aspects of secretory granule exocytosis are identical to the synaptic vesicle mediated neurotransmitter release at the synapse, one major difference lies in the relatively low speed of secretory granule exocytosis (in the range of seconds) compared to synaptic vesicle fusion (in the range of milliseconds) (Ninomiya, *et al.*, 1997). Furthermore, synaptic vesicle exocytosis in the nerve terminal is restricted to a specialised region of the presynaptic membrane called the active zone, while such a specialised region has not yet been identified for the exocytosis of large dense core secretory granules, reviewed by Nicholls (1994).

1.2 Molecular components involved in intracellular membrane fusion events

1.2.1 NSF and SNAPs

The major breakthrough in intracellular membrane fusion was the discovery that a protein sensitive to low concentrations of the sulphhydryl alkylating agent *N*-ethylmaleimide (NEM) called the NEM-sensitive fusion factor/protein (NSF) was required for a late step in vesicular transport of vesicular stomatitis virus glycoprotein (VSV-G) protein from one Golgi-cisterna to another (Block, *et al.*, 1988; Glick and Rothman, 1987; Malhorta, *et al.*, 1988; Orci, *et al.*, 1989; Rothman, 1996). These data were derived from a cell-free assay measuring transport of VSV-G from a "donor" Golgi population, derived from a mutant CHO cell-line that lacks a glycosylating enzyme, to an acceptor Golgi of wild-type CHO cells in which the VSV-G is then modified by addition of radioactive *N*-acetylglucosamine (Balch, *et al.*, 1984; Braell, *et al.*, 1984; Fries and Rothman, 1980; Rothman, 1996). Dissection of this transport step led to the identification of sequential intermediates and resulted in the identification of proteins involved in budding and fusion of the vesicles (Orci, *et al.*, 1989).

The finding that NEM-inactivation of transport could be restored by complementing with fresh cytosol, indicated that there was a cytosolic pool of NSF and provided a functional assay for its purification (Block, *et al.*, 1988). NSF is a 76 kDa protein that forms trimers and has two ATP-binding domains (Whiteheart, *et al.*, 1994). These domains are characteristic for a family of ATPases (Confalonieri and Duguet, 1995) of which another member called p97 (Peters, *et al.*, 1990) has also been implicated in intracellular membrane fusion events (Acharya, *et al.*, 1995; Latterich, *et al.*, 1995; Rabouille, *et al.*, 1995). The sequence of NSF revealed (Wilson, *et al.*, 1989) that there was a functionally exchangeable homologue in yeast, called sec18 which has been shown to be required for transport between the ER and the Golgi (Kaiser and Schekman, 1990). This finding provided evidence for the idea that the same fundamental machinery operates in yeast and animal cells (Ferro-Novick and Jahn, 1994). NSF was found to be involved in many other transport steps from the ER to the plasma membrane and in endocytosis both in mammals and in yeast suggesting that intracellular membrane fusion uses a "universal" molecular machinery throughout the cell (Beckers, *et al.*, 1989; Diaz, *et al.*, 1989a; Haas and Wickner, 1996; Rabouille, *et al.*, 1995; Robinson, *et al.*, 1997; for reviews see Whiteheart and Kubalek, 1995; Hay and Scheller, 1997).

NSF is found both in the cytosol and on membranes. Binding of NSF to membranes was shown to be mediated by soluble NSF-attachment proteins called SNAPs (Clary, *et al.*, 1990; Weidman, *et al.*, 1989). In mammalian cells, SNAPs exist in three isoforms: α-SNAP and γ-SNAP are ubiquitously expressed while β-SNAP is a brain-specific isoform (Whiteheart, *et al.*, 1993) which is similar to α-SNAP. The yeast homologue sec17 (Clary, *et al.*, 1990) is functionally equivalent to mammalian α-SNAP (Griff, *et al.*, 1992).

1.2.2. The SNARE-hypothesis

Following the discovery of the SNAPs, the search began for the binding site for SNAP on the membrane, the so-called SNAP-receptor (Wilson, *et al.*, 1992). Experiments using affinity purification with recombinant NSF and α-SNAP in the presence of non-hydrolysable ATP led to the isolation of SNAP-receptors, or SNAREs from brain extracts (Söllner, *et al.*, 1993b). One of these proteins, VAMP (Vesicle-Associated-Membrane-Protein, also known as synaptobrevin) was a synaptic vesicle protein (Baumert, *et al.*, 1989; Trimble, *et al.*, 1988) and two other proteins, syntaxin and SNAP-25 (Synaptosome-Associated-Protein-25kDa) were proteins of the presynaptic membrane (Bennett, *et al.*, 1992b; Oyler, *et al.*, 1989). Interestingly, VAMP had already been identified as a target for tetanus and botulinum-B neurotoxins (Schiavo, *et al.*, 1992). Subsequently, it has been shown that these three proteins and some of their isoforms are the targets of the clostridial neurotoxins (Huttner, 1993; Niemann, *et al.*, 1994). VAMP is cleaved by tetanus toxin and botulinum toxin (BoNT) D, B and G, syntaxin by BoNT C and SNAP-25 by BoNT A, E and C (for reviews see Montecucco and Schiavo, 1995; Niemann, *et al.*, 1994). Poisoning of synaptic terminals by these neurotoxins leads to an inhibition of neurotransmitter-release (Montecucco and Schiavo, 1995) providing functional evidence for a central role of the SNAREs in synaptic vesicle fusion .

Many more SNAREs have since been cloned both in yeast and mammals and SNAREs have been shown to be required for many intracellular fusion events (Bock and Scheller, 1997). The SNAREs that are associated with vesicles are called v-SNAREs (or vesicle membrane SNAREs) while the presynaptic membrane SNAREs are called t-SNAREs (or target membrane SNAREs). Although the primary sequence of the v- and t-SNAREs is not very well conserved, they have a number of structural and topological features in common (Hay and Scheller, 1997). The VAMP-like v-SNAREs and the syntaxin-homologues are type I-transmembrane proteins with a large

cytoplasmic domain and a very short luminal domain (Bennett, *et al.*, 1993; Bennett and Scheller, 1993). The cytoplasmic N-terminal region of these proteins contains predicted coiled-coil domains which have been implicated in protein-protein interactions (Hayashi, *et al.*, 1994; Kee, *et al.*, 1995). SNAP-25 and a new isoform, SNAP-23, are palmitoylated and associated with the cytoplasmic face of the membrane (Oyler, *et al.*, 1989; Ravichandran, *et al.*, 1996). It has been proposed that the interaction of v- and t-SNAREs determines the specificity of the vesicular targeting and/or fusion process (Calakos, *et al.*, 1994; Rothman and Warren, 1994). Specificity might be determined by the interaction of different v-SNAREs or t-SNAREs with each other in multivalent complexes (Hay and Scheller, 1997).

A complex containing NSF, SNAP and SNAREs can be extracted from membranes using non-ionic detergents (Wilson, *et al.*, 1992). This "fusion particle" sediments at 20S and dissociates upon ATP-hydrolysis of NSF (Wilson, *et al.*, 1992). In the absence of NSF and SNAPs the SNAREs can form a SDS-resistant complex that sediments at 7S (Hayashi, *et al.*, 1994). VAMP only binds weakly to syntaxin or SNAP-25 alone, but tightly to the complex of syntaxin and SNAP-25 (Hayashi, *et al.*, 1994). The complex of VAMP, syntaxin and SNAP-25 binds α-SNAP (McMahon and Südhof, 1995) which in turn can recruit NSF.

NSF-dependent dissociation of the SNARE-complex is thought to be required during exocytosis (Söllner, *et al.*, 1993a) and other membrane fusion events at some stage leading up to targeting and/or the fusion process itself (Hay and Scheller, 1997; Martin, 1997). It has also been suggested that NSF might have a chaperoning function and is involved in the reactivation of the SNAREs (Morgan and Burgoyne, 1995a). Indeed, it has been shown that NSF interacts with isolated syntaxin and induces a conformational change that might be required for "activation" of the SNARE (Hanson, *et al.*, 1995).

Other proteins associate with the SNAREs and regulate the SNARE-interaction. Regulation of the SNARE interaction is required: for example, v-SNAREs will inevitably end up in target membranes and have to be retrieved to the donor membrane. These recycling SNAREs as well as newly synthesised SNAREs have to be kept in an inactive form in order to ensure the fidelity of vesicle targeting and prevent non-specific fusion. One candidate for such a negative regulator is Munc-18, also called n-sec1/rb-sec1, a soluble 67 kDa protein that binds to syntaxin and prevents *in vitro* interaction with SNAP-25 and VAMP (Garcia, *et al.*, 1994; Hata, *et al.*, 1993; Pevsner, *et al.*, 1994, Hata and Südhof; 1995). Another protein implicated in the regulation of the SNARE interaction is synaptotagmin. Synaptotagmin (also known as p65) is an integral vesicle membrane protein with a luminal N- and a cytoplasmic C-terminus. A C2-domain in the cytoplasmic portion of the protein (Sutton, *et al.*, 1995) is responsible for its calcium and phospholipid binding characteristics (Schiavo, *et al.*, 1996 and references therein). Synaptotagmin binds to syntaxin (Bennett, *et al.*, 1992b) and SNAP-25 (Schiavo, *et al.*, 1997) and also binds β-SNAP (Schiavo, *et al.*, 1995). It has been suggested that synaptotagmin may act as a calcium-dependent fusion clamp preventing NSF-dependent fusion in the absence of calcium (DeBello, *et al.*, 1993; Kelly, 1995). Different members of the synaptotagmin family show different sensitivities to calcium (Li, *et al.*, 1995; Ullrich, *et al.*, 1994a). Another family of proteins binding to the SNARE-complex are the complexins (McMahon, *et al.*, 1995). Complexins compete with α-SNAP for binding to the SNARE complex and might regulate the sequential interactions of α-SNAP and synaptotagmin with t-SNAREs (McMahon, *et al.*, 1995).

1.2.3 Rab proteins

Low molecular weight GTPases of the rab-family in mammals and the sec/ypt-family in yeast have been implicated in vesicular transport based on a combination of biochemical studies and genetic analysis. More than 40 mammalian members have so far been identified (Novick and Zerial, 1997). Rab proteins undergo a cycle of GDP/GTP-exchange and GTP-hydrolysis and thus exist in a GTP-bound or active form and a GDP-bound inactive form (Bourne, 1988; Bourne, *et al.*, 1990). This nucleotide-cycle is coupled to a membrane association/dissociation cycle (Pfeffer, 1994). Cytosolic GDP-rabs are associated with a protein called GDI (GDP-dissociation inhibitor) which mediates the rab-delivery to and removal from the membrane (Araki, *et al.*, 1990; Ullrich, *et al.*, 1993; Pfeffer, 1994). Rab-insertion into the membrane requires geranyl-geranylation typically of two cysteines in the C-terminus, a reaction catalysed by a Rab geranyl-geranyl transferase (GGTase) (Casey and Seabra, 1996). The newly synthesised non-prenylated rabs are complexed with a Rab escort protein (REP) that presents the rabs to the GGTase (Shen and Seabra, 1996) and is also able to deliver prenylated rab to the membrane (Alexandrov, *et al.*, 1994). Membrane association is accompanied by catalysed exchange of GDP for GTP by a Guanine nucleotide exchange factor (GEF) after displacement of GDI (Dirac-Svejstrup, *et al.*, 1997). GTP-hydrolysis stimulated by a GAP (GTPase activating protein) returns the rab into the inactive GDP-bound form, that can be recycled into the cytosolic pool by GDI (Dirac-Svejstrup, *et al.*, 1994; Ullrich, *et al.*, 1994b). GTP-bound rabs can interact with effector proteins that are thought to mediate the rab-function (Novick and Zerial, 1997; Shirataki, *et al.*, 1993; Stenmark, *et al.*, 1995; Wang, *et al.*, 1997).

Rab proteins were proposed to act as molecular switches in a proof-reading step in vesicular transport (Novick and Zerial, 1997; Pfeffer, 1994). However the exact stage at which these proteins act and their mechanism of action is only now being unravelled. Some rab proteins have been implicated in the organisation of the

cytoskeleton (Peranen, *et al.*, 1996) while others may be involved in the budding of vesicles (Benli, *et al.*, 1996; see Novick and Zerial, 1997, for review). The synaptic vesicle associated protein rab3 has an inhibitory role in synaptic vesicle fusion with the presynaptic membrane (Südhof, 1997), while other rab proteins, for example rab5, stimulate fusion (Bucci, *et al.*, 1992). It has also been suggested that rab proteins regulate the formation of multimeric protein complexes (Novick and Zerial, 1997) and several recent observations suggest a role for some rabs as upstream regulators of SNARE-interaction (Lian, *et al.*, 1994; Lupashin and Waters, 1997; Mayer and Wickner, 1997; Rothman and Söllner, 1997; Søgaard, *et al.*, 1994). In particular, the yeast rab1 homologue Ypt1 has been shown to be required for interaction of the t-SNARE Sed5 with the v-SNARE Bos1 by displacing the N-sec1 homologue Sly1 (Lupashin and Waters, 1997). Ypt1 was also required for association of Bos1 and Sec22 and the *ypt1* mutant phenotype could be overcome by overexpression of bos1 and sec22 (Lian, *et al.*, 1994). Another rab-like GTPase, Ypt7 was required for docking of yeast vacuoles after priming with Sec18 and Sec17 (Mayer and Wickner, 1997). Thus, rab proteins might ensure correct docking by activating specific SNAREs at the right time and at the right place and thereby ensure the fidelity of vesicle targeting.

1.3 Biogenesis of regulated secretory granules in neuroendocrine cells.

1.3.1 Morphological appearance of secretory granules.

As mentioned above, secretory granules are found in many different cell types including exocrine, endocrine, neuronal, neuroendocrine cells and cells of the blood lineage and immune system. Secretory granules vary in size and content, but they all share an electron-opaque, dense core, that gives them a granular appearance (Caro and Palade, 1964). The dense core consists of highly concentrated and condensed secretory proteins in an osmotically inert state (Apps, 1997; Farquhar and Palade, 1981). Some

cell-types contain more than one type of granule. For example, polymorphonuclear leukocytes contain "azurophilic" and "specific" granules (Bainton and Farquhar, 1968) and growth hormone and prolactin were found to be packaged in separate granules within the same cell in cow anterior pituitary (Fumagalli and Zanini, 1985). In Aplysia, pro-ELH (egg-laying hormone) is cleaved in the Golgi and the N- and C-terminal processing intermediates are sorted into two distinct populations of secretory granules (Sossin, *et al.*, 1990).

In a given tissue, the size of the secretory granules is characteristic for each cell-type (Burgess and Kelly, 1987). Thus mammotrophs, somatotrophs, gonadotrophs and thyrotrophs, all found in the anterior pituitary, can be discriminated on the basis of the diameter of their secretory granules which is 350μm, 600-900μm, 200μm, 150μm respectively (Smith and Farquhar, 1966). The size of the secretory granules seems to be related to their content. Granules containing mainly growth hormone for example are larger than granules containing mainly secretogranin II (Hashimoto, *et al.*, 1987) and FSH-containing secretory granules were twice as large as LH-containing granules in the same cell-type (Inoue and Kursomi, 1984).

The biophysical state of the secretory granule core has been shown to respond to changes in voltage and it has been suggested that the decondensation of the core-matrix is a consequence of potential gradients that arise during exocytosis (Nanavati and Fernandez, 1993). Decondensation of the core is necessary for the release of peptides and neurotransmitters that are trapped in the matrix.

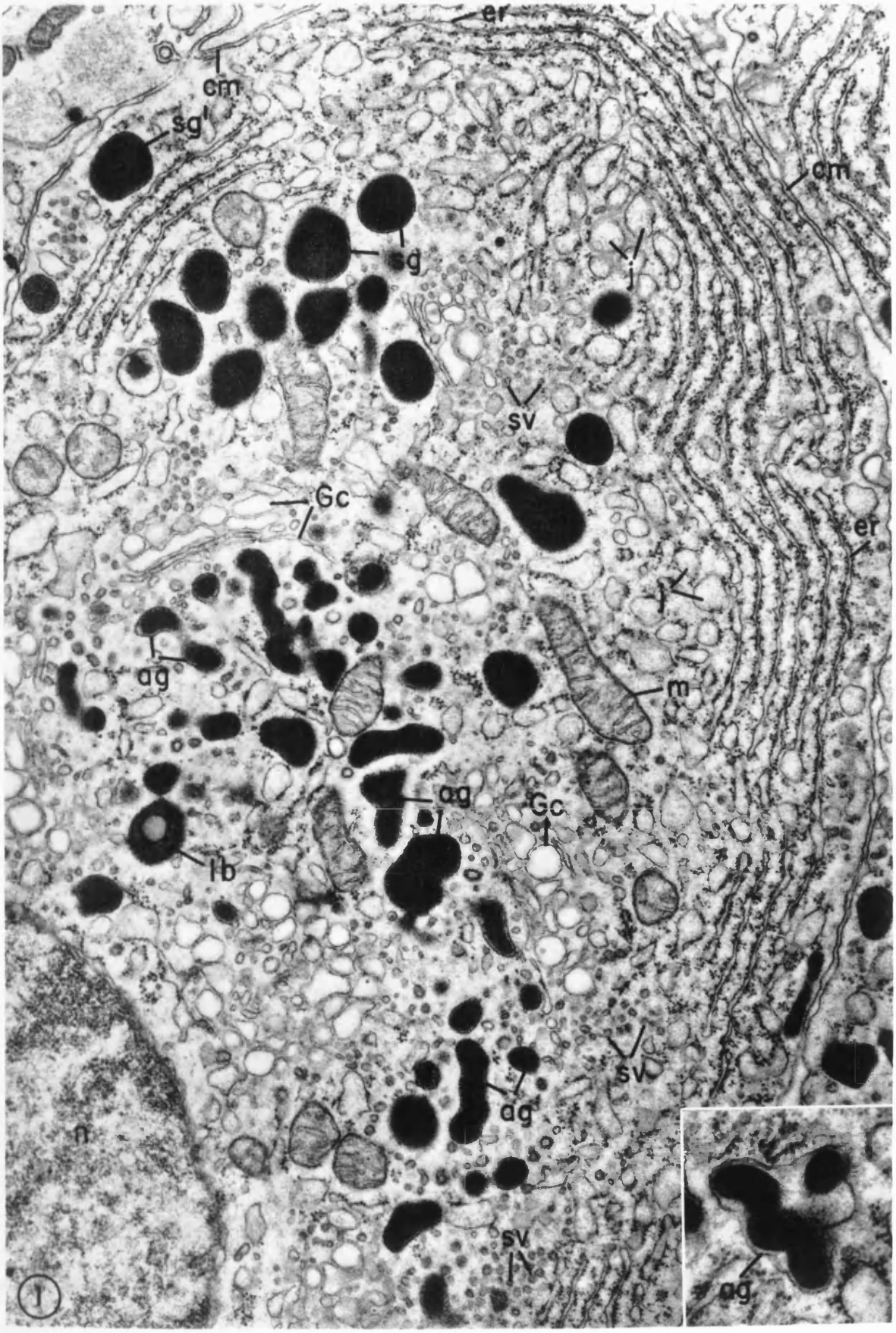
Profiles of secretory granules that contain several dense cores within one delimiting membrane have been observed by electron microscopy in neuroendocrine cells and in leukocytes (see insert Figure 1/2) (Bainton and Farquhar, 1966; Smith and Farquhar, 1966). Careful studies based on autoradiography of electron micrographs taken of pituitary mammotrophs pulse-labelled with [^{3}H]-labelled amino acids and chased up to 4 hours, led the authors to suggest that these "aggregated granules" arise by coalescence (fusion) of several (3 to 5) nascent granules (Salpeter and Fraquhar,

Figure 1/2: *Morphological appearance of secretory granules in mammotrophs.*

Mammotroph cell from the anterior pituitary gland of a lactating rat. These cells are rapidly synthesizing, concentrating and discharging mammotrophic (lactogenic) hormone; they show abundant peripheral arrays of rough-surfaced ER (er) parallel to the cell membrane (cm), a large juxtanuclear Golgi complex (Gc), many profiles of forming granules (aggregating granules, ag) and relatively few mature secretory granules (sg). Immature secretory granules vary in size and shape and are concentrated in the core of cytoplasm circumscribed by the Golgi cisternae. Mature granules are rounded or ovoid, more uniform in size (diameter, 600 μm), and are found primarily between the Golgi complex and ER or along the cell membrane (sg'). The inset depicts a polymorphous immature secretory granule, which appears to be formed by fusion of several smaller granules.(j), junctional elements of the ER; (sv), smooth-surfaced vesicles; (lb), lytic body.

Specimen fixed in 1% OsO4 inphosphate buffer (pH 7.6) and embedded in Araldite. Section doubly stained with uranyl and lead. x 24,000; inset, x 40,000.

Reproduced with permission from (Smith *et al.*, 1966).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1981). These irregular, polymorphous, aggregated granules round up and become ovoid, mature granules (Figure 1/2) (Smith and Farquhar, 1966). An increase in size from nascent or immature secretory granules to mature secretory granules has also been described in PC12 cells (Tooze, *et al.*, 1991), while β-granules from pancreatic β-islet cells do not enlarge during maturation (Halban and Irminger, 1994). A partial coat of clathrin has been shown on granules in β-cells of the endocrine pancreas and in neuroendocrine AtT20 cells. The significance of this coat will be discussed below (Orci, *et al.*, 1984a; Orci, *et al.*, 1985b; Tooze and Tooze, 1986).

1.3.2 Content of secretory granules in endocrine and neuroendocrine cells.

The composition of dense core secretory granules has been studied for over 20 years and reflects the dual nature of the secretory granule as a transport vesicle and a storage organelle (Apps, 1997; Winkler, *et al.*, 1986). Secretory granules of endocrine and neuroendocrine cells undergo a maturation process that is characterised by a gradual acidification of the granule interior (Hutton, 1982; Johnson, *et al.*, 1980; Johnson and Scarpa, 1975; Russell and Holz, 1981). Acidification is the driving force for neurotransmitter uptake (Johnson, 1988; Johnson, *et al.*, 1985) and is essential for efficient processing of prohormones (Orci, *et al.*, 1986; Orci, *et al.*, 1987). The proton concentration inside the secretory granule is controlled by the ion permeabilities of the granule membrane and by the activity of an ATP-dependent proton pump of the vacuolar type (Al-Awqati, 1986; Forgac, 1989). The secretory granule membrane thus contains proteins involved in the bioenergetic functions of the granule including the vacuolar H^+-ATPase and protein complexes responsible for $Na^{2+}/2H^+$ antiport and Ca^{2+}-uptake (Njus, *et al.*, 1986). The granule membrane also contains a transporter called VMAT1 (vesicular monoamine transporter 1) for catecholamine-uptake (Henry, *et al.*, 1994) and SV2 (synaptic vesicle protein 2) (Buckley and Kelly, 1985), a protein that may function as a transporter of nucleotides or polyanions in general (for review

see Apps, 1997). Cytochrome b561 is another component of the granule membrane that is unique to this organelle (Perin, *et al.*, 1988). Cytochrome b561 mediates the chemi-osmotically driven electron transport from a cytosolic pool of ascorbic acid to an intravesicular pool of semi-dehydro ascorbic acid and thus provides reducing equivalents to dopamine-β-hydroxylase and α-peptide amidase (Njus, *et al.*, 1986). Other proteins of the secretory granule membrane, including SNAREs and rab proteins, are involved in the targeting and fusion of the secretory granule to the plasma membrane (Darchen, *et al.*, 1990; Papini, *et al.*, 1994; Tagaya, *et al.*, 1996; Tagaya, *et al.*, 1995; for review see Apps, 1997).

The granule matrix contains secretory proteins including hormones, neuropeptides, prohormone convertases (PC) and other enzymes involved in either prohormone processing or neurotransmitter metabolism, as well as catecholamines and high concentrations of calcium and ATP (Apps, 1997; Halban and Irminger, 1994; Winkler, *et al.*, 1986). Secretory proteins are 200 fold more concentrated in secretory granules compared to the Golgi complex (Salpeter and Fraquhar, 1981). Some secretory proteins, for example insulin (in the presence of zinc ions), have the ability to form crystals (Steiner, 1973) while others are present as an amorphous aggregate (Chanat and Huttner, 1991).

A common component of the secretory granule matrix of endocrine, neuroendocrine and neuronal cells are the chromogranins. These proteins belong to a family of acidic proteins that are very abundant and constitute 87% of the total soluble proteins of chromaffin granules (Fischer-Colbrie, *et al.*, 1987; Huttner, *et al.*, 1991). Although these proteins have been studied for over 20 years, their function is still not understood. Chromogranins have been shown to be cleaved by prohormone convertases and their processing products have been suggested to act in a similar manner to neuropeptides (Arden, *et al.*, 1994; Hoflehner, *et al.*, 1995; Kirchmair, *et al.*, 1994; Kirchmair, *et al.*, 1993; Natori and Huttner, 1994). For example, secretoneurin, a peptide derived from secretogranin II stimulates dopamine release from

brain slices (Saria, *et al.*, 1993). Finally one member of the granin family, secretogranin V, also called 7B2, has been implicated in chaperoning the transport and modulating the activity of the prohormone convertase PC2 (Braks and Martens, 1994; Martens, *et al.*, 1994). It has also been suggested that the chromogranins act as helper proteins by coaggregating secretory proteins that have a lesser ability to aggregate and hence promote sorting of such proteins into the secretory granules (see below) (Huttner and Natori, 1995).

1.3.3 Acidification of secretory granules

The pH of acidic compartments can be visualised by electron microscopy by uptake of a basic derivative of dinitrophenol, 3-(2,4-dinitroanilino)-3'-amino-N-methyldipropylamine (DAMP) followed by immunocytochemistry with a monoclonal anti-dinitrophenol antibody (Anderson and Orci, 1988). Using this method a pH of 6.5 was determined for the TGN-lumen in fibroblasts. (Anderson and Pathak, 1985). More recently the pH of the TGN was measured by two different methods in living cells and values of 6.17 and 6.45 respectively were determined (Kim, *et al.*, 1996; Seksek, *et al.*, 1995). Secretory granules of endocrine and neuroendocrine cells and tissues are characterised by an acidic luminal pH in the range of 5.0 to 5.5 as measured by uptake of [^{3}H] or [^{14}C]-methylamine into isolated granules (Carty, *et al.*, 1982; Hutton, 1982; Johnson, *et al.*, 1980; Johnson and Scarpa, 1975; Russell and Holz, 1981).

The enzyme that is responsible for the proton-pumping both in the secretory and in the endocytic pathway is the vacuolar H^{+}-ATPase (Al-Awqati, 1986; Forgac, 1989; Nelson, *et al.*, 1988). This H^{+}-pump consists of 2 subdomains of which one catalyses ATP-hydrolysis (V_1) and the other forms the proton channel (V_0) (Al-Awqati, 1986; Forgac, 1989). An accessory subunit Ac45 has been found in chromaffin granules but its function remains unknown (Supek, *et al.*, 1994). The vacuolar ATPase generates a proton motive force that manifests itself in the generation of a membrane potential

and/or a pH-gradient the extent of which is determined by the permeability of the membrane for ions (especially anions) (Njus, *et al.*, 1986). If anions are able to follow the proton-flow, they counteract the build-up of an inwardly positive membrane potential and favour the generation of a pH-gradient. Other biophysical determinants are equally important. For example, in some cell-types, the presence of the Na^+/K^+-ATPase in the membrane of the early endosome has been shown to be responsible for the higher pH of the early endosome (pH 6.2) in comparison to late endosomes (pH 5.5) which do not contain this electrogenic exchanger (Cain, *et al.*, 1989; Fuchs, *et al.*, 1989).

The proton pumping activity of the vacuolar ATPase is the driving force for many basic functions fulfilled by the secretory granule: First, the uptake of neurotransmitters (catecholamines) into the granules is catalysed by a vesicular amine transporter in exchange for protons (Johnson, 1988; Johnson, *et al.*, 1985). Second, the transfer of electrons needed for the activity of dopamine-β-hydroxylase from cytosolic ascorbate via cytochrome b562 to luminal ascorbate depends on the membrane potential generated by the proton pump (Njus, *et al.*, 1986; Njus, *et al.*, 1983). Third, the processing of prohormones by some PC-enzymes, especially by PC2, is facilitated at acidic pH (Orci, *et al.*, 1986; Orci, *et al.*, 1987) due to a low pH-optimum of this enzyme (Arden, *et al.*, 1994; Davidson, *et al.*, 1988; Shennan, *et al.*, 1991; Shennan, *et al.*, 1995). Acidification also plays an important role for sorting by favouring aggregation of regulated secretory proteins and exclusion of other proteins in the TGN (Chanat, *et al.*, 1991). Furthermore, binding of ligands to receptors is regulated by the luminal pH of the compartments. This is illustrated by the binding of lysosomal enzymes to the mannose-6-phosphate receptor at pH 6.2 (in the TGN) which are then released at pH 5.5 (in late endosomes; Hille-Rehfeld, 1995).

The importance of acidification in the secretory pathway has been demonstrated for example with the use of weak bases, like ammonium chloride and chloroquine (Moore, *et al.*, 1983) or with monensin (Orci, *et al.*, 1984b). Weak bases accumulate

inside acidic compartments and neutralise the pumping-activity of the ATPase. The discovery of a class of fungal metabolites, the bafilomycins and concanamycins, that act as highly specific inhibitors of the vacuolar H^+-ATPase (Bowman, *et al.*, 1988; Droese, *et al.*, 1993), has provided a more specific way to assess the role of the vacuolar H^+-ATPase both in the endocytic (Clague, *et al.*, 1994; Johnson, *et al.*, 1993) and the biosynthetic pathways (Henomatsu, *et al.*, 1993; Reaves and Banting, 1994; Xu and Shields, 1994; Yilla, *et al.*, 1993). Thus it has been shown for example, that the formation of a vesicular intermediate in the endocytic pathway, called endocytic carrier vesicle or multivesicular body, was inhibited by bafilomycin (Clague, *et al.*, 1994). It also has been shown that bafilomycin inhibits secretion of prolactin to the plasma membrane in GH3 cells possibly by interfering with transport either at the TGN or in the ISG. Bafilomycin treatment resulted in a decrease in dense core vesicles and the appearence of large prolactin-containing vacuoles that were partially clathrin coated (Henomatsu, *et al.*, 1993). It is therefore conceivable that acidification also plays a role in the membrane remodelling of the secretory granule.

1.3.4 Proteolytic processing in neuroendocrine cells.

Most polypeptide hormones are initially synthesized as larger precursors that undergo limited proteolysis beyond the removal of the signal peptide in the ER to yield bioactive peptides (Halban and Irminger, 1994; Hook, *et al.*, 1994). Endoproteolytic cleavage usually occurs at a dibasic amino acid site although cleavage can also take place at a single basic amino acid residue (for review see Devi, 1991). The most common processing sites found in mammals are Lys-Arg and Arg-Arg. Subsequent to endoproteolysis, basic residue extensions at the C- and N-termini are removed by carboxypeptidase E/H (CpE) (Fricker, 1988) and aminopeptidase (Gainer, *et al.*, 1984) respectively. The peptides may then be amidated by the peptidyl α-amidating mono-oxygenase (PAM) at a glycine residue (Eipper, *et al.*, 1992).

The first direct evidence for the conversion of prohormones into active hormones came from the work of Don Steiner's laboratory on proinsulin in 1967 (Steiner, *et al.*, 1967; Steiner and Oyer, 1967). Proinsulin conversion could be mimicked *in vitro* by limited proteolysis with trypsin followed by carboxypeptidase treatment (Kemmler, *et al.*, 1973; Steiner, *et al.*, 1974). Originally, cathepsins derived from lysosome contaminations of the secretory granule preparations were erroneously identified as the conversion protease. However, the breakthrough came in 1987/1988 when Hutton and colleagues identified and characterised two enzymatic activities that converted proinsulin to insulin in a calcium and acidic pH-dependent manner (Davidson, *et al.*, 1988). This finding was followed by genetic studies in yeast and led to the discovery of a family of endoproteases of which the yeast kex2-protein is the prototype (Halban and Irminger, 1994).

Kex2 is a membrane bound, calcium-dependent serine protease that is required for processing of pro-α-factor and pro-killer toxin in yeast (Fuller, *et al.*, 1989). Kex2 and the kex-like endoproteases belong to the same family as the prokaryotic serine protease subtilisin and contain other members such as furin and the proprotein or prohormone convertases (PC-enzymes) (Barr, 1991; Steiner, 1991). The first mammalian homologue of kex2 was identified by computer sequence comparison as the fur-gene (Barr, *et al.*, 1991b; Roebroek, *et al.*, 1986). Its gene-product furin (also called PACE) is ubiquitously expressed (Seidah, *et al.*, 1994). Other members were subsequently identified which belong to the PC enzyme family. Seven members of this family have so far been described (Hook, *et al.*, 1994; Seidah, *et al.*, 1994; Seidah, *et al.*, 1996, and references therein). Expression of PC1/PC3 and PC2 is restricted to endocrine and neuroendocrine tissues including the brain (Seidah, *et al.*, 1994). Other members of the family are PACE4, PC4, PC5, PC6A and PC6B (Hook, *et al.*, 1994; Seidah, *et al.*, 1994 and references therein) and the most recently described PC7 which is also called LPC, PC8 or SPC7 (Seidah, *et al.*, 1996, and references therein).

All members share a common domain structure. The highest region of homology is found in the catalytic domain (Seidah, *et al.*, 1994; Seidah, *et al.*, 1993). The C-terminal region is most variable and includes a transmembrane domain in the case of kex2, furin and PC6B whilst PC1/3 and PC2 have a region which is predicted to form an amphipathic helix (for review see Halban and Irminger, 1994). In the case of CpE a similar motif is involved in association with the membrane (Fricker, *et al.*, 1990). Both PC1 and PC2 have an acidic pH-optimum (Bailyes, *et al.*, 1992; Shennan, *et al.*, 1991; Zhou and Lindberg, 1993) while furin is most active at neutral pH (Molloy, *et al.*, 1992). The activity of some PC-enzymes is also dependent on calcium (for review see Halban and Irminger, 1994).

The intracellular site of prohormone processing has remained controversial: processing is thought to commence either in the TGN (Schnabel, *et al.*, 1989; Xu and Shields, 1993) or only after packaging of secretory proteins into ISGs (Orci, *et al.*, 1987; Tooze, *et al.*, 1987). All PC-enzymes are synthesised as inactive precursors. Activation involves removal of the N-terminal pro-region which may act as a chaperone-like domain in the folding and intracellular transport of the enzymes (Molloy, *et al.*, 1994; Taylor, *et al.*, 1997) and as a competitive inhibitor of the proteolytic activity (Anderson, *et al.*, 1997). Removal of the pro-region can be autocatalytical and involve either an intramolecular (kex2, furin) or intermolecular reaction (PC2) (Anderson, *et al.*, 1997; Matthews, *et al.*, 1994). The rate of precursor activation varies between PC-enzymes. While pro-PC1 conversion begins in the ER, pro-PC2 activation is only initiated in the TGN and continues in the secretory granule (Benjannet, *et al.*, 1993; Seidah, *et al.*, 1994). Both the transport of PC2 along the secretory pathway (Benjannet, *et al.*, 1993) and its autocatalytic activation (Matthews, *et al.*, 1994) are regulated by its association with the granin 7B2 (Braks and Martens, 1994). 7B2 shares distant homology with the potato inhibitor I family of protease inhibitors and the pro-7B2, but not cleaved 7B2, inhibits PC2 activity *in vitro* (Martens, *et al.*, 1994; Zhu and Lindberg, 1995). Thus PC2-activation and activity

might depend on 7B2-conversion which can be catalysed *in vitro* by furin (Paquet, *et al.*, 1994).

1.3.5 Formation of secretory granules

The best-studied transport vesicles are characterised by a proteinaceous coat. This coat consists of clathrin and adaptor proteins in the case of the clathrin-coated vesicles and of coatomer in the case of COP-I coated vesicles (Robinson, 1997; Schekman and Orci, 1996). These coat-proteins self-assemble and, at least in the case of clathrin, this self-assembly is thought to drive the deformation of the membrane into a vesicle while adaptor proteins are involved in cargo selection (Pearse and Robinson, 1990). The only coat proteins identified so far on the secretory granule (clathrin and adaptor complex AP1), which only partially cover the membrane, are thought to mediate vesicle formation from the ISG rather than formation of the ISG from the TGN (Dittie, *et al.*, 1996). Many regulated secretory proteins, for example the granins, aggregate under the conditions of the TGN (low pH, high calcium) (Shennan, *et al.*, 1994; Chanat and Huttner, 1991). It has been suggested that budding of secretory granules does not require any coat proteins but that the aggregated proteinaceous core of the granule is able to provide the driving force for vesicle formation in a way analogous to the budding of a viral particle (Urbé, *et al.*, 1997). This hypothesis predicts that there have to be specific interactions between the granule core and the granule membrane that mirror the binding of viral nucleocapsid proteins to the membrane bound envelope proteins. In this way the secretory granule core could mould its own vesicle, driven only by the binding of granule membrane proteins with luminal aggregated content.

Sorting of proteins into clathrin-coated pits at the plasma-membrane is mediated by receptors (Robinson, 1994; Schmid, 1992) and by analogy it has been proposed that there may be a receptor responsible for sorting of regulated secretory proteins into

secretory granules (Halban and Irminger, 1994; Urbé, *et al.*, 1997). Studies demonstrating correct sorting of heterologous regulated secretory proteins derived for example from exocrine tissues (trypsinogen), into the secretory granules of endocrine or neuroendocrine cells (Burgess, *et al.*, 1985; Chu, *et al.*, 1990) suggest that if such a receptor exists, it would have to recognise a highly conserved sorting signal. The high degree of sequence diversity amongst regulated secretory proteins makes it unlikely that this motif corresponds to a linear amino acid sequence determinant as in the case of receptor mediated endocytosis (Kirchhausen, *et al.*, 1997). However, it has been shown that the proregion of prosomatostatin is necessary for sorting and acts as a dominant signal when linked to α-globin by rerouting this soluble protein to the regulated secretory pathway (Sevarino, *et al.*, 1989; Stoller and Shields, 1989).

More recent studies have focused on an N-terminal disulphide loop structure found in proopiomelanocortin (POMC), provasopressin, prooxytocin, proenkephalin, prodynorphin, chromogranin (Cg) A and B (Cool, *et al.*, 1995; Huttner, *et al.*, 1991). This amphipathic loop structure plays a role in the sorting of POMC to secretory granules and may represent a structural sorting motif (Cool, *et al.*, 1995). The importance of this loop is supported by data showing that DTT treatment of PC12 cells, resulting in the disruption of the disulphide bond in CgB, leads to constitutive secretion of CgB (Chanat, *et al.*, 1993).

Aggregation of regulated secretory proteins in the TGN has been suggested to play a central role in sorting of regulated secretory proteins by excluding non-aggregating soluble proteins from the budding secretory granule (Chanat, *et al.*, 1991; Urbé, *et al.*, 1997). Overexpression of proteins with a high propensity for aggregation has been shown to increase the sorting efficiency of regulated secretory proteins with a lower propensity to aggregate (Natori and Huttner, 1996) and it was suggested that this is due to heterophilic interactions leading to coaggregation (Huttner and Natori, 1995). If aggregation is an underlying feature of sorting into the secretory granule, the putative sorting receptor would not have to interact with all the molecules that are to be included,

but possibly only with peripheral aggregated molecules. Some granule matrix proteins, such as CgB (Pimplikar and Huttner, 1992), exist as membrane associated isoforms and it has been suggested that the membrane associated forms of such proteins act as receptors in the sorting process (Huttner, *et al.*, 1991). Interaction with the granule matrix would then rely both on homophilic interaction (between membrane bound and soluble isoforms) and heterophilic interaction by coaggregation between these "nucleating" complexes and other regulated secretory proteins. It has recently been shown that the membrane associated form of carboxypeptidase E (CpE), an enzyme involved in the generation of biologically active peptides (Fricker, 1988), can function as a sorting receptor for POMC (Cool, *et al.*, 1997). However these observations are currently debated (Thiele, *et al.*, 1997) and it seems unlikely that this protein is a universal sorting receptor as the absence of CpE in a mouse mutant CpE^{fat}/CpE^{fat} does not result in abnormal insulin processing or secretion (Irminger, *et al.*, 1997).

Unlike COP-I vesicles which continue to form in the presence of GTPγS (Melancon, *et al.*, 1987), secretory granule formation is inhibited under these conditions (Tooze, *et al.*, 1990b). Based on this initial observation, it has been shown that heterotrimeric G-proteins regulate the formation of secretory granules and constitutive secretory vesicles from the TGN (Barr, *et al.*, 1992; Barr, *et al.*, 1991a; Leyte, *et al.*, 1992). Another GTPase of the arf-family (ARF1) has also been shown to be involved in the formation of constitutive secretory vesicles and secretory granules from isolated TGN-membranes and in the formation of growth hormone (GH) - and prolactin (PRL)-containing secretory vesicles in semi-intact cells (Barr and Huttner, 1996; Chen and Shields, 1996). It has been suggested that ARF1 might act through phospholipase D (PLD) either by recruiting cytosolic factors to a patch of acidic phospholipids in the membrane of the forming bud or by initiating a signalling cascade (Boman and Kahn, 1995). Indeed, PLD-activity has been shown to stimulate secretory vesicle formation from the TGN in semi-permeabilised GH_4C_1 cells (Chen, *et al.*, 1997).

Phosphatidylinositol transfer protein (PITP) has also been implicated in the formation of both regulated and constitutive secretory vesicles in PC12 cells (Ohashi, *et al.*, 1995). It has been suggested that PITP presents polyphosphatidylinositols (PtdIns) to various phosphatidylinositol-kinases and thus contributes to the local generation of acidic phospholipids in the membrane (Cunningham, *et al.*, 1995; Kauffmann Zeh, *et al.*, 1995; Liscovitch and Cantley, 1995). PtdIns(4,5)P_2 has been shown to regulate the nucleotide bound state of ARF (Randazzo and Kahn, 1994; Terui, *et al.*, 1994), stimulate PLD activity (Liscovitch, *et al.*, 1994) and thus might also contribute to the recruitment of cytosolic factors to the forming bud (Liscovitch. and Cantley, 1995). There have also been several indications that both serine (Austin and Shields, 1996; Simon, *et al.*, 1996; Xu, *et al.*, 1995; Xu, *et al.*, 1996) and tyrosine phosphorylation events (Solimena, *et al.*, 1996; Wasmeier and Hutton, 1996) play a role in regulating the formation of regulated secretory granules. Interestingly, an as yet unidentified cytosolic phosphoprotein acts as a modulator upstream of the heterotrimeric G-proteins (Ohashi and Huttner, 1994).

1.3.6 Membrane remodelling during maturation

The temporal sequence of budding of immature secretory granules and sorting of regulated secretory proteins has been the subject of much discussion (Arvan and Castle, 1992). In some cells, for example in β-cells of the endocrine pancreas, sorting takes place predominantly from the ISG while in other cells, for example in PC12 cells, sorting is largely completed in the TGN (Arvan and Castle, 1992; Urbé, *et al.*, 1997). It has been suggested that whether sorting is predominantly TGN-based (also called "sorting by entry") or ISG-based ("sorting by retention") might depend on the propensity of the prohormones to form aggregates and the relative rates of prohormone synthesis and vesicle formation in the TGN of each cell-type (Urbé, *et al.*, 1997).

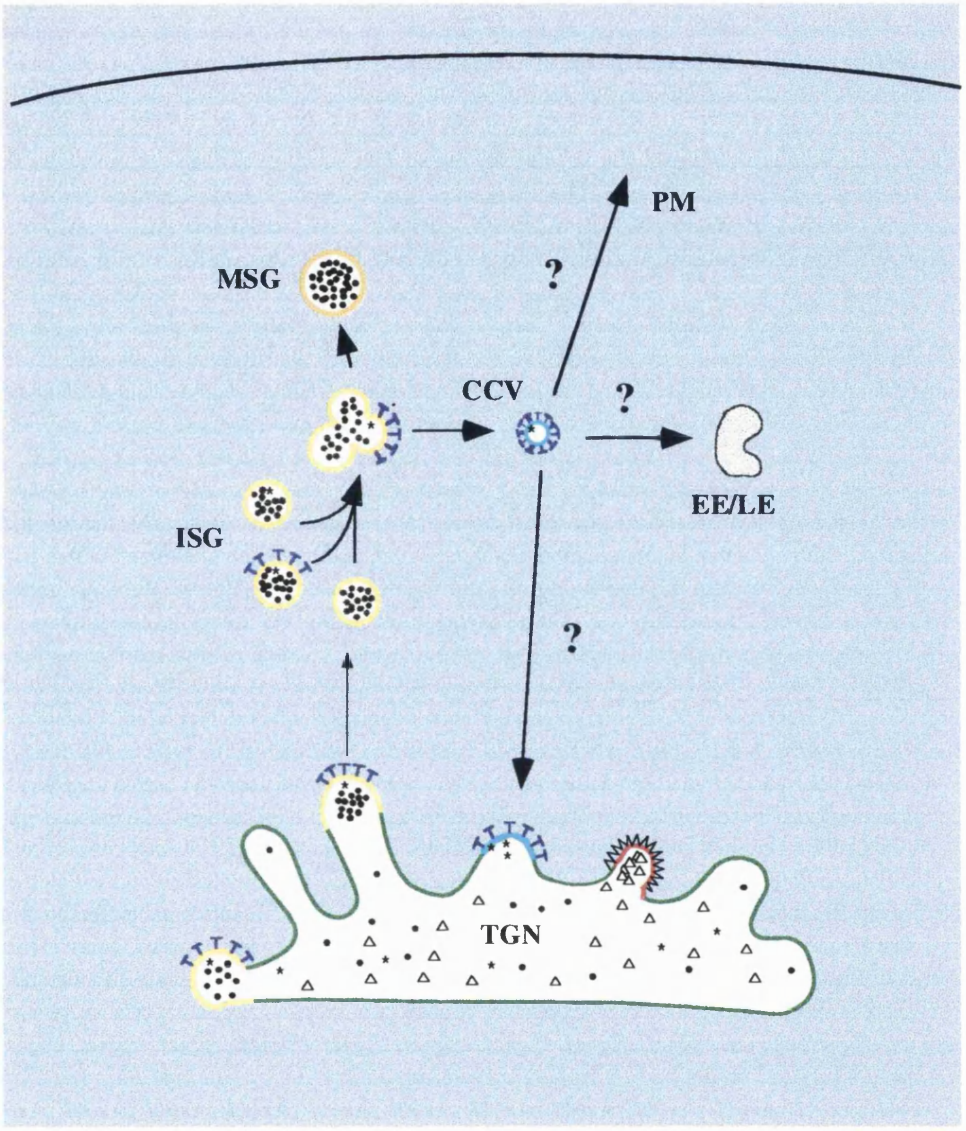


Figure 1/3: *Membrane remodelling during maturation*

Immature secretory granules (ISG), trans-Golgi network (TGN), mature secretory granules (MSG), clathrin coated vesicles (CCV), early endosomes (EE), late endosomes (LE), plasma membrane (PM).

Sorting from the ISG is thought to involve clathrin. It has been shown that the clathrin seen on ISGs (Orci, *et al.*, 1984a; Orci, *et al.*, 1985b; Tooze and Tooze, 1986) is bound through the adaptor complex AP1 (Dittie, *et al.*, 1996) which also mediates clathrin binding to the TGN (Robinson, 1997). Clathrin-coated vesicles budding from the ISG would then be targeted to one of three possible destinations (see Figure 1/3): First, proteins that are involved in the formation of the ISG might be recycled back to the TGN. Second, lysosomal proteins that have not been efficiently excluded during sorting at the TGN, might be targeted to an endosomal compartment. In β-cells (Kuliawat and Arvan, 1994) and in GH_4C_1 cells (Waguri, *et al.*, 1995) lysosomal enzymes have been shown to enter ISGs. Cathepsin B (but not cathepsin L) was removed from the secretory granules as it was absent from MSGs (Kuliawat, *et al.*, 1997). Finally, some proteins might be removed from the ISG and secreted in a "constitutive-like" way (Arvan and Castle, 1992). This might be the case for the C-peptide which is generated by proinsulin processing and excluded from the insulin crystal (Kuliawat and Arvan, 1992).

1.3.7 Homotypic fusion of immature secretory granules

Secretory granule maturation in some neuroendocrine cells is characterised by an increase in size (Salpeter and Farquhar, 1981; Tooze, *et al.*, 1991). In particular, immature secretory granules in PC12 cells had a measured dense core diameter of 79.9 +/- 12.8 nm compared to the MSG (120.0 +/- 11.7 nm) as determined on electron micrographs (Tooze, *et al.*, 1991). The nascent or immature secretory granules in these cells are smaller, of an irregular shape and a wider size-distribution while the mature secretory granules are larger and of uniform size. It has been mentioned above (section 1.3.1) that secretory granules with more than one dense core have been observed by electron microscopy (Bainton and Farquhar, 1966; Smith and Farquhar, 1966) (Figure 1/2). In addition to these morphological data, it has also been shown by differential centrifugation analysis of ISGs and MSGs in isopycnic solutions that MSGs have a

higher density than ISGs (Tooze, *et al.*, 1991). The respective calculated values for ISG and MSG were 385 +/- 19 and 1340 +/- 73 Svedberg (Tooze, *et al.*, 1991). This increase in size and density from ISG to MSG was taken as an indication for a homotypic fusion event between ISGs during maturation. From the morphological and biochemical data described above, it was proposed that 3 to 5 ISGs fuse with each other to form one MSG (Tooze, *et al.*, 1991) (Figure 1/3). Given the spherical nature of both ISGs and MSGs, such a fusion event would result in an excess of membrane and it was suggested that this membrane would be removed by clathrin coated vesicle budding (Tooze and Stinchcombe, 1992; Urbé, *et al.*, 1997).

1.4 Purpose of study

At the start of this study no information was available on the homotypic fusion of immature secretory granules. Hence, the main goal of this thesis was to establish a cell-free assay that would allow the reconstitution and characterisation of homotypic ISG fusion. The cell-lines used in this study, PC12, a cell-line derived from a rat pheochromocytoma (Heumann, *et al.*, 1983), and PC12/PC2 (Dittié and Tooze, 1995), are a good model for the study of the regulated secretory pathway. Fusion of two distinct populations of ISGs was to be monitored by measuring content mixing based on the endoproteolytic processing of [^{35}S]-sulphate labelled secretogranin II (SgII), contained in one population, by the prohormone convertase PC2, contained exclusively in the other ISG population. In order to establish this assay, the processing of SgII by PC2 in isolated ISGs was to be first characterised. The cell-free fusion assay was then to be used to investigate the molecular components involved in homotypic fusion of ISGs.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Cell culture

Reagents used for cell culture were supplied by Gibco-BRL Europe or Sigma. Plastic flasks and dishes for cell culture were obtained from Falcon or Nunc.

2.1.2 Chemicals

Chemicals were supplied by Merck, Sigma, Boehringer Mannheim or Pharmacia unless indicated otherwise. Reagents for the ATP-regenerating system and nucleotides were supplied by Boehringer Mannheim. Radiochemicals, carrier free [^{35}S]-sulphate as a disodium salt (1-10^{6} mCi/mmol and 10 mCi/ml), [^{125}I]-protein A (30 mCi/mg) and [^{14}C]-labelled molecular weight markers for SDS-PAGE were supplied by Amersham. Protein A-sepharose (CL-4B) was supplied by Pharmacia. Dried skimmed milk (Marvel, 1.1% (w/w) fat) for immunoblotting was supplied by Premier Brands UK Ltd (Stafford, UK). Protogel (30% (w/v) Acrylamide + 0.8% (w/v) Bisacrylamide) was supplied by National Diagnostics (UK). Bradford protein assay reagent and molecular weight markers for SDS-PAGE were supplied by Bio-Rad (UK). Ponceau S and Dowex 1X2 ion-exchange resin were from Serva (UK). Ready safe scintillation fluid and all ultra-centrifuge tubes were from Beckman GmbH (UK). Moviol 4-88 was supplied by Hoechst (UK). Concanamycin A was a gift from Ciba Geigy (Basel, Switzerland).

Films for autoradiography were from Kodak (XAR5). Nitrocellulose membranes were supplied by Schleicher and Schuell GmbH (UK), cryotubes by Nunc (UK) and sterile filtration units by Nalgene (UK).

2.1.3 Antibodies

Commercial secondary antibodies:

Horse-radish peroxidase (HRP) -conjugated anti-rabbit and anti-mouse IgGs were supplied by Amersham (UK). Alkaline Phosphatase (AP) -conjugated anti-rabbit and anti-mouse IgGs were supplied by Bio-Rad (UK). Cy3- and Fluorescein-conjugated donkey anti-rabbit and anti-mouse IgGs were supplied by Jackson Immuno Research Laboratories Inc. (UK).

All other antibodies used and generated in this study are listed in Table 2/1.

2.1.4 Recombinant proteins

Recombinant L-chains of botulinum neurotoxins A, C and D, His-tagged NSF and His-tagged α-SNAP were obtained from Dr. G. Schiavo. His-tagged rab3-GDI was prepared and donated by Dr. F.A. Barr.

Table 2.1: *Antibodies*

Antibody	raised against	Source	Application	Reference
4BF (pc)	PC2 (C-term.)	I. Lindberg	WB	
175 (pc)	SgII (C-term.)	S.A. Tooze	WB, IP, IF	(Dittié&Tooze,1995)
100 (pc)	SgII (N-term.)	S.A. Tooze	IP	(Dittié&Tooze, 1995)
67 (pc)	SgII (preCS2)	this study	WB	
68 (pc)	SgII (preCS2)	this study	WB	
69 (pc)	SgII (preCS2)	this study	WB, IP, IF, EM	
71 (pc)	SgII (CS2)	this study	WB	
72 (pc)	SgII (CS2)	this study	WB	
4E11/6 (mc)	SgII (preCS2)	this study	IF	
HPC1 (mc)	syntaxin 1	G. Schiavo	WB	(Inoue, *et al,* 1992)
anti-SNAP-25 (pc)	SNAP-25 (C-term.)	G. Schiavo	WB	
anti-VAMP2 (pc)	VAMP2	WAKO, USA	WB	
6E6 (mc)	NSF	G. Stenbeck	WB	
1946 (pc)	α-SNAP	G. Stenbeck	WB	
anti-p47 (pc)	p47	H. Kondo	WB	
anti-p97 (pc)	p97	H. Kondo	WB	
anti-GDI (pc)	GDI	J.E. Rothman	WB	(Elazar, *et al.*, 1994)
STO 31 (pc)	rab11 (C-term.)	S.A. Tooze	WB	
anti-rab6 (pc)	rab6 (C-term.)	Santa Cruz, UK	WB	

WB=Western Blotting, IP=Immunoprecipitation, IF=Immunofluorescence, EM=Electron microscopy, pc=polyclonal, mc=monoclonal, C-term.=carboxy-terminal, N-term.=amino-terminal, CS2=cleavage site number 2

2.2 Methods

2.2.1 Maintenance and labelling of cells.

Maintenance and storage of PC12 and PC12/PC2 cells

PC12 cells clone 251 were originally obtained from Dr. H. Thoenen (Martinsried, Germany) (Heumann, *et al.*, 1983). PC12 cells are a neuroendocrine cell-line derived from a pheochromocytoma of adrenal medulla. A PC12/PC2 cell-line has been established by Dr. A. S. Dittié. This cell-line stably expresses the endoprotease PC2 and has been characterised in (Dittié and Tooze, 1995).

PC12 and PC12/PC2 cells were grown in DMEM supplemented with 10% horse serum and 5% foetal calf serum in humidified incubators at 10% CO_2 and 37°C. Stock PC12 and PC12/PC2 cells were passaged routinely every 5 to 7 days when confluent and plated at a dilution of 1:6 in 175 cm^2 flasks. For sulphate-labelling experiments, cells were plated on 15 cm diameter dishes at a dilution of 1:6 and then used 4 to 7 days after passage. For large scale preparations of ISGs and cytosol, cells were plated on 24 x 24 cm dishes at a dilution of 1:6 and used 6 to 7 days after passage. For immunofluorescence, cells were grown at a dilution of 1:3 on glass coverslips that had been coated with poly-D-lysine (molecular weight 70,000-150,000; 1 mg/ml solution in H_2O) and used 2-3 days after passage. Cells were passaged using sterile technique as follows. Each 175 cm^2 flask of confluent cells was washed once in 10 ml of PBS (140 mM NaCl, 2.7 mM KCl, 5.4 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2) prewarmed to 37°C. The washed cells were then incubated for 5 min at 37°C in 5 ml of 0.25% (w/v) trypsin, 100 mM EDTA pH 7.2 prewarmed to 37°C. To remove the cells from the plastic, the flask was repeatedly tapped against the palm of one hand and 9 ml of medium were added to inactivate the trypsin. Using a 10 ml glass pipette the cells were removed from the flask and pelleted by centrifugation for 5 min at 1000 rpm in a Heraeus Megafuge 1.0 at room temperature. Cell pellets were resuspended in

1.5 ml of growth medium using a glass Pasteur pipette. To disperse clumped cells, it was necessary to pass cells 10 to 20 times through a Pasteur pipette which had been flamed to narrow the opening at the tip. The cell suspension was checked under a phase-contrast microscope, diluted at 1:6 in growth medium prewarmed to 37°C and then plated out on to fresh flasks and dishes. PC12 and PC12/PC2 cells were only used for experiments between passages 9 and 22.

For long-term storage, PC12 and PC12/PC2 cells were grown to confluency, harvested as described above and resuspended in 10 ml of growth medium containing 10% dimethylsulfoxide. Aliquots of these cells were frozen in 1 ml cryotubes at -70°C for one week and then transferred to a liquid nitrogen storage unit. When new cells were required, low passage number (7 to 9) cells were rapidly thawed at 37°C, diluted in growth medium prewarmed to 37°C, and plated out on flasks. The medium was changed the next day.

[^{35}S]-sulphate labelling of PC12 and PC12/PC2 cells

Stock solutions required for pulse-labelling of PC12 and PC12/PC2 cells with [^{35}S]-sulphate and for subcellular fractionation

The following stock solutions were required: DMEM minus sulphate (DMEM-S: DMEM containing no sulphate and only 1% of the normal levels of cysteine and methionine, plus 1% dialysed horse serum and 0.5% dialysed foetal calf serum. Serum was dialysed against PBS overnight to remove any sulphate present which would lower the incorporation of radioactive sulphate during the pulse-labelling. Aliquots of dialysed serum were stored at -20°C.); Tris buffered saline (TBS: 4.5 mM KCl, 137 mM NaCl, 0.7 mM Na_2HPO_4, 1.6 mM Na_2SO_4 and 25 mM Tris-HCl pH 7.4); TBS-PMSF (TBS and 0.5 mM PMSF); TBS-pi (TBS and 0.5 mM PMSF, 10.5 μM Leupeptin); HB (250 mM sucrose, 10 mM HEPES-KOH pH 7.2, 1 mM

$Mg(OAc)_2$, 1 mM EDTA pH 7.2);.HB-PMSF (HB with 0.5 mM PMSF); HB-pi (HB and 0.5 mM PMSF, 10.5 μM Leupeptin)

Pulse-chase labelling with [^{35}S]-sulphate.

PC12 cells grown on 15 cm diameter dishes to 80% confluence were incubated in 20 ml DMEM-S in a humidified 37°C and 10% CO_2 incubator for 20 min in order to deplete the endogenous sulphate from the cells. This medium was then removed and replaced by 5 ml of DMEM-S to which 5 mCi of [^{35}S]-sulphate had been added (final concentration: 1 mCi/ml). Labelling was for 5, 7, 10 or 30 min on a rocking platform in an incubator (37°C, 10% CO_2) depending on the experiment. The effective labelling time can be estimated to be approximately 2 min less than this given that sulphate uptake into cells, its subsequent conversion into activated nucleotide cosubstrate PAPS (3'-phosphoadenosine 5'-phosphosulphate), and the translocation of PAPS into the TGN takes approximately 2 min (Baeuerle and Huttner, 1987). Sulphation of tyrosine residues (Baeuerle and Huttner, 1987) and carbohydrates (Kimura, *et al.*, 1984) are TGN-specific modifications. In PC12 cells the major sulphated proteins are secretogranin I (also called chromogranin B (CgB)) and secretogranin II (SgII), targeted to the secretory granules (Rosa, *et al.*, 1985), and heparin sulphate proteoglycan (hsPG) which is constitutively secreted (Gowda, *et al.*, 1989). To terminate the labelling, the dish of cells was placed on an ice-cold aluminium plate, the medium was aspirated and replaced by 10 ml ice-cold TBS to stop further incorporation of [^{35}S]-sulphate. The cells were washed another 2 times with TBS before being harvested.

In some experiments cells were labelled as above and chased *in vivo* as follows: at the end of the labelling period the medium was aspirated at room temperature and replaced with 20 ml of prewarmed (to 37°C) growth medium containing 1.6 mM Na_2SO_4. The presence of the excess sulphate in the chase medium prevents further incorporation of [^{35}S]-sulphate during the chase period. The dishes of cells were then placed back into the incubator (37°C, 10% CO_2) until the end of the

chase period. The dishes were then placed on ice and the cells were washed three times with ice cold TBS.

Harvesting PC12 and PC12/PC2 cells

Pulse-labelled cells were scraped from their dishes using a silicone rubber scraper in 10 ml TBS-PMSF and transferred into a conical tube on ice. Scraped cells were then pelleted by centrifugation at 800 rpm (Heraeus megafuge 1.0) for 7 min at 4°C. Each cell pellet, corresponding to 2 dishes of cells, was resuspended in 6 ml of ice-cold HB-pi and the cells were pelleted by centrifugation at 1800 rpm (Heraeus megafuge 1.0) for 7 min at 4°C. These cell pellets were diluted with one volume of ice-cold HB-pi and resuspended 5 times with a blue Gilson tip.

2.2.2 Subcellular fractionation

Preparation of a post-nuclear supernatant (PNS)

Homogenisation of PC12 and PC12/PC2 cells

Using a 1 ml syringe, washed and scraped PC12 cells in HB-PMSF (250 mM sucrose, 10 mM HEPES pH 7.2, 1 mM $Mg(OAc)_2$, 1 mM EDTA pH 7.2.; 0.5 mM PMSF) were passed 7 times through a 21 gauge needle 1.5 inches in length. A 4 µl aliquot of cell suspension was mixed with an equal volume of trypan blue and examined under a light microscope at 40x magnification. At this stage more than 80% were single cells while less than 20% were broken. The resulting cell suspension was homogenised in 1 ml aliquots using 3 to 5 passages (back and forth) through an ice-cold cell cracker with a 18 µm clearance as described in (Tooze and Huttner, 1992). Breakage of cells was checked by analysing a 4 µl aliquot of cell homogenate diluted with the same volume of trypan blue under a light microscope. Optimal breakage was achieved with less than 2% unbroken cells (by trypan blue exclusion) and a minimum

of broken nuclei. The cell-cracker was then washed out with 400 µl of HB and this wash was pooled with the cell homogenate.

Preparation of a post nuclear supernatant (PNS)

Nuclei and unbroken cells were pelleted by centrifugation in 15 ml conical tubes at 2800 rpm for 7 min at 4°C using a swing out rotor in a Heraeus megafuge 1.0. The resulting supernatant was transferred to a clean 15 ml conical tube taking care not to disturb the nuclear pellet and avoiding the particulate layer floating on top of the PNS. If necessary, this centrifugation was repeated for another 3 min to clear the PNS of any particulate debris. The PNS contained approximately 10 mg/ml of protein as determined by Bio-Rad protein microassay using bovine IgG (reagent grade) as a standard.

Preparation of cytosol (RBC, PC12, HeLa)

Preparation of PC12 cytosol

PC12 cells were grown to confluency on nine 24 x 24 cm dishes, placed on an ice-cold metal-plate, washed twice with 25 ml of ice-cold TBS and scraped off the dish in 20 ml TBS-pi using a silicone rubber scraper. The following manipulations were all carried out on ice and centrifugations were at 4°C. Cells were pelleted for 7 min at 800 rpm in a Heraeus megafuge 1.0 and the pellets were resuspended in 24 ml HB-pi. In some cases 1 mM ATP was added to the HB to stabilise ATP-binding proteins. The cell-suspension was distributed onto six 15 ml conical tubes and the cells were pelleted by centrifugation at 1800 rpm for 7 min in a Heraeus megafuge 1.0. The cell pellets, now containing the equivalent of 1.5 dishes, were resuspended in an equal volume of HB-pi. The cells were homogenised and a PNS was prepared as described above. The PNS was then transferred into a plastic tube with screw cap lid and centrifuged for 30 min at 18,000 rpm (30,000 g) using a fixed angle Ti70.1 rotor in a Beckman Ultracentrifuge.

The supernatant was transferred into a fresh tube and the remaining membranes were pelleted by centrifugation for 1.5 hrs at 35,000 rpm (100,000 g) using the same rotor. The clear supernatant was transferred into fresh tubes and 0.5 ml aliquots of this cytosol were stored in liquid nitrogen. Cytosol was used within 3 months. Before use, cytosol was thawed quickly for 3 min at 37°C and transferred on ice. The protein concentration of this PC12 cytosol was determined using the Bio-Rad microassay and was typically between 8 and 13 mg/ml.

Preparation of HeLa cytosol

HeLa S3 cells were obtained from ICRF central services, London, UK. HeLa cells (1.8×10^9) were pelleted by centrifugation at 1000 rpm (Heraeus megafuge) and washed twice with TBS-PMSF. Cell pellets were then resuspended in 0.2 volumes of HB-pi and left on ice for 5 min to allow the cells to swell. The cells were then pelleted by centrifugation at 1000 rpm (Heraeus megafuge 1.0) and the pellets were resuspended in an equal volume of HB-pi and homogenised as described above for PC12 cells except that 20 passages through the cell cracker were necessary to achieve optimal breakage. Preparation of the cytosol from the homogenate as well as storage was as described for PC12 cells. The HeLa cytosol contained typically between 5 and 10 mg/ml total protein.

Preparation of rat brain cytosol

Rat brains either from Wistar or Sprague-Dawley male or female rats were provided by the ICRF Animal facility, London, UK. Six total rat brains were weighed (ca. 10 g) and washed 3x in HB. All manipulations were at 4°C. Peripheral blood vessels were removed as far as possible. The brains were transferred to a glass-teflon homogeniser and one volume of HB-pi was added. The brains were homogenised with 4 to 6 strokes using a variable speed electrically operated glass-teflon homogeniser (Janke and Kunkel, Ika Labortechnik, Germany) and the breakage was checked by analysing the homogenate under a light microscope. The brain homogenate was transferred to plastic

tubes with screw caps and centrifuged at 18,000 rpm for 30 min using a Ti70.1 rotor in a Beckman Ultracentrifuge. The supernatant was transferred to clean tubes and centrifuged again at 35,000 rpm using the same rotor. The cytosol was stored in 0.5 ml aliquots under liquid nitrogen and used within 3 months. The protein concentration of this cytosol was typically between 10 and 20 mg/ml.

Preparation of ISGs and TGN-membranes

Separation of post-TGN vesicles from the TGN

To separate post-TGN vesicles from the TGN the following procedure was used (Tooze and Huttner, 1992). 1.3 ml of PC12 or PC12/PC2 PNS were loaded on to linear sucrose gradients of 0.3 M (5.5 ml) to 1.2 M (6.0 ml) sucrose in 10 mM HEPES-KOH (pH 7.2) prepared in "Ultra Clear" tubes for the Beckman SW40 rotor. After loading, the gradients were centrifuged in a Beckman SW40 rotor at 25,000 rpm (110,000 g_{av}) and 4°C for 15 min, timed after reaching the set speed. Fractions of 1 ml were collected from the top to the bottom of the gradients using a Labconco auto densi flow gradient collector. The pellet was resuspended in 0.5 ml HB and pooled with fraction 13. Aliquots of the gradient fractions were either precipitated with 10% TCA and resuspended in Laemmli sample buffer or directly mixed with Laemmli sample buffer and analysed by SDS-PAGE. Fractions 1 to 4 contained post-TGN vesicles including the ISGs and fractions 9 to 11 contained the TGN-membranes (Figure 3/2).

Equilibrium gradient centrifugation on sucrose gradients

To separate the ISGs further from other vesicles, in particular from constitutive secretory vesicles containing sulphated hsPG, fractions 1 to 4 from the velocity sucrose gradients centrifugation were pooled and loaded onto sucrose step gradients of 0.8 M (1 ml), 1.0 M (2 ml), 1.2 M (2 ml), 1.4 M (2 ml) and 1.6 M (1 ml) sucrose in 10 mM HEPES-KOH (pH 7.2) prepared in "Ultra Clear" tubes for the

Beckman SW40 rotor. To purify the TGN-membranes further, fractions 9 to 11 from the velocity sucrose gradients centrifugation were pooled, diluted with 1 ml 10 mM HEPES-KOH (pH 7.2) and loaded onto equivalent sucrose step gradients. After loading, the gradients were centrifuged in a Beckman SW40 rotor at 25,000 rpm (110,000 g_{av}) and 4°C for 5.5 hours or longer (overnight). Fractions of 1 ml were collected and analysed as described above. Fractions 7 to 9 contained the ISGs and fractions 6 to 8 contained the TGN-membranes (Figure 3/2). ISG-pools and TGN-pools were frozen in 1 ml aliquots under liquid nitrogen for long-term storage.

2.2.3 Protein biochemistry

Precipitation of proteins

Stock solutions required for protein precipitation

Trichloroacetic acid (100% (w/v) TCA: add 227 ml H_2O to 500g); PBS-HG (PBS + 10 µg/ml haemoglobin); Ethanol:diethyl-ether (1:1); unbuffered Laemmli sample buffer (62.5 mM Tris (unbuffered), 3% (w/v) SDS, 3.3% (v/v) β-mercaptoethanol, 10% (w/v) glycerol, 0.015% (w/v) bromophenol blue); Acetone (-20°C).

TCA-precipitation of proteins

Proteins were precipitated with 10% TCA as follows: Protein containing samples were diluted with PBS-HG to 900 µl and 100 µl of TCA was added, mixed and incubated for 1 hr or over night on ice. Haemoglobin was added to ensure that efficient precipitation occured in samples with low protein concentration. The precipitated proteins were pelleted at 13,000 rpm for 30 min in an Eppendorf microfuge at 4 °C. The supernatants were carefully aspirated and the pellets were washed with 200 µl of ethanol:diethyl-ether. The pellets were centrifuged for 5 min as above, the supernatant was aspirated and the residual solvent was allowed to evaporate for 5 min.

The pellets were then resuspended in 30 µl of unbuffered Laemmli sample buffer and vortexed until completely dissolved. 3 µl of unbuffered 1M Tris was added to neutralise samples which turned yellow, due to residual TCA.

Acetone precipitation

Acetone precipitation was overnight at -20°C as follows: samples were diluted to 1 ml with PBS-HB (in 5.4 ml cryotubes) mixed with 4 ml of acetone (-20°C; 80% (v/v) final concentration). After precipitation overnight at -20°C, the precipitated proteins were recovered by centrifugation for 15 min at 2000 g at 4°C. The pellets were resuspended in Laemmli sample buffer and analysed by SDS-PAGE. Acetone precipitation was avoided if the samples contained large amounts of potassium salts (> 50 mM).

SDS-Polyacrylamide gel electrophoresis

Stock solutions required for SDS-PAGE

Stock solutions were as follows: 4x Lower buffer (1.5 M Tris-HCl pH 8.8, 0.4% SDS), 4x Upper buffer (0.5 M Tris-HCl pH 6.8, 0.4% SDS); AMBA (Protogel: 30% (w/v) Acrylamide + 0.8% (w/v) Bisacrylamide); TEMED; 10% (w/v) APS (Ammonium peroxodisulphate); 10x Running buffer (1.9 M glycine, 250 mM Tris, 10% (w/v) SDS, pH 8.8); Laemmli sample buffer (62.5 mM Tris-HCL pH 6.8, 3.3% (v/v) 2-mercaptoethanol, 3% (w/v) SDS, 10% (w/v) glycerol, 0.015% (w/v) bromophenol blue); 5x Laemmli sample buffer; Stain (50% (v/v) methanol, 10% (v/v) acetic acid, 1% (w/v) coomassie blue R-250); Destain (20% (v/v) isopropanol, 20% (v/v) acetic acid); 1 M sodium salicylate. All solutions were made in dH_2O.

SDS-polyacrylamide gel electrophoresis

SDS-PAGE was performed using a modified Laemmli system (Lee and Huttner, 1983) on 1.5 mm thick Bio-Rad minigels (Mini-Protean II electrophoresis cell), or 20 x 16 cm Bio-Rad gels (Protean II xi vertical electrophoresis cell). The tables shown below give recipes for 40 ml of resolving gel and 20 ml of stacking gel. These volumes are enough for 6 minigels or 1 standard gel, both of 1.5 mm thickness. 12% resolving gels were used whenever proteins smaller than 30 kDa were to be resolved.

Resolving gel	7.5%	12 %
4x Lower Buffer	10.00 ml	10.00 ml
AMBA	10.00 ml	16.00 ml
dH_2O	19.78 ml	13.78 ml
TEMED	20 µl	20 µl
10% APS	200 µl	200 µl

Table 2/2: *Resolving gel mix for SDS-PAGE*

Stacking Gel:	3.75%
4x Upper Buffer	5.00 ml
AMBA	2.50 ml
dH_2O	12.28 ml
TEMED	30 µl
10% APS	200 µl

Table 2/3: *Stacking gel mix for SDS-PAGE*

Samples were resuspended in 30 µl or 40 µl of 1x Laemmli sample buffer for loading on 15 lane or 10 lane minigels respectively. For loading on 15 lane standard gels, samples were resuspended in 75 µl Laemmli sample buffer. All samples were heated for 5 min in a boiling water bath and then immediately loaded using a Hamilton syringe. Molecular weight markers prepared in sample buffer were run in a separate lane, and any empty lanes were loaded with 1x sample buffer.

Minigels were electrophoresed first at 90 V for 15 minutes (stacking) and then at 120 V for 1 hr until the sample buffer dye front had reached the bottom of the resolving gel. Large gels were electrophoresed overnight at 12 mA per gel (150 mA x hrs).

After electrophoresis, gels were fixed for 15 min in stain, washed once in destain and then shaken in destaining solution overnight. For rapid destaining (1 to 2 hours), Dowex 1X2 ion-exchange resin was added to the destain. After destaining gels were photographed if necessary and either dried or subjected to fluorography. Gels to

be fluorographed were washed in dH_2O, shaken in dH_2O for 10 min, then shaken for a further 30 min in 1 M sodium salicylate solution prior to drying.

Gels were placed on a single sheet of Whatman 3MM paper, covered with plastic film, then dried under vacuum at 80°C for 2 hours. Dried gels were exposed at -80°C to preflashed Kodak XAR5 film for 16 hrs to 4 weeks. Quantitation was usually obtained by densitometry of the autoradiographs using the NIH image software package.

Western blotting

Solutions required for western blotting

The following solutions were used: Transfer buffer (20 mM Tris, 150 mM glycine, 20% (v/v) methanol); Ponceau S (2% (w/v) in 3% (w/v) TCA); Blocking buffer (5% (w/v) skimmed milk powder in PBS with 0.2% (w/v) Triton-X100)

Transfer of proteins onto nitrocellulose

Samples were subjected to SDS-PAGE as described above and then transferred to nitrocellulose membrane as follows: the gel was placed on top of a sheet of Whatman 3MM paper wetted with transfer buffer. A sheet of nitrocellulose membrane (0.2 μM pore size) was cut to the same size as the gel, wetted with dH_2O and layed over the gel. A further piece of Whatman 3MM paper, wetted with transfer buffer, was placed over the nitrocellulose membrane. Air bubbles were removed using a 10 cm rubber roller. The blot was sandwiched between two nylon pads in the tray of a plate electrode blotter (Idea Scientific, UK). The assembled blot was placed inside the blotting chamber which was then filled to the top with transfer buffer. Transfer was for 60 min at 20 Volts at 4°C. After transfer, the nitrocellulose membrane was stained with Ponceau S and rinsed with distilled water to check for efficient transfer. For storage, the nitrocellulose membrane was incubated for 1 hr or over night in blocking buffer to

block non-specific protein binding sites and then sandwiched between parafilm and frozen at -20°C.

2.1.4 Immunological methods

Coupling of peptides to KLH

Stock solutions required for coupling peptides with glutaraldehydes

Peptide solution (10 mg/ml); KLH (keyhole limpet hemacyanin, 73 mg/ml, Pierce); 0.1 M $NaHCO_3$ (made up fresh before use); glutaraldehyde (25%, Agar Scientific Ltd); Glycine ethyl ester (1M, pH 8.0 with NaOH), Acetone (-20°); PBS.

Coupling of preCS2 peptide with glutaraldehyde (coupling to lysines)

6 mg of the preCS2 peptide (KEENSRENPF) was mixed with 6 mg KLH (82.2 µl) and diluted to 3 ml with 0.1M $NaHCO_3$ in a Corex glass tube. Glutaraldehyde was added to a final concentration of 0.05% and the solution was mixed overnight at room temperature. The remaining glutaraldehyde was quenched with glycine ethyl ester, added to a final concentration of 0.1M, and shaken for 30 min at room temperature. The coupled peptide was precipitated with 4 volumes of acetone at -70°C overnight and pelleted by centrifugation for 20 min at 10,000 g in a swing-out rotor at -20°C. The pellet was resuspended at 1 mg/ml KLH in PBS with a blue Gilson tip and frozen in 1 ml aliquots at -20°C.

Stock solutions required for coupling of peptides with MBS

Peptide solution (10 mg/ml); Citronic anhydride (10 mg/ml); 1M sodium phosphate pH 7.2 (72 ml 2M Na_2HPO_4 and 28 ml 2M NH_2PO_4), MBS (m-Maleimidobenzoyl-N-hydroxysuccimide ester (Pierce, UK), 25 mg/ml in DMF); β-mercaptoethanol, KLH (keyhole limpet hemacyanin (KLH), 73 mg/ml (Pierce)); PBS.

Coupling of peptide CS2 to KLH with MBS (coupling to cysteines)

Peptide coupling with MBS was carried out as described in Harlow and Lane (1988). To block free amino groups in the peptide, a solution of the CS2 peptide (CSRENPFKR) was adjusted to pH 8.5 and slowly mixed with an equal volume of citronic anhydride whilst keeping the pH between 8 and 9 with NaOH. This solution was then incubated for 1 hr at room temperature after which 100 µl of 1 M sodium phosphate pH 7.2 was added. For activation of the peptide, MBS was added, with continous mixing using a magnetic stirrer, to a final concentration of 5 mg/ml and the solution was incubated at room temperature for 30 min. To inactivate any remaining maleimide groups on the MBS, β-mercaptoethanol was added to a final concentration of 35 mM and incubated for 1 hr at room temperature. 5 mg of the carrier protein, KLH, was added to the activated peptide and incubated, for 3 hrs at room temperature, with stirring. The uncoupled peptide was removed by dialysis against PBS.

Generation of antibodies

The coupled peptides were emulsified in Freund's Adjuvant and injected into rabbits which were subsequently bled according to ICRF standard protocol. All work with animals was carried out by the staff of the ICRF animal unit.

Immunoblotting

Solutions required for immunoblotting

The following solutions were used: PBS; Blocking buffer which is equivalent to washing buffer (5% (w/v) low-fat milk powder in PBS with 0.2% Triton-X100); TBS; TBS-0.2% T-X100 (TBS with 0.2% Triton-X100), TBS-0.5% T-X100 (TBS with 0.5% Triton-X100); BCIP (5'-bromo 4'-chloro-3-indoyl-phosphate, 1 tablet (25 mg) in 0.5 ml 100% DMF (*N,N*-dimethylformamide), stored at -20°C); NBT (nitro blue tetrazolium, 1 tablet (10 mg) in 1 ml dH_2O, stored at -20°C); AP-buffer (100

mM Tris-HCL, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$); AP-developing solution (10 ml AP-buffer with 33 µl BCIP and 330 µl NBT (made up just before use); ECL-developing reagents(Amersham); [^{125}I]-protein A (0.4 µCi/ml in TBS-0.5% TX100).

Immunoblotting

Proteins were transferred onto nitrocellulose membranes as described above (Western blotting) and the nitrocellulose membranes were incubated in blocking buffer for 1 hr at room temperature or overnight at 4°C to block non-specific protein binding sites. The primary antibody was diluted in blocking buffer and added to the blocked blot. For competition experiments, primary antibodies were first pre-incubated for 30 min at room temperature with the appropriate peptide antigen at a concentration of 0.05 to 0.2 µg/ml. Incubation of the blot with the antibody was either at room temperature for 2 hrs or overnight at 4°C. Blots were then washed three times in washing buffer for 10 min per wash and incubated with secondary antibody diluted in washing buffer. If not indicated otherwise, the secondary antibody was conjugated to Horse radish peroxidase (HRP). In some cases the secondary antibody was coupled to alkaline phosphatase (AP). Incubation with the secondary antibody was at room temperature for 1 hr. Blots were then washed in washing buffer for 10 min per wash.

Blots incubated with secondary antibodies coupled to AP were then washed with TBS to remove the phosphate-buffer based milk-solution and then rinsed twice with AP-buffer. Blots were then incubated with freshly made AP-developing solution for 2 to 10 min in the dark. The reaction was stopped by several washes with dH_2O and the blots dried between two pieces of cardboard before being photographed.

Blots incubated with secondary antibodies coupled to HRP were washed twice for 3 min with TBS to remove the blocking-buffer and then incubated for 1 min in HRP-developing solution (1:1 freshly mixed solutions 1 and 2). Blots were placed on a glass plate, after excess of the solution was removed by touching the edge of the blot to a tissue, and covered with cling-film. Blots were then immediately exposed to

preflashed Kodak XAR5 film for 5 sec, 30 sec and 2 min and quantitated by densitometry. If necessary, intermediate or longer exposures were also obtained.

As an alternative to secondary antibodies blots were incubated with [^{125}I]-protein A solution for 1 hr at room temperature and washed with TBS-0.2%TX100 until no background counts could be detected using a hand held gamma counter.

Immunoprecipitation

Stock solutions required for immunoprecipitation

Lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.3% Triton-X100, 5 mM EDTA); 5x Lysis-buffer (500 mM Tris-HCl pH 7.5, 750 mM NaCl, 1.5% Triton-X100, 25 mM EDTA); 10 mM Tris-HCl pH 7.5; RIPA-buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% (w/v) Triton X-100, 0.1% (w/v) SDS, 1% (w/v) sodium deoxycholate); Protein A-sepharose (10% (w/v) suspension in dH2O).

Immunoprecipitation from overnight labelled PC12 and PC12/PC2 cells

Unless indicated otherwise, all steps were performed at room temperature. PC12 or PC12/PC2 cells grown on 15 cm diameter dishes were labelled overnight with 1 mCi [^{35}S]-sulphate in 20 ml of DMEM-S to which 10% of normal growth medium was added. The labelled cells were washed, harvested and a PNS made as described above for pulse-labelled cells. Aliquots of this PNS were lysed with either lysis buffer or RIPA-buffer and incubated for 10 min on ice. The samples were precleared by centrifugation for 10 min at 13,000 rpm in an Eppendorf microfuge at 4°C to remove all insoluble material. 5µl of first antibody and 20-50 µl of protein A-sepharose were added to these precleared and lysed samples. The samples were then rotated, for 2 hrs or overnight, on a wheel at 4°C. The immunoprecipitates were pelleted for 20 sec at 13,000 rpm in an Eppendorf microfuge and the beads washed twice with lysis buffer or RIPA-buffer and once with 10 mM Tris pH 7.5. The immunoprecipitated material was

eluted from the beads by boiling in Laemmli sample buffer and analysed by SDS-PAGE and fluorography.

Immunoprecipitation as a read-out for cell-free fusion

Standard immunoprecipitations with Ab 69, from samples that have been incubated for processing in 50 mM MES pH 5.5 and 0.3 M sucrose, were carried out as described above with the following modifications: To neutralise the pH and facilitate solubilisation of the aggregated granule matrix proteins, samples were diluted with 0.2 volumes of 5x lysis buffer (giving a final concentration of 100 mM Tris pH 7.5). Lysis was performed at room temperature by shaking the samples for 10 min. Insoluble material was removed by 10 min centrifugation at 13,000 rpm in an Eppendorf microfuge at 4°C. For standard immunoprecipitations from cell-free fusion assay incubations, 30 µl of Ab 69 or 20 µl of Ab 175 and 150 µl of protein A-sepharose were used per sample.

Indirect immunofluorescence

Stock solutions required for indirect immunofluorescence

The following solutions were used: PBS; Formaldehyde fixative solution (16% Paraformaldehyde solution (methanol-free, TAAB) was diluted to 3% with PBS and stored at -20°C as 10 ml aliquots); 10x gelatin stock solution (2 g of gelatin was dissolved in 100 ml of H_2O and stored at -20°C in 10 ml aliquots); Blocking solution (PBS-0.2% gelatin: gelatin 10x stock diluted ten-fold with PBS prior to use); Quench solution (50 mM NH_4Cl in PBS); Permeabilisation solution (0.2% Triton X-100 in PBS); Moviol mounting medium (6g analytical grade glycerol was placed in a 50 ml plastic tube and 2.4 g Moviol 4-88 added while stirring. After addition of 6 ml dH_2O the solution was left for 2 hrs at room temperature. Following this, 12 ml of 0.2 M Tris-HCl pH 8.5 was added and the solution was incubated for 10 min at 50°C with

stirring. The moviol was clarified by centrifugation at 5000 g for 15 min and stored either at 4°C or aliquoted and stored at -20°C)

Indirect immunofluorescence

Cells were grown on poly-D-lysine coated coverslips (22 x 22 mm) in individual wells of a 6 well culture plate. The cells were washed three times with 2 ml of PBS, then fixed in 2 ml of formaldehyde fixative solution for 10 min. Fixed cells were washed in 2 ml of PBS, and the fixative quenched by a 10 min incubation in 2 ml of quench solution. The cells were then washed three times with 2 ml of PBS. To allow access of antibodies to intracellular, cytosolic and luminal, epitopes the fixed cells were incubated for 4 min in the permeabilisation solution and then washed twice with PBS.

Cells were preincubated for 10 min in blocking solution and the primary antibodies were diluted in blocking solution. The coverslips were placed cell side facing down, onto 80 µl of antibody solution which was placed on a strip of parafilm in a moist chamber. The cells were incubated with antibody for 20 min at room temperature. Coverslips were then transferred back to their wells and the cells were washed three times with blocking solution. Incubation with the secondary antibody conjugated to a fluorophore, was as above, followed by three, 5 min washes in blocking solution. This protocol was also used for double-labelling. In this case, the two primary antibodies were mixed as were the secondary antibodies. The coverslips were then rinsed with PBS, dipped into dH_2O, drained of excess liquid by touching the edges against a tissue and placed, cell side down, on to a 20µl drop of Moviol mounting medium on a clean coverslip. Coverslips were left to dry at room temperature overnight.

Immunogold labelling for electron microscopy

Cryo-fixation of cells, specimen preparation and immunogold labelling were performed by Steve Gschmeissner in the electron microscopy unit (head of the unit: Nassar Hajibagheri) at ICRF, London according to standard techniques.

Specimen preparation

Cells were fixed on the dish in 2% paraformaldehyde/0.2% monomeric glutaraldehyde (Stock solutions: 16% paraformaldehyde and 8% monomeric glutaraldehyde, TAAB) for 1 hr at room temperature before being carefully scraped off the dish with a silicone rubber scraper and pelleted by centrifugation at 1000 g. The supernatant fixative was removed, 2% paraformaldehyde added and the cells stored overnight at 4°C. The cells were then washed three times in Sørensens buffer (100 mM sodium phosphate pH 7.4) and resuspended in 10% gelatine at 37°C, pelleted and cooled on ice to allow the gelatine to solidify. The specimen was trimmed to size and cryoprotected in 2.3 M sucrose for at least 3 hrs after which it was mounted onto a metal stub and rapidly frozen in liquid nitrogen. Ultrathin (100 μm) cryosections were cut on a Leica Ultracut S microtome, picked up from the knife with methylcellulose/sucrose (2% methylcellulose (25 centipoises) and 2.3M sucrose) and transferred to carbon and formvar coated grids. The grids were stored, cell side down, in a petri dish on 2% gelatine at 4°C.

Immunogold labelling

The gelatine on which the grids were placed was heated to 40°C for 10 min and then transferred onto liquid drops of the following solutions and incubated for the indicated times at room temperature if not stated otherwise:

1) 0.02 M glycine	3 x 1 min
2) 0.1% BSA	5 min
3) antibody 69 diluted 1:200 in 1% BSA at 4°C	overnight

4) wash in 0.1% BSA	4x 1 min
5) protein A gold (10 mm) diluted 1:50 in 1% BSA	30 min
6) wash in 0.1% BSA	5 min
7) wash in PBS	4x 5 min
8) post-fix in 1% glutaraldehyde	5 min
9) wash in PBS	2x 5 min
10) wash in dH_2O	4x 2 min
11) negative stain with saturated aqueous uranyl acetate	5 min
12) rinse in dH_2O	
13) 4% methyl cellulose, 0.4% uranyl acetate at 4°C	5 min

The sections were then embedded in 4% methyl cellulose, 0.4% uranyl acetate and examined and pictures were taken with a JEOL 1200FX (Japanese Electron Optical Limited) electron microscope.

2.2.5 Cell-free assays

Processing of SgII by PC2

Processing in isolated ISGs and TGN

Aliquots of 125µl of "isolated ISGs" (ca 75 µg) or "isolated TGN" (ca 50 µg) were adjusted to 50 mM KOAc, 1 mM $Mg(OAc)_2$ and 20 mM HEPES, Tris or MES buffered at a variable pH (as indicated in figures) in a final volume of 375 µl. In some experiments the KOAc and the $Mg(OAc)_2$ were replaced by KCl and $MgCl_2$. ISGs or TGN were supplemented with an ATP-regenerating system (Tooze and Huttner, 1990) where indicated and incubated at 4°C or 37°C for indicated periods of time. Nigericin, valinomycin, concanamycin (stock solutions in ethanol) and NH_4Cl were added at 4°C, 15 min before incubation at 37°C. The samples were cooled at 4°C and the membranes were recovered by ultracentrifugation at 100,000g for 1 hour at 4°C. The pellets were resuspended in Laemmli sample buffer and analysed as described

above by 12% SDS-PAGE, followed by fluorography and autoradiography (Lee and Huttner, 1983).

The [^{35}S]-sulphate radioactivity was quantified with a PhosphorImager and the ImageQuant Software package (Molecular Dynamics). The efficiency of processing was assessed by individual quantitation of the [^{35}S]-sulphate labelled processing products that could be resolved on a 12% gel and expressed as percent of the sum of the values obtained for SgII and each SgII-derived band. Alternatively (Chapter 4), ISG pellets were lysed in Lysis buffer or RIPA-buffer, precleared by centrifugation for 10 min at 13,000 rpm in an Eppendorf centrifuge and subjected either to immunoprecipitation with antibody 69 or to sequential immunoprecipitation with antibody 175 followed by antibody 100. Immunoprecipitated proteins were then either analysed by 7.5 or 12 % SDS-PAGE, fluorography, autoradiography and densitometry as described above, or dissolved in scintillation fluid and analysed with a scintillation counter to determine the number of cpm by scintillation counting.

Processing in semi-intact cells-

PC12/PC2 cells (one 15 cm dish) were pulse-labelled for 5 min with [^{35}S]-sulphate, or pulse-labelled and chased for 15 min as described above or pulse-labelled for 5 min with [^{35}S]-sulphate and then incubated for 15 min to two hours in a 20°C waterbath in HEPES-buffered chase medium (D-MEM in which the bicarbonate is replaced by 55 mM HEPES-NaOH pH 7.0, 1.6 mM Na_2SO_4). Cells were then placed on an ice-cold metal plate and washed with ice-cold KGlu-buffer (20 mM HEPES pH 7.2, 120 mM KGlutamate, 20 mM KOAc, 2 mM EGTA) containing 0.1% BSA. Cells were permeabilised using 6 passages through a 21 gauge needle followed by 2 passages through a cell-cracker (EMBL workshop) with an 18μm clearance. The cells were washed several times with KGlu-buffer, distributed into five aliquots and finally resuspended in 120 μl processing buffer (50mM KOAc, 1 mM $Mg(OAc)_2$, 20 mM HEPES pH 7.2 or 20 mM MES pH 6.2 or 5.5). An ATP-regenerating system and nigericin (100 nM final) were added where indicated. After 10 min preincubation on

ice, the cells were incubated for 45 min at 37°C, cooled for 5 min on ice and pelleted for 30 min at 4200 rpm in a Heraeus Megafuge 1.0. The pellets were resuspended in lysis buffer and a heat-stable fraction was prepared as follows: Lysed samples were boiled for 5 min in a waterbath, cooled down for 5 min at room temperature and precipitated proteins were removed by centrifugation for 5 min at 13,000 rpm in an Eppendorf centrifuge. The supernatant was transferred to a clean cryotube and the heat-stable proteins were acetone precipitated, resuspended in Laemmli sample buffer and analysed by 12% SDS-PAGE followed by fluorography.

Cell-free fusion assay

Stock solutions required for the cell-free assay

The following stock solutions were required: 10x FB (Fusion buffer: 200 mM HEPES, pH 7.2; 500 mM KOAc, 30 mM $MgCl_2$, 10 mM DTT; frozen in 1 ml aliquots at -20°C); 10 mM HEPES pH 7.2; ATP-regenerating system (see below); low pH buffer (50 mM MES pH 5.5 in 0.3 M sucrose); 5x Lysis-buffer (500 mM Tris-HCl pH 7.5, 750 mM NaCl, 1.5% Triton-X100, 25 mM EDTA); Protein A-sepharose (10% (w/v) suspension in dH_2O)

ATP regenerating and ATP depletion systems

The systems used to regenerate or deplete ATP were previously described by (Davey, *et al.*, 1985). For ATP regeneration the following stock solutions were required: 100 mM ATP (made to pH 7 with NaOH), 800 mM creatine phosphate, and 3200 U/ml creatine phospho kinase, all three stored at -20°C in 100 µl aliquots. Prior to use these were rapidly thawed and kept on ice. Equal volumes of ATP, creatine phosphate and creatine phosphokinase were mixed on ice (30x stock solution) and then used immediately.

For ATP-depletion, the following system was used. Stock solutions of 200 mM D-glucose (60x stock) and 1500 U/ml hexokinase (60x stock) were stored in 25 µl aliquots at -20°C. Prior to use these were rapidly thawed and kept on ice. Equal volumes of D-glucose and hexokinase were mixed on ice and used immediately. AMP-PNP was made up as a stock solution at 10 mM in H_2O and stored at -20°C

Reconstitution of ISG fusion in a PNS

PC12 cells (usually six 15 cm dishes) were pulse-labelled with [^{35}S]-sulphate or pulse-labelled and chased and the cells were washed, harvested and homogenised as described above. A PNS was obtained, diluted with HB-PMSF to 2 ml final volume and kept on ice. Aliquots of PC2-ISGs were thawed rapidly at 37°C (3 min), diluted with 2 volumes of 10 mM HEPES-KOH pH 7.2 and pelleted by centrifugation in a SW55 Beckman rotor at 29,000 rpm for 1 hr 5 min. The pellets were resuspended in HB-PMSF at 50 fold the concentration of the original ISG pool. If not indicated otherwise, standard conditions were as follows: 100 µl of the PC12 PNS were used per sample (condition) and were supplemented with 10 x FB (to a final concentration of 20 mM HEPES-KOH pH 7.2, 50 mM KOAc, 3 mM $MgCl_2$, 1 mM DTT), ATP-regenerating system (30-fold dilution) and 10 µl PC2-ISGs in a final volume of 120 to 160 µl. The tube was vortexed very carefully to mix the contents and the cell free reactions were incubated at 4°C or 37°C for 30 min (standard conditions). After the incubation the samples were transferred on ice and diluted with low pH buffer to a final volume of 800 µl. The samples were preincubated for 30 min on ice to equilibrate the pH in the ISGs with the buffer and then transferred to 37°C for 90 min to allow processing of SgII by PC2 to take place. After this processing incubation, the samples were placed on ice and 200 µl of 5x lysis buffer was added to each sample. The samples were resuspended for 10 min by shaking vigorously on an automated shaker. The lysed samples were precleared and subjected to immunoprecipitation as described above. The amount of [^{35}S]-sulphate labelled p18 that was immunoprecipitated was analysed by 12% SDS-PAGE, followed by fluorography and

exposure to a pre-flashed Kodak XAR5 film for 16 hrs to 3 days. Quantitation of [^{35}S]-sulphate labelled p18 was by densitometry using the NIH image package. If not indicated otherwise, the PC2-independent signal was subtracted from the signal obtained in the presence of PC2-ISGs.

Analysis of the cell-free assay by velocity gradient centrifugation

To identify the fused ISGs by subcellular fractionation, a cell-free assay was performed as described above either in the presence or absence of PC2-ISGs and incubated as usual for 30 min at 37°C. Samples were then incubated for 5 min on ice and identical samples were pooled. Usually 8 identical samples were used for each condition. These samples were diluted with ice-cold HB-PMSF to 1.3 ml and loaded at 4 °C on top of linear sucrose gradients of 0.3 M (5.5 ml) to 1.2 M (6.0 ml) sucrose in 10 mM HEPES-KOH (pH 7.2) prepared in "Ultra Clear" tubes for the Beckman SW40 rotor. After loading, the gradients were centrifuged in a Beckman SW40 rotor at 25,000 rpm (110,000 g_{av}) and 4°C for 15 min, counting from reaching the set speed. Fractions of 1 ml were collected from the top to the bottom of the gradients using a gradient collector. Aliquots of the gradient fractions were TCA-precipitated and resuspended in Laemmli sample buffer analysed by SDS-PAGE. 800 µl of each fraction was diluted with 10 mM HEPES-KOH pH 7.2 to 0.34 M sucrose and the membranes were pelleted at 29,000 rpm in a SW55 rotor for 1 hr 5 min. The membrane pellets were resuspended in 100 µl of 0.3 M sucrose buffered with 50 mM MES pH 5.5, incubated for 30 min on ice, followed by 90 min incubation at 37°C. The membranes were then diluted with 10 mM HEPES-KOH pH 7.2 to 800 µl volume, 200 µl of 5x lysis buffer were added and the samples were lysed and analysed as described above for the standard fusion assay.

Pre-treatment of PC2-ISGs with trypsin

PC2-ISGs were thawed rapidly at 37°C and distributed into Beckman microfuge tubes (500 µl per tube). 0.5 µl of trypsin stock solution (1 mg/ml) were

added either together or without 25 µl of Soybean trypsin inhibitor (STI: 1 mg/ml). Samples were incubated for 15 min at room temperature. To stop cleavage, the samples were placed on ice and PMSF and STI were added to the samples. The ISGs were then pelleted for 1 hr 5 min at 45,000 rpm in a TLA45 rotor in a TL-100 Beckman table top centrifuge. Each ISG-sample was resuspended in 10µl HB-PMSF and used in a cell-free fusion assay.

Pre-treatment of PC2-ISGs with botulinum neurotoxins

PC2-ISGs were thawed rapidly at 37°C and distributed into Beckman microfuge tubes (500 µl per tube). ATP-regenerating system and fusion buffer were added to the ISG-pool. Recombinant L-chains of various botulinum neurotoxins were added at a final concentration of 20 nM and the ISGs were incubated for 30 min at 37°C to allow cleavage by the toxins to take place. Samples were then placed on ice and the ISGs were pelleted and resuspended as described above for trypsin treated ISGs..

Cell-free assay using a membrane pellet

Preparation of a membrane pellet

To obtain a membrane pellet, 100 µl of PNS were diluted with 100 µl of HB-PMSF and layered on top of a 1 ml sucrose cushion (0.5 M sucrose in 10 mM HEPES-KOH pH 7.2) in polycarbonate tubes for the Beckman SW55 rotor. The membranes were pelleted by centrifugation for 1 hr 5 min at 29,000 rpm and at 4°C in a SW55 rotor. The supernatant was removed and the membranes were resuspended in 100 µl cytosol or HB with a yellow Gilson tip. An ATP-regenerating system, 10x FB and PC2-ISGs were added as described for the standard cell-free assay using a PNS.

Pre-treatment of the PNS with N-Ethylmaleimide (NEM)

In order to obtain NEM-treated membranes, 2 to 6 µl of a freshly made 50 mM NEM-stock solution (1 and 3 mM final concentrations) were added to 100 µl PNS.

The samples were mixed immediately, incubated for 10 min on ice and quenched by addition of 2 to 6 µl of 60 mM glutathione (1.2 and 3.6 mM final concentrations) for 15 min on ice. The membranes were then pelleted through a sucrose cushion as described above. Cytosol was NEM-treated in the same way as described for the PNS. Control samples were treated with glutathione only.

Cell-free assay using sucrose gradient fractions

For some experiments, the PNS was first subjected to velocity controlled sucrose centrifugation to separate the TGN and post-TGN vesicles. 1 ml fractions were collected from such gradients as described above and 400 µl of each fraction was diluted to 0.34M sucrose in duplicates. The membranes contained in each fraction were pelleted by centrifugation for 1 hr 5 min at 29,000 rpm in a SW55 rotor and resuspended with a yellow Gilson tip in 100 µl of cytosol or HB-pi. An ATP-regenerating system and 10x FB were added as described for the standard incubation. 10 µl PC2-ISGs were added to one of the two samples, while the other sample received HB-PMSF only. Fusion incubation and all further manipulations were as described above.

Assay for cell-free formation of ISGs

To analyse whether ISGs form in the cell-free assay, PC12 cells were labelled by a 7 min pulse of [^{35}S]-sulphate and a PNS was obtained as described above. An ATP-regenerating system and 10x FB were added to this PNS and samples were incubated either at 4°C or at 37°C for 30 min. GTPγS (100 µM final concentration, 10 mM stock in H_2O at -20°C) was added to some incubations. Cell-free incubations were the transferred on ice and three identical conditions were pooled and loaded at 4°C onto a linear sucrose gradients of 0.3 M (5.5 ml) to 1.2 M (6.0 ml)

sucrose in 10 mM HEPES-KOH (pH 7.2) prepared in "Ultra Clear" tubes for the Beckman SW40 rotor. After loading, the gradients were centrifuged in a Beckman SW40 rotor at 25,000 rpm (110,000 g_{av}) and 4°C for 15 min, counting from reaching the set speed. Fractions of 1 ml were collected from the top to the bottom of the gradients using a Haakle-Buchler gradient collector. Aliquots of the gradient fractions were TCA-precipitated, resuspended in Laemmli sample buffer and analysed by 7.5% SDS-PAGE and fluorography.

Chapter 3: PH-dependent processing of secretogranin II by the endopeptidase PC2 in isolated ISGs

3.1 Objective

The focus of this chapter will be the characterisation of processing of SgII by the endopeptidase PC2 in ISGs isolated from PC12/PC2 cells, a PC12 cell-line that is stably transfected with PC2 (Dittié and Tooze, 1995). SgII (Mr ~ 86 000) has 6 dibasic cleavage sites (Lys-Arg) that are utilised by the prohormone convertase PC2 (Figure 3/1) (Tooze, *et al.*, 1994). PC12 cells express neither PC1 nor PC2, although they do express 7B2 (Seidah, *et al.*, 1994). A stable cell-line derived from PC12 cells has recently been developed and processing of SgII by PC2 has been characterised in these PC12/PC2 cells (Dittié and Tooze, 1995).

Processing of SgII by PC2 in intact PC12/PC2 cells has been previously described and the resulting processing products have been characterised (Dittié and Tooze, 1995). SgII is sulphated on a single tyrosine residue (Tyr 126) (Lee and Huttner, 1983) and this sulphation-site is preserved in all five N-terminal PC2 processing products (Figure 3/1). In this study only the sulphated processing products of SgII will be monitored as these can be readily identified after [^{35}S]-sulphate labelling of the PC12/PC2 cells. Isolation of the ISGs facilitates the manipulation of the external milieu and the analysis of the requirements for processing in ISGs. The information gained from such experiments will be the basis for a cell-free assay measuring fusion of ISGs described in chapters 4 to 7. Furthermore the question whether SgII processing begins in the TGN or the ISG will be addressed by comparing processing in the TGN and in ISGs either in isolated organelles or in semi-intact cells.

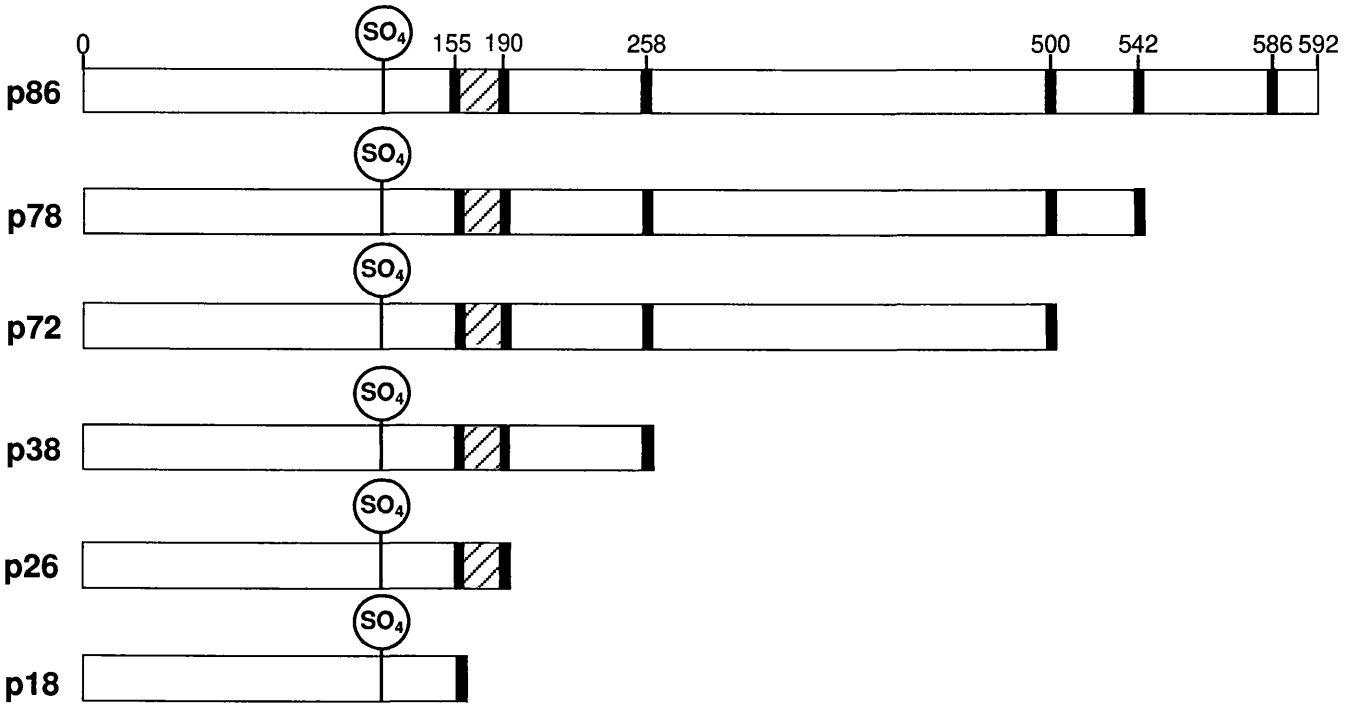


Figure 3/1: *Schematic representation of SgII and sulphated processing products.*

p86 refers to full-length SgII (Mr~86,000). p78, p72, p38, p26 and p18 refer to SgII-derived processing products of Mr~ 78,000, 72,000, 38,000, 26,000, and 18,000 respectively. Black bars indicate dibasic cleavage sites (Lys-Arg) utilized by PC2. The hatched area between two cleavage sites corresponds to the position of secretoneurin. SgII is sulphated on Tyr 126 (SO_4).

3.2 Isolation of ISGs from sulphate labelled PC12/PC2 cells.

ISGs were isolated by subcellular fractionation of PC12/PC2 cells that had been labelled by a 5 min [^{35}S]-sulphate pulse followed by a 15 min chase as described in (Tooze and Huttner, 1992). This protocol allows selective labelling of the regulated secretory proteins in ISGs and serves throughout this study as the operational definition of an ISG. The three major sulphated proteins in PC12/PC2 cells are secretory proteins: a constitutively secreted heparin sulphated proteoglycan (hsPG) (Gowda, *et al.*, 1989) and two proteins belonging to the granin family of secretory granule proteins, chromogranin B (CgB) and secretogranin II (SgII) (Gerdes, *et al.*, 1989; Rosa, *et al.*, 1985). All three proteins are sulphated in the TGN (Baeuerle and Huttner, 1987; Kimura, *et al.*, 1984; Rosa, *et al.*, 1985) and when pulse-labelled with [^{35}S]-sulphate are detected in constitutive secretory vesicles and ISGs respectively after a chase period of 15 min (Tooze and Huttner, 1990). ISGs containing [^{35}S]-sulphate labelled proteins can be separated first from the compartment from which they are formed, the TGN, by velocity controlled sucrose gradient centrifugation and second from constitutive secretory vesicles by an equilibrium sucrose gradient centrifugation (Tooze and Huttner, 1990).

To obtain ISGs containing [^{35}S]-sulphate labelled SgII, PC12/PC2 cells were depleted of sulphate, then pulse-labelled for 5 min with [^{35}S]-sulphate and chased for 15 min in normal growth medium supplemented with excess non-radioactive sodium sulphate as described in (Tooze and Huttner, 1992). The cells were washed, scraped from the dishes, homogenised and a post-nuclear supernatant (PNS) was obtained. This PNS was subjected to velocity controlled centrifugation on linear sucrose gradients (0.3 M to 1.2 M) to resolve the post-TGN vesicles from the TGN. Fractions from such gradients were analysed by SDS-PAGE and fluorography. A typical fluorogram from such an experiment is shown in Figure 3/2a. Figure 3/2c shows fractions of a the same type of gradient loaded with a PNS derived from PC12/PC2

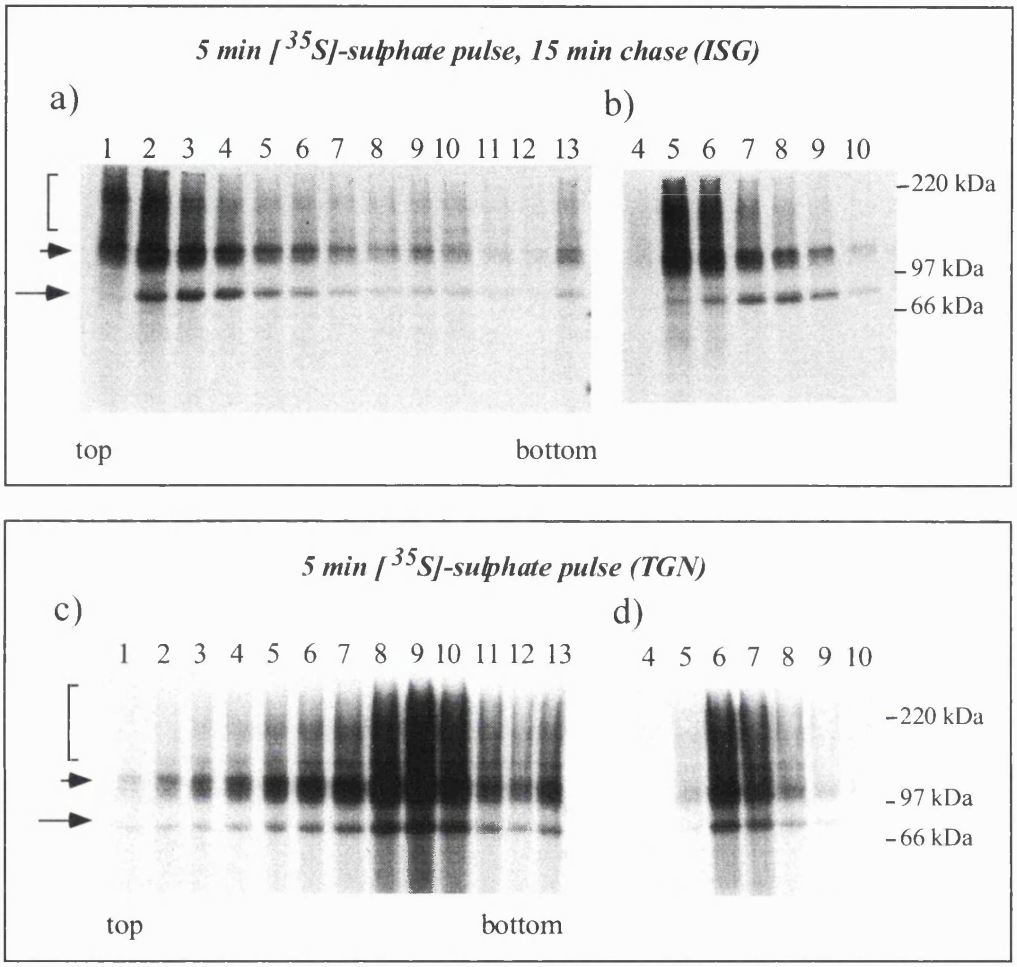


Figure 3/2: *Isolation of ISGs and TGN from sulphate labelled PC12/PC2 cells.*

PC12/PC2 cells were pulse-labelled for 5 min with [^{35}S]-sulphate and chased for 15 min (a, b) or only pulse-labelled for 5 min (c, d). A PNS was obtained and subjected to velocity controlled centrifugation on linear sucrose gradients (0.3 M to 1.2 M) to resolve the post-TGN vesicles from the TGN (a, c). Fractions were analysed by SDS-PAGE and fluorography. Note that TGN-membranes are found in fractions 8-10 (c) whilst the ISGs are recovered in fractions 2-4 (a). ISG- and TGN-containing fractions were combined into respective pools and subjected to equilibrium gradient centrifugation using step gradients prepared from 0.8, 1.0, 1.2, 1.4 and 1.6 M sucrose (b, d). Fractions from these gradients were analysed as above. TGN-membranes are recovered in fractions 6-8 (d) and ISGs are found in fractions 7-9 (b). The bracket indicates the hsPG, the arrowhead CgB and the arrow SgII.

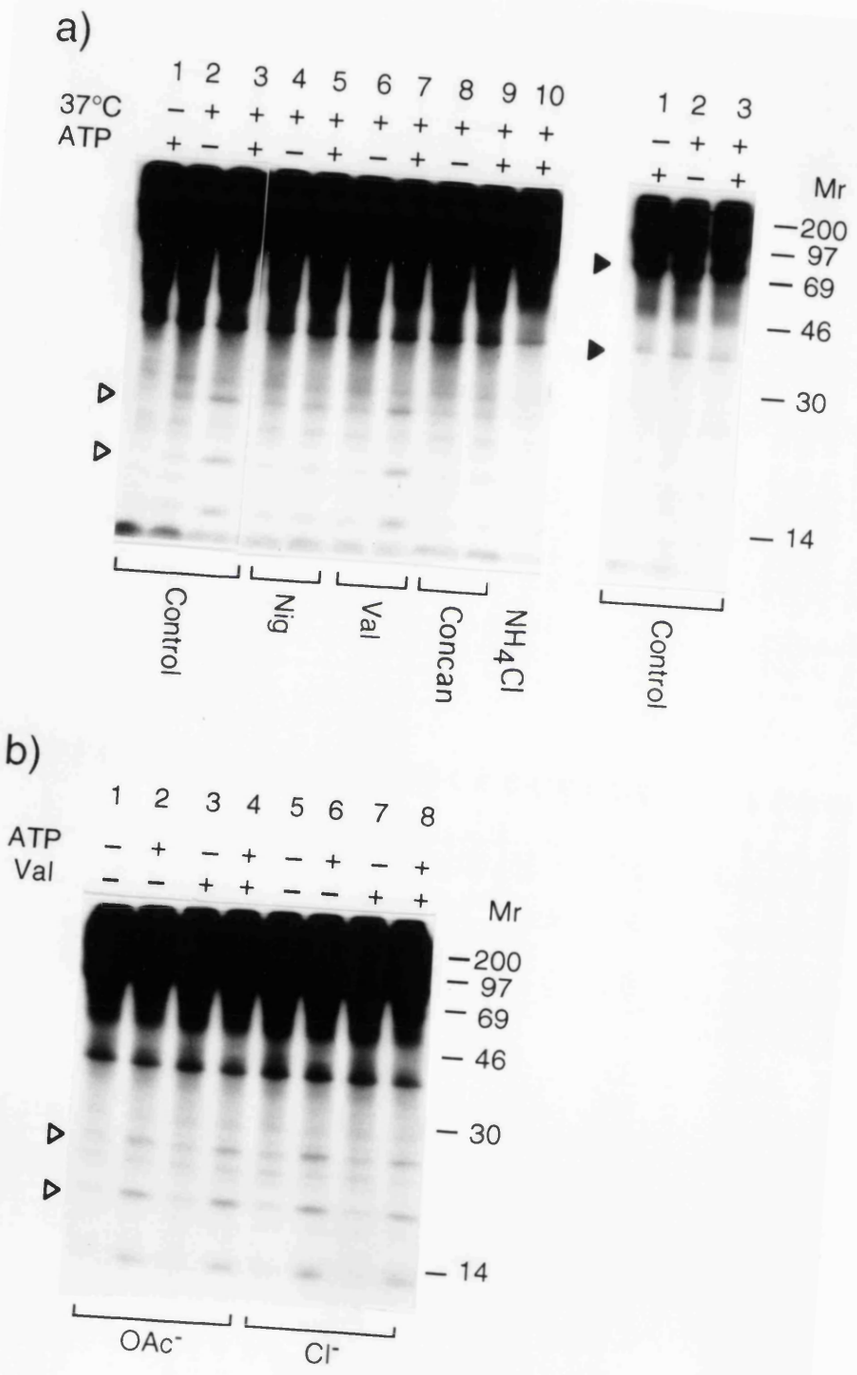
cells labelled by a 5 min [^{35}S]-sulphate pulse only, to show the distribution of the TGN-membranes. Note that TGN-membranes are found in fractions 8-10 (Figure 3/2c), while the ISGs are recovered in fractions 2-4 (Figure 3/2a) (Tooze and Huttner, 1992). ISG- and TGN-containing fractions were combined into respective pools and subjected to equilibrium gradient centrifugation using step gradients prepared from 0.8, 1.0, 1.2, 1.4 and 1.6 M sucrose. Fractions from these gradients were likewise analysed as above by SDS-PAGE and fluorography. Figure 3/2b and d show such fluorograms. Fractions 7 to 9 (Figure 3/2b) containing the peak of the SgII labelled by a 5 min [^{35}S]-sulphate pulse and a 15 min chase were combined (ISG-pool). Note that the ISGs identified by the peak of SgII are separated from the hsPG-containing constitutive secretory vesicles (Figure 3/2b, fractions 5 and 6). In contrast, SgII and hsPG labelled by a 5 min [^{35}S]-sulphate pulse are found in the same peak (Figure 3/2d, fractions 6 and 7) (Tooze and Huttner, 1990). The ISG-pool was used as starting material for the studies described in this chapter.

3.3 Processing of SgII in isolated ISGs requires activation of the vacuolar H^+-ATPase.

ISGs were isolated by subcellular fractionation from PC12/PC2 cells that had been labelled by a 5 min [^{35}S]-sulphate pulse followed by a 15 min chase as described above and in Chapter 2. Isolated ISGs were incubated in a neutral pH buffer (20 mM Tris or HEPES pH 7.2, 50 mM KOAc, 1 mM $Mg(OAc)_2$) in the presence or absence of an ATP-regenerating system (+/- ATP) at 4°C or 37°C for 45 min. After incubation at 4°C analysis of the sulphated proteins by 12% SDS-PAGE and autoradiography revealed a prominent band representing full-length SgII (p86) and p38 previously identified as a SgII-derived processing fragment (Figure 3/3a, lane 1) (Dittié and Tooze, 1995). Further characteristic SgII processing fragments, p26 and p18, appeared only upon incubation at 37°C in the presence of ATP (Figure 3/3a, compare lane 2 and

Figure 3/3: *Processing of SgII in isolated ISGs requires activation of the vacuolar H^+ -ATPase.*

(a) [^{35}S]-sulphate labelled ISGs were isolated from PC12/PC2 cells and incubated in 20 mM Hepes pH 7.2, 50 mM KOAc, 1 mM $Mg(OAc)_2$ for 45 min at 4°C or 37°C. Lane 1-3 are shown twice, in the right panel a lighter exposure is shown. An ATP-regenerating system and nigericin (Nig, 100nM), valinomycin (Val, 250nM), concanamycin (Concan, 100nM), and NH_4Cl (10mM), all final concentrations, were added where indicated. ISGs were recovered by ultracentrifugation and analysed by 12% SDS-PAGE and autoradiography. Open triangles indicate the position of the SgII-processing products p26 and p18, filled triangles indicate the full length SgII (p86) and the SgII-processing product p38. **(b)** [^{35}S]-sulphate labelled ISGs were isolated from PC12/PC2 cells and incubated in 20 mM Hepes pH 7.2, 50 mM KOAc, 1 mM $Mg(OAc)_2$ (lanes 1-4) or in 20 mM Hepes pH 7.2, 50 mM KCl, 1 mM $Mg(Cl)_2$ (lanes 5-8) for 45 min at 37°C. An ATP-regenerating system and/or valinomycin (1μM final concentration) were added where indicated. ISGs were analysed as above. Mr is expressed as $x10^{-3}$.


a)
1 2 3 4 5 6 7 8 9 10
37°C
ATP
1 2 3
Mr
200
97
69
46
30
14
Control
Nig
Val
Concan
NH4Cl
Control
b)
1 2 3 4 5 6 7 8
ATP
Val
Mr
200
97
69
46
30
14
OAc-
Cl-

3). The sulphated band of approximately 12 kDa is not derived from SgII and has not yet been identified (Dittié and Tooze, 1995). Full length SgII and the high molecular weight processing products p78 and p72 are largely masked by a sulphated proteoglycan (hsPG). Unfortunately this proteoglycan smear interferes with an accurate quantitative analysis of the processing-efficiency. The relative efficiency of processing under different conditions was therefore assessed by quantitation of the sulphated end product p18 (see below).

Processing of various substrates by the endopeptidase PC2 has been studied *in vitro* in several different cell- and granule-lysates, and has been shown to have a characteristic acidic pH-optimum (pH 5.0-5.5) (Arden, *et al.*, 1994; Davidson, *et al.*, 1988; Shennan, *et al.*, 1991; Shennan, *et al.*, 1995). Chromaffin-granules and other secretory granules in a variety of tissues contain a H^+-ATPase of the vacuolar type (Al-Awqati, 1986; Nelson, *et al.*, 1988). This enzyme plays a central role in the acidification of the dense-core secretory granules (Cidon and Nelson, 1983; Cidon and Nelson, 1986; Moriyama and Nelson, 1987; for review see Nelson, *et al.*, 1988). Therefore, it seemed likely that ATP might stimulate processing in isolated ISGs by activating the vacuolar H^+-ATPase of the ISG, thereby leading to a decrease in the intragranular pH. Consistent with this hypothesis, inhibition of the vacuolar H^+-ATPase by the fungal metabolite concanamycin A (a specific inhibitor of v-type H^+-ATPases (Droese, *et al.*, 1993) abolished the ATP-dependent processing (Figure 3/3a, lane 8 and 9). Furthermore, inclusion of nigericin was sufficient to eliminate the appearance of SgII-fragments in accordance with its function of facilitating proton-release in exchange for potassium-uptake (lane 4 and 5). In contrast, the K^+-ionophore valinomycin, which uncouples the membrane potential from the proton pumping activity, had no effect on processing (lane 6 and 7). This suggests that the observed effect of ATP on processing results from the establishment of an acidic pH in the lumen of the granule and is neither dependent on, nor limited by the generation of a membrane-potential. As expected addition of NH_4Cl, a weak base that accumulates in

acidic membrane compartments where it is protonated leading to alkalinization of the intragranular milieu, also prevented processing (Figure 3/3a, lane 10).

Previous work by others on the bioenergetics of chromaffin granules has shown a requirement for chloride anions in the generation of a pH-gradient across the granule membrane (Al-Awqati, 1986; Cidon and Nelson, 1983; Johnson, *et al.*, 1985). Chloride is acting as a permeant counterion to the protons pumped by the vacuolar ATPase and allows acidification by counteracting the build-up of a membrane potential that would inhibit further proton influx. Interestingly, replacement of the acetate anions in the incubation buffer by chloride anions did not further increase the extent of processing, suggesting that the conductance of the PC2-ISG membrane for acetate is sufficient to dissipate any membrane-potential that might limit maximal acidification (Figure 3/3b, see also Johnson and Scarpa, 1976).

3.4 Comparison of the time course of processing in intact cells and in isolated ISGs.

The profile of the processing products obtained by incubation of isolated ISGs was reminiscent of that observed *in vivo* (Dittié and Tooze, 1995). Figure 3/4 shows a direct comparison of a time course of processing in intact PC12/PC2 cells pulse-labelled with [^{35}S]-sulphate for 5 min and chased for various lengths of time and the sequential appearance of processing products in isolated ISGs incubated at pH 7.2 in the presence of ATP. The kinetics of both p26 and p18 appearance as well as p18 accumulation in isolated ISGs under these conditions appeared to be very similar to the time course in intact cells. At early time-points, t=30 min of chase in intact cells which corresponds to t=15 min in isolated ISGs, only very little p18 was generated. It has previously been shown that the half-time of maturation for secretory granules in PC12 cells is approximately 45 min (Tooze, *et al.*, 1991), therefore up to 90 min of chase time the granules containing the labelled proteins can be considered as ISGs at various

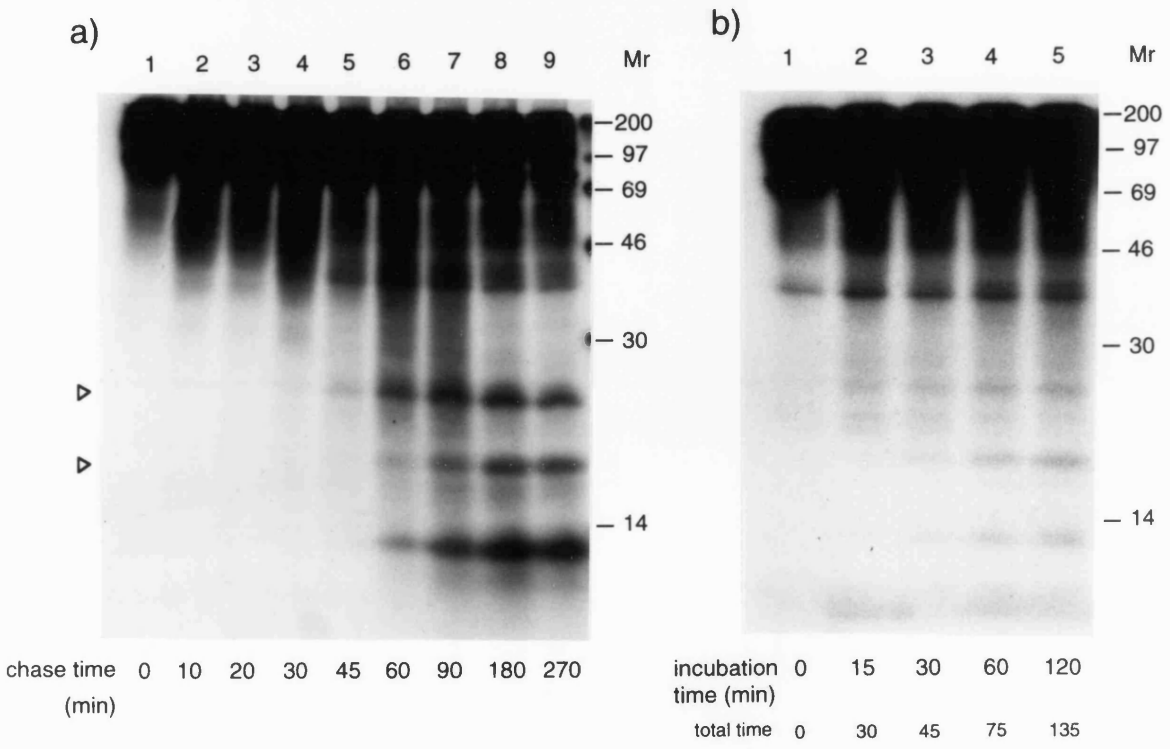


Figure 3/4: *Processing in intact cells and in isolated ISGs.*

(a) PC12/PC2 cells were pulse-labelled for 5 min with [^{35}S]-sulphate and chased for indicated length of time. The cells were lysed, a heat-stable fraction was prepared and the heat-stable proteins were subjected to 12% SDS-PAGE followed by autoradiography. **(b)** PC12/PC2 cells were labelled with a 5 min [^{35}S]-sulphate pulse followed by a 15 min chase. The [^{35}S]-sulphate labelled ISGs were isolated and incubated in 20 mM Hepes pH 7.2, 50 mM KOAc, 1 mM $Mg(OAc)_2$ at 37°C in the presence of an ATP-regenerating system for various length of time (incubation time). The equivalent chase-time (15 min chase in vivo + incubation time in vitro = total time) is indicated to facilitate the comparison of the in vivo and in vitro time course. ISGs were recovered by ultracentrifugation and analysed by 12% SDS-PAGE and autoradiography. Open triangles indicate the position of the SgII-processing products p26 and p18. Mr is expressed as $x10^{-3}$.

stages of maturation. The appearance of p26 and p18 *in vitro* within this time window nicely paralleled the time course *in vivo* and suggests that processing can indeed be reconstituted in isolated ISGs. At later time-points (t=90-180 min) the accumulation of p18 reached a plateau in intact cells and processing seemed to come to an end (as observed in isolated ISGs incubated at pH 5.5 (see below)). Given the limitations imposed by our inability to resolve p86, p78, p72 and the proteoglycan it was estimated that roughly 44% of the radioactivity remained within the p86 band and 14% was found in p18 after 270 min of chase time in intact cells.

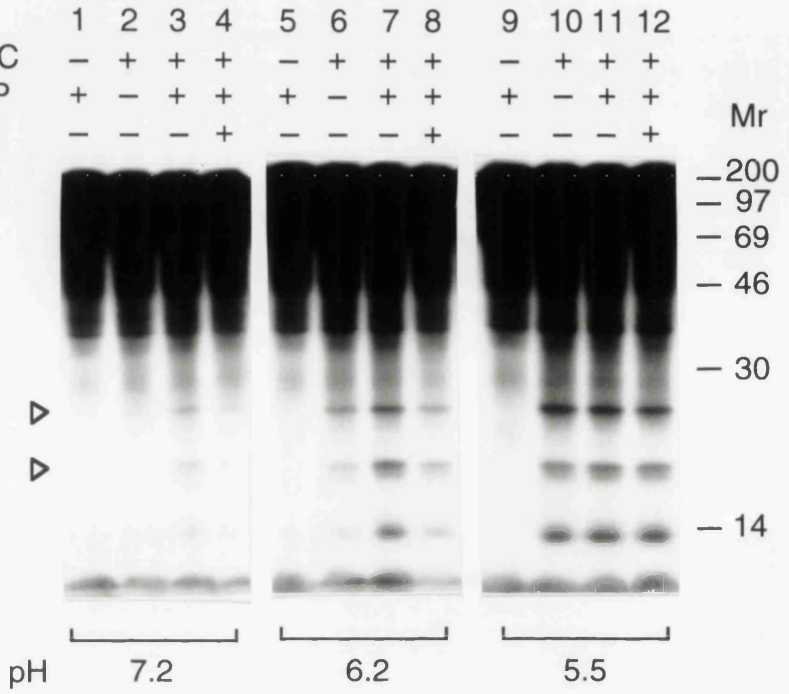
3.5 Acidification of the ISG lumen is sufficient for SgII-processing by PC2.

The data shown in Figure 3/3 suggested that processing in isolated ISGs solely depends on the establishment of an acidic intragranular pH. As all manipulations leading to the isolation of ISGs were carried out at 4°C in the absence of ATP, conditions under which the H^+-ATPase would be expected to be inactive, this might result in the neutralisation of the intragranular pH through equilibration with the buffer (10 mM HEPES, pH 7.2) used in the sucrose solutions. If ATP is only required to re-acidify the ISG lumen after isolation then it should be possible to observe ATP-independent processing after preincubating the ISGs in a low pH buffer. Figure 3/5 shows that processing can indeed be observed at 37°C in the absence of ATP in ISGs that have been pre-equilibrated at pH 6.2 for an hour on ice (lane 6). Addition of ATP to these granules enhanced processing by approximately 2-fold (Figure 3/5a, compare lanes 6 and 7 and Figure 3/5b) as compared to the close to 4-fold stimulation by ATP observed in granules which had been kept at pH 7.2 (compare lanes 2 and 3 and Figure 3/5b). Similar amounts of processing were observed after pre-equilibration at pH 5.5 compared to pH 6.2 in the presence of ATP (Figure 3/5a, compare lanes 7 and 11), however the ATP-dependent stimulation of processing was much reduced (Figure 3/5a, lanes 10 and 11 and Figure 3/5b). Inclusion of nigericin during the incubation at 37°C

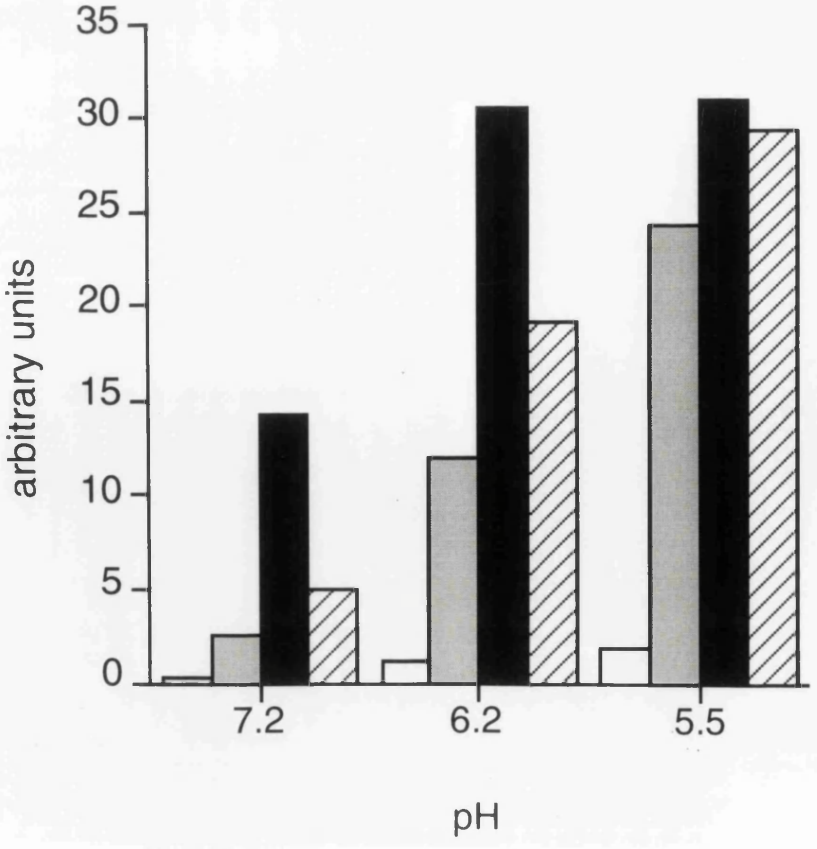
Figure 3/5: *Acidification of the ISG lumen is sufficient for SgII-processing by PC2.*

PC12/PC2 cells were labelled with a 5 min [^{35}S]-sulphate pulse followed by a 15 min chase. **(a)** ISGs were prepared from the labelled cells and preincubated in 20 mM Hepes pH 7.2, 50 mM KOAc, 1 mM $Mg(OAc)_2$ (lanes 1-4), 20 mM MES pH 6.2, 50 mM KOAc, 1 mM $Mg(OAc)_2$ (lanes 5-8) or 20 mM MES pH 5.5, 50 mM KAc, 1 mM $Mg(OAc)_2$ (lanes 9-12) for one hour at 4°C. Nigericin (Nig) was added to a final concentration of 100 nM and an ATP-regenerating system was added where indicated. The samples were incubated for 45 min at 4°C or 37°C. ISGs were recovered by ultracentrifugation and the samples were analysed by 12% SDS-PAGE followed by autoradiography. Triangles in (a) indicate the position of the SgII-processing products p26 and p18. Mr is expressed as $x10^{-3}$. **(b)** The 18 kDa band (p18) in (a) was quantitated by PhosphorImager and the extent of processing for each condition (open bars, 4°C, +ATP; grey bars, 37°C, -ATP; black bars, 37°C, +ATP; hatched bars, 37°C, +ATP, +Nigericin) is shown in arbitrary units.

a)


1 2 3 4 5 6 7 8 9 10 11 12
37°C
ATP
Nig
Mr
200
97
69
46
30
14
pH
7.2
6.2
5.5

b)


arbitrary units
35
30
25
20
15
10
5
0
7.2
6.2
5.5
pH

resulted in a signal which was comparable to the processing in the absence of ATP, suggesting that the intragranular pH was largely equilibrated with the external pH after an hour incubation at 4°C (Figure 3/5a, compare lanes 2 and 4, 6 and 8, 10 and 12 and Figure 3/5b).

3.6 The kinetics of SgII processing depend on the granular pH.

Interestingly, the extent of processing obtained after 45 min incubation in the presence of ATP in a pH 7.2-buffer (Figure 3/5a, lane 3) was significantly less than the extent observed in pH 5.5-buffer in the presence or absence of ATP (Figure 3/5a, lane 10, 11) but was comparable to the processing obtained in pH 6.2-buffer in the absence of ATP (Figure 3/5a, lane 6). In order to see whether this result reflects different kinetics of processing at different pH-values, a time course of processing in ISGs at pH 7.2 in the presence of ATP was compared with the processing in ISGs that had been pre-equilibrated in pH 5.5-buffer. Figure 3/6a shows that processing in granules that have been acidified by the activity of the vacuolar H^+-ATPase was indeed characterised by a much slower rate than processing in granules pre-equilibrated in pH 5.5-buffer.

The data presented in Figure 3/6a suggested a half-time of p18 accumulation of approximately 20 min at pH 5.5 compared to a much slower rate at pH 7.2 in the presence of ATP. As p18 appeared to be an end-product of processing (both *in vivo* and *in vitro*) it can be suggested that the half time of SgII processing by PC2 in isolated ISGs at pH 5.5 is approximately 20 min. Given that acidification of a small vesicle like the ISG can be expected to reach a steady state equilibrium within minutes (Al-Awqati, 1986), the slow kinetics of SgII-processing at pH 7.2 in the presence of ATP indicates that the intragranular pH in ISGs generated by the vacuolar H^+-ATPase does not reach the optimal value for PC2-processing, and thus the kinetics reflect the slower processing-rate of PC2 at pH-values above its pH-optimum (pH 5.0-5.5).

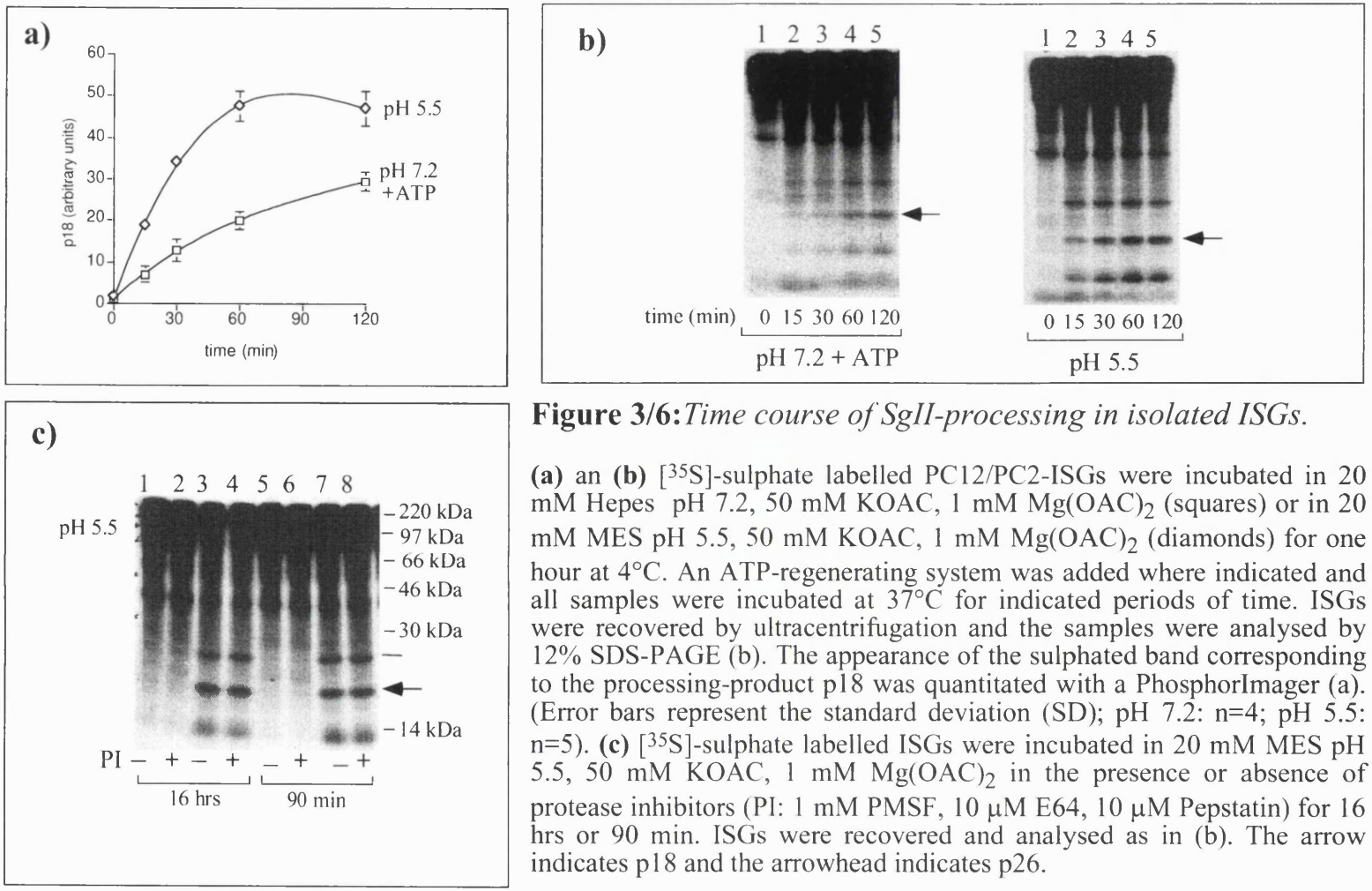


Figure 3/6: *Time course of SgII-processing in isolated ISGs.*

(a) an **(b)** [^{35}S]-sulphate labelled PC12/PC2-ISGs were incubated in 20 mM Hepes pH 7.2, 50 mM KOAC, 1 mM $Mg(OAC)_2$ (squares) or in 20 mM MES pH 5.5, 50 mM KOAC, 1 mM $Mg(OAC)_2$ (diamonds) for one hour at 4°C. An ATP-regenerating system was added where indicated and all samples were incubated at 37°C for indicated periods of time. ISGs were recovered by ultracentrifugation and the samples were analysed by 12% SDS-PAGE (b). The appearance of the sulphated band corresponding to the processing-product p18 was quantitated with a PhosphorImager (a). (Error bars represent the standard deviation (SD); pH 7.2: n=4; pH 5.5: n=5). **(c)** [^{35}S]-sulphate labelled ISGs were incubated in 20 mM MES pH 5.5, 50 mM KOAC, 1 mM $Mg(OAC)_2$ in the presence or absence of protease inhibitors (PI: 1 mM PMSF, 10 μM E64, 10 μM Pepstatin) for 16 hrs or 90 min. ISGs were recovered and analysed as in (b). The arrow indicates p18 and the arrowhead indicates p26.

A representative set of data from one of the experiments used to generate the curve in Figure 3/6a, is shown in Figure 3/6b. From such experiments and lighter exposures, it could be estimated that after 120 min of incubation at pH 5.5 approximately 40% of the radioactivity remained in p86 while the p18 band accounted for about 16% of the total radioactivity. This result was comparable to the extent of conversion observed in intact cells pulse labelled for 5 min with [^{35}S]-sulphate and chased for 270 min (see Figure 3/4). Further incubation for 16 hrs at 30°C did not yield more labelled p18 (Figure 3/6c, compare lanes 3,4 and 7,8).

3.7 Estimation of the intragranular pH using SgII processing as an intrinsic pH indicator.

From the observations made in Figures 3/5 to 3/6 it seemed possible to use the processing of SgII by PC2 as an intrinsic pH-indicator for ISGs. This type of "functional measurement of vesicle acidification" (Mellman, *et al.*, 1986) has the advantage that the acidification event takes place in an unequivocally defined organelle and therefore does not rely on the purity of the preparation. In other words, the observed processing of SgII labelled with a 5 min pulse of [^{35}S]-sulphate followed by a 15 min chase by definition reflects an ISG-specific event (as ISGs are defined by this labelling-procedure). Figure 3/7 shows the result of the pH-calibration of SgII processing (solid line). Isolated ISGs were incubated in buffers of different pH-values for 45 min at 37°C, and the sulphated product p18 was quantitated to generate a standard-curve. To ensure complete equilibration nigericin was added to these incubations. The rate of processing was linear for up to 45 min at 37°C within the range of pH-values used for this calibration.

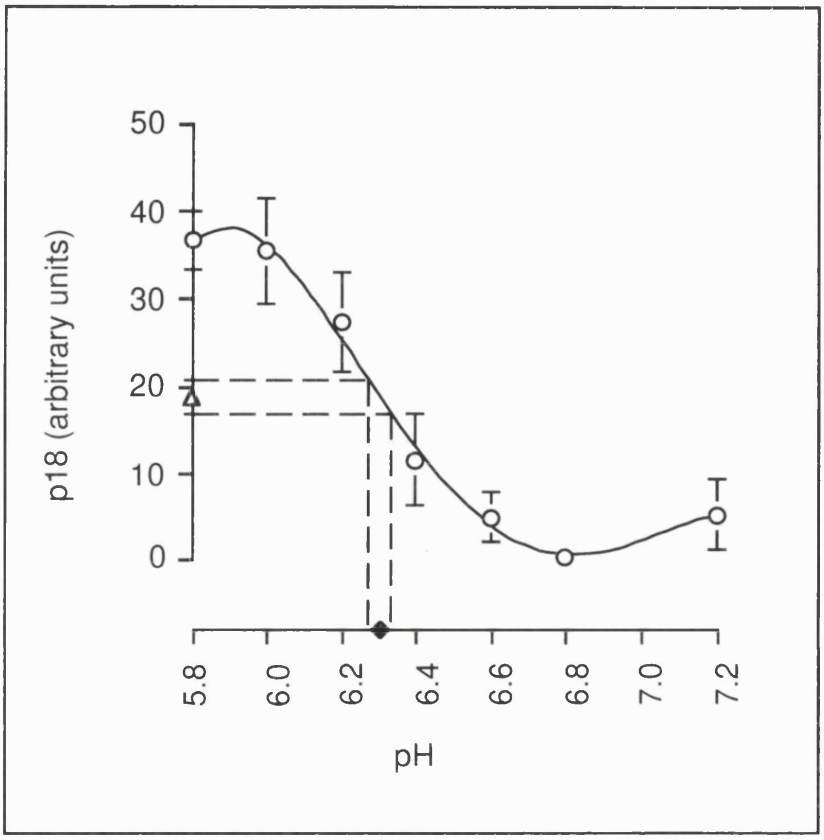


Figure 3/7: *pH in isolated immature secretory granules.*

[^{35}S]-sulphate labelled ISGs were incubated in buffers of different pH-values from 5.8 to 7.2 for 45 min at 37°C in the presence of 100 nM nigericin, and the sulphated product p18 was quantitated with a PhosphorImager to generate a standard-curve. The extent of SgII-processing in ISGs incubated at pH 7.2 in the presence of ATP was determined by quantitating p18 and the result (Δ18.82 +/- 1.90 arbitrary units) was fitted on the standard-curve (error bars indicate SD; n=6)

In the same experiment the extent of SgII-processing in the presence of ATP at pH 7.2 was determined, and the result of the p18 quantitation from this sample was fitted on the standard-curve. The extent of SgII processing observed in isolated ISGs under these conditions corresponds to the extent of processing in ISGs buffered at pH 6.3 +/- 0.1 (see Figure 3/7, ◆). It is therefore proposed that ISGs have a pH-gradient close to 1.0 and an intragranular pH of 6.3 +/-0.1 in a buffer with a physiological pH of 7.2 in the presence of ATP. Interestingly, this value is comparable to the pH of the TGN as determined in an elegant study by Seksek et al. (pH 6.17+/-0.02) (Seksek, *et al.*, 1995) and more recently by Kim et al. (pH 6.45 +/- 0.03) (Kim, *et al.*, 1996). In contrast, mature secretory granules isolated from different neuroendocrine cell types, including chromaffin cells, have been reported to be far more acidic (pH 5.0-5.5) (Hutton, 1982; Johnson and Scarpa, 1975).

3.8 pH-dependent processing of SgII cannot be observed in isolated TGN.

Given the similarity between the pH-values reported for the TGN (Kim, *et al.*, 1996; Seksek, *et al.*, 1995) and the ISGs (Figure 3/7), it was interesting to ask whether processing of SgII by PC2 could actually take place in the TGN. Pulse-chase experiments in PC12/PC2 cells have shown that [^{35}S]-sulphate labelled SgII is not processed prior to its exit from the TGN (Dittié and Tooze, 1995). However this result could be simply a consequence of the short residence time of the labelled substrate in the TGN: it was previously shown that vesicles form at the TGN with a half-time of approximately 5 min (Tooze and Huttner, 1990). It has been shown above that processing of SgII by PC2 at a lumenal pH of 6.3 is, in comparison, a relatively slow process (the $t_{1/2}$ cannot be measured from Figure 3/6 as a plateau is not yet reached after 120 min but it can be estimated to be longer than 30 min).

In order to address this question, the TGN was isolated from PC12/PC2 cells labelled by a 5 min [^{35}S]-sulphate pulse (Tooze and Huttner, 1992) to analyse whether processing of SgII could occur in this isolated TGN. It is important to note that conditions are maintained to prevent vesicle formation. TGN was isolated using gradient centrifugations as described in Figure 3/2. The TGN membranes were recovered in fractions 6-8 of the equilibrium gradient (figure 3/2d). ISGs were isolated in parallel from PC12/PC2 cells labelled for 5 min with [^{35}S]-sulphate and chased for 15 min (see Figure 3/2).

First, ISGs and TGN were incubated at pH 7.2 in the absence or presence of ATP, under conditions that are unlikely to sustain the budding of vesicles (in the absence of cytosol and GTP (Xu and Shields, 1993)). As before (see Figure 3/3) SgII processing was observed in an ATP-dependent manner in ISGs but no genuine processing fragments could be detected in the TGN even after a long exposure (Figure 3/8a). The 16 kDa protein detected in the TGN-membranes has not been observed in intact cells and has not further been characterised. To ensure that acidification of the TGN was not limited by the generation of a membrane-potential (Glickman, *et al.*, 1983) processing was also analysed both in the presence of chloride-anions and in the presence of valinomycin (Figure 3/8a, lanes 3,6,9 and 12). The only low molecular weight [^{35}S]-sulphate labelled protein appearing under these conditions was the 16 kDa protein (Figure 3/8a).

Although the H^+-ATPase of the TGN is likely to share at least several subunits with the ISG H^+-pump (Moriyama and Nelson, 1989), it is possible that the H^+-ATPase is inactive after isolation of the TGN. The TGN was therefore preincubated in pH 6.2 or pH 5.5-buffer in the presence of nigericin in order to acidify the TGN-lumen. Even under these conditions no evidence for processing of SgII in this compartment was obtained (Figure 3/8b).

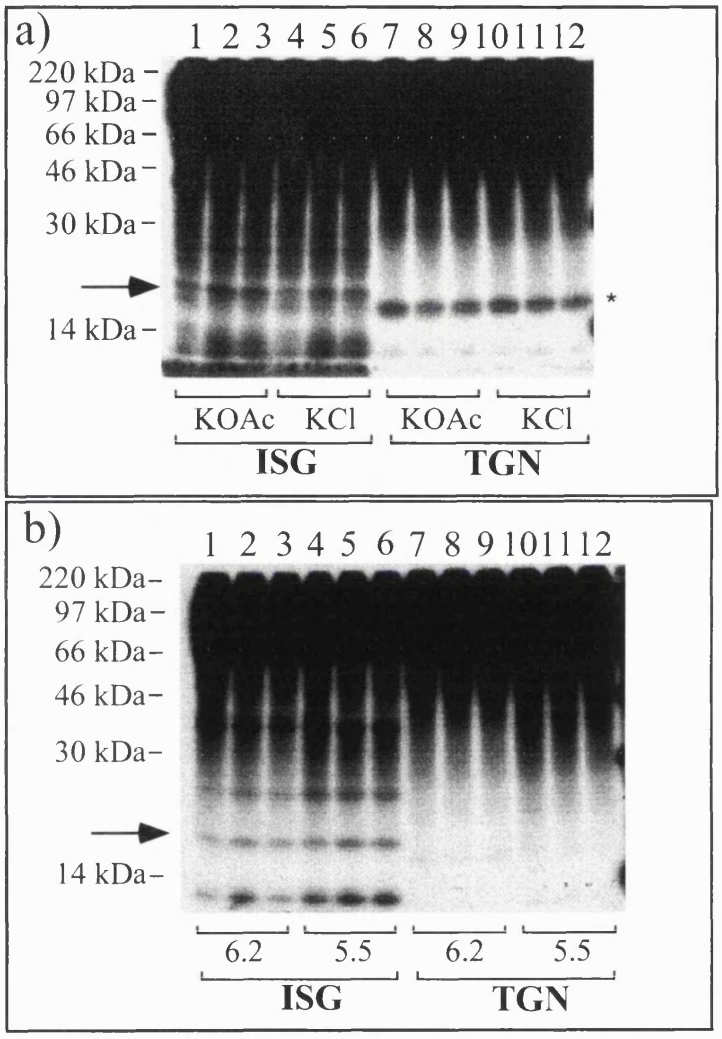


Figure 3/8: *pH-dependent processing of SgII cannot be observed in isolated TGN.*

(a) [^{35}S]-sulphate labelled ISGs (lanes 1-6) and [^{35}S]-sulphate labelled TGN (lanes 7-12) were isolated in parallel and preincubated in either 20 mM HEPES pH 7.2, 50 mM KOAc, 1 mM $Mg(OAc)_2$, (lanes 1-3 and 7-9), or 20 mM HEPES pH 7.2, 50 mM KCl, 1 mM $MgCl_2$ (lanes 4-6 and 10-12) for 1 hour at 4°C. Samples were incubated for 45 min at 37°C in the presence (lanes 3, 6, 9, and 12) or absence of valinomycin (100 nM) in the absence (lanes 1,4,7 and 10) or presence of an ATP-regenerating system. ISGs were analysed as described in Chapter 2. **(b)** [^{35}S]-sulphate labelled ISGs (lanes 1-6) and [^{35}S]-sulphate labelled TGN (lanes 7-12) were isolated in parallel and preincubated in either 20 mM MES, 50 mM KOAc, 1 mM $Mg(OAc)_2$, pH 6.2 (lanes 1-3 and 7-9) or pH 5.5 (lanes 4-6 and 10-12) for 1 hour at 4°C. Nigericin (100 nM) was added to lanes 3, 6, 9, and 12 and an ATP-regenerating system to all lanes but 1, 4, 7 and 10. All samples were incubated for 45 min at 37°C. ISGs were recovered and analysed as in (a). Arrows indicate the position of the SgII-processing products p18 and the star indicates the unknown band of approximately 16 kDa.

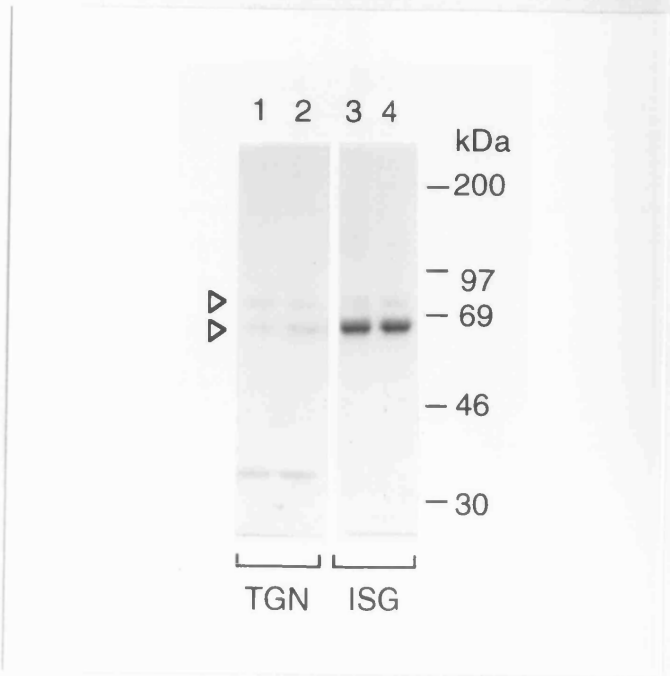


Figure 3/9: *Relative abundance of pro-PC2 and mature PC2 in the TGN and in the ISG.*

Isolated ISGs (100 µg total protein) and isolated TGN (100µg total protein) were incubated as in Figure 3/8b either in 20 mM MES pH 6.2, 50 mM KOAc, 1 mM $Mg(OAc)_2$ (lanes 1and 3) or 20 mM MES pH 5.5, 50 mM KOAc, 1 mM $Mg(OAc)_2$ (lanes 2 and 4) for 1 hour at 4°C. After addition of both 100 nM nigericin and an ATP-regenerating system the samples were transfered to 37°C for 45 min. The proteins were recovered by TCA-precipitation, separated by 7.5% SDS-PAGE and subjected to immunoblotting with antiserum 4BF. Bound antibody was detected with [^{125}I]-protein A and analysed by autoradiography. Mr is expressed as x10-3.

	TGN		ISG	
	pH 6.2	pH 5.5	pH 6.2	pH 5.5
pro-PC2	11678	9835	13947	13450
PC2	10735	16988	107356	110174
PC2/pro-PC2 +/-S.E.M.(3)	1.22+/-0.09	2.28+/-0.42	8.78+/-1.65	8.89+/-1.33

Table 3/1: *Quantitation of pro-PC2 and PC2 in TGN and ISG enriched fractions and determination of the ratio of PC2 to pro-PC2*

The amount of pro-PC2 and PC2 detected in TGN and ISG fractions for the representative experiment shown in Figure 3/9 was quantitated and is shown in arbitrary units. Three independent experiments were carried out in duplicates and the mean ratio of PC2 to pro-PC2 was determined

3.9 Determination of the relative abundance of pro-PC2 and mature PC2 in TGN and ISGs

Activation of PC2 requires cleavage of the pro-form to the mature form. The lack of PC2-activity in the TGN might thus reflect the state of PC2-maturation in this compartment. The relative ratios of the pro- and mature PC2-forms in TGN and ISGs were therefore analysed after incubation at pH 6.2 or pH 5.5 for 45 min (identical conditions to Figure 3/8b, lanes 3, 6, 9 and 12). Immunoblotting of TGN and ISGs (equal amounts of protein loaded) with an antibody directed against the C-terminus of PC2 suggested that pro-PC2 and PC2 are found at a 1:1 ratio in the TGN, whereas in the ISGs the mature form of PC2 is enriched approximately 9 times over pro-PC2 (Figure 3/9 and Table 3/1). Although a larger proportion of PC2 is mature in the ISGs, the lack of SgII-processing in the TGN cannot be explained by the absence of mature PC2 in this compartment. It should however be noted that PC2 is enriched by 3 to 6 fold in ISGs as compared to the TGN: the increased concentration of PC2 upon packaging into ISGs might enhance PC2-conversion and be crucial for PC2-activity towards SgII.

3.10 Processing of SgII by PC2 can be observed in semi-permeabilised cells after a 20°C block

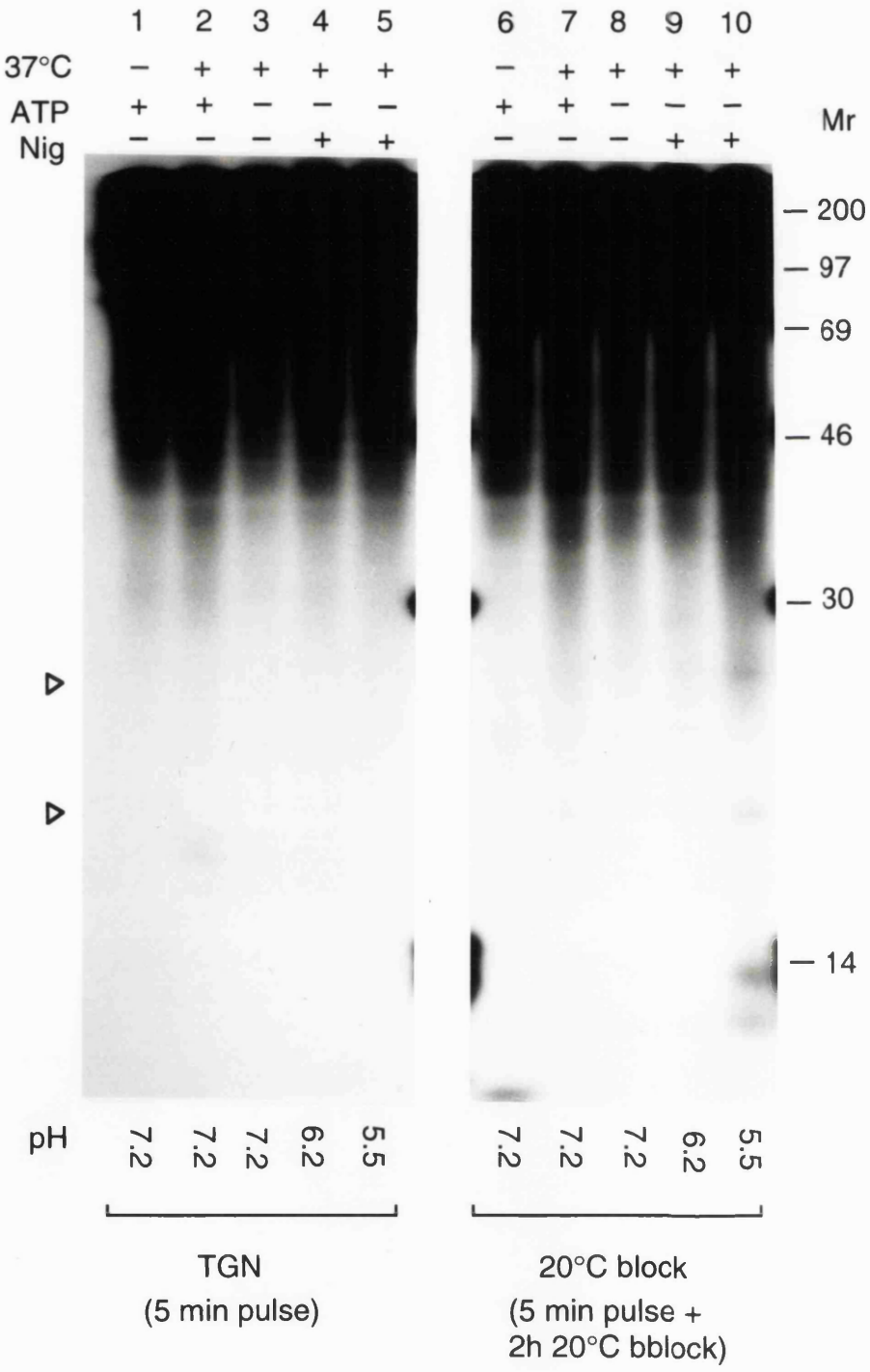
Recently Xu et al. (1993) showed that prosomatostatin processing by PC2 in permeabilised GH3 cells can be uncoupled from budding of secretory vesicles from the TGN using a 20°C block. Processing in the TGN was solely ATP-dependent whereas formation of secretory vesicles needed the addition of both ATP and GTP (Xu and Shields, 1993). The requirement of ATP for prosomatostatin processing in the TGN was circumvented by incubation of the permeabilised cells in acidic buffers in the presence of a protonophore (Xu and Shields, 1994). As shown above, processing of SgII by PC2 was not observed in isolated TGN after similar treatments (Figure 3/8).

This discrepancy leads one to wonder whether the TGN, defined by a 5 min pulse of [^{35}S]-sulphate is functionally equivalent to the TGN in cells blocked for 2 hours at 20°C. Processing in semi-intact cells that had been previously pulse-labelled for 5 min and chased at 20°C for 2 hours was therefore compared with cells that had been permeabilised immediately after the 5 min [^{35}S]-sulphate pulse. The semi-intact cells were washed extensively to remove the cytosol and incubated under conditions equivalent to the experiments in Figures 3/3 and 3/5. At pH 5.5 in the presence of nigericin the processing products p18 and p26 were clearly visible only in cells that had been blocked at 20°C (Figure 3/10 compare lanes 5 and 10). At pH 7.2 in the presence of ATP the signals for p18 and p26 were much weaker but still recognisable (lane 7). In contrast in cells that had only been pulse-labelled a band of approximatively 16 kDa appeared at pH 7.2 in the presence of ATP, but no p26 or p18 was detected even after prolonged exposure (lane 2).

When SgII processing at pH 5.5 in semi-intact cells subjected to a 20°C block was compared directly with processing in semi-intact cells labelled by a 5 min [^{35}S]-sulphate pulse followed by a 15 min chase (ISG), the time course of processing in the 20°C block compartment was very similar to the time course in the ISGs, indicating that the conditions in the lumen of the compartments were comparable at least with respect to processing (Figure 3/11). Furthermore, if the length of the 20°C block incubation was varied, processing of SgII could be detected at pH 5.5 in semi-permeabilised cells as soon as 30 min after initiation of the 20°C block following the 5 min [^{35}S]-sulphate pulse (Figure 3/12).

Figure 3/10: *Processing of SgII in semi-permeabilised cells after a 20°C block.*

PC12/PC2 cells were labelled with a 5 min [^{35}S]-sulphate pulse and either permeabilised immediately (lanes 1-5) or chased for 2 hrs at 20°C prior to permeabilisation (lanes 6-10). The semi-permeabilised cells were washed to remove the cytosol and incubated for 45 min in buffers of pH 7.2, 6.2 or 5.5 (see Figure 3). An ATP-regenerating system (ATP) and nigericin (Nig, final concentration 100 nM) were added where indicated 10 min before the incubation at 37°C. After incubation the cells were lysed, a heat-stable fraction was prepared and the heat-stable proteins were analysed by 12% SDS-PAGE and autoradiography. Triangles indicate the position of the SgII-processing products p26 and p18. Mr is expressed as x10^{-3}.


1 2 3 4 5
6 7 8 9 10
37°C
ATP
Nig
Mr
200
97
69
46
30
14
pH
7.2 7.2 7.2 6.2 5.5
7.2 7.2 7.2 6.2 5.5
TGN
(5 min pulse)
20°C block
(5 min pulse +
2h 20°C bblock)

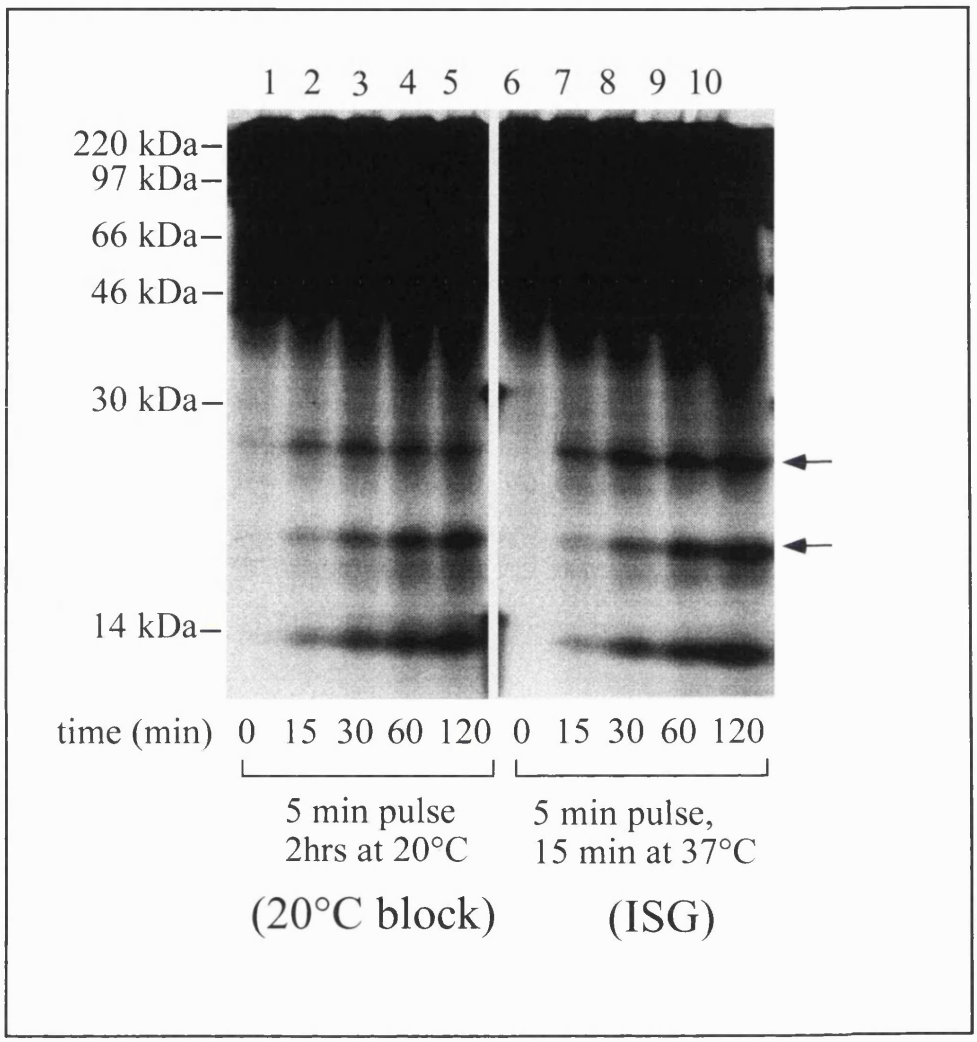


Figure 3/11: *Comparison of SgII processing in ISGs and in the 20° block compartment.*

PC12/PC2 cells were labelled with a 5 min [^{35}S]-sulphate pulse and chased either for 2 hrs at 20°C (lanes 1-5) or for 15 min at 37°C (lanes 6-10) prior to permeabilisation. The semi-permeabilised cells were washed to remove the cytosol and incubated for 45 min in a pH 5.5 buffer (see Figure 3/10). After incubation for indicated length of time, the cells were lysed, a heat-stable fraction was prepared and the heat-stable proteins were analysed by 12% SDS-PAGE and autoradiography. Arrows indicate the position of the SgII-processing products p26 and p18.

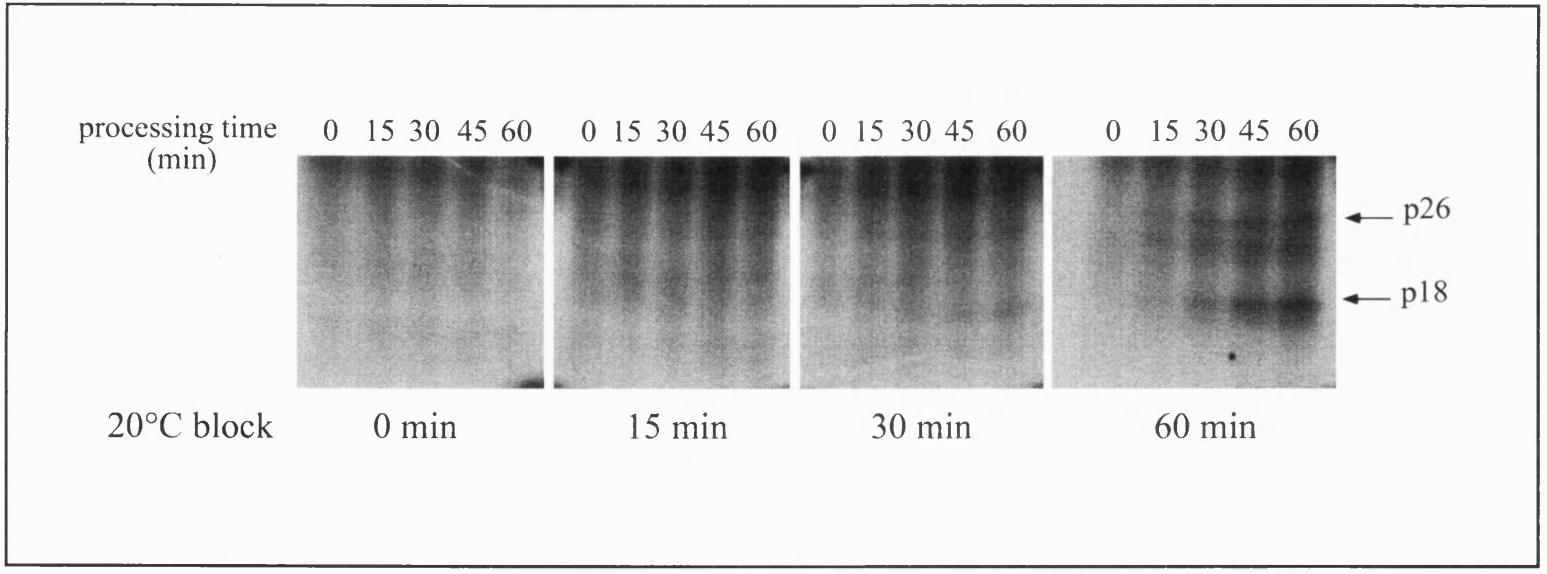


Figure 3/12: *Processing of SgII in the 20°C block compartment.*

PC12/PC2 cells were labelled with a 5 min [^{35}S]-sulphate pulse and either permeabilised immediately or chased for 15, 30, or 60 min at 20°C (20°C block) prior to permeabilisation. The semi-permeabilised cells were washed to remove the cytosol and incubated for 45 min in a pH 5.5 buffer (see Figure 3/10). After incubation for indicated length of time (processing time) , the cells were lysed, a heat-stable fraction was prepared and the heat-stable proteins were analysed by 12% SDS-PAGE and autoradiography. Arrows indicate the position of the SgII-processing products p26 and p18.

3.11 Aberrant processing of SgII in detergent-solubilised isolated ISGs

To standardise the extent of SgII processing in ISGs in different environments. would be useful to measure processing of SgII by PC2 under conditions that are independent from the bioenergetic parameters of the secretory granule membrane and from the physical state of the granule core. To this end, ISGs from [^{35}S]-sulphate labelled PC12/PC2 cells were solubilised in 0.1 % or 1% Triton-X100 and then adjusted to 100 mM NaOAc pH 5.5 containing 5 mM $CaCl_2$. PC2 has been shown to process CgA, another granin, under these conditions (Arden, *et al.*, 1994). Surprisingly the pattern of [^{35}S]-sulphate labelled processing-products was altered if the ISGs had been detergent solubilised prior to incubation (Figure 3/13a, lanes 4 and 5). The SgII-product of Mr ~38,000 was still detectable to some degree, but the end product p18 was barely visible. Two new bands of intermediate molecular weight to p26 and p18 appeared. Another detergent, digitonin, gave similar results as shown in Figure 3/13b. Again two bands with intermediate apparent molecular weight to p26 and p18 appeared in addition to minor amounts of p18. Thus the use of detergents is not appropriate for comparison with the processing efficiency in intact ISGs. The changed processing pattern in the presence of detergents could be caused by a conformational change in SgII in the presence of detergent leading to exposure of different processing sites that are not utilised in intact ISGs.

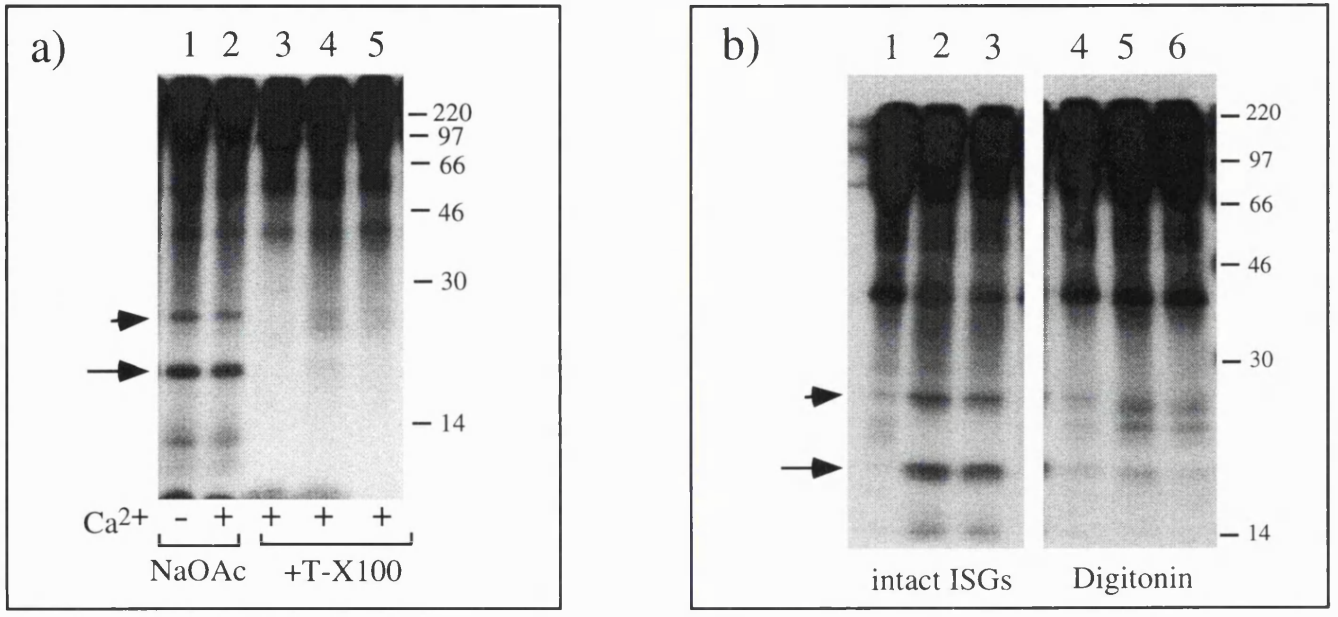


Figure 3/13: *Processing in detergent-solubilised ISGs.*

(a) [^{35}S]-sulphate labelled ISGs were isolated from PC12 /PC2 cells, diluted with 50 mM MES pH 5.5 buffer and pelleted by centrifugation. The pellets were resuspended in 100 mM NaOAc pH 5.5 with (lanes 2 and 3-5) or without (lane 1) 5 mM $CaCl_2$, in the absence (lanes 1,2) or presence of 0.1% (lanes 3,4) or 1% (lanes 5) Triton-X100. The samples were incubated for 90 min at 4°C (lane 3) or 37°C (all other lanes). The proteins were TCA-precipitated and analysed by 12% SDS-PAGE and autoradiography. **(b)** ISGs were prepared and pelleted as in (a). The pellets were resuspended in either SDS-sample buffer (lane 1), 0.3 M sucrose/50 mM MES pH 5.5 (lanes 2,3) or 110 mM NaOAc pH 5.5, 5.5 mM $CaCl_2$ in the absence (lane 4) or presence of 0.1% (lane 5) or 0.5% (lane 6) digitonin. Samples were incubated for 45 min at 4°C (lane 1,4) or 37°C. The proteins were analysed by 12 % SDS-PAGE and autoradiography. The arrowhead indicates p26 and the arrow indicates p18.

3.13 Discussion

3.13.1 Processing of SgII in isolated ISGs

A PC12 cell line that is stably transfected with the endopeptidase PC2 was previously established by Dittié et al. (1995). Pulse-chase experiments using radioactive sulphate in combination with antibody-mapping were used to show that in these PC12/PC2 cells SgII was converted by PC2 to distinct lower molecular weight products (Dittié and Tooze, 1995) including secretoneurin (Kirchmair, *et al.*, 1993; H. Winkler and S.A. Tooze, unpublished data). In this chapter it was demonstrated that processing of SgII to an identical set of products can be observed in isolated ISGs derived from PC12/PC2 cells. The sequential appearance of the different processing-products correlated well with the results obtained from the time course in intact cells (Dittié and Tooze, 1995; and Figure 3/4).

Addition of ATP to isolated ISGs at pH 7.2 stimulated processing by approximately 4-fold. This ATP-induced processing could be completely abolished by either inhibition of the vacuolar H^+-ATPase with concanamycin A or by neutralising its activity through inclusion of a K^+/H^+-ionophore or a weak base. These results are in good agreement with the previous finding by others that proinsulin-conversion can be observed in immature β-granules isolated from rat pancreatic islets (Rhodes, *et al.*, 1987). Both PC2 and PC1 (also known as PC3) are required for conversion of proinsulin to insulin (Bailyes, *et al.*, 1992; Bennett, *et al.*, 1992a; Davidson, *et al.*, 1988). Rhodes et al. (1987) also observed a protonophore-sensitive stimulation of processing by ATP and concluded that the ATP-effect reflected a requirement for an acidic intragranular pH. The use of the specific inhibitor concanamycin A unequivocally demonstrates that stimulation of processing by ATP is indeed due to activation of the vacuolar H^+-ATPase.

3.13.2 SgII is not completely processed in PC12/PC2 cells.

Processing of SgII by PC2 does not result in complete conversion of the full length SgII to p18 either in isolated ISGs or in intact PC12/PC2 cells (Dittié and Tooze, 1995; and Figure 3/4). The final extent of processing obtained in isolated ISGs at pH 5.5 was however similar to the extent of processing observed in intact cells. A possible explanation for the incomplete processing of SgII both *in vitro* and *in vivo* in PC12/PC2 cells might be that a population of full-length SgII, and processing intermediates, remains or becomes inaccessible to PC2 as a result of the progressive condensation of the granule core. This could explain why SgII processing does not reach completion even after prolonged storage in the secretory granules.

Variations in the extent of SgII processing have been observed in other cell types (Kirchmair, *et al.*, 1993). PC12 cells are derived from a pheochromocytoma and the processing efficiency in the PC12/PC2 cells is therefore best compared with chromaffin cells that express both SgII and PC2 endogenously. Interestingly, the processing efficiency observed here in PC12/PC2 cells is very comparable to that observed in bovine chromaffin cells where up to 50% of the proprotein remains unprocessed (Fischer-Colbrie, *et al.*, 1987). However, it cannot be excluded that the expression level or the activity of the PC2-chaperone 7B2 is a limiting factor for SgII processing in PC12/PC2 cells. 7B2 has been shown to play a principal role in the regulation of PC2 (Braks and Martens, 1994; Zhu and Lindberg, 1995) and is present in PC12 cells (Seidah, *et al.*, 1994) and in PC12/PC2 cells (I. Lindberg and S.A. Tooze, unpublished data). However, the level of 7B2 in PC12/PC2 cells is significantly less than in rat chromaffin cells. Overexpression of 7B2 has been shown to facilitate the conversion of proPC2 to mature PC2 (Zhu and Lindberg, 1995) and the processed form of 7B2 was able to enhance the *in vitro* cleavage of POMC in lysates from Xenopus intermediate pituitary cells (Braks and Martens, 1994). The majority of PC2 that is stored in secretory granules in PC12/PC2 cells appears to be converted to the mature form (Figure 3/9), hence PC2-activation is not impaired in PC12/PC2 cells.

Further work on the activity of 7B2 on substrate processing in general, and the role of 7B2 in PC2 dependent SgII-processing in PC12/PC2 cells in particular, will be required to fully address this question.

3.13.3 Processing of SgII in the TGN

The intriguing observation that processing cannot be observed in the TGN, defined operationally as the sulphation compartment but can be observed in the 20°C block compartment might point to a fundamental difference in the functional characteristics of the TGN in a cell at steady state and in a cell that has been subjected to a temperature block. Secretory proteins accumulate at 20°C and it is conceivable that microdomains are formed within the TGN in cells incubated at 20°C that mimic the environment of the secretory granule. In these microdomains, the local concentration of PC2 and SgII (or any other granule enzyme and its substrate) might be greater than in the TGN of a cell at steady state. Coaggregation of enzyme and substrate might be crucial for efficient proteolytic activity. This could explain why PC2 does not appear to be active in the TGN of PC12/PC2 cells although approximately 50% of PC2 is in the mature form (Figure 3/9).

Alternatively the passage from the TGN to the 20°C block environment or ISG-environment could result in the removal of inhibitory factors which may limit PC2-activity. The PC2-chaperone 7B2 has been shown to inhibit PC2 activity *in vitro* (van Horssen, *et al.*, 1995). The full-length protein (intact 7B2) was effective as an inhibitor, whereas the cleaved protein was not. It has been proposed that cleavage of 7B2 precedes PC2-conversion (Braks and Martens, 1994; Zhu and Lindberg, 1995). However, it is conceivable that the C-terminal fragment of 7B2 to which the inhibitory properties of the protein are confined (Martens, *et al.*, 1994; van Horssen, *et al.*, 1995) does not dissociate from the mature form of PC2 in the milieu of the TGN. Alternatively another, as yet unidentified, factor might influence PC2-activity in the

TGN of PC12/PC2 cells. This point could be addressed in the future by further analysing the activation of PC2 in the secretory pathway of PC12/PC2 cells.

3.13.4 The pH of immature secretory granules

Although the internal pH of mature secretory granules (MSGs) in a variety of tissues has been studied in detail by others (Hutton, 1982; Johnson, *et al.*, 1980; Johnson and Scarpa, 1975) very few data are so far available on the lumenal pH of ISGs. Conventional methods for pH-measurements, such as accumulation of radioactively labelled weak bases in acidic compartments, are amenable only when large quantities of easily purified vesicles (e.g. chromaffin granules from adrenal medulla) are available (Hutton, 1982; Johnson, *et al.*, 1980; Johnson and Scarpa, 1975; Martens, *et al.*, 1994; Russell and Holz, 1981; van Horssen, *et al.*, 1995). The following two reasons in particular contribute to the difficulty in obtaining data on the internal pH of ISGs: (1) ISGs in any cell-type constitute a short kinetic intermediate in the biogenesis of secretory granules (half-time of maturation: 45 min) and therefore represent a very small proportion of the total secretory granule population; (2) it is difficult to obtain a pure preparation of ISGs that is absolutely free of any contamination from the large pool of MSGs.

In this chapter the pH of isolated ISGs was measured *in vitro* . The use of an endogenous pH-dependent reaction in the ISG, i.e. processing of a granule-specific protein (SgII) by a granule-specific enzyme (PC2), as an indicator for internal pH eliminates the possibility that the results might be attributed to a contaminating membrane-population. In addition, the ISG is operationally defined by a pulse-chase labelling protocol, thereby strictly limiting all observations to the population of secretory granules that contain SgII labelled by a 5 min [^{35}S]-sulphate pulse followed by a 15 min chase. From the observations described in Figure 3/7, a pH-value of 6.3 +/- 0.1 for isolated ISGs derived from PC12/PC2 cells was obtained.

In a recent study, Orci and others correlated the pH in β-cell granules with processing of proinsulin (Orci, *et al.*, 1994). This study is very informative regarding the pH-dependence of the endoproteolytic cleavages that underlie proinsulin-processing at steady state. The pH of the granules was measured by immunolocalisation of DAMP (3-(2,4-dinitroanilino)-3'-amino-*N*-methyldipropylamine), a weak base that accumulates in acidic compartments (Anderson and Orci, 1988). Processing of proinsulin in these granules was followed by double-labelling with an antibody that specifically recognises proinsulin. Immature β-cell granules were identified by their high proinsulin content. The mean pH of these proinsulin rich granules was found to be 6.3 and can be taken to support the data presented here.

The similarity of the pH-value obtained both for the immature β-cell granules and ISGs isolated from PC12/PC2 cells to the pH determined by others for the TGN (Kim, *et al.*, 1996; Seksek, *et al.*, 1995) suggests that acidification below pH 6.3 is not a direct consequence of budding from the TGN but requires additional factors and/or events to reach the final pH of 5.5 that is characteristic for MSGs. Previous studies have suggested that maturation in PC12 cells involves fusion of several ISGs to form one MSG that is characterised by both a larger size and a greater density (Tooze, *et al.*, 1991; Tooze and Stinchcombe, 1992), an event that will be described in detail in the following chapters of this thesis. It was suggested that the partial clathrin-coat seen on ISGs in a variety of cell-types (Orci, *et al.*, 1985a; Tooze and Tooze, 1986) functions to remove excess membrane via clathrin-coated vesicle formation (Tooze, *et al.*, 1991). It is tempting to speculate that acidification to a value below that of both the TGN and the ISG might require membrane fusion and retrieval of excess membrane. These membrane remodelling events could allow both a concentration of the vacuolar H^+-pumping ATPase (via an increase in linear density) and removal of proteins with a potentially adverse activity on acidification (e.g. $Na^{+/}K^+$-ATPase (Cain, *et al.*, 1989; Fuchs, *et al.*, 1989).

Chapter 4: Cell-free ISG fusion: Optimisation of quantitative measurements of SgII processing

4.1 Objective

The aim of the following four chapters is to describe how the information gained in Chapter 3 on the processing of the secretory granule protein SgII by the endopeptidase PC2 in isolated ISGs has been used to design and establish a cell-free assay to measure fusion of ISGs. Homotypic fusion of ISGs has been postulated (see Chapter 1) based on two fundamental observations:

(1) Morphological analysis of EM-micrographs taken of mammotroph cells has revealed irregular granule profiles with several dense cores contained within one delimiting membrane (Bainton and Farquhar, 1966; Smith and Farquhar, 1966; and Figure 1/2).

(2) Biochemical and morphological studies on PC12 cells that described two distinct populations of secretory granules showed that the "younger" population, the ISGs, was characterised by a smaller diameter and by a lower sedimentation-value. From this study it was proposed that 3-5 ISGs would fuse to give rise to one MSG with a larger diameter and a higher sedimentation value.

Both these observations constitute circumstantial evidence for ISG fusion. However, the only fusion event between granules that has so far been reconstituted *in vitro* is the calcium-triggered fusion of sea urchin egg (mature) secretory granules (Vogel and Zimmerberg, 1992), while homotypic fusion of ISGs has not yet been documented.

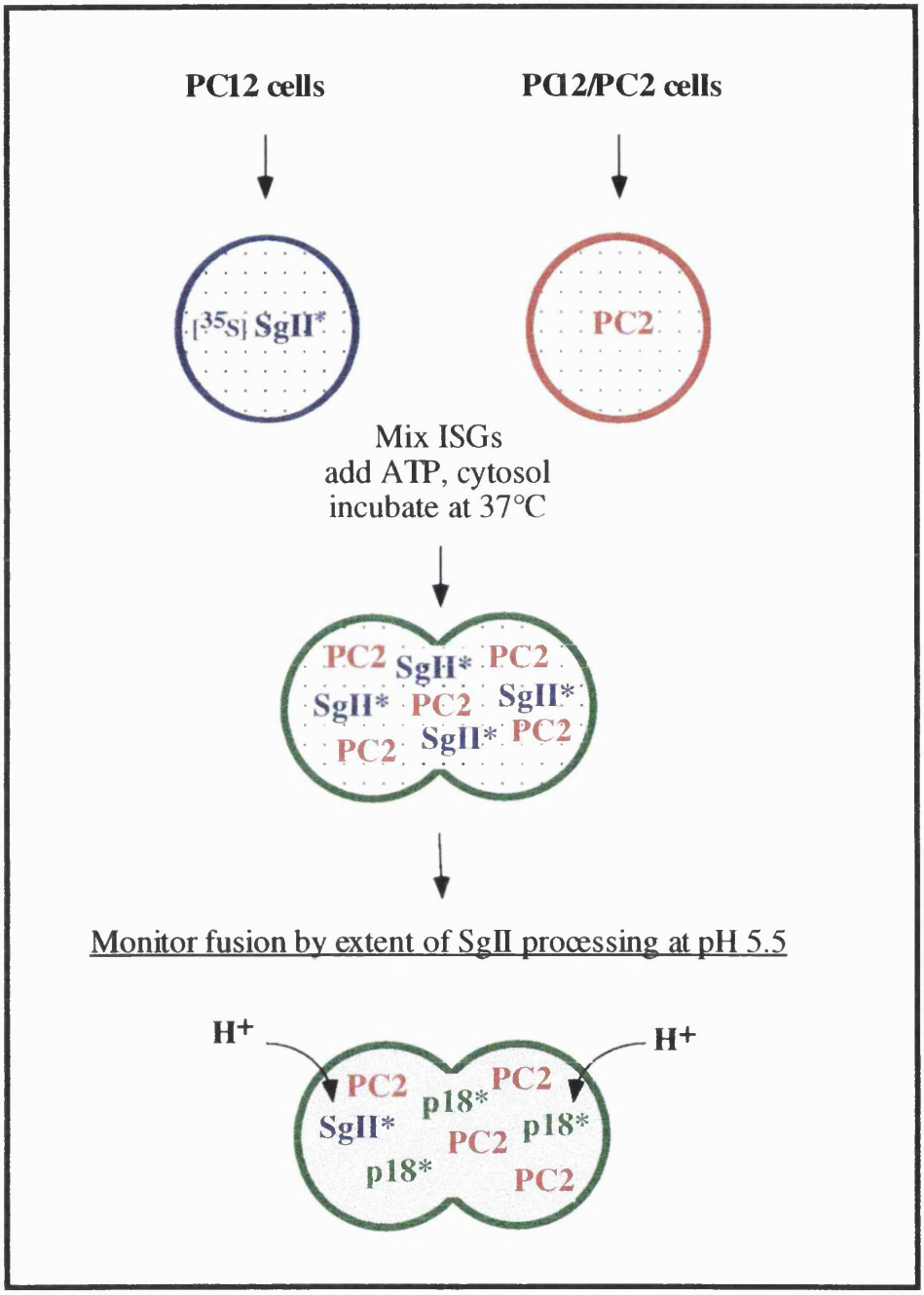


Figure 4/1: *Cell-free fusion of immature secretory granules*

ISGs derived from [^{35}S]-sulphate labelled PC12 cells and ISGs isolated from PC12/PC2 cells are incubated at 37°C in the presence of cytosol and an ATP-regenerating system. Upon fusion, [^{35}S]-sulphate labelled (*) SgII and PC2 can mix in the same secretory granule. Incubation at 37°C in a pH 5.5-buffer enhances SgII processing by PC2 and results in accumulation of the [^{35}S]-sulphate labelled (*) processing endproduct p18.

In the following chapters, I will describe an assay that reconstitutes the fusion of ISGs *in vitro*. This cell-free assay (Figure 4.1) is based on content mixing of two distinct populations of ISGs: (1) ISGs derived from PC12/PC2 cells, containing the prohormone convertase PC2 and (2) ISGs derived from PC12 cells that have been labelled by a [^{35}S]-sulphate pulse followed by a 15 min chase and therefore contain [^{35}S]-sulphate labelled SgII but no proteolytic enzyme PC2. Upon fusion of these ISGs with each other, the enzyme PC2 should be able to cleave SgII and give rise to [^{35}S]-sulphate labelled proteolytic products as described in Chapter 3. As PC2-activity is strongly dependent on an acidic pH (Chapter 3) and it was important to be able to measure fusion under conditions that will also inhibit proton-pump driven acidification of the ISG lumen (for example in the absence of ATP, or in the presence of NEM), experiments were designed so that the initial fusion incubation was followed by a second incubation at pH 5.5 for which processing was optimal (Chapter 3).

This second processing incubation is thus an integral part of the read-out of the fusion assay. The conditions for which processing of SgII in isolated ISGs was optimal have been determined in Chapter 3. However it was also apparent that the quantitative assessment of processing was impaired by the proteoglycan smear underlying the processing end-product p18. As will be seen below (section 4.2), the same problems were naturally encountered when attempting to use processing as a read-out for fusion. In a first attempt to circumvent this problem, use was made of two existing and well-characterised antibodies to analyse the processing products whilst focusing on the possibility of obtaining a fast read-out by scintillation counting of immunoprecipitates (section 4.3). Finally antibodies specific to the processing end-product p18 were obtained and characterised (sections 4.4 to 4.8). One of these p18-specific antibodies (Ab 69) was successfully applied to the quantitative measurement of processing as a read-out for fusion of ISGs as will be described in Chapter 5.

4.2 Cell-free ISG fusion: Analysis of the whole extract

Two important facts had to be taken into consideration before deciding on the details of the fusion assay protocol: 1) The efficiency of the fusion event was completely unknown and might be very low, and 2) The population of ISGs being able to fuse might be very small, that is the time window during which ISGs are fusogenic was unknown and again might be very small.

Although some preliminary experiments were based on mixing two isolated ISG-populations with rat brain or PC12 cell cytosol, to increase the chance of detecting fusion, it was decided to routinely use one of the ISG-populations in a "pure" form , these being derived from PC12/PC2 cells, and add these ISGs to a PNS of PC12 cells labelled with a 10 min [^{35}S]-sulphate pulse followed by a 15 min chase. A 10 min pulse rather than a 5 min pulse was chosen to enable a larger amount of [^{35}S]-sulphate to be incorporated.

Thus, ISGs were isolated from PC12/PC2 cells and added along with an ATP-regeneration system to a PNS prepared from [^{35}S]-sulphate labelled PC12 cells. The buffer conditions were adjusted to 20 mM HEPES pH 7.2, 100 mM KOAc, 2 mM $Mg(OAc)_2$, 1 mM DTT in a final volume of 120 µl and the samples were incubated at 37°C for 45 min. The samples were then underlayed with 0.5 ml of 0.3 M sucrose in 50 mM MES pH 5.5 and the membranes were pelleted for 1 hr by centrifugation at 100 000 g at 4°C (during which the ISGs equilibrate to pH 5.5) before being incubated for 45 min at 37°C. For comparison a PNS of PC12/PC2 cells identically labelled was prepared in parallel and incubated under the same conditions as the PC12 PNS. After the incubations the proteins were solubilised in SDS-sample buffer and separated by SDS-PAGE. While the SgII in PC12/PC2 cells gave rise to the processing products described in Chapter 3 including p26 and p18 (Figure 4.2a, lane 2), such sulphated small molecular weight cleavage products were not apparent in the fusion assay reaction (Figure 4.2a, lane 1).

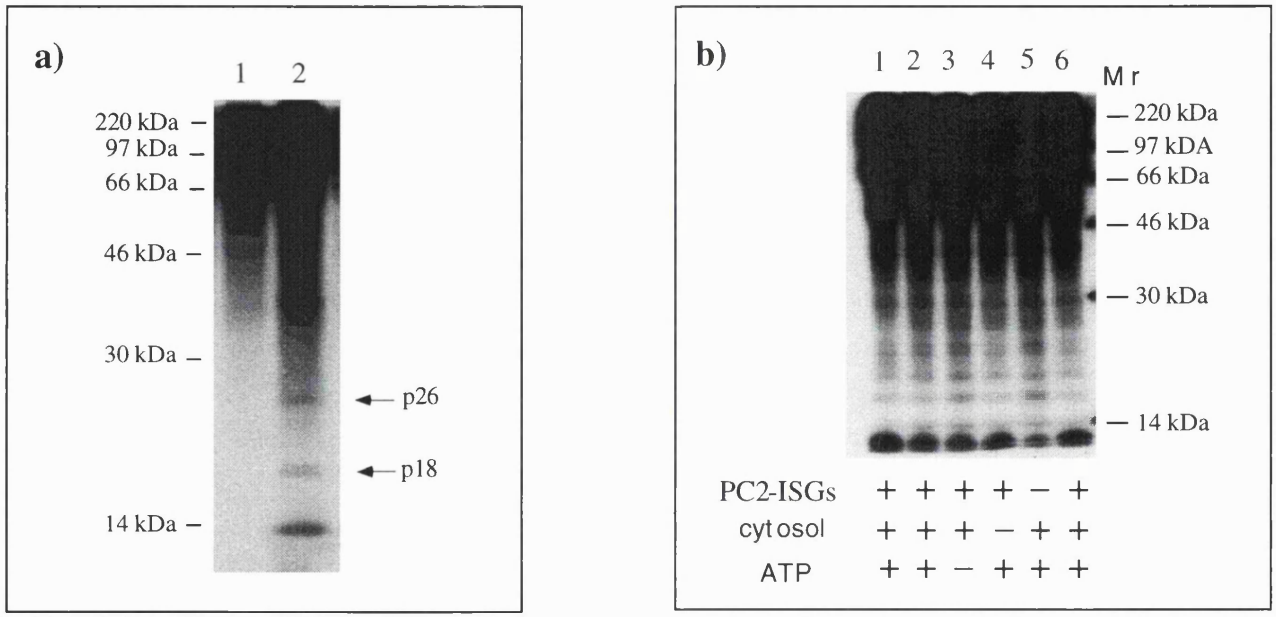


Figure 4/2: *Cell-free fusion - analysis of the whole extract*

(a) A PNS derived from PC12 cells labelled by a 10 min [^{35}S]-sulphate pulse followed by a 15 min chase was mixed with ISGs isolated from PC12/PC2 cells and incubated in the presence of an ATP-regenerating system for 45 min at 37°C (lane 1). A PNS was prepared from PC12/PC2 cells labelled in the same way and incubated for 45 min at 37°C in the presence of an ATP-regenerating system (lane 2). The samples were laid onto a 50 mM MES pH 5.5 buffered sucrose cushion, pelleted by centrifugation and incubated for 45 min at 37°C. The proteins were analysed by 12% SDS-PAGE and autoradiography. The processing products p26 and p18 are indicated. **(b)** ISGs were isolated from PC12 cells labelled by a 35 min pulse of [^{35}S]-sulphate followed by a 15 min chase as well as from unlabelled PC12/PC2 cells. The ISGs were mixed and cytosol (2.2 mg/ml final concentration) and an ATP-regenerating system were added where indicated. Samples were incubated for 90 min at 4°C (lane 1) or 37°C (other lanes). The samples were analysed as in (a).

In order to label a broader spectrum, that is a larger pool of PC12 ISGs, the pulse-time for the [^{35}S]-sulphate labelling of the PC12 cells was increased to 35 min, followed as above by a chase of 15 min. Furthermore, the fusion incubation was increased to 90 min. While this protocol resulted in multiple low molecular weight bands, none of these were specific to the addition of PC2-containing ISGs. Figure 4/2b illustrates this point (compare lanes 5 and 2) from an experiment in which two isolated ISG-populations were mixed with rat brain cytosol (2.2 mg/ml final concentration), in the presence of an ATP-regenerating system and 20 mM HEPES, pH 7.2, 50 mM KOAc, 2.5 mM $Mg(OAc)_2$, 1 mM DTT, in a final volume of 360 µl. Similar results were obtained with experiments using a crude PNS while a smaller amount of ISG-proteins were analysed this way to prevent overloading of the SDS-PAGE.

4.3 Analysis of SgII processing by immunoprecipitation using Ab 100

As the above experiments gave negative results, I decided to use immunoprecipitation in order to reveal the specific processing products that might be too low to detect or hidden under the background of irrelevant minor sulphated proteins. This would overcome the limitations of sample-size dictated by the maximum amount of total protein that can be resolved on a gel and might speed up the analysis by scintillation counting of the immunoprecipitated [^{35}S]-sulphate labelled material.

As a first attempt, a well-described antibody (Ab 100) raised against the N-terminus of SgII (Figure 4/3) was used for immunoprecipitation of the N-terminal sulphated processing products. As this antibody also recognises the full-length SgII, a second well-characterised antibody (Ab 175) raised against the C-terminus of SgII (Figure 4/3) was used to preclear the full-length protein from the lysate before immunoprecipitation with Ab 100.

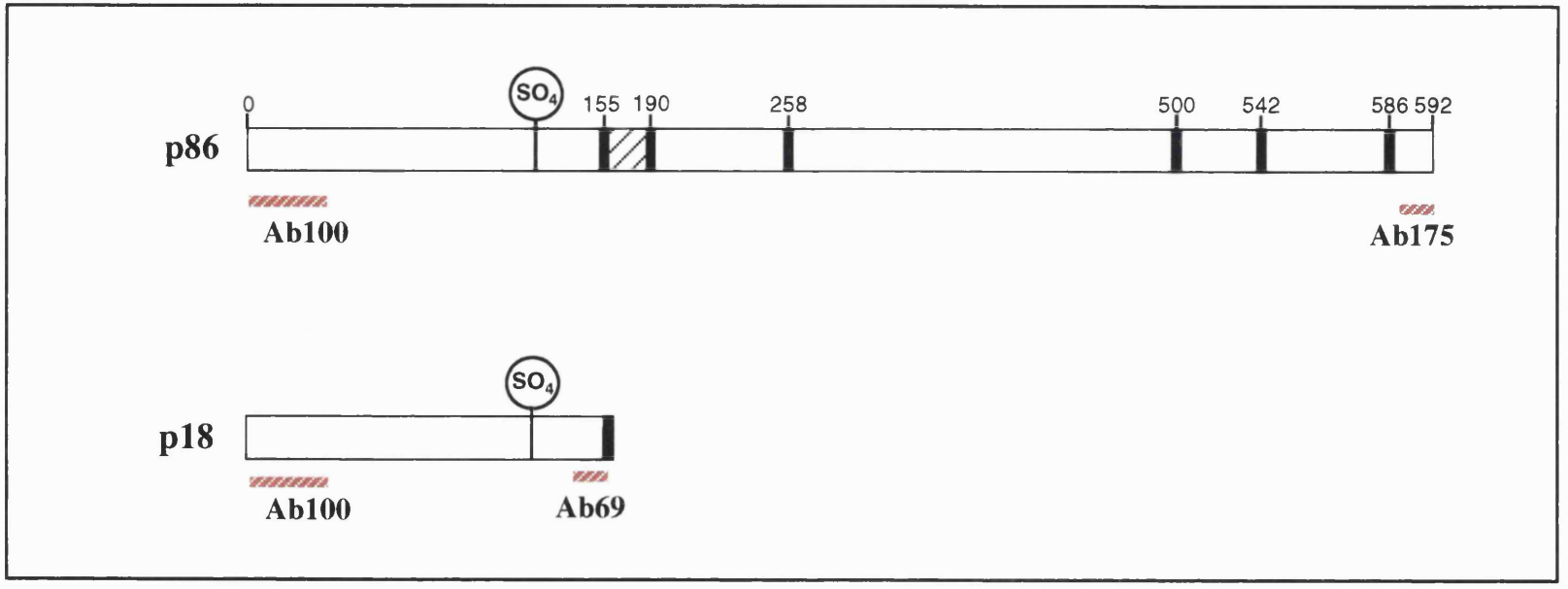


Figure 4/3: *Schematic representation of SgII and p18.*

Full-length SgII (p86) and the sulphated processing endproduct p18 are represented. The black bars indicate dibasic processing sites (Lys-Arg) utilized by PC2. Secretoneurin corresponds to the hatched area. Both p86 and p18 are sulphated on a tyrosin (SO_4). Ab100 is raised against the N-terminal 17mer and recognises both p86 and p18, whereas Ab175 is raised against a C-terminal pentamer and only reacts with p86 and not with p18. Ab69 is directed against the C-terminal end of p18 and is specific for p18.

This preclearing step was important to allow a read-out by scintillation counting of the immunoprecipitate in order to overcome the problems of detecting a potentially low signal by SDS-PAGE and fluorography. Figure 4/4a shows the sulphated SgII-derived processing products precipitated by Ab 100 from a PC12/PC2 cell lysate of cells labelled overnight with [^{35}S]-sulphate. Preclearing the lysate with 10 µl of Ab 175 decreased the signal for full-length SgII (p86) precipitated with Ab 100 by 50% (compare lanes 1 and 2) while preclearing with an additional 10 µl of Ab 175 did not remove any more p86 (lanes 2 and 3). The amount of p18 precipitated with Ab 100 also decreased slightly (20%), most likely due to non-specific losses from the additional manipulation. The result from this procedure was a net increase of 30% in the ratio of p18 to p86.

To assess whether this protocol was applicable to analysis of processing by scintillation counting, PC12/PC2 cells were labelled by a 10 min [^{35}S]-sulphate pulse followed by a 15 min chase, homogenised and a PNS was obtained. ISGs were isolated as described in Chapter 3 (section 3.2) and samples of the ISGs were diluted with pH 5.5 buffer and pelleted by centrifugation for 1 hr at 100,000 g. The ISGs were then incubated for various length of time under processing conditions (0.3 M sucrose, 50 mM MES pH 5.5, see section 3.5), solubilised in lysis buffer and subjected to the sequential immunoprecipitation with 30 µl Ab 175 followed by 40 µl Ab 100 as described above. The immunoprecipitates were analysed by scintillation counting and the result of such a time course is illustrated in Figure 4/4b. The signal for Ab 100 did increase by about 50%, while the signal for Ab 175 decreased rapidly to about 20% of the starting material after a 45 min incubation. Processing of SgII in PC12/PC2-ISGs could therefore be monitored successfully by this procedure.

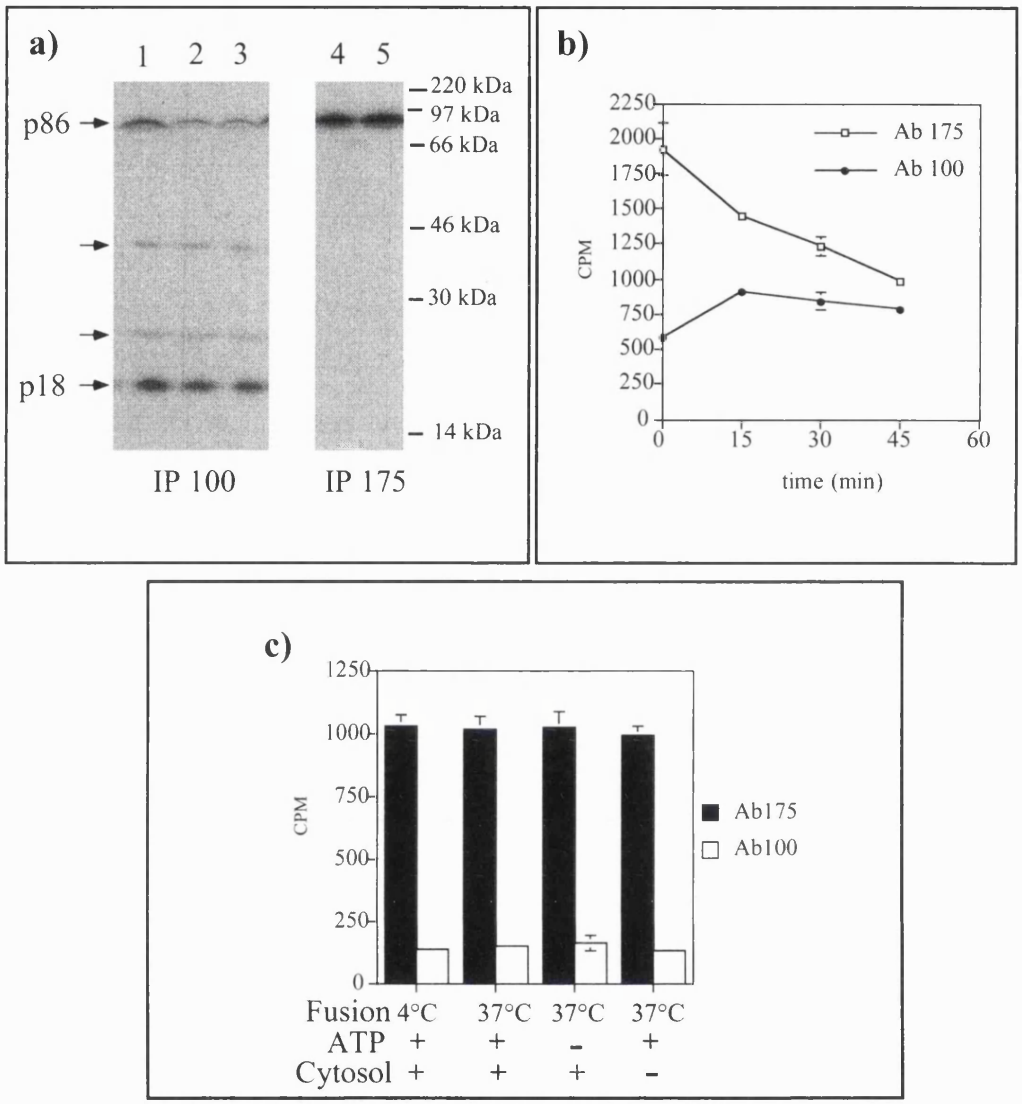


Figure 4/4: *Analysis of SgII processing by immunoprecipitation using Ab100*

(a) Ab100 was used for immunoprecipitation from a lysate prepared from overnight [^{35}S]-sulphate labelled PC12/PC2 cells that had been precleared either with 10 (lane 2), 20 µl (lane 3) Ab175 (IP 175, lanes 4 and 5) or mock-treated (lane 1). The sulphated processing products p38, p26 and p18 as well as p86 (SgII) are indicated with arrows. **(b)** ISGs were isolated from PC12/PC2 cells labelled by a 10 min [^{35}S]-sulphate pulse followed by a 15 min chase and incubated for various length of time under processing conditions. The ISGs were solubilised in lysis buffer and subjected to sequential immunoprecipitation with 30 µl Ab 175 followed by 40 µl Ab100. The immunoprecipitates were analysed by scintillation counting. **(c)** ISGs were prepared as in (b) but from PC12 cells, mixed with unlabelled PC12/PC2-ISGs, and incubated at 4°C or 37°C in the presence or absence of cytosol and an ATP-regenerating system. Samples were then incubated under processing conditions and subjected to immunoprecipitations as in (b). Error bars indicate SD (n=3).

Fusion of [^{35}S]-sulphate labelled PC12 ISGs with PC12/PC2-ISGs was however not observed with this approach, either because the signal to noise ratio of these experiments was too low or the fusion conditions were not yet optimised. Figure 4/4c shows a representative experiment in which PC12 ISGs were prepared in the same way as described for PC12/PC2-ISGs in Figure 4/4b. These labelled ISGs were mixed with unlabelled PC12/PC2-ISGs, in the presence or absence of cytosol and an ATP-regenerating system. No ATP-dependent increase in p18 nor any decrease in p86 was observed. This line of research was subsequently discontinued as the development of a specific antibody against p18 provided a more promising approach.

4.4 Characterisation of antibodies specific to p18

In order to simplify the protocol and improve the signal to noise ratio, two peptides derived from the C-terminal sequence of the SgII processing product p18 were designed for generation of antibodies specific for p18 (see also Figure 4/3) : (1) preCS2 which does not contain the dibasic cleavage site and (2) CS2, in which the cleavage site (in bold) was retained, and which contains an additional cysteine residue for MBS coupling to KLH (see Chapter 2). *In vivo* the Lys-Arg pair may be removed during maturation in the granule by a carboxypeptidase (Fricker, 1988), however no mass spectroscopic analysis was available to indicate whether this was the case for p18.

preCS2 KEENSRENPF

CS2 CSRENPF**KR**

Both peptides were synthesised and purified by central service facilities at ICRF and then coupled to KLH via the N-terminal lysine in the case of preCS2 and via the N-terminally added cysteine in the case of CS2. These conjugates were then used for injection into three rabbits per peptide at the ICRF facility.

Figure 4/5: *Immunoblot with antibodies against pre-CS2 and CS2*

A PNS (50 µg/lane) was obtained from PC12/PC2 cells and the proteins were separated by 12% SDS-PAGE and transferred onto nitrocellulose. Antigens were detected with polyclonal antibodies (bleed 3, lane 1; bleed 4, lane 2; bleed 5, lane 3) raised against pre-CS2 (Ab 67, 68, 69; 1:250) and CS2 (Ab 70, 71, 72; 1:250) or with Ab 175 (1:200). Detection was with a secondary antibody coupled to alkaline phosphatase. The position of p18 is indicated with an arrow.

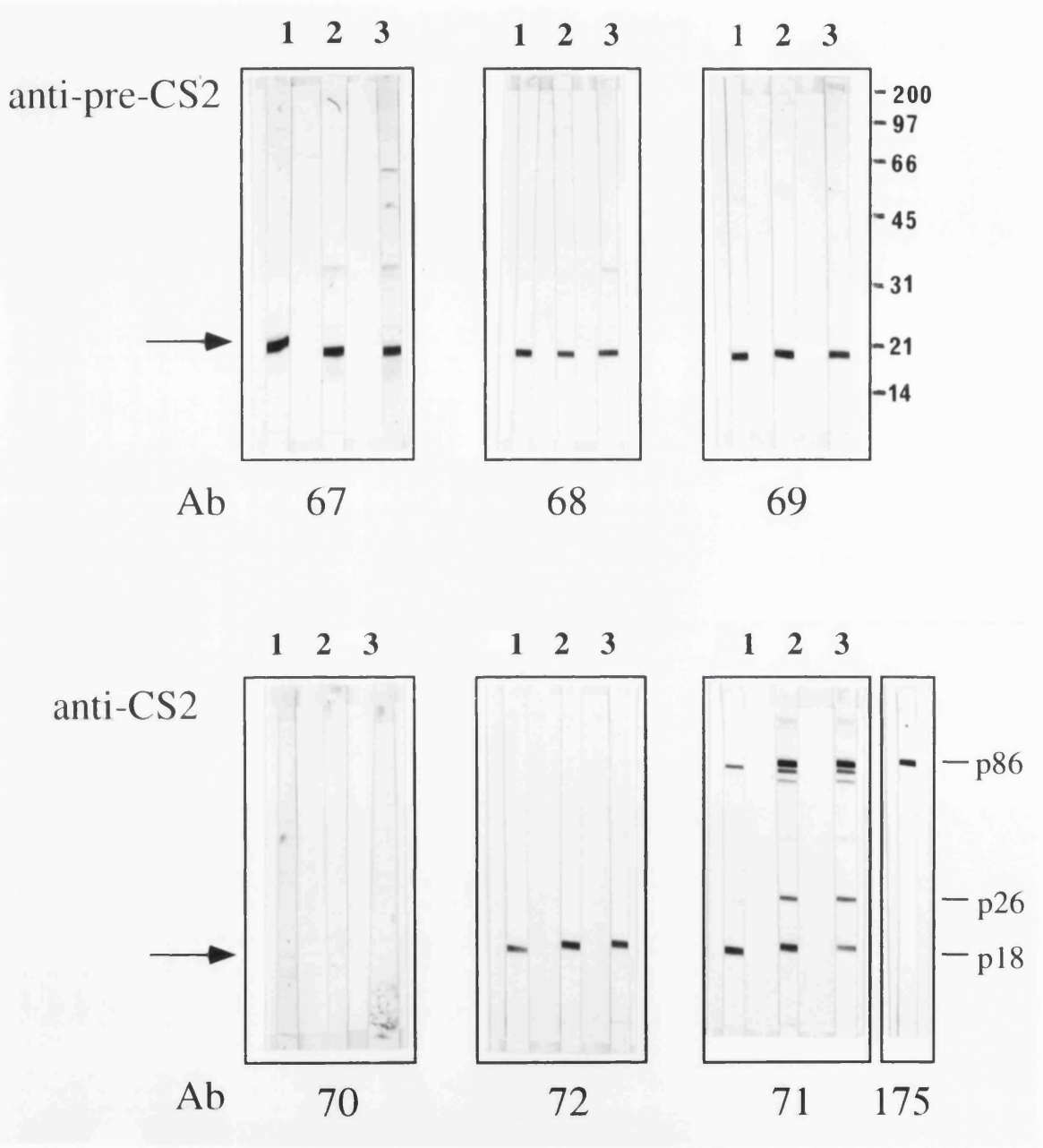
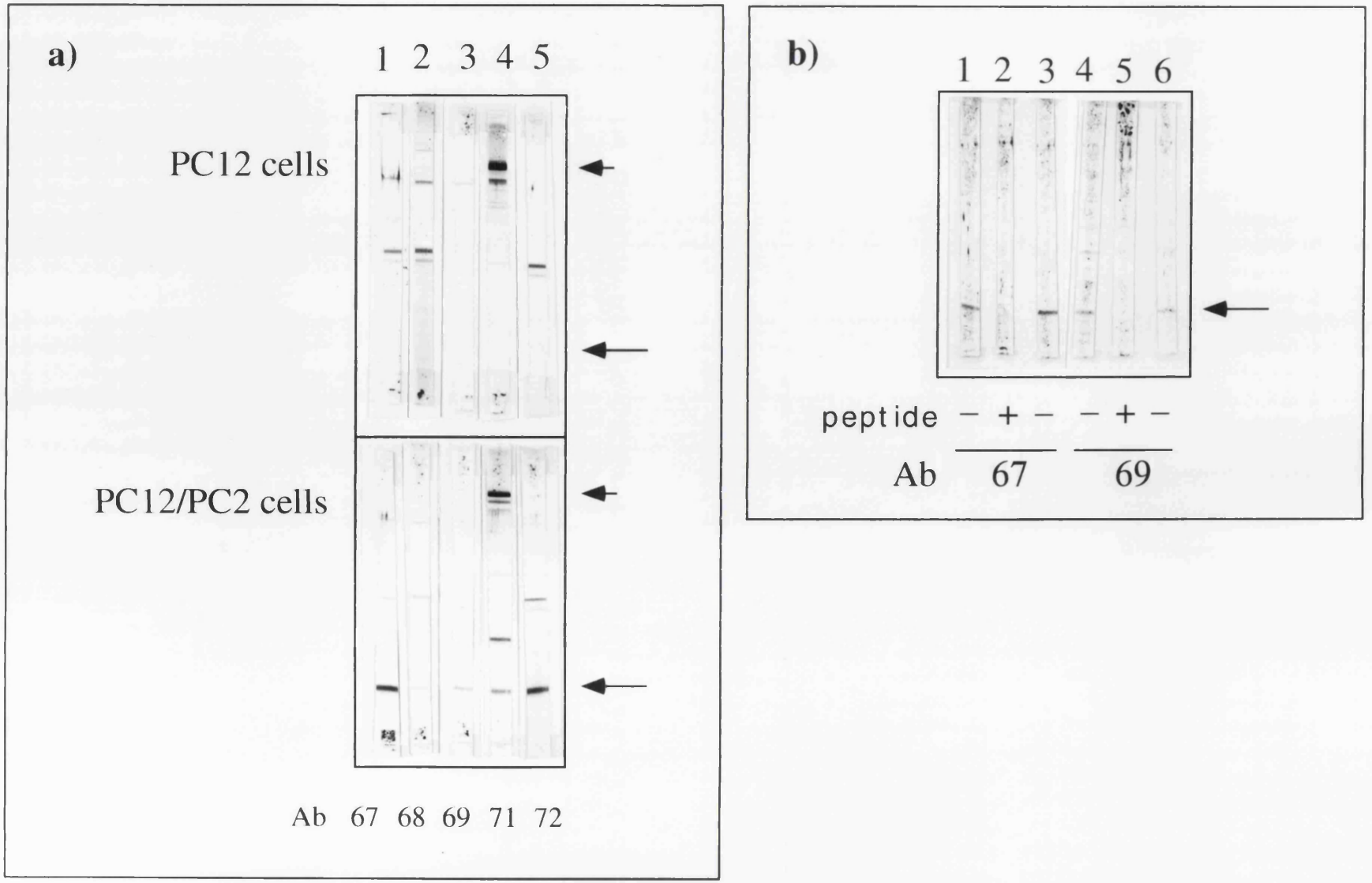

anti-pre-CS2
1 2 3
1 2 3
1 2 3
200
97
66
45
31
21
14
Ab
67
68
69
anti-CS2
1 2 3
1 2 3
1 2 3
p86
p26
p18
Ab
70
72
71
175

Figure 4/6: *Antibodies raised against preCS2 specifically recognise p18*

(a and b) A PNS (50 μg/lane) was obtained from PC12 or PC12/PC2 cells, and the proteins were separated by 12% SDS-PAGE and transferred onto nitrocellulose. .... **(a)** Antigens were detected with polyclonal antibodies 67, 68, 69, 71 or 72 at a dilution of 1:250 (bleed 6). **(b)** Antibodies 67 and 69 were pre-incubated for 30 min at room temperature either in the presence (lane 2) or absence (lane1, 3) of 0.05 μg/μl pre-CS2 peptide. Preincubated antibodies were used for detection of the antigens at a dilution of 1:500 (lane 1, 2) or 1:1000 (lane 3). Detection was with a secondary antibody coupled to alkaline phosphatase. The position of p18 is indicated with an arrow.


a)
1 2 3 4 5
PC12 cells
PC12/PC2 cells
Ab 67 68 69 71 72
b)
1 2 3 4 5 6
peptide − + − − + −
Ab 67 69

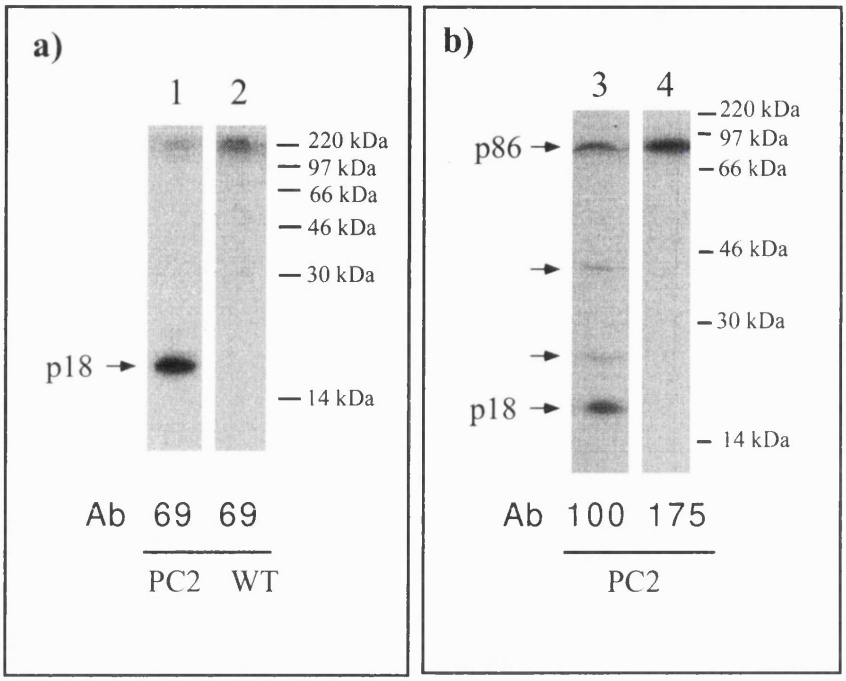


Figure 4/7: *Immunoprecipitation of p18 from PC12/PC2 cells.*

(a) A PNS was prepared from [^{35}S]-sulphate labelled PC12/PC2 (PC2) and PC12 (WT) cells and subjected to immunoprecipitation with Ab 69 and analysed by SDS-PAGE and autoradiography. **(b)** An immunoprecipitate with Ab 100 and 175 from [^{35}S]-sulphate labelled PC12/PC2 (PC2) cells is shown for comparison. Arrows indicate the position of full-length SgII (p86) and the sulphated processing products p38, p26 and p18.

The resulting polyclonal antisera were tested first by immunoblotting on a PNS derived from PC12/PC2 cells. As shown in Figure 4/5, all three antibodies generated against the preCS2 peptide, Ab 67-69, recognised a band of 18 kDa in PC12/PC2 cells. Of the antibodies raised against the CS2 peptide, Ab 72 recognised p18 very well, whereas Ab 71 labelled both the full-length SgII and all processing products (an immunoblot with Ab 175 is shown for comparison). Ab 70 did not reveal any specific bands.

The specificity of the p18 band was assessed by two experiments. First the immunoblotting result on a PC12/PC2 cell PNS was compared with a PC12 PNS. As shown in Figure 4/6a for bleed #6 of Ab 67, 68, 69, 71 and 72, the band for p18 was specific to PC12/PC2 cells. Second, the signal for p18 was competed by preincubation of the antibodies with the appropriate peptide as shown for antibodies 67 and 69 in Figure 4/6b.

The antisera were also tested by immunoprecipitation. A PNS was prepared from overnight [^{35}S]-sulphate labelled PC12/PC2 and PC12 cells, solubilised in lysis buffer and subjected to immunoprecipitation with the antibodies. Only Ab 69 was able to specifically precipitate p18 from PC12/PC2 cells (Figure 4/7, lane 1). An immunoprecipitate with Ab 100 and 175 is shown for comparison (lane 3 and 4). As expected, no signal was obtained in immunoprecipitates from PC12 cells (lane 2).

Finally the antisera were tested by immunofluorescence on PC12/PC2 cells and PC12 cells. Antisera 67, 68, 69 and 72 gave a specific granular staining pattern by immunofluorescence that was only seen in PC12/PC2 cells. A representative picture for Ab 69 is shown in Figure 4/8. No specific staining was seen in PC12 cells (compare a and b with c). A set of monoclonal mouse antibodies was also generated against the preCS2 peptide and these antibodies gave a similar staining pattern by immunofluorescence (Figure 4/9a, Ab 4E11/6). Interestingly, when PC12/PC2 cells were co-stained with Ab 175 and Ab 4E11/6, the resulting double label showed a more peripheral staining pattern for the anti preCS2 while the Ab 175 was more perinuclear

Figure 4/8: *Indirect immunofluorescence with antibody 69*

PC12/PC2 cells (a, b) and PC12 cells (c) were fixed with 3% PFA and permeabilised as described in Chapter 2. The cells were stained with antibody 69 diluted at 1:500 followed by a secondary antibody coupled to CY3. A granular staining can be seen in PC12/PC2 but not in PC12 cells.

Figure 4/9: *Indirect immunofluorescence with a monoclonal antibody raised against pre-CS2.*

PC12/PC2 cells were fixed and permeabilised as described in Chapter 2. The cells were stained with a monoclonal antibody raised against pre-CS2 (4E11/6) diluted 1:200 (a) and with Ab175 (1:200, b).

Figure 4/10: *Immunogold labelling on ultrathin cryosections (1)*

PC12/PC2 cells were fixed in 2% PFA, 0.2% glutaraldehyde as described in Chapter 2. Ultrathin cryosections were cut and stained with antibody 69 (1:500) followed by Protein A-gold (10 nm). Staining can be seen over the surface of dense cores. Arrows indicate the plasma membrane of three individual cells. (m, mitochondrium; n, nucleus; bar: 200 nm)

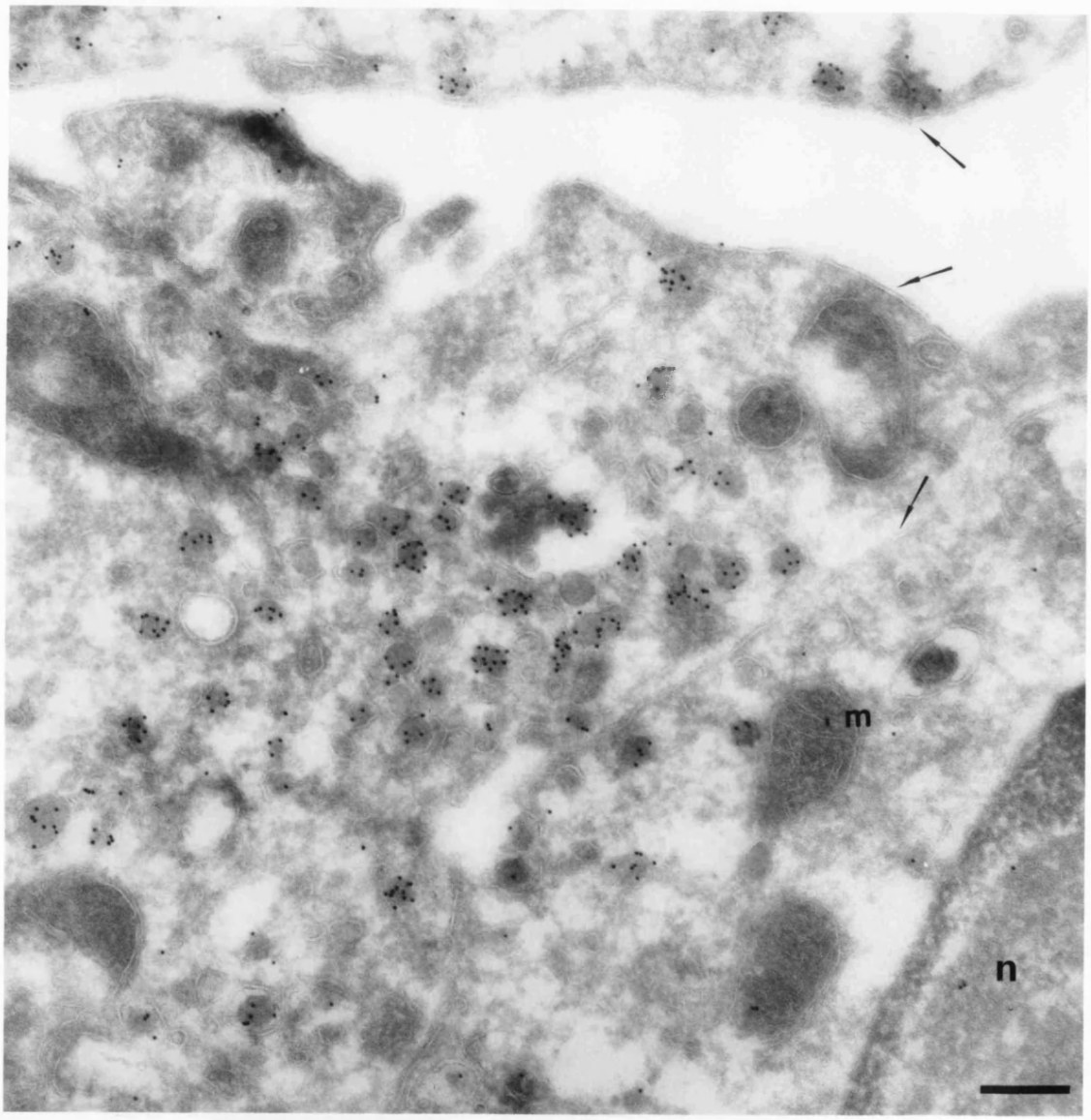
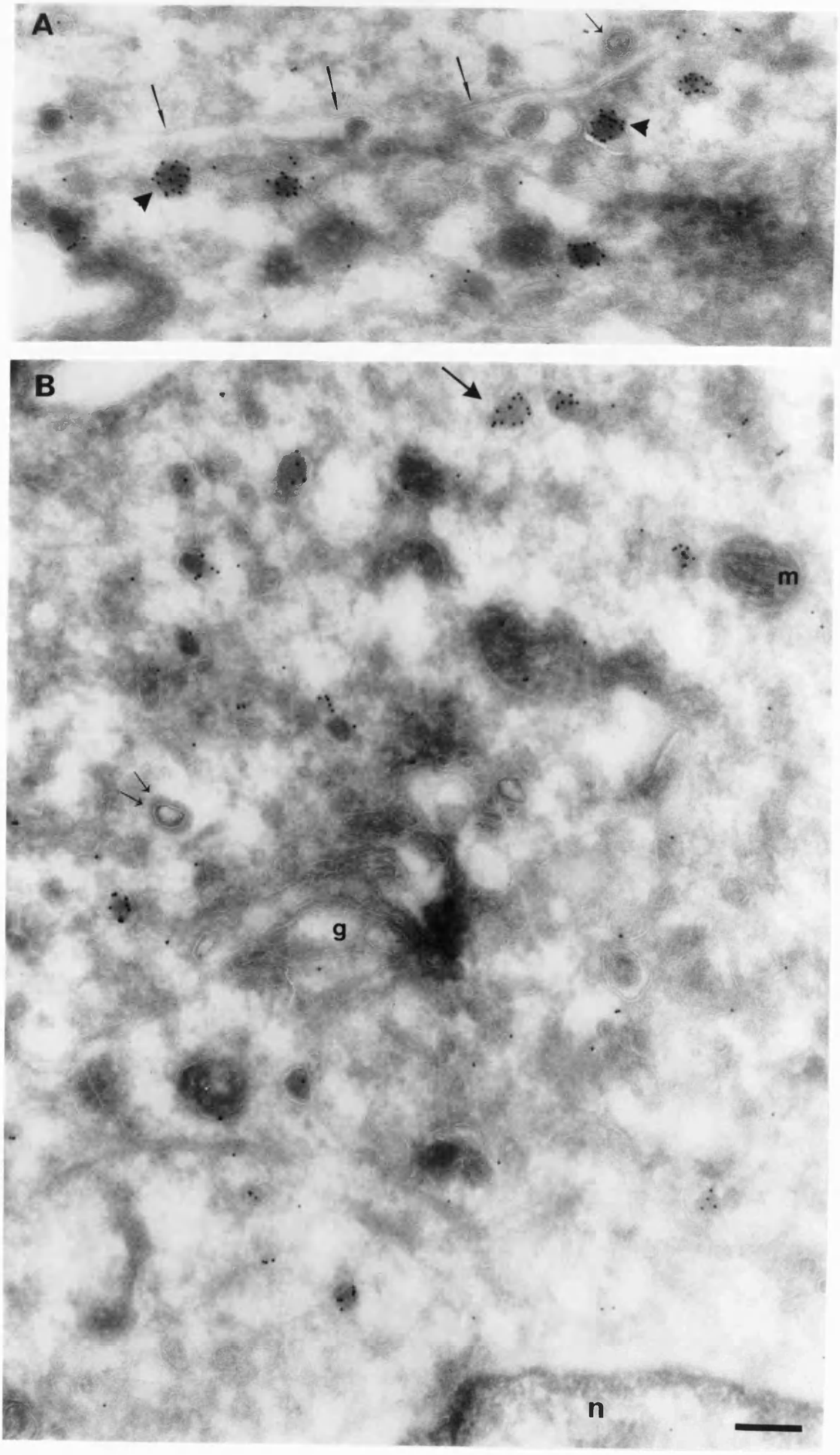

m
n

Figure 4/11: *Immunogold labelling on ultrathin cryosections (2)*

PC12/PC2 cells were prepared and sectioned as described in Chapter 2. Ultrathin sections were stained with antibody 69 (1:500) followed by Protein A-gold (10 nm). **(A)** Staining can be seen over the surface of dense cores close to the plasma membrane (arrowheads). Long arrows indicate the plasma membrane of the neighbouring cell. The small arrow indicates a clathrin coated vesicle that is not stained. **(B)** Overview of a cell with stained dense-cored granules in the periphery (arrow) and an unstained clathrin coated vesicle (small arrows) in the Golgi area (g). (m, mitochondrium; n, nucleus; bar: 200 nm)


A
B
m
g
n

(Figure 4/9b). This staining pattern suggests that antibodies raised againt preCS2 preferentially recognise mature secretory granules in which the concentration of p18 is expected to be highest in PC12/PC2 cells. This result also indicates that SgII processing during maturation leads to a gradual loss of the antigenic epitope for Ab 175 in PC12/PC2 cells.

The specificity of the staining pattern seen by immunofluorescence was confirmed at the ultrastructural level by immunogold labelling of ultrathin cryosections. All polyclonal preCS2 antibodies strongly stained the dense cored granules (Figure 4/10; Ab 69 is shown) especially those at the cell periphery (Figure 4/11).

4.5 Analysis of SgII processing by immunoprecipitation of p18 using Ab 69

As shown above (Figure 4/7) only one antibody, Ab 69, was able to immunoprecipitate p18 from PC12/PC2 cells. To test the use of this antibody to measure processing of SgII, ISGs were isolated from PC12/PC2 cells and from PC12 cells that had been labelled for 10 min with [^{35}S]-sulphate and chased for 15 min. PC12 ISGs served as a negative control as no processing of SgII takes place in PC12 cells. The isolated ISGs were pelleted by centrifugation and resuspended in rat brain cytosol in 20 mM HEPES, pH 7.2, 50 mM KOAc, 2.5 mM $MgCl_2$, 1 mM DTT (all final concentrations) and in the presence of an ATP-regenerating system. These conditions were chosen to mimic the conditions of a fusion assay: The samples were first incubated for 30 min (fusion incubation) either at 4°C or at 37°C, equilibrated for 1 hr at 4°C in 0.3 M sucrose, 50 mM MES pH 5.5 and were then incubated under processing conditions either at 4°C or at 37°C for 45 min. The samples were solubilised in lysis buffer (100 mM Tris, pH 7.5, 150 mM NaCl, 0.3% (w/v) Triton-X100, 5 mM EDTA) and used for immunoprecipitation with the Ab 69. The immunoprecipitates were dissolved in scintillation fluid and the amount of radioactive p18 was determined

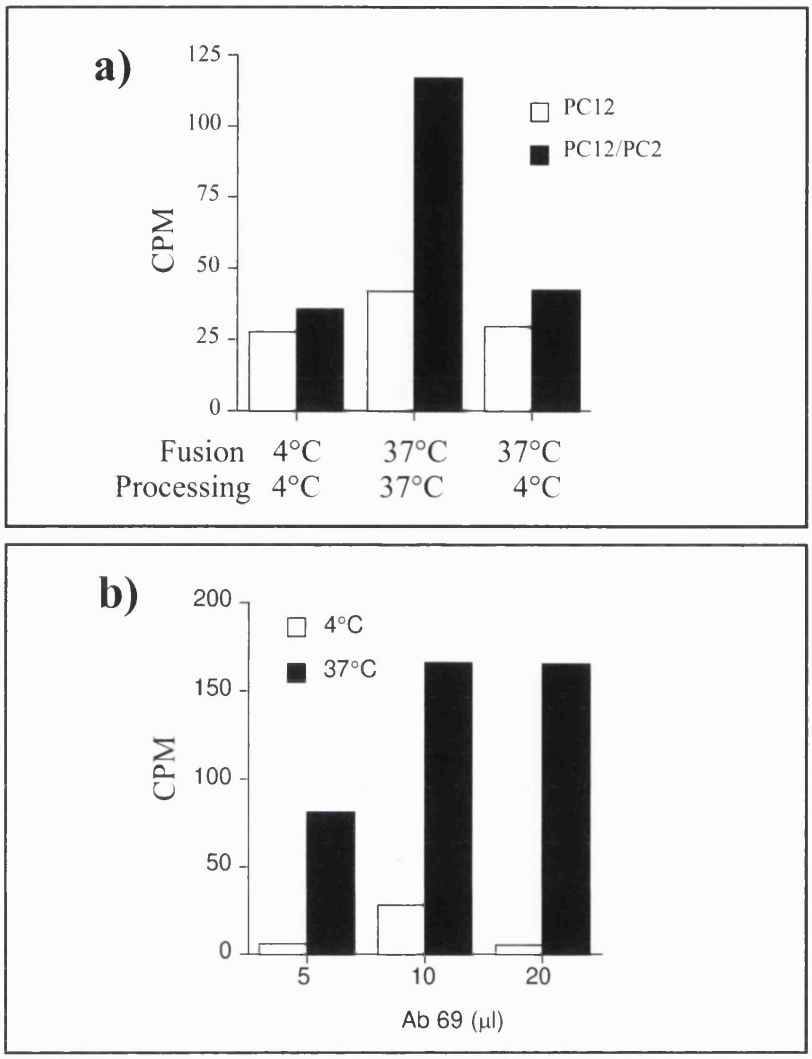


Figure 4/12: *Analysis of SgII processing by immunoprecipitation of p18 using Ab 69*

(a) ISGs were isolated from PC12 and PC12/PC2 cells labelled for 10 min with [^{35}S]-sulphate and chased for 15 min. ISGs were pelleted by centrifugation, resuspended in rat brain cytosol and incubated for 30 min at 4°C or 37°C in fusion buffer and in the presence of an ATP-regenerating system (Fusion). The samples were then equilibrated for 1 hr at 4°C in 0.3 M sucrose, 50 mM MES pH 5.5 and incubated either at 4°C or at 37°C for 45 min (Processing). The samples were lysed, and subjected to immunoprecipitation with Ab69. The immunoprecipitates were analysed by scintillation couting. **(b)** ISGs were isolated from PC12/PC2 cells labelled by a 10 min pulse of [^{35}S]-sulphate followed by a 13 min chase and incubated as in (a). Both the fusion and the processing incubation were either carried out on ice (4°C) or at 37°C. Samples were lysed and the proteins were immunoprecipitated with 5, 10 or 20 µl of Ab69.

by scintillation counting. The signal for PC12-ISGs and PC12/PC2-ISGs incubated at 4°C for processing was more or less identical, however incubation at 37°C under both fusion and processing conditions produced a clear increase in signal from PC12/PC2-ISGs but not from PC12-ISGs as expected if processing had occured (Figure 4/12a). This signal was not observed if the samples were left on ice during the processing incubation, indicating that very little if any processing took place during the fusion reaction which was performed at neutral pH.

In order to estimate the amount of Ab 69 needed to quantitatively immunoprecipitate the p18 generated in a fusion assay, [^{35}S]-sulphate labelled PC12/PC2-ISGs were used for a preliminary titration. Knowing the amount of antibody required per PC12/PC2-ISG incubated under fusion and processing conditions would then allow the calculation of the amount needed for a fusion assay: The maximal amount of p18 generated in a fusion assay containing a given number of (PC12 and PC12/PC2) ISGs will always be less or equal to the amount generated in the same number of PC12/PC2-ISGs. ISGs were therefore isolated from PC12/PC2 cells labelled by a 10 min pulse of [^{35}S]-sulphate followed by a 13 min chase and incubated under similar conditions to Figure 4/12a. As the fusion incubation does not contribute to the generation of p18 (see Figure 4/12a), the fusion incubation time was reduced to 10 min to simplify the experiment. The amount of ISGs in this assay was equivalent to about a third of the amount used later in fusion assays and served as a rough guideline for the initial experiments. Both the fusion and the processing incubation were either carried out on ice or at 37°C. Samples were lysed and increasing amounts of Ab 69 were added in the presence of excess of protein A-sepharose. Figure 4/12b shows that the antibody immunoprecipitated the p18 only from samples incubated at 37°C and that 10 µl of antibody were sufficient to immunoprecipitate all the p18.

In order to maximise the chance for detecting fusion, efforts were concentrated on fusion assays based on a PNS derived from PC12 cells and ISGs isolated from PC12/PC2 cells. To test the suitability of Ab 69 for the read-out of those experiments, a

PNS was prepared from PC12/PC2 cells and from PC12 cells (as a control) that had been labelled by a 10 min pulse of [^{35}S]-sulphate followed by a 15 min chase. The samples were individually incubated for 30 min at 4°C or 37°C in the presence or absence of ATP and an ATP-regenerating system in fusion buffer. The samples were then diluted with low pH buffer, preequilibrated one hour on ice and incubated 45 min for processing to occur. The samples were lysed and subjected to immunoprecipitation with Ab 69. Immunoprecipitates were analysed both by SDS-PAGE and by scintillation counting. Figure 4/13a illustrates the cpm that were obtained by immunoprecipitation with Ab 69. Surprisingly, incubation in the absence of ATP gave the highest signal. The reason for this can be seen in Figure 4/13b which is an autoradiograph of the SDS-PAGE analysis of the immunoprecipitates. The amount of p18 generated in the PC12/PC2 PNS was identical in all three conditions, in agreement with the previous observation that SgII processing in ISGs buffered at pH 5.5 is ATP-independent (section 3.5). However, samples incubated in the absence of ATP (lanes 6) revealed that a large amount of a high molecular weight, sulphated material was also immunoprecipitated with Ab 69. This material is most probably due to the hsPG non-specifically sticking to the protein A-sepharose (see below). This background was also seen for the PNS derived from PC12 cells, however, as expected, no p18 was detected (lane 3). Unfortunately, the high molecular weight background proved impossible to eliminate and was present in variable amounts in all immunoprecipitations independent on the assay conditions. In the following chapters the immunoprecipitations were therefore all analysed by SDS-PAGE and the signal for p18 was determined by fluorography followed by densitometry of exposed X-ray films.

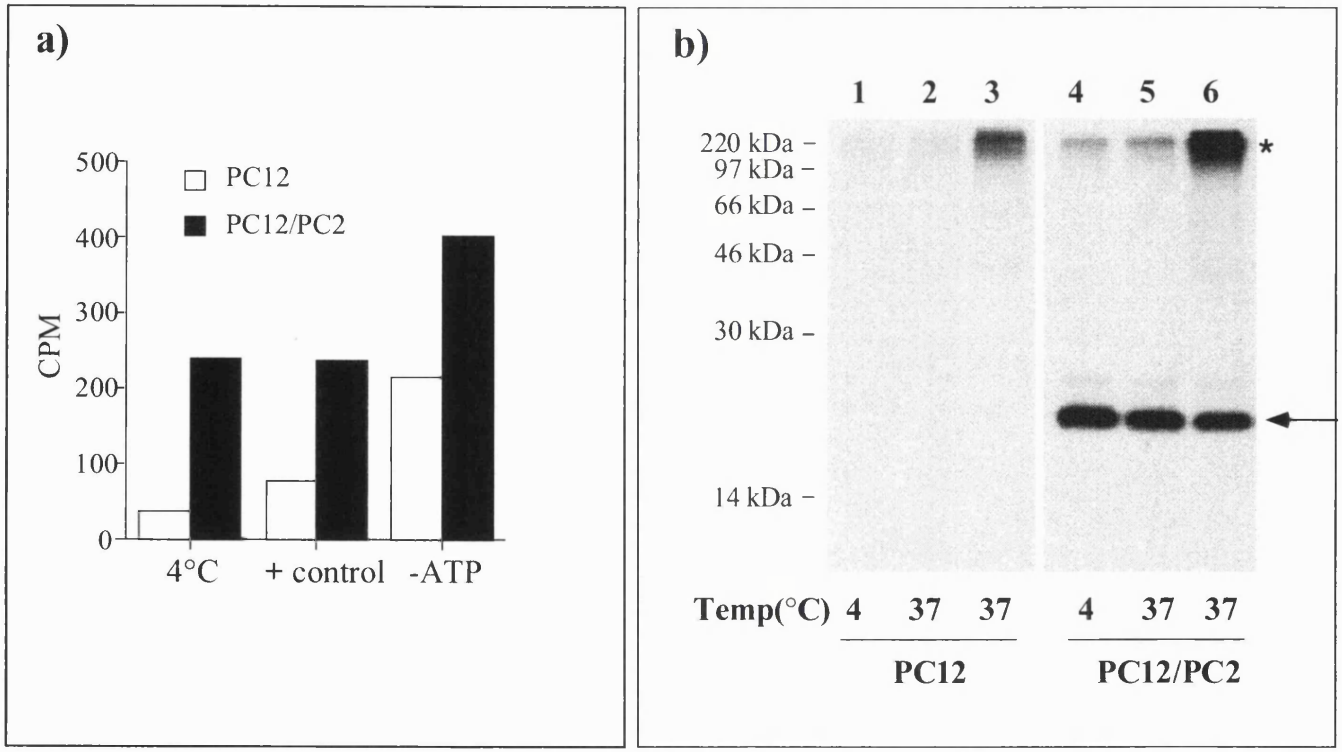


Figure 4/13: *Immunoprecipitation with Ab 69 from mock fusion incubations.*

A PNS was prepared from PC12/PC2 cells and from PC12 cells that had been labelled by a 10 min pulse of [^{35}S]-sulphate followed by a 15 min chase. The samples were individually incubated for 30 min at 4°C (4°C, lane 1) or 37°C (control, lane 2) in the presence (control) or absence of an ATP-regenerating system (-ATP, lane 3) in fusion buffer before being diluted with low pH buffer, pre-equilibrated one hour on ice and incubated 45 min at 37°C for processing to occur. The samples were lysed and subjected to immunoprecipitation with Ab 69. Immunoprecipitates were analysed both by 12% SDS-PAGE (**b**) and by scintillation counting (**a**).

4.6. Discussion

The data presented in this chapter served to establish a method that allows the quantitation of SgII processing by PC2 in ISGs as a read-out measurement of homotypic fusion of ISGs. Analysing the complete sulphated protein pattern proved to be unsuccessful for detecting fusion. SgII processing was therefore quantitated by immunoprecipitating the processing products. The use of Ab 100 was suboptimal as this antibody also reacted with the non-processed SgII. Interestingly the amount of SgII precipitated with Ab 175 decreased rapidly and reproducibly under processing conditions and could be used to monitor processing (Figure 4/4b, see also Table 5/1, section 5.10). However two reasons pointed against this approach. First, it has been shown in Chapter 3 (Figure 3/2), that a small amount of p86 has already been converted to p72 and p38 in ISGs at the point of isolation and this first processing step has not been well characterised. Second, the loss of full-length substrate is less easy to assess than the gain of a processing product, as a loss could be due to non-specific proteolysis.

Therefore, SgII processing was assessed by measuring the accumulation of the processing end product, p18, and the antibody 69 provided the perfect tool for this. This antibody specifically recognised p18 suggesting that this epitope is not exposed or does not exist in the right conformation in the full-length non-processed protein. The cleavage site at the C-terminus of p18 seemed to be recognised by PC2 last as p18 only appears after all other sulphated intermediates (see Chapter 3, Figure 3/4). This indicates that access of this site to PC2 might be restricted until p26 has been generated. The fact that Ab 69 only recognises p18 and not p86 even under denaturing conditions, suggests that the antibody recognises the free C-terminal end of p18 which is only exposed after cleavage. It has been mentioned above that the C-terminal dibasic cleavage site can be removed by a carboxypeptidase (Fricker, 1988). It is possible that Ab 69 which was raised against the preCS2 peptide, from which the dibasic site is missing, favours the conformation resulting from the activity of CpE.

Immunocytochemistry indicated that antibodies raised against preCS2 might be good markers for MSGs. In contrast Ab 175, raised against the very C-terminus, preferentially labelled ISGs in the perinuclear region of the PC12/PC2 cells. This correlates well with the fact that the most C-terminal cleavage site is the most exposed and most likely used first. The cleavage removes a pentapeptide which includes four of the six amino acids that compose the peptide that Ab 175 was raised against. The cleavage therefore destroys the epitope recognised by Ab 175..

Ab 69 was successfully used to immunoprecipitate p18 generated under fusion assay conditions in a PC12/PC2 PNS derived from cells labelled by a 10 min [^{35}S]-sulphate pulse and a 15 min chase. The amount of p18 immunoprecipitated with Ab 69 from a PC12/PC2 PNS was the same whether the fusion reaction was carried out at 4°C or at 37°C (4/13b). This indicates that the processing signal does not depend on any processing that could theoretically already occur during the fusion reaction. Furthermore, no signal was observed if the processing incubation was carried out on ice (Figure 4/12a). This is in agreement with experiments described in Chapter 3 (section 3.6) showing that processing in a pH 7.2 buffer in the presence of ATP is very slow compared to processing in a pH 5.5 buffer (Figure 3/6). The processing incubation following fusion will therefore ensure that SgII processing reaches a plateau that will be characteristic for a given amount of fusion, irrespective of the conditions of the fusion incubation.

Chapter 5: Reconstitution of immature secretory granule fusion in a cell-free assay.

5.1 Objective

Having determined that Ab 69 was suitable to measure SgII processing in ISGs as a potential read-out (Chapter 4), I will now describe the characterisation of a cell-free assay for ISG-fusion. This data will provide the first direct evidence for this fusion event and pave the way for a biochemical analysis.

Figure 5/1 illustrates the standard protocol for the cell-free fusion assay. Briefly PC12 cells are labelled with [^{35}S]-sulphate and a PNS is prepared in HB (10 mM HEPES pH 7.2, 1 mM $Mg(OAc)_2$, 1 mM EDTA, 250 mM sucrose, 0.5 mM PMSF). The length of pulse and chase may be varied to analyse different subpopulations of ISGs (see Chapter 7). To this PNS are added ISGs isolated from PC12/PC2 cells (referred to as PC2-ISGs), an ATP-regenerating system and an appropriate fusion buffer (FB: 20 mM HEPES pH 7.2, 50 mM KOAc, 3 mM $MgCl_2$, 1 mM DTT). The samples are incubated for 30 min at 37°C to allow fusion to occur, diluted 4-5 fold with ice-cold pH 5.5 buffer (50 mM MES pH 5.5, 0.3 M sucrose) and incubated on ice to equilibrate the pH. This and the following incubation at 37°C are part of the read-out of the assay as fusion is not supported under these conditions (see below). Samples are then lysed in lysis buffer (100 mM Tris, pH 7.2; 150 mM NaCl; 0.3% Triton-X100; 5 mM EDTA) in the presence of protease inhibitors, precleared by centrifugation and the supernatants used for immunoprecipitation with Ab 69. The immunoprecipitates are solubilised in SDS-sample buffer and the proteins are separated on SDS-PAGE followed by fluorography. Quantitation is by autoradiography followed by densitometry.

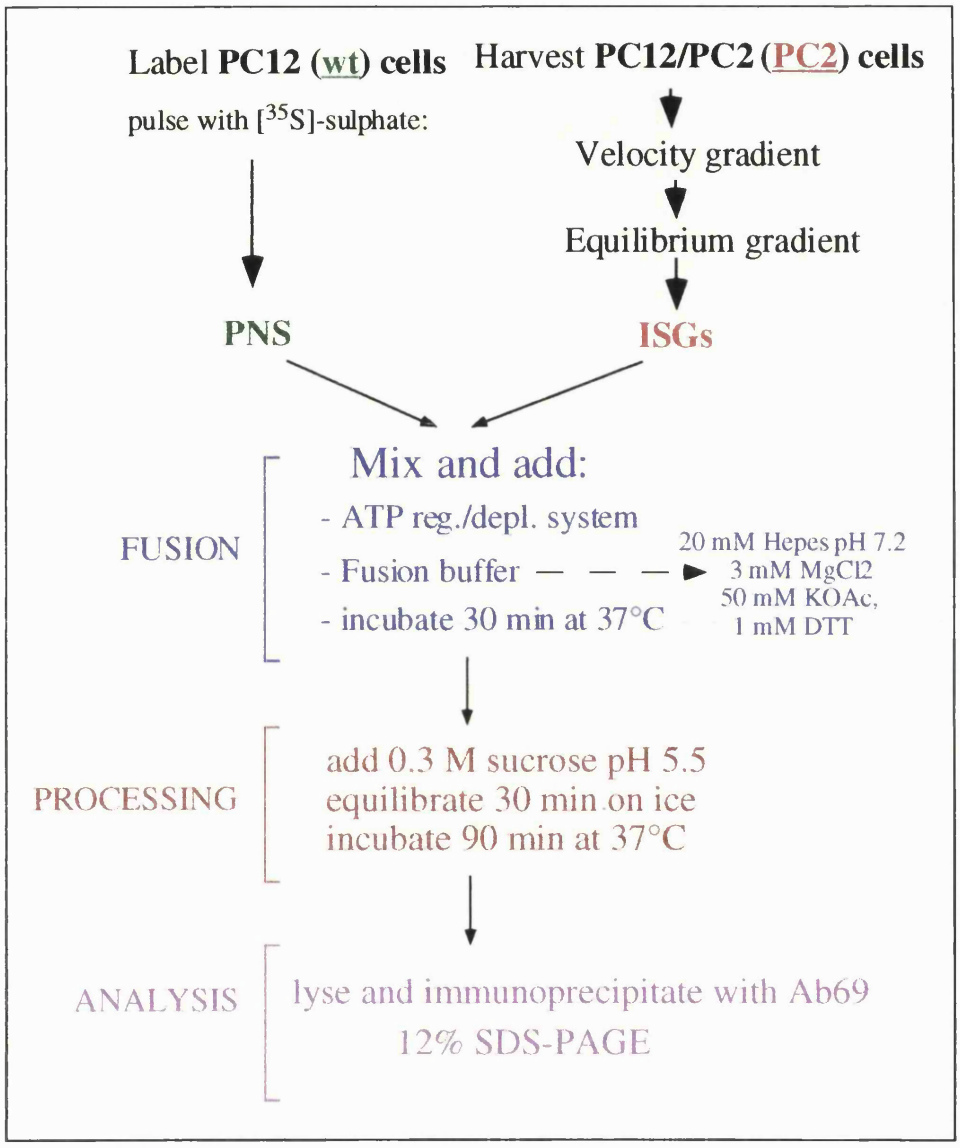


Figure 5/1: *Flow-chart of the cell-free fusion assay*
(see text and Chapter 2 for details)

5.2 Fusion of ISGs is temperature dependent and does not take place during the processing incubation

Initial experiments were carried out with a PNS from PC12 cells labelled by a 10 min [^{35}S]-sulphate pulse followed by a 15 min chase. To give an idea of the ratio of "donor" material, derived from enzyme containing PC12/PC2 cells, and "acceptor" material, derived from the [^{35}S]-sulphate labelled PC12 cells, it should be noted that PC2-ISGs obtained from a quarter (Figure 5/2) or a whole (in all other figures if not indicated otherwise) 15 cm diameter dish of cells were added to a PNS derived from one third of a 15 cm dish of PC12 cells.

The samples were either incubated at 4°C or 37°C in the presence or absence of PC2-ISGs during the fusion reaction and processed as described in section 5.1. After a long exposure time (1 month), it was possible to detect a signal for p18 that was clearly stronger when the samples had been incubated at 37°C but only if the incubation did include PC2-ISG (Figure 5/2a). The PC2-independent generation of p18 indicated that there was an endogenous enzyme capable of utilising SgII as a substrate and able to generate p18 during the processing incubation of the fusion assay.

As mentioned above (4.1) it was conceivable that the time window during which ISGs were fusogenic was very short and in order to increase the probability of working within the relevant time window and thereby maximise the signal, all experiments described below were carried out using PC12 cells labelled by a 30 min pulse of [^{35}S]-sulphate unless indicated otherwise. Under these conditions the amount of p18 generated in the presence of PC2-ISGs was significantly increased and exposure times of the autoradiographs could be reduced to 1-3 days (Figure 5/2b). Omission of the antibody from the immunoprecipitation showed that p18 was specifically bound by the antibody. In contrast, the high molecular weight smear was bound to the protein A-sepharose beads (Figure 5/2b lanes 3 and 4).

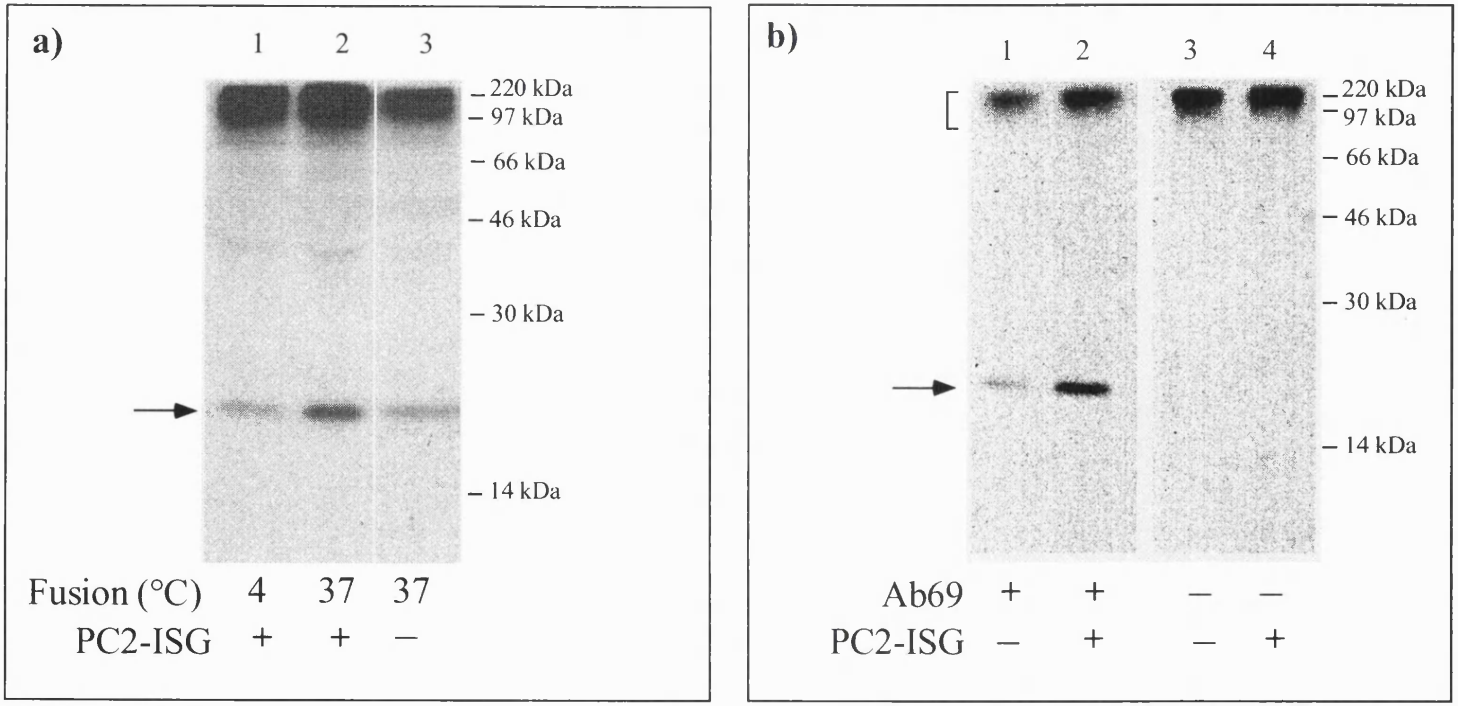


Figure 5/2: *Reconstitution of ISG fusion*

(a) A PNS was derived from PC12 cells labelled by a 10 min [^{35}S]-sulphate pulse and a 15 min chase. Fusion incubations were performed in the presence (lane 1, 2) or absence (lane 3) of PC2-ISGs for 30 min at 37°C (lane 2, 3) or on ice (4°C). Samples were further incubated in low pH buffer for processing and analysed by immunoprecipitation with Ab 69 followed by 12% SDS-PAGE. **(b)** A PNS was derived from PC12 cells labelled by a 30 min [^{35}S]-sulphate pulse. Standard fusion reactions were performed as in (a) in the absence (lane 1, 3) or presence (lane 2, 4) of PC2-ISGs and analysed by immunoprecipitation with Ab69 and Protein A-sepharose. In lanes 3 and 4 the Ab 69 was omitted.

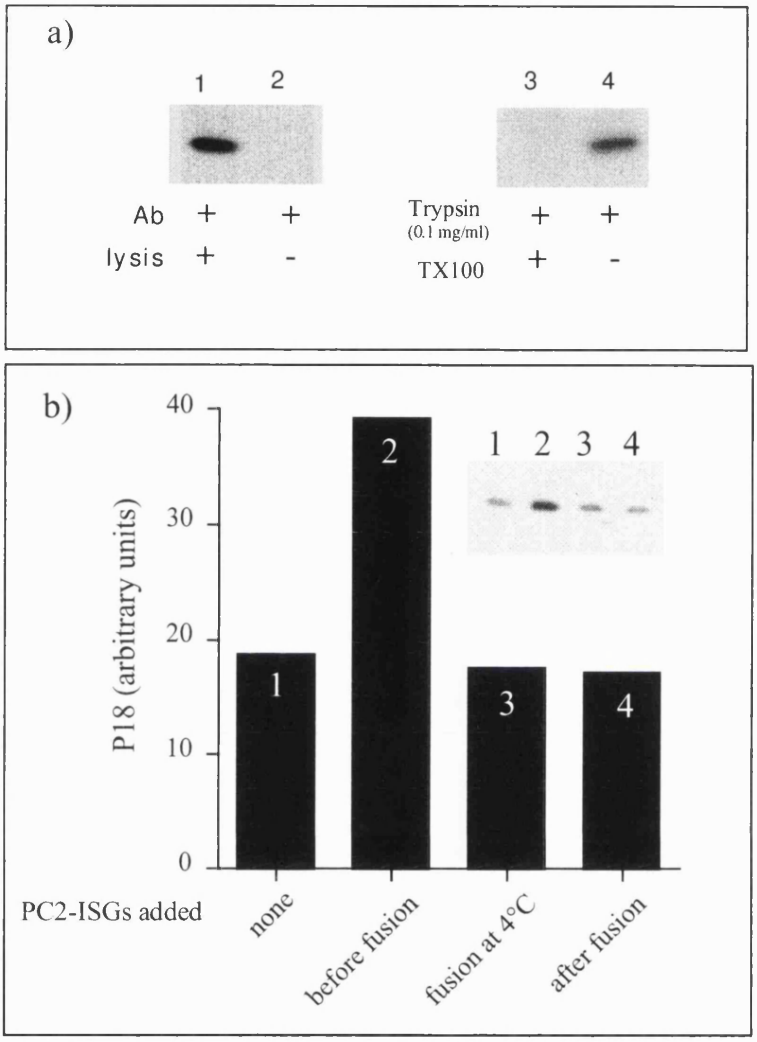


Figure 5/3: *Cell-free fusion of ISGs does not take place during the processing incubation*

(a) A PNS was prepared from PC12 cells labelled by a 30 min [^{35}S]-sulphate pulse and standard fusion reactions were carried out as described in Figure 5/1. After the processing incubation, the samples were incubated for 15 min at 37°C with Trypsin in the absence (lane 4) or presence (lane 3) of 0.3% Triton-X100 (TX100). Samples were either solubilised in lysis-buffer (lane 1, 3, 4) or not (lane 2) prior to immunoprecipitation with Ab69. Only the area of the molecular weight range of p18 is shown. **(b)** A PNS was prepared from PC12 cells labelled by a 30 min [^{35}S]-sulphate pulse and incubated under standard conditions either in the presence (lane 2 and 3) or absence (lane 1 and 4) of PC2-ISGs at 37°C or at 4°C (lane 3). After the fusion incubation, PC2-ISGs were added to the sample in lane 4. Subsequent manipulations and analysis were as described in Chapter 2.

In the following figures, results illustrated by autoradiographs of SDS-PAGE are therefore restricted to the area of the autoradiograph in the molecular weight range of p18.

No p18 was recovered if the samples were not lysed at the end of the incubations, indicating that p18 was indeed generated and retained inside a membrane bound compartment (Figure 5/3a, lanes 1 and 2). This was confirmed by demonstrating that p18 was protected against trypsin digestion in the absence of detergent (Figure 5/3a, lanes 3 and 4). No specific (PC2-dependent) fusion signal was observed when the PC2-ISGs were added at the end of the fusion incubation and were only present during the processing incubation (Figure 5/3b). Thus the processing incubation does not support fusion of ISGs.

To obtain some information about the endogenous processing activity present in PC12 cells that produces background levels of p18, a PNS derived from PC12 cells labelled by a 30 min [^{35}S]-sulphate pulse was fractionated by velocity controlled sucrose density centrifugation. As described in detail in Chapter 3, this procedure allows the separation of TGN and post-TGN vesicles including ISGs and is used as a first step to isolate the PC2-ISGs used in the fusion assay. Fractions of this gradient were diluted to 0.34 M sucrose, the membranes were pelleted by centrifugation at 100,000 g for 1 hr and resuspended in PC12 cytosol (see Chapter 6). An ATP-regenerating system and fusion buffer were added and samples were incubated for 30 min at 37°C. Figure 5/4 shows the distribution of the p18 generated independently of PC2 and demonstrates that the proteolytic activity is located in post-TGN-vesicles containing the [^{35}S]-sulphate labelled SgII (fractions 2-4) rather than in the TGN (fractions 8-10).

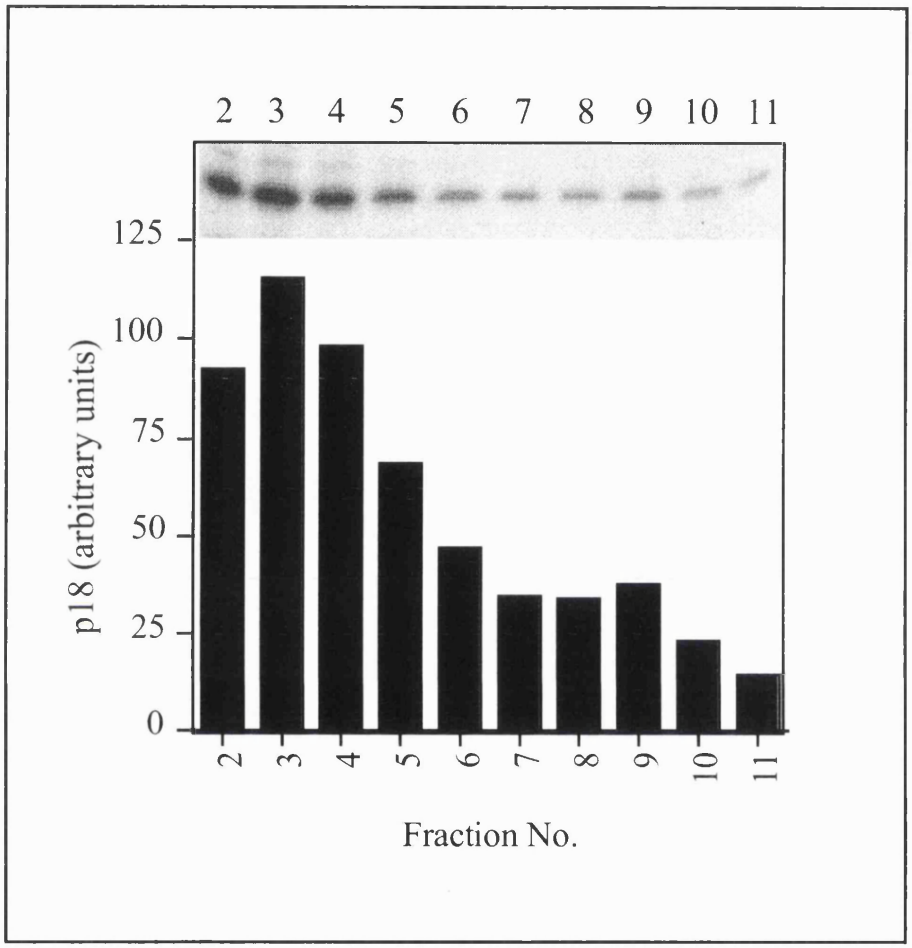


Figure 5/4: *Distribution of p18 generated independently of PC2*

A PNS was derived from PC12 cells labelled by a 30 min [^{35}S]-sulphate pulse and fractionated by velocity controlled sucrose density centrifugation. The fractions were diluted to 0.34 M sucrose, and the membranes were pelleted and resuspended in PC12 cytosol. An ATP-regenerating system and fusion buffer were added and samples were incubated for 30 min at 37°C. Processing incubation and analysis were as described in Figure 5/1. The amount of immunoprecipitated [^{35}S]-sulphate labelled p18 was analysed by SDS-PAGE and quantitated by densitometry.

5.3 Fusion of ISGs is ATP-dependent and requires ATP-hydrolysis

A PNS was prepared as described above, from PC12 cells labelled by a 30 min [^{35}S]-sulphate pulse, and PC2-ISGs were added in the presence or absence of an ATP-regenerating system. Fusion of ISGs was strictly ATP-dependent, and addition of hexokinase in the presence of glucose completely abolished the fusion signal (Figure 5/5a). The non-hydrolysable analogue AMP-PNP was unable to support fusion, suggesting that ATP-hydrolysis was required for ISG-fusion (Figure 5/5b). Inclusion of GTPγS in the incubation had only a slight inhibitory effect on fusion.

The above experiments were carried out in the presence of 50 mM KOAc. A coarse titration of the KOAc indicated a clear requirement for salt while higher concentrations were inhibitory to fusion (Figure 5/6).

5.4 Titration of the PC2-ISGs

PC12 cells were labelled by a 30 min [^{35}S]-sulphate pulse and a PNS was obtained and incubated in the presence of an ATP-regenerating system and fusion buffer. Increasing amounts of PC2-ISGs were titrated into the fusion assay. Figure 5/7a shows a representative experiment. The amount of p18 generated reaches a plateau after addition of 20 µl PC2-ISGs. A second round of immunoprecipitation with Ab 69 showed that the first immunoprecipitation was quantitative and sufficient (Figure 6/7a, 2nd IP). A more detailed titration is shown in Figure 5/7b. 20 µl of PC2-ISGs correspond to the ISGs isolated from 2x 15 cm diameter dish of cells and these were added to a PNS obtained from one third of a 15 cm diameter dish of PC12 cells. In general, between 10 and 20 µl of PC2-ISGs were added to the fusion assay.

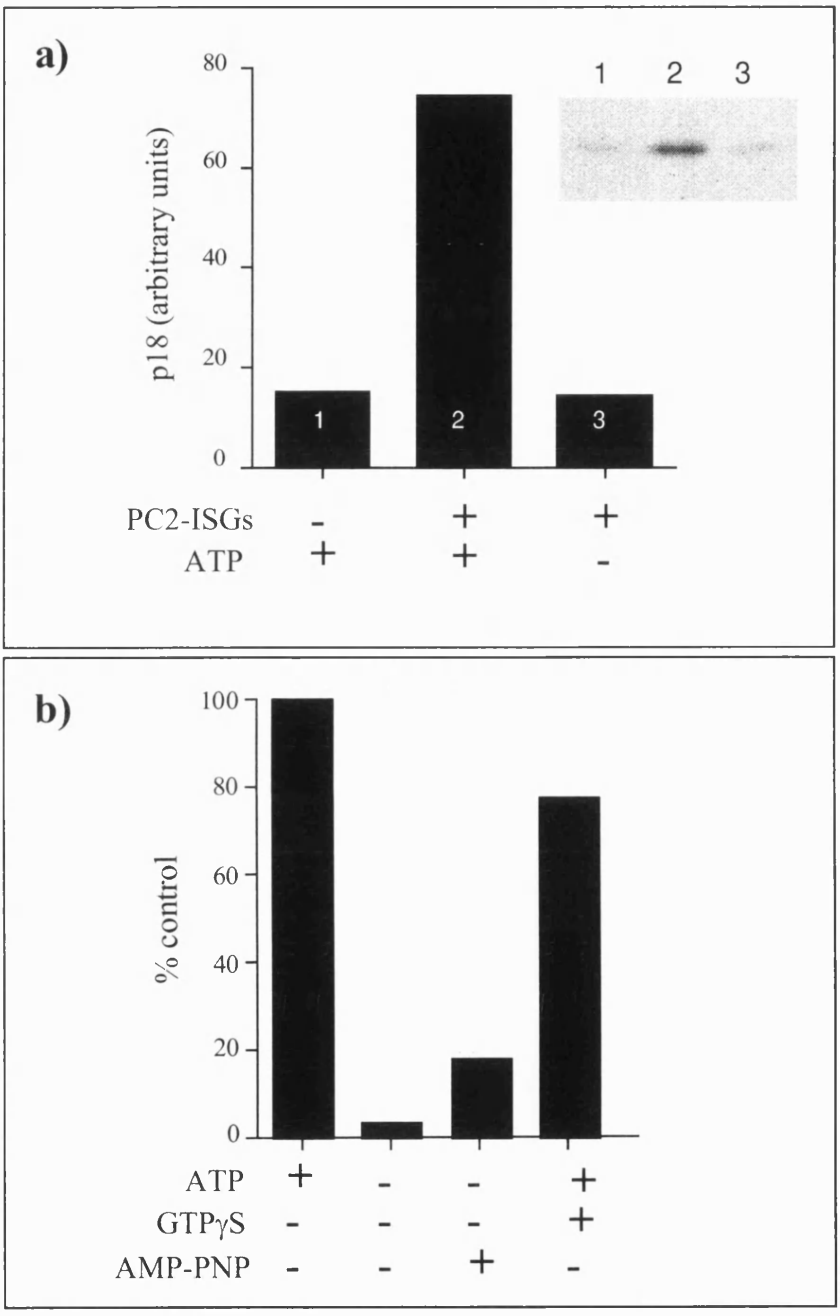


Figure 5/5: *Fusion of ISGs is ATP-dependent*

(a) A PNS was prepared from PC12 cells labelled for 30 min with [^{35}S]-sulphate. Fusion reactions were carried out for 30 min at 37°C in the absence (lane 1) or presence (lane 2 and 3) of PC2 ISGs and in either the presence of an ATP-regenerating system (lanes 1 and 2) or ATP-depletion system (lane 3). **(b)** A PNS was prepared from PC12 cells labelled for 30 min with [^{35}S]-sulphate. Fusion reactions contained PC2-ISGs, an ATP-regenerating system (+ATP), an ATP-depletion system (-ATP), AMP-PNP (100 μM), or GTPγS (+ GTPγS, 100 μM). Further manipulations and analysis were as described in Figure 5/1.

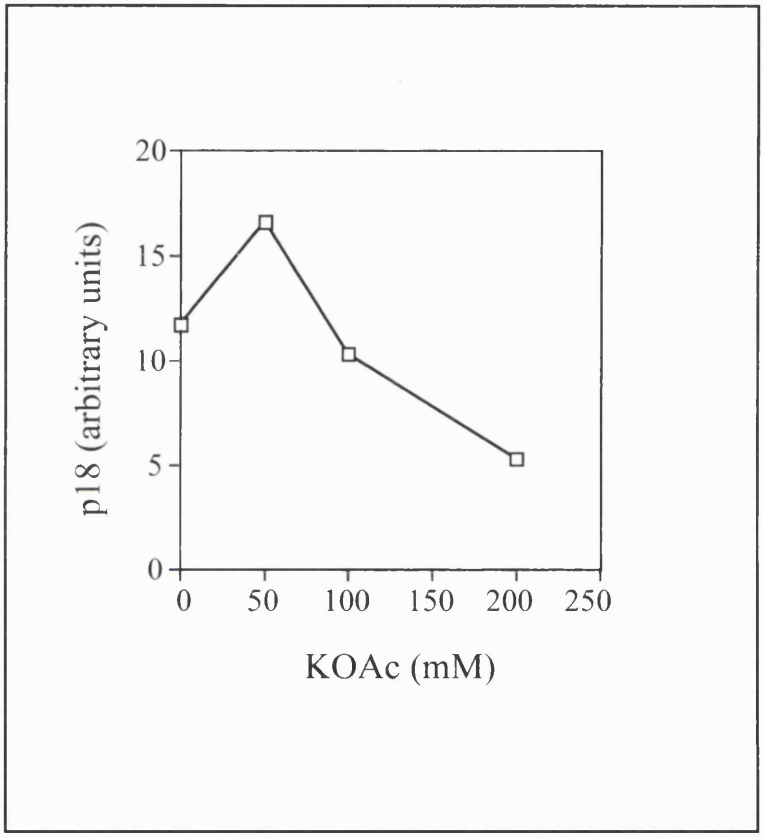


Figure 5/6: *Salt requirement of cell-free ISG fusion*

A PNS was derived from PC12 cells labelled by a 30 min [^{35}S]-sulphate pulse and cell-free fusion reactions were performed as described in Figure 5/1 except that the concentration of KOAc in the fusion buffer was varied from 0 to 200 mM. Further manipulatins and analysis were as described in Figure 5/1.

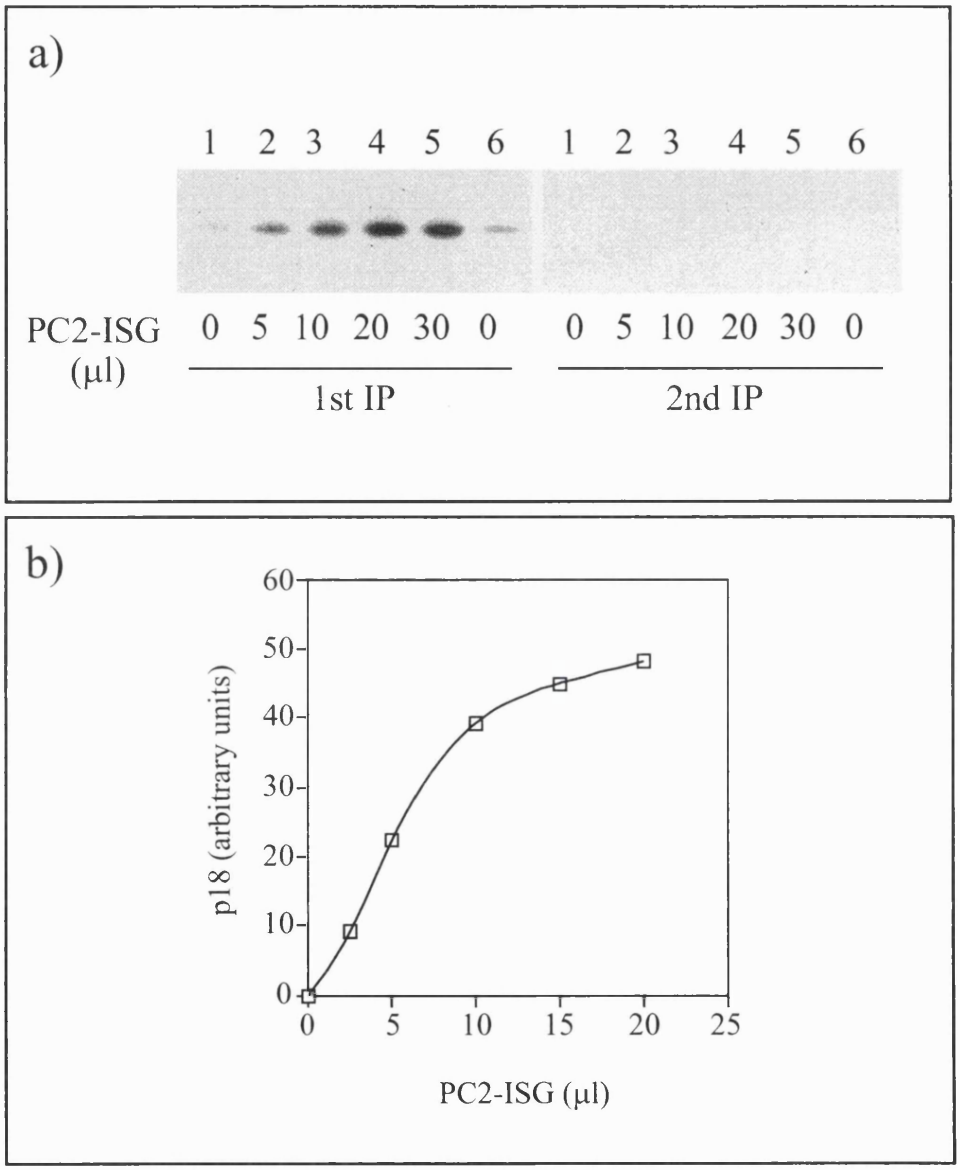


Figure 5/7: *Titration of PC2-ISGs*

(a) A PNS was derived from PC12 cells labelled by a 30 min [^{35}S]-sulphate pulse. PC2-ISGs were isolated by subcellular fractionation. pelleted and resuspended 50x concentrated in buffer. PC2-ISGs were added to the PNS and supplemented with an ATP-regenerating system. Further incubations and analysis were as described in Figur e 5/1. The [^{35}S]-sulphate labelled p18 was immunoprecipitated with Ab69 (1st IP) and the supernatant was subjected to a second round of immunoprecipitation with Ab 69 (2nd IP). **(b)** A PNS was obtained as in (a) and fusion reactions contained increasing amounts of PC2-ISGs. Analysis was as described in (a) and the PC2-independent signal was subtracted.

5.5 Time course of Fusion

A PNS was prepared as described above from PC12 cells labelled by a 30 min [^{35}S]-sulphate pulse. An ATP-regenerating system and fusion buffer were added and samples were incubated for varying length of time at 37°C in the presence or absence of PC2-ISGs, equilibrated at pH 5.5 and incubated for 45 min at 37°C. As shown in Figure 5/8, p18 accumulates over time in a PC2-dependent manner and reaches a plateau after approximately 30 min. This time point was used in all following experiments as a standard condition. The PC2-independent p18 signal does not increase with the fusion time, indicating that the endogenous proteolytic enzyme is converting SgII during the processing incubation rather than during the fusion incubation (see also Figure 5/9a).

5.6 Time course of processing

For initial fusion experiments the incubation time for processing was arbitrarily kept at 45 min to achieve a good processing signal whilst keeping the manipulations as short as possible. It was however important to determine whether this incubation time was sufficient to reach a maximum signal for the read-out of fusion.

PC12 cells were labelled as usual by a 30 min [^{35}S]-sulphate pulse. A PNS was obtained and incubated in the presence of an ATP-regenerating system and PC2-ISGs for 30 min at 37°C. After equilibration on ice in low pH buffer for 60 min, samples were incubated for different lengths of time at 37°C to allow processing to occur (Figure 5/9a). The time course reveals that SgII processing occurs with different kinetics in fused "hybrid"-ISGs than in isolated PC2-ISGs: For comparison a curve showing a time course of processing in isolated PC2-ISGs derived from cells labelled by a 10 min [^{35}S]-sulphate pulse followed by a 15 min chase is shown in parallel in Figure 5/9b. While 50% of the maximum processing signal is reached after 15 min

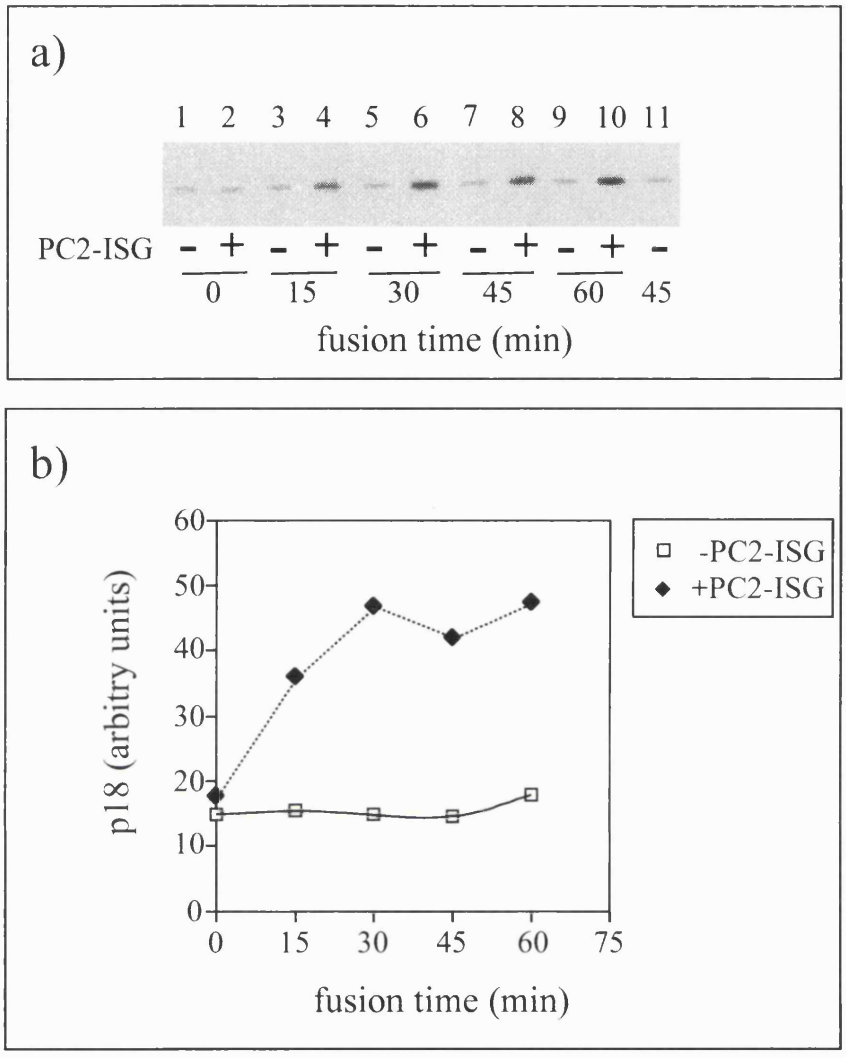


Figure 5/8: *Time course of fusion*

(a) Fusion reactions containing a PNS derived from PC12 cells labelled by a 30 min [^{35}S]-sulphate pulse, an ATP-regenerating system and fusion buffer were incubated in the absence or presence of PC2-ISGs for various length of time. Further incubations for processing and analysis were carried out as described in Figure 5/1. **(b)** The [^{35}S]-sulphate labelled, immunoprecipitated p18 shown in (a) was quantitated by densitometry.

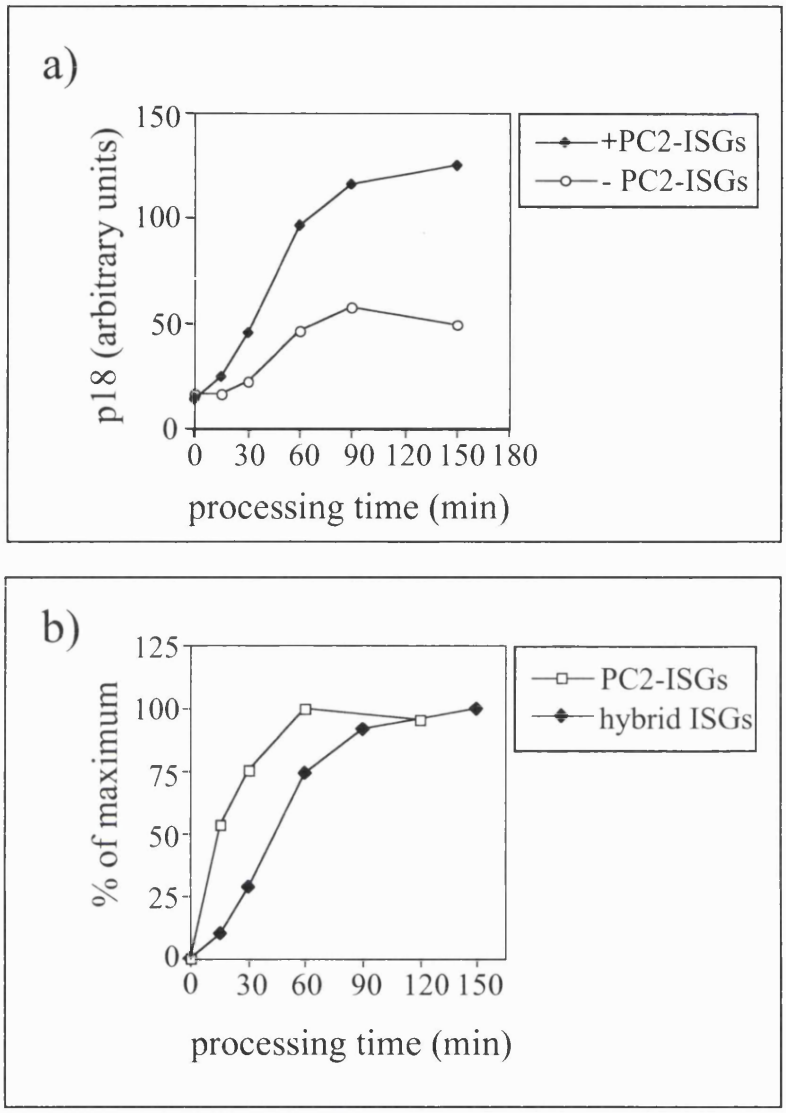


Figure 5/9: *Time course of processing in fused ISGs*

(a) Standard fusion reactions were carried out in the absence or presence of PC2-ISGs using a PNS derived from PC12 cells labelled by a 30 min [^{35}S]-sulphate pulse. The samples were equilibrated with low pH buffer for 1 hr on ice and incubated for various length of time to allow processing to occur. Analysis of p18 was as described in Figure 5/1. **(b)** Comparison of the processing kinetics in fused ISGs (hybrid ISGs, see (a)) and in isolated PC2-ISGs (Figure 3/6).

incubation in PC2-ISGs, the processing signal resulting from fusion is less than 10% of the maximum signal by that time point (Figure 5/9b) and only reaches a plateau after 90 min (Figure 5/9a). The PC2-independent signal increases with time during the processing incubation, in agreement with the observations made in Figure 5/8 (Figure 5/9a). Subsequently, the standard incubation time for processing was therefore increased to 90 min.

5.7 Fusion of ISGs requires a trypsin sensitive membrane associated protein.

All characterised intracellular membrane fusion reactions depend on proteins. Some of these proteins required for fusion appear to be membrane associated as even mild protease digestion of the membrane-bound compartments leads to inhibition of fusion. To test whether this was also the case for the homotypic fusion of ISGs, PC2-ISGs were treated for 15 min at 37°C with trypsin in the presence or absence of soy bean trypsin inhibitor and then re-isolated by centrifugation at 100,000 g for 1 hour. The ISGs were then resuspended in a PNS derived from [^{35}S]-sulphate-labelled PC12-cells and incubated under standard conditions (ATP-regenerating system, fusion buffer, 30 min fusion at 37°C). As seen in Figure 5/10 a small concentration of trypsin (1 μg/ml, corresponding to approximately 0.5 μg trypsin to 50 μg ISG protein) completely abolished the fusion signal, indicating that some proteins either associated with the ISG membrane or integral secretory granule membrane proteins, are essential for fusion to take place.

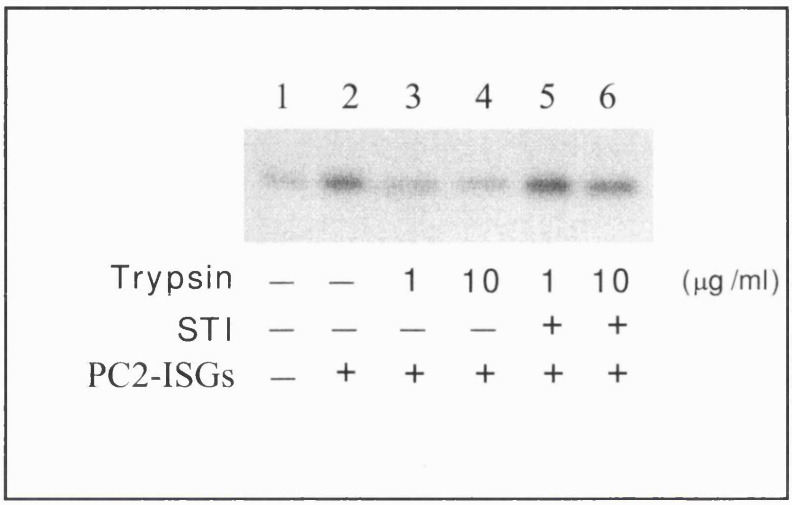


Figure 5/10: *Trypsin sensitivity of cell-free ISG fusion*

PC12/PC2 ISGs were pretreated for 15 min at 37°C with 1 or 10 μg/ml trypsin in the absence or presence of soy bean trypsin inhibitor (STI) as described in Chapter 2. The ISGs were pelleted by centrifugation, resuspended in buffer and added together with an ATP-regenerating system to a PNS derived from PC12 cells labelled for 30 min with [^{35}S]-sulphate. Standard fusion and processing reactions were performed and analysed as described in Figure 5/1.

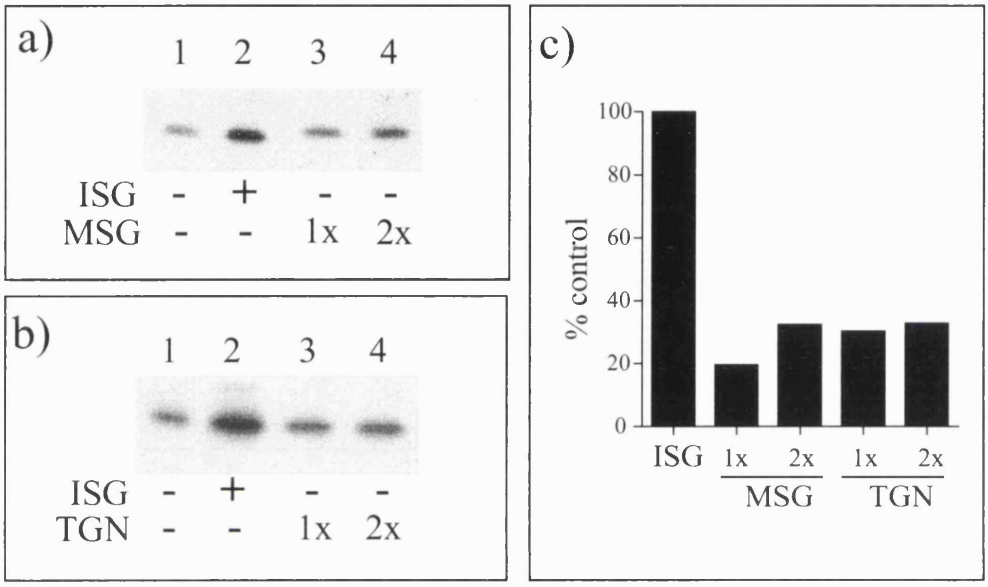


Figure 5/11: *Specificity of cell-free ISG fusion*

Standard fusion reactions were performed using a PNS derived from PC12 cells labelled for 30 min with [^{35}S]-sulphate in the presence of either PC2-ISGs (lane 2), an equal volume (1x, lane 3), twice the volume (2x, lane 4) of PC2-MSGs (a), PC2-TGN-membranes (b) or buffer only (lane 1). Further manipulations and analysis was as described in Figure 5/1. The results shown in (a) and (b) were normalised to the control condition (lane 2) after background (lane 1) subtraction and expressed as a percentage.

5.8 Specificity of the fusion assay: Neither MSGs nor TGN can fuse with ISGs

It was mentioned above that the ISG-pool isolated from PC12/PC2 cells was not pure and contained other membranes. In order to see whether fusion is specific to ISGs or whether possible trace contaminations of other PC2-containing compartments of the late secretory pathway contribute to the fusion signal measured in this assay, the PC2-ISGs were substituted by MSGs and TGN. First, MSGs were isolated from PC12/PC2 cells using the same two consecutive gradients as described for PC2-ISGs with the only difference that the denser fractions 10 to 12 of the equilibrium gradient were pooled rather than fractions 7 to 9. These MSG were then added instead of PC2-ISGs to the fusion assay with all other parameters remaining unchanged. Figure 5/11a shows that addition of MSGs only results in a small signal, that might be due to a residual fusion activity in maturing secretory granules. Second, TGN-membranes were isolated from PC12/PC2 cells by velocity centrifugation only (see Figure 3/2c). Addition of these TGN-membranes to the fusion assay resulted only in a small signal even if the amount of TGN-membranes added was doubled. Experiments shown in (a) and (b) were quantitated and normalised for comparison (Figure 5/12c). The fusion signal measured in the cell-free assay is therefore not due to a contaminating membrane population, but specifically reflects fusion of ISGs.

5.9 Competition of fusion with unlabelled PC12-ISGs

If ISGs undergo a limited number of fusion events, as suggested by the uniform size of MSGs, it might then be possible to compete the fusion signal by adding a source of ISGs that does not contain any [^{35}S]-sulphate labelled SgII nor any PC2. To test this, unlabelled PC12-ISGs (referred to as wt-ISGs, i.e. wild-type ISGs) were titrated into a fusion assay and as shown in Figure 5/13 were able to compete with the PC2-ISGs for fusion. This decrease in p18 accumulation unequivocally demonstrates

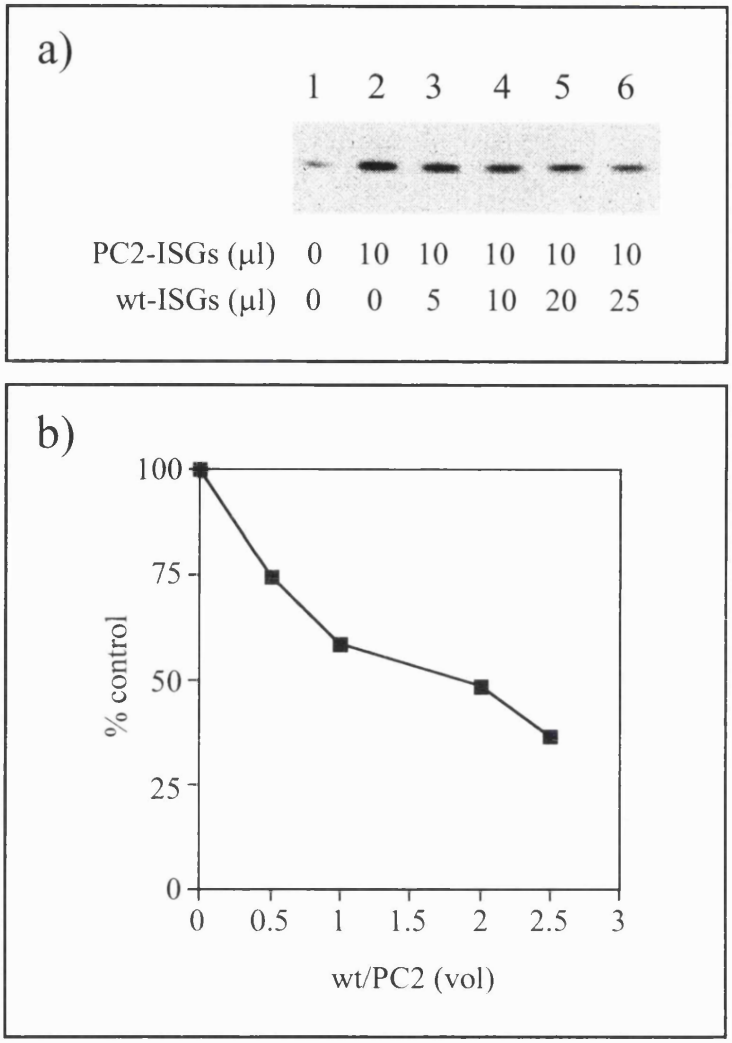


Figure 5/12: *Competition of fusion with unlabelled wt-ISGs*

(a) A PNS was derived from PC12 cells labelled for 30 min with [^{35}S]-sulphate and fusion incubations were carried out in the presence or absence (lane 1) of PC2-ISGs to which various amounts of PC12-ISGs (wt-ISGs) were added. Standard incubations and analysis were as described in Figure 5/1. **(b)** Quantitative analysis of (a). The amount of p18 generated in the absence of PC2-ISGs was subtracted and the PC2-dependent signal was expressed as percentage of the control (lane 2) and plotted against the ratio of wt-ISGs/PC2-ISGs.

that isolated wt-ISGs added to the assay fuse with either the labelled wt-ISGs or the PC2-ISGs or with both. It is however important to consider that fusion of unlabelled wt-ISGs adds unlabelled substrate and it is possible that the competition curve reflects the competition of the additional unlabelled SgII with the [^{35}S]-sulphate labelled SgII for PC2-interaction and cleavage rather than the competition of ISGs with each other.

5.10 Determination of the fusion efficiency

Ideally one would like to compare the amount of p18 generated as a result of fusion with the amount of p18 which could be generated under conditions where all the substrate in the assay has access to the enzyme. Theoretically such a situation can be produced by detergent lysis of the membranes. In the case of SgII processing by PC2 however this leads to a different processing pattern as has been shown in section 3.13. The best system for comparison was therefore the quantitation of processing inside PC2-ISGs containing both PC2 and labelled SgII, incubated under the conditions of the fusion assay.

Therefore, PC12/PC2 cells were labelled by a 30 min [^{35}S]-sulphate pulse, a PNS was obtained and incubated under the conditions of the fusion assay. The relative processing efficiency in the PC2-ISGs contained in this PNS was calculated by relating the amount of p18 generated to the amount of starting material which was determined by immunoprecipitation of full-length SgII (p86) with the Ab 175. This value is not identical to the absolute processing efficiency for which all intermediate processing products would also have to be taken into account. Alternatively the relative processing efficiency was calculated from the decrease in the amount of p86 that was immunoprecipitated before and after the fusion assay. The processing efficiency in PC2-ISGs was then compared to the relative processing efficiency resulting from the fusion of PC2-ISGs with ISGs contained in a PNS derived from PC12 cells labelled for 30 min with [^{35}S]-sulphate (Table 5/1). From these results the fusion efficiency

was determined by comparison of the relative processing efficiencies and was estimated to be in the range of 30%.

	PC2-ISGs	fused wt/PC2-"hybrid" ISGs
p86 in starting material (st)	76.03 +/- 3.5	62.73
p86 after incubations (fin)	23.6	46.76
p18 after incubations	66.1 +/- 5.8	15.48
(1) $\frac{p18 \times 100}{p86st}$	89.9 % +/- 15.4%	24.68 %
(2) $\frac{p86st - p86fin \times 100}{p86st}$	68.96 %	25.46 %
Fusion efficiency (1)	28.40 %	
Fusion efficiency (2)	36.92 %	

Table 5/1: *Determination of the fusion efficiency*

PC12/PC2 cells and PC12 cells were labelled for 30 min with [^{35}S]-sulphate. A PNS was obtained from both cell-lines and used for two parallel fusion assays, with the only difference that 20 µl unlabelled PC2-ISGs were added to the PC12 PNS and none were added to the PC12/PC2 PNS. The fusion incubation was in the presence of an ATP-regenerating system and was processed as described above. The amount of p18 generated was determined by immunoprecipitation with Ab 69, while the amount of p86 before (starting material = st) and after the fusion reaction (final = fin), was determined by immunoprecipitation with Ab 175. Amounts of p18 and p86 are indicated as arbitrary units. Processing efficiency was determined for each case by two ways: (1) the amount of p18 expressed as percentage of p86 in the starting material and (2) the difference between p86 before and after the assay expressed as percentage of p86 in the starting material. Fusion efficiency was calculated as a ratio of the processing efficiency in the fusion assay (in "hybrid" fused ISGs) to the processing efficiency in PC2-ISGs.

5.11 Discussion

5.11.1 A cell-free assay for ISG fusion

In this chapter it has been shown that homotypic fusion of ISGs can be reconstituted in a cell free system. The signal detected by immunoprecipitation of p18 truly reflects the extent of fusion for the following reasons. No p18 was immunoprecipitated if the membranes had not been lysed and all of it was protected against protease digestion demonstrating that the p18 was generated and contained inside a membrane bound compartment (Figure 5/3a). Furthermore, addition of unlabelled PC12-ISGs, which did not contain either enzyme or labelled substrate, were able to efficiently compete the fusion assay (Figure 5/12).

Competition could arise from two scenarios both of which demand that the unlabelled PC12-ISGs undergo fusion with either or both the other ISG-populations in the assay. First, competition could be based on the fact that ISG fusion is saturable in the sense that each ISG can only participate in a fixed number of fusion events. The larger the number of ISGs that can fuse with each other, the more unlabelled PC12 ISGs have to be added to observe a competition. It has been proposed that between 3 and 5 ISGs fuse with each other (Tooze, *et al.*, 1991). The competition curve in figure 5/12 is compatible with two ISGs fusing with each other. Second, competition could also result through substrate competition. In this case the unlabelled SgII added by fusing PC12-ISGs competes with the radioactive substrate for enzyme. The apparent competition should disappear if the processing incubation time is increased. The incubation time in Figure 5/12 was only 45 min and it is therefore likely that the observed competition is at least partly due to substrate competition. Experiments with extended processing times should make it possible to discriminate between these two alternatives and might indicate how many ISGs fuse with each other in the cell-free assay.

ISG fusion was strictly ATP-dependent in common with other intracellular fusion events and hydrolysis of ATP was required as ATPγS did not support fusion (Figure 5/5). ISG fusion was also dependent on the integrity of some membrane associated granule protein(s) as even mild protease-treatment of the PC2-ISGs abolished fusion (Figure 5/11). Like ATP-dependence, this is one of the general requirements for intracellular membrane fusion events. Fusion of ISGs was optimal at 50 mM KOAc whilst experiments have not been done to test whether it is the cation or the anion or both that are required (Figure 5/6). A similar salt requirement has also been described for homotypic endosome fusion (Colombo, *et al.*, 1992). Homotypic fusion of endosomes has been shown to depend on calcium under some conditions (Mayorga, *et al.*, 1994). Homotypic fusion of sea urchin (mature) secretory granules is also calcium dependent as is the fusion of secretory granules with the plasma-membrane (Knight and Baker, 1985; Vogel, *et al.*, 1992). The buffer used for this fusion assay does not contain either calcium or any calcium chelating agents and preliminary results indicate that homotypic ISG fusion is not regulated by calcium (Lesley Page, unpublished observations).

The efficiency of ISG fusion was determined by comparison of the relative processing efficiency in fused ISGs with the relative processing efficiency in PC2-ISGs (Figure 5/12): The calculated fusion efficiency of approximate 30% should be seen as a rough estimate. While this might indicate that only a third of the ISGs in the assay undergo fusion, the accuracy of this estimation will depend on a large number of partially unknown parameters in particular on the number of fusion events each ISG can participate in.

5.11.2 Specificity of the fusion assay

An important feature of this assay is the use of radioactive sulphate to label the content of one population of ISGs and it is this feature that allows the use of a crude fraction as half of the starting material for the assay (the other population being ISGs isolated by subcellular fractionation from PC12/PC2 cells). The radioactive sulphate is incorporated into secretory proteins, including SgII, in the TGN and this labelled SgII is then packaged exclusively into budding ISGs. In the first type of experiment described in this chapter (Figure 5/2), the [^{35}S]-sulphate was chased for 15 min, so that all of the SgII labelled was contained in ISGs (Tooze, *et al.*, 1991). The isolation of the PC2-ISGs was based on a subcellular fractionation protocol that was initially calibrated with a sulphate labelled PNS derived from PC12 cells labelled by a 5 min [^{35}S]-sulphate pulse followed by a 15 min chase. Therefore the PC2-ISGs and the PC12 ISGs containing the sulphate-labelled SgII in the fusion assay are equivalent and the fusion event observed in that experiment could be described as truly homotypic.

All other experiments described in this chapter are based on a 30 min [^{35}S]-sulphate pulse in order to increase the population of ISGs containing labelled SgII and to maximise the signal. It could be argued that in this case a fraction of the [^{35}S]-sulphate labelled SgII resides in the TGN and that the cell-free assay measures homotypic fusion of TGN-membranes contaminating the ISG-fraction with the [^{35}S]-sulphate labelled TGN in the PNS. While this point will be further discussed in Chapter 7, a few important points ought to be made here.

First, analysis of the distribution of SgII labelled by a 5 min [^{35}S]-sulphate pulse suggests that less than 1% of the TGN-membranes cofractionate with the ISG in the first purification step (Figure 3/2c). Second, only a small fraction of the labelled substrate, approximately one sixth of the total [^{35}S]-sulphate-labelled SgII, resides in the TGN while the bulk is incorporated into ISGs. This can be deduced from the data of Tooze et al. that suggest a half time of 5 min for budding of ISGs from the TGN

(Tooze and Huttner, 1990). Third, it has been shown in this chapter that addition of isolated TGN membranes derived from PC12/PC2 cells did not result in a fusion signal (Figure 5/11). It is therefore also unlikely that the added PC2-ISGs fuse with the [^{35}S]-sulphate labelled TGN if the reverse reaction, PC2-TGN fusing with PC12-ISGs, is not observed. Finally, it has been shown in Chapter 3 that SgII processing was not observed in the TGN, unless the cells were previously incubated at 20°C. Thus, in order to generate a processing signal, contaminating TGN-membranes in the ISG-preparation would have to fuse with the TGN in the PNS and then form new ISGs that now would contain PC2 and [^{35}S]-sulphate labelled SgII.

Is this fusion event specific to ISGs or do all secretory granules undergo homotypic fusion independent of their maturation stage? Homotypic fusion has been described for secretory granules derived from sea urchin eggs (Vogel and Zimmerberg, 1992). In this chapter we have shown that isolated MSGs were unable to fuse with ISGs (Figure 5/11). This is reminiscent of fusion assays analysing the fusion of compartments of the endocytic pathway where it was found that early endosomes fuse with each other but do not fuse with late endosomes (Aniento, *et al.*, 1993). This issue will be further discussed in Chapter 7.

5.11.3 SgII-processing as an enzymatic read-out independent of fusion

The ISG fusion assay described here is a two step assay. The first incubation is the actual experiment in which ISG fusion takes place. The second incubation is part of the read-out, similar to an enzymatic colour-reaction used to visualise the amount of antibody bound in an immunoblot. In the case of the ISG-fusion assay, the enzyme used is PC2 and the substrate is SgII, the two of which only meet as a function of fusion. It is crucial that fusion does not continue during the "development" of the assay, i.e. the processing reaction. Indeed it has been shown in Figure 5/3b that no

fusion signal is observed if the PC2-ISGs are added after the fusion reaction, although the processing incubation time is 3 times longer than the fusion incubation time. The factors that ensure that fusion does not take place during this incubation are most likely the acidic pH and the dilution factor. The pH of 5.5 is much more acidic than the pH of the cytosol and most cytosolic proteins are denatured and precipitate under these conditions.

Processing of [^{35}S]-sulphate labelled SgII took longer to reach an endpoint in fused "hybrid" ISGs than in PC2-ISGs (as described in Chapter 3) (Figure 5/9b). This might be due to the fact that in "hybrid" ISGs the enzyme PC2 not only processes the PC12-ISG derived [^{35}S]-sulphate labelled SgII but also the unlabelled SgII contained in the PC2-ISGs. For the hypothetical situation of one PC2-ISG fusing with one PC12-ISG, only half of the total processed SgII at any one time will give rise to [^{35}S]-sulphate labelled p18. In other words, assuming a constant activity of PC2 in fused and in non-fused ISGs, and complete mixing of labelled and unlabelled content proteins, including SgII, one would predict that complete processing of the labelled SgII would take twice as long in a fused "hybrid" ISG as in a PC2-ISG. The apparent lag-period before accumulation of [^{35}S]-sulphate labelled starts in "hybrid" ISGs might indicate that PC2 first processes the unlabelled substrate in its proximity before starting to process the labelled substrate derived from the PC12-ISG core.

Processing seemed to increase further even after 90 min incubation time but at a very slow rate such that incubating for an additional hour only yielded an additional 10% of signal (Figure 5/9a). A 90 min incubation time was therefore chosen as a good compromise between a fast assay and a maximal signal.

The PC2-independent signal was generated during the processing reaction (Figure 5/9a) rather than during the fusion incubation (Figure 5/8) indicating that the endogenous proteolytic enzyme might have an acidic pH-optimum. Subcellular fractionation indicated that the endogenous enzyme activity is located in post-TGN vesicles (Figure 5/4). Given that the [^{35}S]-sulphate-labelled SgII is efficiently sorted

into the regulated secretory pathway, this distribution of PC2-independent processing suggests that this enzyme is present in secretory granules. Why is it then that PC2-independent generation of p18 is not observed in overnight [^{35}S]-sulphate labelled PC12 cells (Figure 4/7)? Although the PC12 cell endogenous enzyme has activity at acidic pH, its steady state distribution in the cell could be such that only little enzyme resides in a compartment with a low pH. It is tempting to speculate that this enzyme might be removed from ISGs in a manner similar to furin (Dittie, *et al.*, 1997).

Chapter 6: Towards the identification of molecules involved in the fusion of immature secretory granules

6.1 Objective

The general aspects of the cell-free ISG fusion have been described in detail in Chapter 5. The focus of this chapter is the identification of molecular components involved in ISG-fusion. The approach taken here to identify such factors is based on observations made in other assays measuring intracellular transport events. In order to investigate the requirements of cytosolic factors in this fusion assay, the system was modified such that exogenous cytosol could be added to membranes prepared from a PC12 cell PNS. In some experiments, either the PNS or PC2-ISGs were preincubated before being used in the fusion assay.

6.2 Homotypic fusion of ISGs is not sensitive to Botulinum neurotoxins

It has been shown in Chapter 5 (section 5.7, Figure 5/10), that ISG fusion is sensitive to trypsin digestion of the PC2-ISGs. This suggested that fusion of ISGs required at least one or several membrane associated proteins. While trypsin acts on many proteins, the family of clostridial neurotoxins are Zn^{2+}-proteases with a very high substrate specificity. Each of them cleaves only one single protein with high efficiency, with the exception of BoNT C which cleaves two substrates (Montecucco and Schiavo, 1995). The targets of the clostridial neurotoxins are a group of proteins involved in membrane fusion events called SNAREs (Niemann, *et al.*, 1994). The abbreviation SNARE stands for SNAP-receptor proteins and these SNARE proteins form

complexes with each other that bind SNAP (soluble NSF-attachment protein) and NSF (Rothman, 1996; Söllner, *et al.*, 1993a; Söllner, *et al.*, 1993b).

Originally the SNAREs were divided in two subfamilies, the v-(or vesicle-associated) SNAREs and the t-(or target membrane associated) SNAREs, indicating their relative position in heterotypic fusion events like the exocytotic fusion of synaptic vesicles with the presynaptic membrane (Rothman and Warren, 1994). This discrimination has become more blurred with the identification of SNAREs (e.g. the Golgi-associated SNARE, Gos28 (Nagahama, *et al.*, 1996; Subramaniam, *et al.*, 1996) that do not fit well in either category. Proteins initially identified as t-SNAREs have been found on vesicles (e.g. syntaxin 1 and SNAP-25 (Tagaya, *et al.*, 1996; Tagaya, *et al.*, 1995; Walch-Solinema, *et al.*, 1995)). Furthermore, in the face of an involvement of SNAREs in homotypic fusion events, the v-/t-SNARE nomenclature is inappropriate (Nichols, *et al.*, 1997).

Interaction between v- and t-SNAREs is strongly implicated in the specific interaction between membranes (docking) and in events leading up to the fusion of intracellular membranes including the fusion itself (Rothman and Warren, 1994; Söllner, *et al.*, 1993a). VAMP2, syntaxin 1 and SNAP-25, the SNAREs involved in fusion of synaptic vesicles with the pre-synaptic membrane, are found in a stoichiometric complex in detergent extracts of brain membranes. It has been suggested that this complex represents a docking or fusion intermediate (Söllner, *et al.*, 1993a). The same SNAREs have also been found to be involved in exocytosis of chromaffin granules (Glenn and Burgoyne, 1996) and secretory granules in PC12 cells (Banerjee, *et al.*, 1996). The SNAREs acting in exocytosis are also referred to as (nerve-) "terminal" SNAREs.

Clostridial neurotoxins derive from bacterial strains of the genus clostridium. They include tetanus toxin and the botulinum neurotoxins (BoNT) which have been classified into serotypes A-G. They are expressed as a single polypeptide chain which is proteolytically cleaved to the active di-chain toxin and held together by a disulphide

bond which is required for neurotoxicity (Montecucco and Schiavo, 1995). The heavy chain is essential for the neurospecific binding and translocation of the proteolytically active light chain into the cytosol (Montecucco and Schiavo, 1995). This latter can be expressed as a recombinant protein in bacteria and used in cell-free assays. Alternatively the holo-enzyme can be isolated from bacterial cultures, purified and either used for poisoning competent cells or activated by proteolysis and reduction of the disulphide bond for use in cell-free assays (Schiavo and Montecucco, 1995).

VAMP2 (as well as VAMP1 and cellubrevin/VAMP3) is cleaved by tetanus toxin and the BoNTs serotype B, D, F and G, while syntaxin 1A and 1B as well as syntaxin 2 are cleaved by BoNT serotype C (Montecucco and Schiavo, 1995). SNAP-25 is cleaved by BoNT serotype A and E as well as by C (Foran, *et al.*, 1996; Schiavo, *et al.*, 1993). Other SNAREs are involved in other intracellular fusion events (Hay and Scheller, 1997), but with the exception of the v-SNARE cellubrevin, VAMP1, syntaxin 1b and syntaxin 2 none of the other SNAREs are cleaved by the clostridial neurotoxins (Montecucco and Schiavo, 1995; Niemann, *et al.*, 1994).

VAMP2 has been found on secretory granules of PC12 cells (Papini, *et al.*, 1994) and this integral membrane protein is therefore expected to be found on ISGs. Syntaxin 1 and SNAP-25 are predominantly associated with the plasma membrane in neurones and chromaffin cells, however a small amount has been found on synaptic vesicles and on chromaffin granules (Tagaya, *et al.*, 1996; Tagaya, *et al.*, 1995; Walch-Solinema, *et al.*, 1995). To investigate whether the same SNAREs that are involved in exocytosis also play a role in homotypic granule fusion, the effect of the botulinum neurotoxins on the fusion of ISGs was studied in the following experiment. The activity of the proteases was confirmed by Dr. G. Schiavo in independent experiments. PC2-ISGs were preincubated with the BoNT A, C and D for 30 min at 37°C in the presence of an ATP-regenerating system to break up any SNARE-complexes in the ISG-membrane and thus to ensure that all the SNAREs were accessible to the toxins. The ISGs were then reisolated by centrifugation and added to a fusion incubation

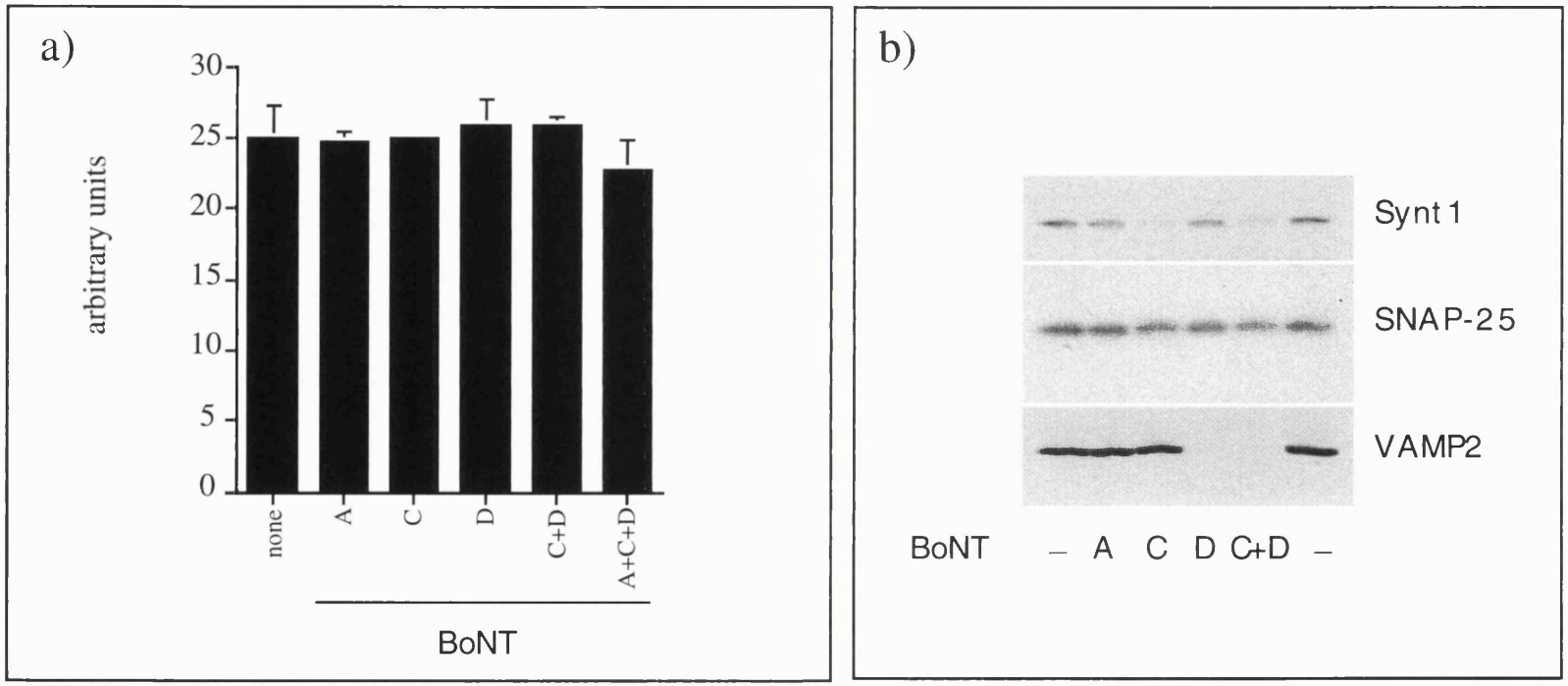


Figure 6/1: *Effect of botulinum neurotoxins (BoNT) on cell-free ISG fiusion*

PC2 ISGs were pretreated for 30 min at 37°C with 20 nM BoNT A, C, D, or, C and D, or A, C and D as described in Chapter 2. **(a)** The ISGs were pelleted by centrifugation, resuspended in buffer and added together with an ATP-regenerating system to a PNS derived from PC12 cells labelled for 30 min with [^{35}S]-sulphate. Standard fusion and processing reactions were performed and analysed as described in Chapter 2. Error bars indicate SD (n=3). **(b)** Aliquots of ISGs pretreated with botulinum neurotoxins as above were analysed by SDS-PAGE and immunoblotting with antibodies against Syntaxin 1 (Synt 1), SNAP-25 and VAMP2.

containing a PNS derived from PC12 cells labelled with a 30 min [^{35}S]-sulphate pulse and an ATP-regenerating system. Figure 6/1a shows that fusion was not inhibited by pretreatment of the PC2-ISGs by either BoNT A, C or D.

Studies on homotypic fusion of yeast vacuoles suggested that homotypic fusion only requires one v-SNARE on one membrane and its cognate t-SNARE on the other membrane (Nichols, *et al.*, 1997) and it could be argued that in the above experiment one of the cognate SNAREs was uncleaved. For example VAMP2 would still be functional on the PC12 ISGs in the PNS and could interact with the syntaxin 1 and SNAP-25 present on the BoNT D-treated PC2-ISGs. Therefore the PC2-ISGs were pretreated with a cocktail of either C and D or all three BoNTs in order to cleave all the SNAREs that interact with each other in exocytosis. Even with all three toxins present no significant inhibition of fusion was observed (Figure 6/1a). Immunoblotting of PC2-ISGs pretreated with BoNTs in parallel revealed complete cleavage of VAMP2 by BoNT D (Figure 6/1b, lanes 4 and 5) and very extensive cleavage of syntaxin 1 by BoNT C (lanes 3 and 5), while surprisingly no cleavage of SNAP-25 either with BoNT C or A could be detected (lanes 2 and 3).

6.3 Fusion of ISGs is cytosol dependent

Cytosol was obtained from PC12 or PC12/PC2 cells by preparing a PNS and removing the membranes by centrifugation (100,000 g) for 1 hour. PC12 cells were [^{35}S]-sulphate labelled as usual and a PNS was prepared. To obtain a membrane fraction, 100 µl of the PNS (equivalent to the amount used per sample in the fusion assay) was laid on top of 1 ml of a 0.5 M sucrose cushion and subjected to centrifugation at 100,000 g for 1 hour. The supernatant was removed and the pellet containing the membranes was resuspended either in buffer or in cytosol. PC2-ISGs and an ATP-regeneration system were added and the samples were incubated for 30 min at 37°C followed by the usual processing incubation and immunoprecipitation. The

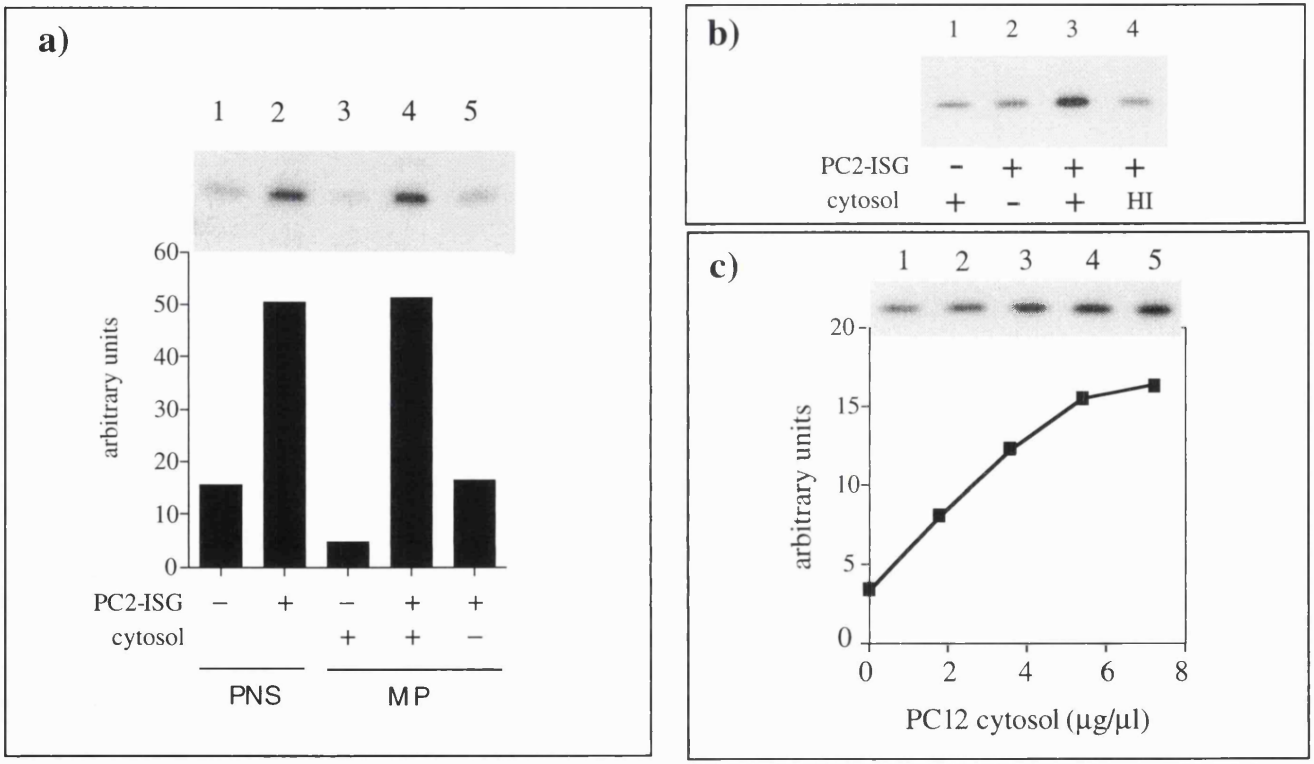


Figure 6/2: *Cytosol-dependence of cell-free ISG fusion*

(a) A PNS was prepared from PC12 cells labelled by a 30 min [^{35}S]-sulphate pulse. An aliquot of this PNS was used to prepare a membrane pellet (MP). The membranes were resuspended in either PC12 cytosol (3.17 mg/ml final concentration) (lane 4) or buffer (lane 5). PC2-ISGs were added either to the PNS (lane 2) or to the MP and Standard fusion reactions were carried out as described in Chapter 2. **(b)** Standard fusion reactions were carried out with a membrane pellet resuspended in PC12 cytosol (5.4 mg/ml final concentration, lane 3) or buffer (lane 2) as in (a) or in heat inactivated (HI) cytosol (lane 4) and analysed as described in Chapter 2. **c)** PC2-ISGs were added to a membrane pellet resuspended either in buffer (lane 1) or PC12 cytosol at final concentrations of 1.8 (lane 2), 3.6 (lane 3), 5.4 (lane 4) or 7.2 (lane 5) mg/ml. Standard fusion reactions were carried out and analysed as described in Chapter 2.

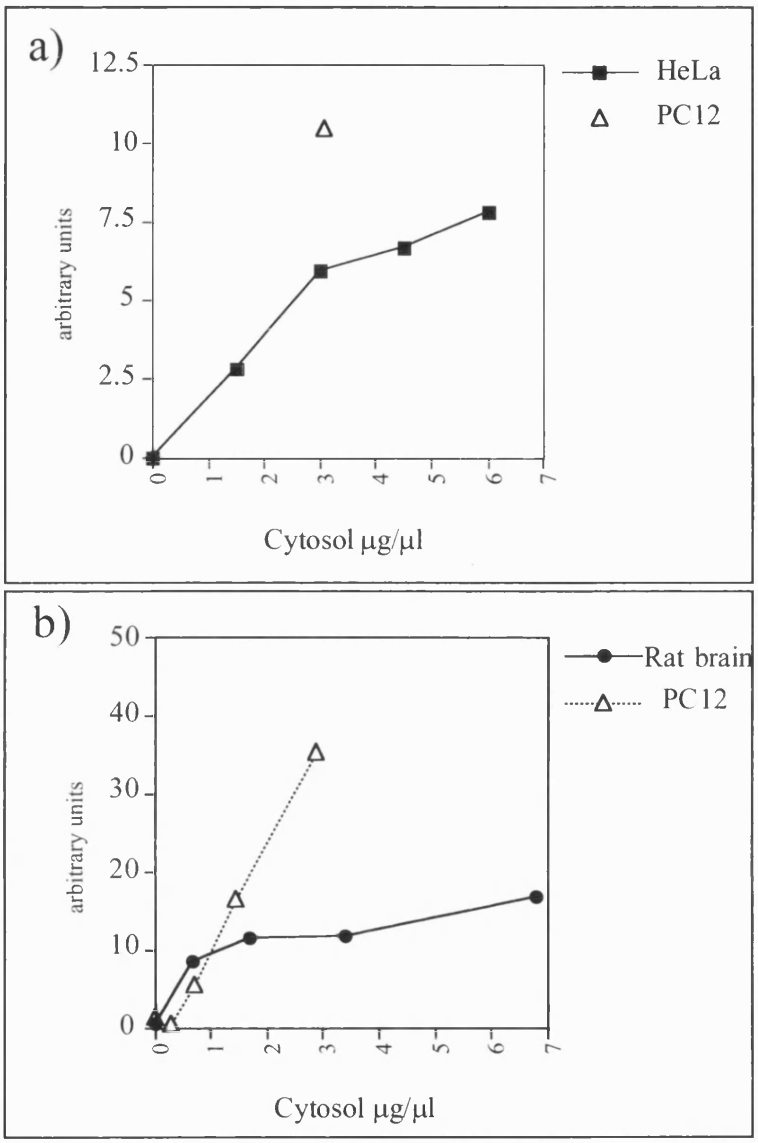


Figure 6/3: *Comparison of cytosols derived from different sources*

(a) A membrane pellet was prepared from a PNS derived from [^{35}S]-sulphate labelled PC12 cells and resuspended in either buffer, cytosol derived from PC12 (3 mg/ml) or HeLa cells (final concentrations: 1.5, 3.0, 4.5, 6.0 mg/ml). PC2-ISGs and an ATP-regenerating system were added and the cell-free incubations incubated for 30 min at 37°C. **(b)** A membrane pellet was prepared from a PNS derived from [^{35}S]-sulphate labelled PC12 cells and resuspended in either buffer, cytosol derived from PC12 cells (0.29, 0.7, 1.4, 2.9, mg/ml) or from Rat brains (final concentrations: 0.7, 1.7, 3.4, 6.8 mg/ml). PC12/PC2 ISGs and an ATP-regenerating system were added and the samples were incubated for 30 min at 37°C. All further manipulations were as described in Chapter 2. The amount of [^{35}S]-sulphate labelled p18 was analysed by SDS-PAGE and fluorography. Quantitation was by densitometry and the cytosol-independent signal was substracted.

fusion signal was compared with the signal obtained in a fusion assay using a total PNS and was found to be very similar (Figure 6/2a, lanes 2 and 4). Fusion was strictly dependent on cytosol (compare lanes 4 and 5). Addition of cytosol in the absence of PC2-ISGs did not result in a fusion signal (lane 3). To ensure that the activity in the PC12 cytosol was due to a proteinaceous activity, an aliquot of cytosol was boiled for 5 min before being used in a fusion assay. Figure 6/2b shows that heat-inactivated cytosol does not support fusion (lane 4). Titration of the PC12 cytosol showed that saturation was only reached at a concentration of 7 mg/ml (Figure 6/2c).

Cytosol derived in a similar way from HeLa cells also supported ISG-fusion. However at a concentration of 3 mg/ml HeLa cytosol supported in the same experiment only about half as much fusion as PC12 cytosol at the same concentration (Figure 6/3a). PC12 cytosol was also compared with cytosol derived from rat brain, a tissue which is specialised for regulated secretion and might therefore be suitable for large scale production of cytosol. However, rat brain cytosol prepared in the same way as PC12 cytosol was very ineffective in supporting ISG fusion (Figure 6/3b). At a half maximal concentration of 3 mg/ml PC12 cytosol gave in the same experiment a two fold higher signal than that observed for rat brain cytosol at 7 mg/ml. .

6.4 GDI removes some but not all rab proteins from ISG membranes

His-tagged rab3-GDI (Sasaki, *et al.*, 1990, Elazar, *et al.*, 1994) was obtained from Dr. Francis A. Barr and was used to investigate whether proteins of the rab-family of small GTPases are involved in homotypic fusion of ISGs. GDI plays an important role in the rab-cycle in allowing the rab proteins to be recycled after use (Pfeffer, 1994). GDI only interacts with the inactive, GDP-bound form of the rab proteins and is able to remove GDP-rabs from membranes (Soldati, *et al.*, 1993; Ullrich, *et al.*, 1993). The cytosolic pool of rab proteins is complexed with GDI

(Regazzi, *et al.*, 1992) and this complex is able to deliver the rabs to their correct membrane location (Dirac-Svejstrup, *et al.*, 1994; Ullrich, *et al.*, 1994b; Ullrich, *et al.*, 1993). Although several mammalian isoforms of GDI proteins exist, rab3 GDI was found to interact more or less indiscriminately with all rab proteins tested so far (Shisheva, *et al.*, 1994; Ueda *et al.*, 1991; Yang, *et al.*, 1994; Nishimura, *et al.*, 1994). Addition of GDI to a number of transport assays had an inhibitory effect and this inhibition was correlated with the removal of rab proteins from the membranes (Elazar, *et al.*, 1994; Ward, *et al.*, 1997; Peter, *et al.*, 1994; Dirac-Svejstrup, *et al.*, 1994). Only a few rab proteins have been detected by GTP-overlay on mature secretory granules of PC12 cells (S. Urbé, L. Huber and S. Tooze, unpublished results and Urbé, 1993). Amongst these, rab3a and rab11 were the only known rab proteins. The pattern for ISGs was slightly more complex, however it cannot be excluded that some of the small GTPases detected by immunoblot or by GTP-overlay in the ISG-fraction were actually derived from contaminating membranes. This might for example be the case for rab6, which has elsewhere been localised both to post-Golgi vesicles (Jasmin, *et al.*, 1992; Tixier-Vidal, *et al.*, 1993) and to the cis and medial Golgi (Antony, *et al.*, 1992; Goud, *et al.*, 1990; Martinez, *et al.*, 1994). Overexpression of a rab6 mutant defective in GTP-hydrolysis revealed a phenotype reminiscent of BFA treatment suggesting a role of rab6 in retrograde transport from the Golgi to the ER and in intra-Golgi transport (Martinez, *et al.*, 1997). The presence of rab11 on ISG-membranes was directly demonstrated by immunoisolation of [^{35}S]-sulphate labelled ISGs (Urbé, *et al.*, 1993). Rab11 is also associated with MSGs, TGN-membranes, constitutive secretory vesicles (Urbé, *et al.*, 1993) and recycling endosomes and has been shown to be implicated in the traffic through the recycling endosomal compartment (Ullrich, *et al.*, 1996). On the other hand, rab3 was clearly depleted in the ISG fractions suggesting that rab3 might associate only at a late stage with the secretory granule (Matteoli, *et al.*, 1991; Urbé, 1993).

Addition of excess GDI has been shown to remove rab proteins from membranes (Soldati, *et al.*, 1993; Ullrich, *et al.*, 1993) either by mass action, that is by

shifting the equilibrium between membrane-bound and cytosolic forms of the rabs, or by competing in some way with membrane delivery of cytosolic rabs. To investigate whether the His-tagged rab3-GDI was able to remove rab proteins from ISG-membranes, ISGs isolated from PC12 cells were incubated for 15 min at 37°C in the presence of increasing amounts of GDI. A PNS was prepared from PC12 cells and incubated with GDI under the same conditions as the ISGs. Membrane- and cytosolic fractions were separated by ultracentrifugation (1 hr, 100,000 g) and the proteins of each fraction were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with antibodies against rab11, rab6, GDI and CgB. The majority of the endogenous GDI was found in the cytosol in agreement with the literature (Pfeffer, 1994), while the bulk of rab11 and rab6 were found on the membranes (Figure 6/4, lane 1, compare a and b). All the rab11 and rab6 in the ISG-fraction were found on the membrane, indicating that the rab proteins in the ISG-fraction are tightly associated with the membrane (lane 7, compare a and b). Incubation of the PNS and the ISGs at 37°C (lane 2 and 8) did not result in a dissociation of rab11 from either total membranes or ISGs (compare lanes 1 and 2, 7 and 8). The same procedure caused a redistribution of some rab6 from the cytosol to the membrane (Figure 6/4a and b, compare lane 1 and 2).

Addition of increasing amounts of GDI to the PNS and to the ISGs removed the majority of rab11 from the membranes (Figure 6/4c), while there was much less of an effect on rab6 (Figure 6/4d): At the highest concentration of GDI (3.3μM) approximately 85% of the rab11 was removed from the membranes in the PNS and 95% from the ISG membranes. At the same GDI concentration only 30% of the rab6 was removed from the membranes in the PNS and 60% from the ISG-membranes. More than 50% of the rab11 was removed at a GDI concentration of 0.65 μM. The distribution of chromogranin B (CgB) indicates that all the secretory granules are pelleted (Figure 6/4a) and that equal amounts of PNS and ISGs were used for each fraction.

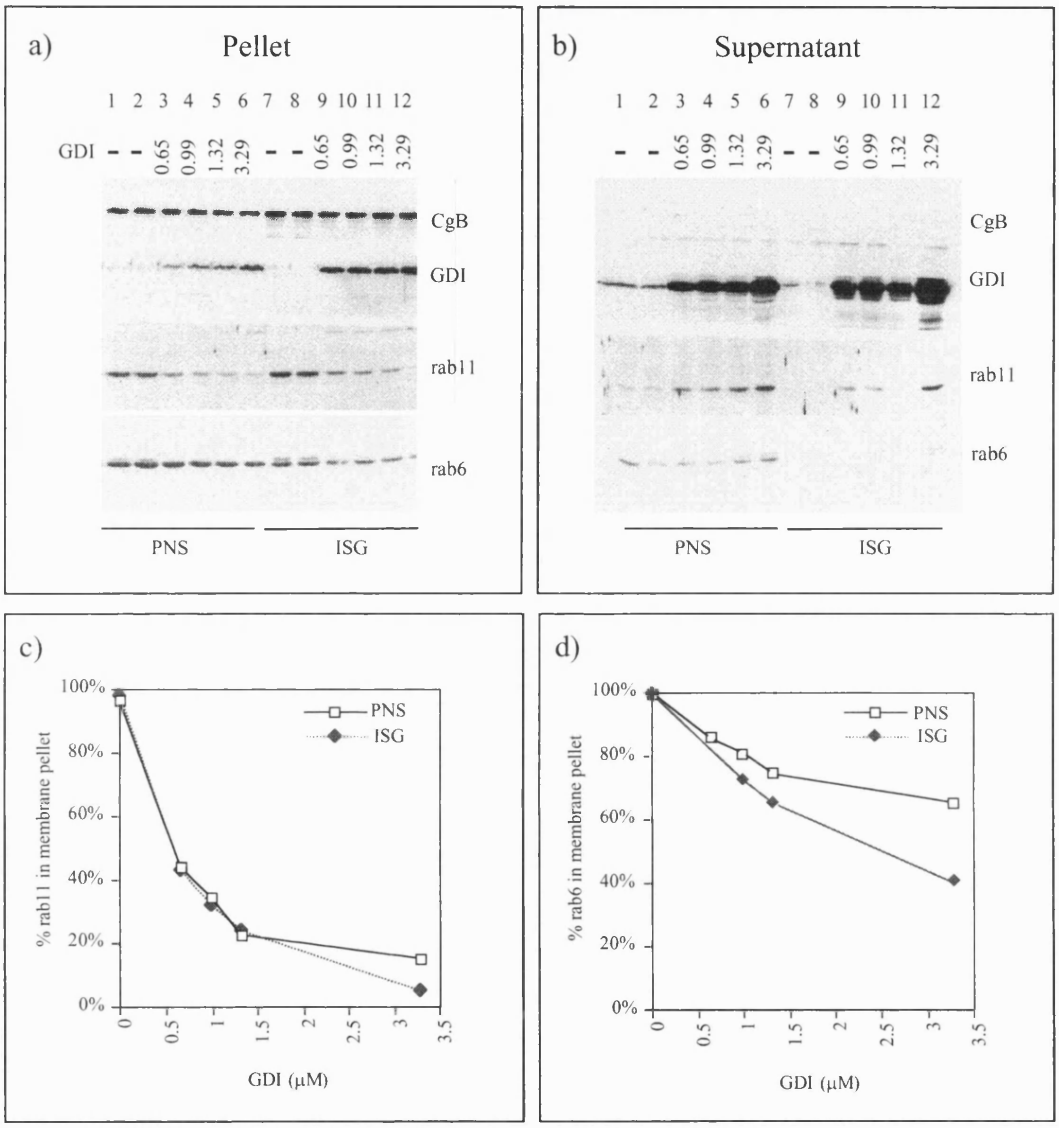


Figure 6/4: *Effect of rab3-GDI on the membrane association of rab11 and rab6*

Recombinant rab3-GDI (final concentration indicated in μM) was added to a PNS (750 μg) or to ISGs (200 μg) derived from PC12 cells and PC12/PC2 cells respectively, and incubated in fusion buffer for 15 min at 37°C (lanes 2-6 and 8-12) or 4°C (lanes 1, 7). The membranes were pelleted by centrifugation and the supernatants were TCA-precipitated. Both pellets (a) and supernatants (b) were analysed by 12% SDS-PAGE and immunoblotting with antibodies against Chromogranin B (CgB), GDI, rab11 and rab6. Detection was by ECL. (c) and (d) The amount of rab11 and rab6 bound to membranes was normalised to the amount bound in absence of GDI on ice (lanes 1. 7).

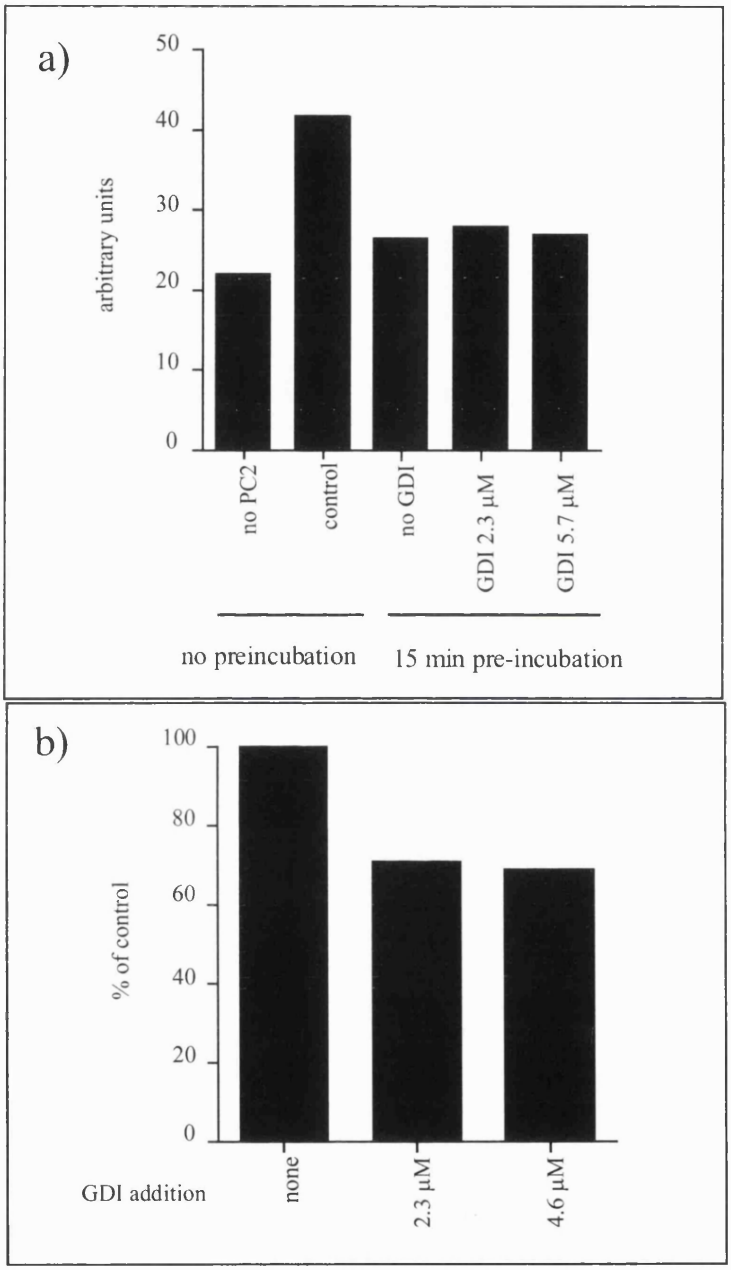


Figure 6/5: *rab3-GDI inhibits cell-free fusion of ISGs*

(a) A PNS made from PC12 cells labelled for 30 min with [^{35}S]-sulphate, and PC2-ISGs were either pre-incubated for 15 min at 37°C in the presence or absence of recombinant rab3-GDI or left on ice (no pre-incubation). PNS and ISGs were mixed and standard fusion reactions were performed and analysed as described in Chapter 2. **(b)** Standard cell free fusion incubations were carried out in the presence or absence of rab3-GDI where indicated (final concentration) and analysed as described in Chapter 2.

6.5 Inhibition of ISG fusion by GDI

It has been shown above that ISG fusion was strictly cytosol dependent and it is possible that one of the cytosolic factors required might be a rab-protein. The cytosolic pool of rab proteins exists as a complex with GDI and it is this complex that delivers the rab-protein to the membrane. Addition of excess GDI can shift the equilibrium of membrane bound and GDI-bound (that is cytosolic) pools of rab proteins and prevent a net delivery of rab proteins to the membrane (Dirac-Svejstrup, *et al.*, 1994). This is especially important if the assay involves several rounds of fusion and requires recycling of rab proteins. To investigate whether addition of excess GDI had an effect on ISG-fusion, a PNS was prepared from PC12 cells labelled for 30 min with [^{35}S]-sulphate and preincubated for 15 min at 37°C in the presence or absence of GDI. ISGs were isolated from PC12/PC2 cells and were preincubated in parallel. PNS and ISGs were then combined, supplemented with an ATP-regenerating system and incubated for 30 min at 37°C. As shown in Figure 6/5a preincubation of the PNS led to a rundown of the fusion assay independently of the addition of GDI. Therefore GDI was next added to the whole fusion incubation (consisting of PNS and PC2-ISGs) prior to transfer to 37°C. Under these conditions 2.3 μM GDI, which was sufficient to remove more than 80% of rab11 (Figure 6/4a and c), resulted in 30% inhibition of ISG-fusion, and doubling the GDI concentration did not have any further effect (Figure 6/5b).

6.6 Fusion of ISGs is NEM-sensitive

A landmark discovery in the field of intracellular membrane fusion is based on the use of the alkylating reagent *N*-Ethylmaleimide (NEM). A large number of fusion assays are inhibited in the mM range by this reagent. Based on this observation the ATPase NSF was identified as a key component of the intracellular fusion machinery (Block, *et al.*, 1988; Malhorta, *et al.*, 1988; Whiteheart, *et al.*, 1994). Recently another NEM-sensitive ATPase with a similar ATP-binding domain as NSF, called p97

(Peters, *et al.*, 1990), has been implicated in some fusion events (Acharya, *et al.*, 1995; Latterich, *et al.*, 1995; Rabouille, *et al.*, 1995) and it is likely that more proteins of this type exist (Confalonieri and Duguet, 1995).

First, the effect of NEM-treatment of the cytosol was investigated. PC12 cytosol was preincubated with 1 mM or 3 mM NEM for 10 min on ice and the excess NEM was quenched with 1.2 mM or 3.6 mM glutathione for 15 min. The results of a representative experiment are shown in Figure 6/6a. Treatment of the cytosol with NEM did not inhibit ISG-fusion, suggesting that the cytosolic factors required for ISG-fusion in this cell-free assay are not NEM-sensitive. Second, the effect of NEM-treatment of the membranes was investigated by preincubating the PNS (derived from PC12 cells labelled by a 30 min [^{35}S]-sulphate pulse) with 3 mM NEM for 10 min on ice, followed by 15 min quenching with 3.6 mM glutathione. The PNS was then centrifuged through a sucrose cushion as described in section 6.3. The recovered membranes were resuspended either in buffer, cytosol or in cytosol treated with NEM in the same way as the PNS. NEM-treatment of both cytosol and membranes resulted in approximately 80% inhibition of fusion (Figure 6/6b). This data indicate that the NEM-sensitive proteins essential for fusion are associated with the PNS-derived membranes. Given that the NEM-sensitive factors identified in other cell-free assays are at least partially cytosolic, untreated cytosol was added to NEM-treated membranes to test if ISG-fusion could be rescued. As shown in Figure 6/6b, untreated cytosol was able to rescue NEM-inhibition to 63% of the control. This suggests that the NEM-sensitive factor(s) associated with the membranes are also present in the cytosol, but are only required if the membrane-associated factors are inactivated.

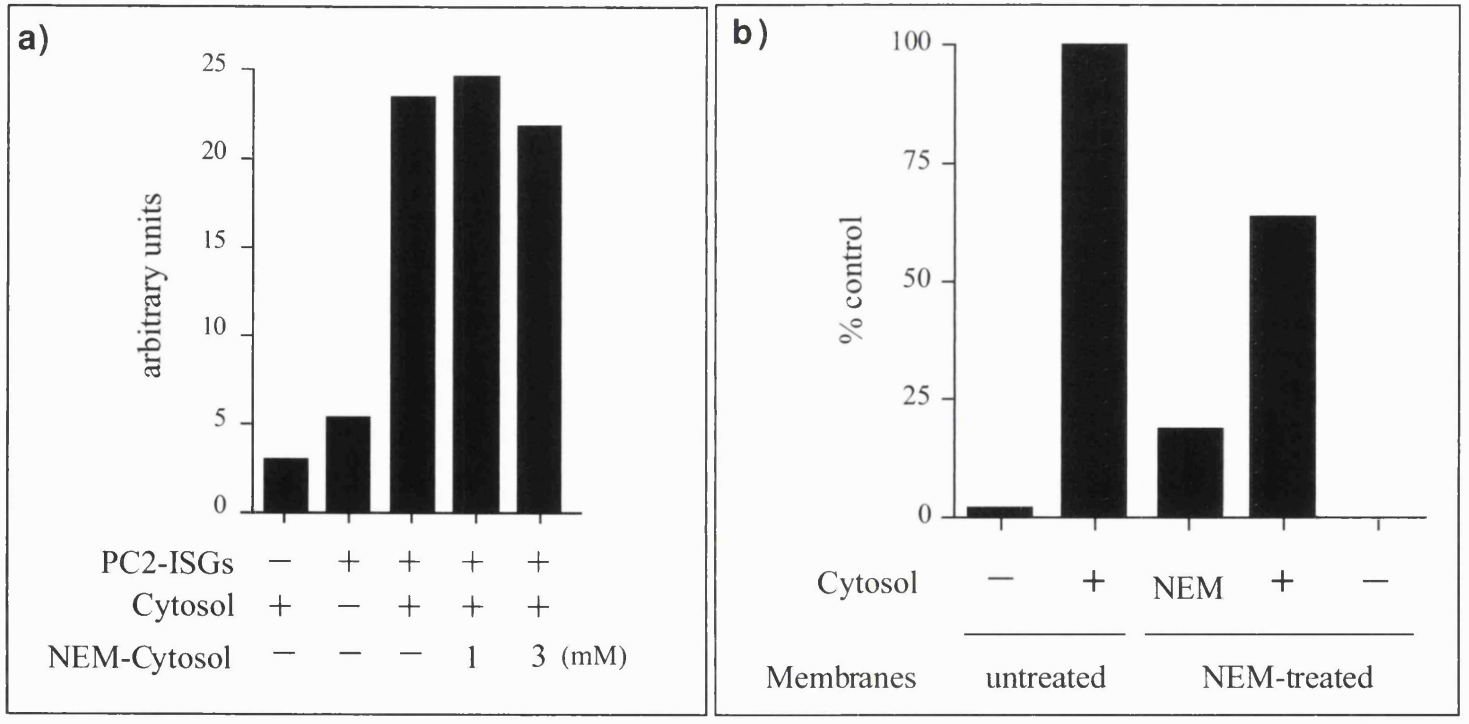


Figure 6/6: *Cell-free fusion of ISGs is sensitive to NEM-treatment*

(a) A membrane pellet was prepared from PC12 cells labelled for 30 min with [^{35}S]-sulphate. The membranes were resuspended in either buffer or cytosol pretreated with 0, 1, or 3 mM NEM for 15 min on ice and quenched for 10 min on ice with 3.6 mM glutathione. Standard fusion incubations were performed and analysed as described in Chapter 2. **(b)** A PNS was derived from PC12 cells labelled for 30 min with [^{35}S]-sulphate and pretreated with 3 mM NEM for 15 min on ice (pretreated) or not (untreated) and quenched for 10 min on ice with 3.6 mM glutathione. Membrane pellets were derived from this PNS and resuspended in either buffer, untreated cytosol, or cytosol pretreated with 3 mM NEM for 15 min on ice and quenched for 10 min on ice with 3.6 mM glutathione. Standard fusion incubations were carried out and analysed as described in Chapter 2.

6.7 Immunoblotting reveals that NSF and α-SNAP are associated with membranes while p97 and p47 are predominantly found in the cytosol

To investigate the relative abundance of the NEM-sensitive ATPases NSF and p97 on the membranes and in the cytosol used in the ISG fusion assay, 50 μg of a PNS, the membrane fraction (P) and supernatant (S) derived from this PNS, 50 μg of a PC2-ISG-sample (ISG), PC12 cytosol (Pcyt) and rat brain cytosol (RBC) were subjected to SDS-PAGE and the proteins were transferred to nitrocellulose. Antibodies against NSF, α-SNAP, p97 and p47, which seems to be essential for p97 interaction with or delivery to the membrane (Kondo, *et al.*, 1997) were used for immunoblotting of this material. Figure 6/7 reveals that the bulk of the NSF and α-SNAP are associated with the membrane pellet (lane 2) while only a little of these proteins are found in the supernatant (lane 3). Indeed, NSF was nearly undetectable in PC12 cytosol that was able to rescue NEM-inhibition of fusion (lane 5). Rat brain cytosol contained more NSF and α-SNAP (lane 6). The majority of p97 on the other hand, was found in the supernatant and in the PC12 cytosol (lanes 3 and 5). Interestingly p47 was exclusively detected in cytosolic fractions. Both NSF, α-SNAP, and p97 were associated with ISGs (lane 4) and NSF appeared to be enriched on ISGs compared to the total membrane fraction (compare lanes 2 and 4). The amount of NSF in the different samples was estimated by comparison with a titration of His-tagged NSF (lanes 7-9; table 6/1): A standard fusion assay contains between 400 and 700 μg of cytosol, therefore the PC12 cytosol in a standard fusion assay contained between 0.08 and 0.14 μg of NSF.

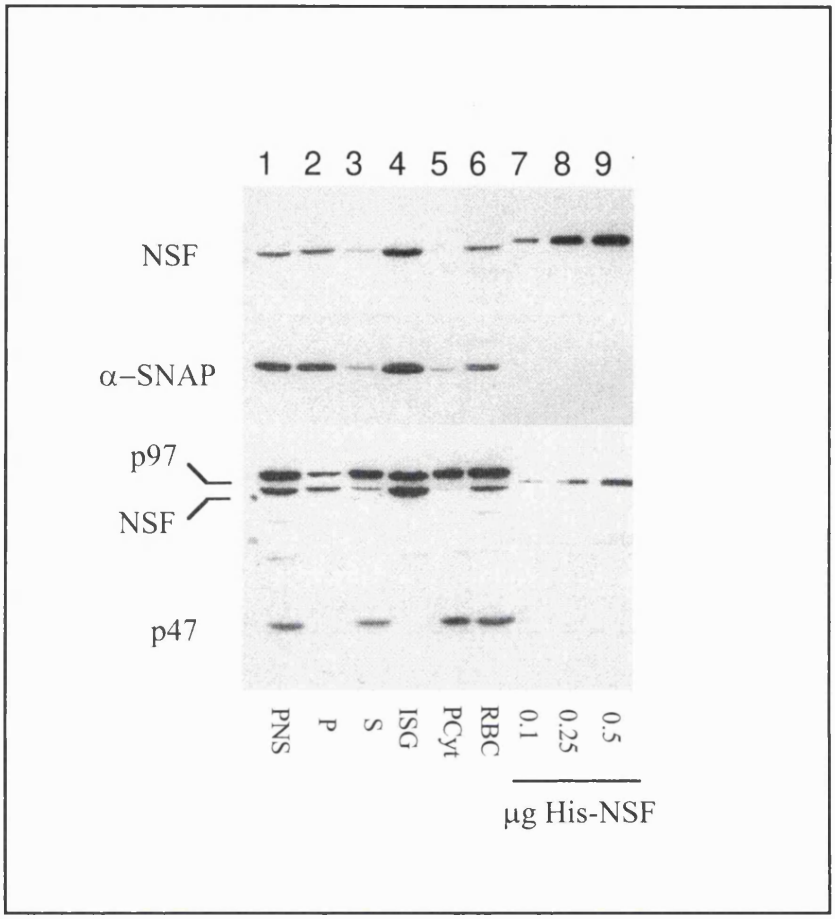


Figure 6/7: *Immunoblot of NSF, α-SNAP, p97 and p47.*

PNS (50 μg), the membrane fraction (P) and supernatant (S) derived from this PNS, PC12/PC2 ISGs (ISG, 50 μg), PC12 cytosol (Pcyt, 50 μg), rat brain cytosol (RBC, 50 μg) and His-tagged NSF were analysed by SDS-PAGE and immunoblotting with antibodies against NSF, α-SNAP, p97 and p47.

	µg NSF / 50 µg total protein
PNS	0.14
Membrane Pellet (P)*	0.16
Supernatant (S)*	0.04
ISG	0.34
PC12 cytosol (PCyt)	<0.01
Rat Brain Cytosol (RBC)	0.14

Table 6/1: *Estimation of the amount of NSF in subcellular fractions*

Samples of PNS (50µg), a membrane pellet and the supernatant derived from this PNS, PC12 cytosol (50µg), PC2-ISGs (50µg), PC12 cytosol (50µg) and rat brain cytosol (50µg) were analysed by SDS-PAGE, transferred to nitrocellulose and subjected to immunoblotting with an antibody against NSF. The amount of NSF in each sample was estimated by comparison with the immunoreactivity obtained for standards of His-tagged NSF loaded on the same gel (see Figure 6/7).

6.8 NSF and α-SNAP are partially able to rescue NEM-inhibition of ISG-fusion.

Recombinant His-tagged NSF and His-tagged α-SNAP were obtained from Dr. G. Schiavo. The activity of both proteins was tested by Dr. G. Schiavo in an independent functional assay which measures the ability of recombinant NSF and α-SNAP to disassemble the synaptic SNARE-complex containing VAMP2 and syntaxin 1.

α-SNAP mediates the binding of NSF to the SNARE complex (Söllner, *et al.*, 1993b). SNAPs have also been shown to stimulate neurotransmitter release from giant squid axons (DeBello, *et al.*, 1995) as well as catecholamine secretion from permeabilised chromaffin cells (Morgan and Burgoyne, 1995b). The effect of NSF and α-SNAP on ISG fusion was investigated in the following experiment. PC12 cells were labelled by a 30 min [^{35}S]-sulphate pulse. An aliquot of this PNS was treated for 10 min on ice with 3 mM NEM followed by quenching with 3.6 mM glutathione for 15 min on ice, while the control sample was only treated with glutathione. A membrane pellet of both untreated and NEM-treated PNS was obtained by centrifugation at 100,000g through a sucrose cushion and the membranes were resuspended in buffer (-cytosol), cytosol (+cytosol) or NEM-treated cytosol, supplemented with an ATP-regenerating system and fusion buffer and incubated in the absence (white bars) or presence (black bars) of 5μg NSF and 5 μg α-SNAP for 30 min at 37°C. Addition of NSF and α–SNAP in the absence of cytosol was not sufficient to support fusion (Figure 6/8a). This is in agreement with the observation that cytosol is absolutely required for fusion and that NEM does not effect the activity of this cytosol. NSF and α-SNAP did not stimulate cytosol dependent fusion but rather inhibited fusion by 40% (Figure 6/8a). In the presence of NEM-treated cytosol this inhibition was even larger. As described above (Figure 6/6b), untreated cytosol was able to rescue NEM-inhibition of NEM-treated membranes (by 60%) but this rescue was not stimulated but rather

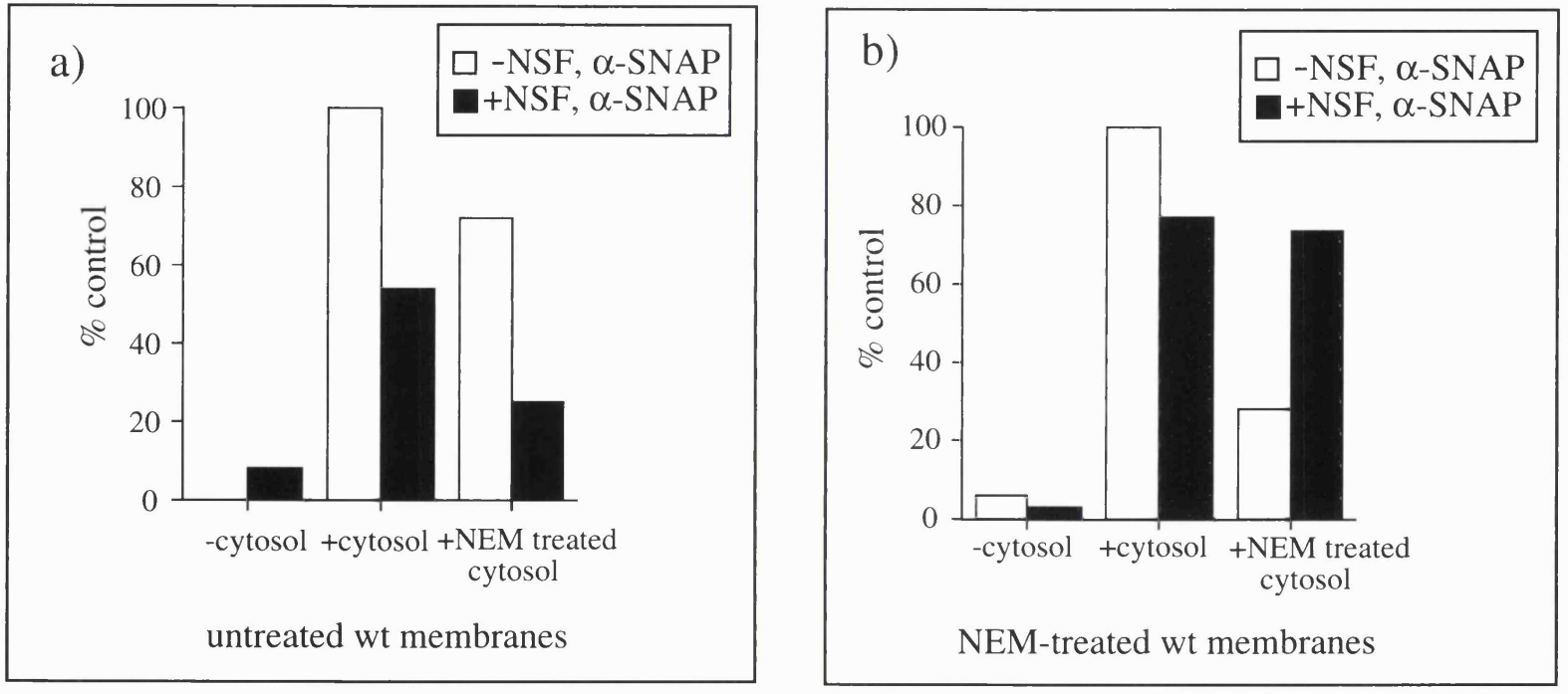


Figure 6/8: *NSF and α-SNAP can partially rescue cell-free ISG fusion.*

PC12 cells were labelled by a 30 min [^{35}S]-sulphate pulse. Samples of this PNS were pretreated either with 3 mM NEM for 10 min on ice and quenched with 3.6 mM glutathione or only quenched with glutathione (untreated). A membrane pellet of both untreated and NEM-treated PNS was obtained and resuspended in buffer (-cytosol), cytosol (+cytosol) or NEM-treated cytosol.

Standard fusion incubations were carried out in the absence (white bars) or presence (black bars) of 5μg NSF and 5 μg α-SNAP. Analysis was as described in Chapter 2.

decreased by addition of NEM and α-SNAP (Figure 6/8b). However if NSF and αSNAP were added to NEM-treated cytosol, NEM-inhibition was partially rescued (by 40%), suggesting that NSF and α-SNAP could partially replace the cytosolic factors that are able to rescue NEM-inhibition.

6.9 Discussion

6.9.1 SNAREs and ISG fusion

Current models envisage that intracellular membrane fusion is preceded by a highly controlled recognition process that ensures that fusion only occurs between the correct membranes. It has been proposed that the specificity of this recognition step relies on SNARE-proteins (Rothman, 1996; Rothman and Warren, 1994). Interaction between cognate SNARE-pairs is thought to be part of the docking mechanism (Söllner, *et al.*, 1993a) and was shown to be required for subsequent fusion (Hay and Scheller, 1997). The best-studied SNARE-proteins are those that were first identified and act in synaptic vesicle exocytosis (Söllner, *et al.*, 1993b). The v-SNARE, on the synaptic vesicle, involved in this fusion event is called VAMP and the t-SNAREs, on the presynaptic membrane, interacting with VAMP are called syntaxin 1 and SNAP-25. These SNAREs were first isolated as the receptors for the soluble NSF attachment proteins (SNAPs) and their role in regulated exocytosis was confirmed by the observation that the inhibition of exocytosis by the clostridial neurotoxins was in fact a direct consequence of cleavage of these SNAREs (Niemann, *et al.*, 1994; Poulain and Molgo, 1992).

The same neurotoxins that block exocytosis in the presynaptic terminal (Poulain and Molgo, 1992) as well as in chromaffin and PC12 cells (Ahnert-Hilgert, *et al.*, 1993), have been used here to investigate whether the so-called terminal, toxin-sensitive SNAREs play a role in a fusion event earlier in the cycle of regulated secretory

granules. Although the majority of syntaxin 1 and SNAP-25 are associated with the plasma membrane (Bennett, *et al.*, 1993; Hay and Scheller, 1997), a small amount of both proteins have been found in synaptic vesicles (Walch-Solinema, *et al.*, 1995) and in chromaffin granules (Tagaya, *et al.*, 1996; Tagaya, *et al.*, 1995). ISGs like MSGs can undergo regulated exocytosis (Tooze, *et al.*, 1991) and although this process has not yet been studied in great detail, it is most likely that they both will use the same basic machinery, whilst their regulation might well differ. Given that both VAMP2 and syntaxin 1 are integral membrane proteins and have been localised to chromaffin granules and to dense core granules in PC12 cells respectively (Tagaya, *et al.*, 1995, Papini, *et al.*, 1994), they are expected to be present in the ISG-membranes. SNAP-25 is not an integral membrane protein but is palmitoylated and a small amount has also been detected on chromaffin granules (Tagaya, *et al.*, 1996). The results presented in this chapter indicated that all three terminal SNAREs are present on ISGs (Figure 6/1b).

Pretreatment of the PC2-ISGs with the BoNT A, C and D in the presence of ATP did not have any effect although all of the VAMP2 and the majority of the syntaxin 1 in the cell had been cleaved (Figure 6/1). This suggests that neither VAMP2 and nor syntaxin 1 are required for fusion of ISGs. Surprisingly cleavage of SNAP-25 by either BoNT A or C was undetectable. Treatment with equivalent toxin concentrations achieved 80% cleavage of SNAP-25 in chromaffin cells (Foran, *et al.*, 1996) and 70% in synaptosomes (Blasi, *et al.*, 1993). The lack of SNAP-25 cleavage was not due to inactive neurotoxins as BoNT C was able to cleave syntaxin 1 and the activity of both toxins had been confirmed in independent experiments by Dr. G. Schiavo.

SNAREs engaged with each other in a 7S "docking"-complex are insensitive to cleavage by BoNT A and D (Hayashi, *et al.*, 1994). It has been suggested that SNAP-25, syntaxin 1 and VAMP2 found on chromaffin granules can form a complex and that this complex can bind αSNAP and NSF (Tagaya, *et al.*, 1995, Tagaya, *et al.*, 1996). However it is not clear whether this complex is present in situ or is assembled in the detergent extract during the preparation of the sample. The pretreatment was therefore

carried out in the presence of ATP, to allow dissociation of any potential SNARE complex by NSF-action and increase their accessibility to the toxins. No exogenous NSF was added to the preincubation and it is possible that the NSF detected in the ISG-fraction (see Figure 6/7) is engaged in some other interaction that prevents it from cycling even in the presence of ATP. As the majority of syntaxin 1 and all of the VAMP2 had been cleaved, it is possible that the uncleaved SNAP-25 was inaccessible to the toxins due to an interaction with another protein that might protect the cleavage site against the toxin.

Over the last couple of years the family of SNARE-proteins has been constantly growing and another 8 VAMP-like and 8 syntaxin-like SNAREs have been identified in EST-database searches (Bock and Scheller, 1997). One of these proteins might yet turn out to be localised to ISGs. What criteria does a putative ISG-SNARE have to fulfil ? One might expect the protein to be specifically expressed in cell-types and tissues with a regulated secretory pathway and be localised to the TGN and ISGs. It is conceivable that such a SNARE might be removed from the ISG by clathrin-dependent budding in the course of maturation. The SNARE could then be returned to the TGN and be reused for the biogenesis for another MSG. A SNARE involved in ISG-fusion might therefore be de-enriched from MSGs while being found in the TGN. A candidate SNARE that might have that distribution is syntaxin 6 (Bock, *et al.*, 1996). This t-SNARE is localised to the TGN and is also found close to clathrin coated buds in the TGN. This has been taken as an indication for the involvement of this protein in TGN to endosome transport (Bock, *et al.*, 1997). Another syntaxin homologue, syntaxin 3 has been found on zymogen granules (Gaisano, *et al.*, 1996) and it has been speculated that this SNARE might be involved either in fusions between mature zymogen granules (Edwardson, *et al.*, 1993) or in fusion of Golgi-derived pro-granules to form immature condensing vacuoles (Lew, *et al.*, 1994).

6.9.2 Cytosolic factors involved in ISG fusion

ISG fusion was shown to be completely cytosol dependent. The cytosol concentration required for a maximal signal was quite high (7 mg/ml) compared to other assays. This might however be dependent on the way the cytosol was prepared. All the cytosols used in this assay were prepared in the absence of salt (except $Mg(OAc)_2$) and this might effectively deplete or inactivate some proteins from the cytosol. Thus, the actual concentration of essential cytosolic factors might be lower than the total protein would suggest.

Although some proteins might be depleted from the cytosol, fusion was still strictly dependent on addition of cytosol. The factor(s) required were not NEM-sensitive as NEM-treated cytosol was more or less as effective as untreated cytosol. Although PC12 cell cytosol was more active, HeLa cytosol was also able to support fusion of ISGs arguing against the requirement of an essential cytosolic factor specific to regulated secretory cells. The fact that HeLa cytosol was not as active as PC12 cell cytosol might be due to the relative abundance of essential or rate-limiting factors required for fusion of ISGs although the titration curve for HeLa cytosol seems to plateau within the same range as PC12 cell cytosol. Alternatively, the difference could be related to the difference in species. The latter could be controlled for by using cytosol from NRK or BHK cells, for example. Rat brain cytosol was inactive and it is possible that this cytosol contains some inhibitory activity that is not present in cell culture cytosol. Interestingly the rat brain cytosol had a much higher concentration of NSF and α-SNAP than the PC12 cell cytosol.

6.9.3 Involvement of rab proteins in ISG-fusion

Potential candidates for the cytosolic factor(s) required for ISG fusion include the rab proteins. These ras-like small molecular weight GTPases have been proposed to act as a molecular switch involved in the quality control of vesicular transport (Novick and Zerial, 1997; Pfeffer, 1994). The function of rab proteins is for the most part still unclear. Some rabs seem to interact with elements of the cytoskeleton or with signal transduction molecules, while it is generally assumed that they co-ordinate and control the assembly of protein complexes on the membranes (Novick and Zerial, 1997). Although they were initially identified as proteins essential for vesicular transport (Salminen and Novick, 1987), for a long time there seemed to be no connection between their function and the seemingly ubiquitous action of NSF (Novick and Brennwald, 1993). A series of recent observations has now shown that some rab proteins act upstream of NSF and suggest that they are involved in the activation of SNAREs (Mayer and Wickner, 1997; Sogaard, *et al.*, 1994). The yeast rab1 homologue Ypt1 was required for association of Bos1 and Sec22 and overexpression of bos1 and sec22 (two SNAREs involved in ER to Golgi) together were able to overcome the *ypt1* mutant phenotype (Lian, *et al.*, 1994). Recent data have also shown convincingly that Ypt1 enables Sed5 to bind Bos1 by displacing the N-sec1 homologue Sly1 (Lupashin and Waters, 1997). Therefore one function of at least some of the rab proteins might lie in the control of SNARE-interactions, adding another layer of control to ensure the fidelity of vesicle targeting.

The cytosolic pool of rab proteins is associated with GDI (Regazzi, *et al.*, 1992). Addition of excess GDI has been shown to remove rabs from membranes (Ullrich, *et al.*, 1993, Soldati, *et al.*, 1993). Excess GDI removed rab11 from ISG-membranes, but was less efficient in removing rab6. The inefficient removal of rab6 might be due to the fact that several GDI-isoforms exist (Sasaki, *et al.*, 1990; Ueda, *et al.*, 1991; Yang, *et al.*, 1994), and while the isoform, rab3-GDI, used in this assay

removes most rab proteins, it might interact better with some than with others. Rab6 was found both in complex with rab3-GDI and in a complex with β-GDI (depending on the cell-type) and could be removed from CHO membranes with small amounts of rab3-GDI (Yang, *et al.*, 1994).

Addition of excess GDI has also been shown to inhibit the fusion of endosomes and lysosomes (Ward, *et al.*, 1997) as well as transport from ER to Golgi (Peter, *et al.*, 1994), cis to medial Golgi (Elazar, *et al.*, 1994) and late endosomes to the TGN (Dirac-Svejstrup, *et al.*, 1994). GDI interacts specifically with the GDP-bound (inactive) form of the rab proteins and plays an essential role in the rab cycle by regulating rab protein distribution between cytosol and membranes (Araki, *et al.*, 1990; Ullrich, *et al.*, 1993, Pfeffer, 1994). GDI delivers rabs to membranes where they undergo exchange of GDP for GTP, and also removes the GDP-rabs from membranes after they have hydrolysed their GTP, thereby returning them to the cytosolic pool (Dirac-Svejstrup, *et al.*, 1994; Ullrich, *et al.*, 1994b). It has been shown that GTP-hydrolysis is not required for rab5 function in endosome fusion (Rybin, *et al.*, 1996). It is therefore likely that GDI effects transport and fusion assays by interfering with the recycling of GDP-rabs from or the recruitment of GDP-rabs to the membrane.

It was shown above that GDI was able to remove 80% of the rab11 from the ISG membranes, but only inhibited fusion by 30% (Figures 6/4 and 6/5). These results suggest that fusion of ISGs does not depend on recruitment to the membrane of rab11 or other rab-proteins interacting with rab3-GDI. It is thus unlikely that the essential cytosolic factor required for ISG-fusion is a rab-protein.

This does however not exclude that the 20% rab11 (or another unknown rab-protein) that were still associated with the membranes are involved in ISG-fusion. As GDI does not interact with the GTP-bound form, this pool might in fact constitute the active (GTP-bound) rab11. It is also possible that rab proteins have an effect on the kinetics of ISG fusion. It has been shown that rab5 is a rate-limiting factor for early endosome fusion (Bucci, *et al.*, 1992) and it would be interesting to study the effects of

GDI on the kinetics of ISG fusion. A definitive answer to the question whether rab11 is involved in ISG fusion will require the addition of the modified (geranyl-geranylated) protein and rab11-mutants defective for GTP-hydrolysis or GDP/GTP exchange to the fusion assay.

6.9.4 An NEM-sensitive factor involved in ISG fusion

NEM has been shown to inhibit a large number of intracellular fusion assays. In most cases the NEM-sensitive factor was a cytosolic protein and NEM-inhibition could be rescued by addition of cytosol (Rothman, 1994). NEM also inhibits ISG fusion and the NEM-sensitive factor seems to be membrane associated as NEM-treatment of membranes was necessary for inhibition while NEM-treatment of the cytosol alone did not have an effect (Figure 6/6). NEM-inhibition of ISG-fusion could be largely overcome by addition of fresh cytosol. Complete rescue was not observed and it is possible that the NEM-inactivated membrane bound factors have to be removed from their binding sites to allow access of active protein from the cytosol. So far attempts to wash the membranes with salt and reconstitute ISG fusion with fresh cytosol have not been successful.

The observation that a large amount of NSF and α-SNAP are bound to the membranes of isolated ISGs (Figure 6/7) is in agreement with the finding of Burgoyne and Williams who detected NSF and α-SNAP on chromaffin granules (Burgoyne and Williams, 1997). NSF has also been shown to be associated with clathrin coated vesicles (Steel, *et al.*, 1996) as well as with pre-docked synaptic vesicles (Hong, *et al.*, 1994). It has been suggested that NSF might bind to vesicles long before docking, possibly during budding, and might have a function prior to fusion in a pre-docking stage (Diaz, *et al.*, 1989a; Scheller, 1995; Wattenberg, *et al.*, 1992). Indeed NSF and α-SNAP were found to act in a priming step early on in the events leading up to exocytosis from chromaffin and PC12 cells (Banerjee, *et al.*, 1996; Mayer, *et al*, 1996;

Chamberlain, *et al.*, 1995). Furthermore in an assay monitoring homotypic fusion of yeast vacuoles the two fusion partners could be incubated and "primed" separately with NSF with the effect that NSF was then no longer required in the fusion reaction (Mayer, *et al.*, 1996). On these grounds, it has been suggested that NSF might prime fusion by breaking up SNARE complexes (Hay and Scheller, 1997). All these data have been taken to indicate a role for NSF prior to formation of the 20S complex (Hay and Scheller, 1997; Morgan and Burgoyne, 1995a). Several potential binding sites for NSF on vesicles have been identified. Recently it has been suggested that NSF can bind to the t-SNARE syntaxin on its own (Hanson, *et al.*, 1995).

What is the NEM-sensitive factor required for fusion of the ISGs? From the data presented here, it can be derived that this protein is present on the membranes and can be recruited from cytosol. NSF is *a priori* a likely candidate and has been shown to be required for a number of different intracellular fusion events including intra Golgi transport (Block, *et al.*, 1988), vesicular transport in yeast (Wilson, *et al.*, 1989), early endosome fusion (Diaz, *et al.*, 1989a), ER to Golgi transport (Beckers, *et al.*, 1989), fusion of Golgi derived vesicles with Golgi cisternae (Rabouille, *et al.*, 1995), transport from early to late endosomes (Robinson, *et al.*, 1997) and lysosome fusion (Ward, *et al.*, 1997), for review see (Whiteheart, *et al.*, 1994).

However, it is possible that NSF is not the only NEM-sensitive factor active in ISG fusion: There is very little NSF and α-SNAP in the cytosol that rescued NEM-inhibition (Figure 6/7) and recombinant NSF and α-SNAP could only rescue about 50% as much as untreated cytosol (Figure 6/8). The same concentrations (and the same ratio) of NSF and α-SNAP have elsewhere been shown to effectively stimulate exocytosis in PC12 cells (Banerjee, *et al.*, 1996). A stimulatory effect of NSF and α-SNAP on ISG fusion were only seen when both the membranes and the cytosol were NEM-treated (Figure 6/8), suggesting that exogenous NSF and α-SNAP can only act in ISG-fusion once all other NEM-sensitive factors are inactivated. Along these lines it is interesting to note an observation made in another NEM-sensitive cell-free assay.

Both NSF and α-SNAP in concert with a third protein p115, as well as p97 together with p47 were able to promote fusion of Golgi derived vesicles to form cisternae. The two systems were however not synergistic (Rabouille, *et al.*, 1995). Recently, Kondo et al. found that α-SNAP in fact competes with p47, the "p97-attachment protein" (Kondo, *et al.*, 1997), for binding to syntaxin 5 on Golgi membranes (Kondo, *et al.*, submitted). Both p97 and p47 were easily detected in PC12 cytosol (Figure 6/7) and it will be interesting to see whether these proteins have a role in ISG fusion.

NSF and p97 belong to a family of ATPases, called the AAA family (ATPases Associated to a variety of cellular Activities) which comprises many members involved in such different cellular functions as cell cycle regulation, assembly, vesicle mediated transport and proteasome function (Confalonieri and Duguet, 1995). NEM-sensitive activities besides NSF (Diaz, *et al.*, 1989a) have for example been implicated in fusion events in the endocytic pathway (Rodriguez, *et al.*, 1994; Robinson, *et al.*, 1997). Furthermore, transport of mannose-6-phosphate receptor from late endosomes to the TGN was also NEM-sensitive, while NSF-depleted cytosol was able to rescue inhibition and NSF was not (Goda and Pfeffer, 1991). The NEM-sensitive factor restoring activity was partially purified and was sensitive to much lower concentrations of NEM (0.25 mM) than described here for ISG fusion. It would therefore be interesting to determine the range of NEM-sensitivity for the ISG fusion assay.

Chapter 7: Characterisation of the time window for fusion of immature secretory granules

7.1 Objective

The experiments in Chapters 5 and 6 were designed to answer the question *how* ISGs fuse with each other. The focus of this chapter will be on the temporal sequence of events, that is on the question *when* the ISGs fuse with each other. The experiments in the previous chapters have been based on a 30 min [^{35}S]-sulphate pulse labelling, thereby maximising the signal but at the same time decreasing the resolution as a large population of ISGs and the TGN could function as possible acceptors. It has been shown in Chapter 5 that MSGs and TGN do not fuse with ISGs (Figure 5/11). In this chapter, the sulphate labelling procedure will be modified such that a narrower time window in ISG maturation can be considered. The relationship between fusion competence and "age" (maturation time) will be investigated using these time-defined ISG-subpopulations as acceptors (containing the labelled substrate) in the fusion assay. To determine the most fusogenic acceptor membranes, the labelled material will also be fractionated on sucrose gradients and the relative fusogenicity of the subcellular fractions will be tested. Finally, the fused compartments will be analysed by subcellular fractionation and compared to the labelled starting material in order to characterise the fusion product.

7.2 ISGs rapidly loose their ability to fuse with each other after budding from the TGN

In order to label a smaller population of ISGs, the [^{35}S]-sulphate-pulse was reduced to 10 min and the [^{35}S]-sulphate-labelled SgII was chased out of the TGN by various lengths of chase with excess "cold" (non-radioactive) sulphate. A PNS was prepared from PC12 cells labelled either by a 10 min [^{35}S]-sulphate pulse or by a 10 min pulse followed by a 10 min, 20 min or 60 min chase. To this PNS were added PC2-ISGs, an ATP-regenerating system and fusion buffer. Incubation for fusion was as in the standard protocol (Figure 5/1) for 30 min at 37°C. The amount of p18 generated was determined by immunoprecipitation with Ab 69 and the relative amount of starting material was quantitated by immunoprecipitation of full-length SgII (p86) with Ab 175 from one tenth of the PNS. The relative fusion efficiency was calculated as the ratio of p18 to p86. A representative experiment is illustrated in Figure 7/1a and shows that the relative fusion efficiency decreases rapidly with time such that after 10 min of chase time the fusion efficiency had declined by half when compared to a 10 min pulse. After 1 hr of chase time fusion became undetectable.

As the half-time of exit from the TGN has been estimated to be 5 min (Tooze and Huttner, 1990), half of the labelled SgII in cells labelled by a 10 min [^{35}S]-sulphate pulse might be expected to have left the TGN already. This was verified by subjecting a PNS derived from PC12 cells labelled by a 10 min [^{35}S]-sulphate pulse to the velocity controlled sucrose gradient centrifugation that was also used as a first step in the isolation of ISGs (section 3.2). As described in Chapter 3, this gradient serves to separate the ISGs away from the TGN. Figure 7/1b shows that indeed nearly half of the 10 min [^{35}S]-sulphate pulse labelled SgII is found on top of the gradient in the position of the ISGs (fractions 2-5). For comparison, if the cells were labelled by a 5 min [^{35}S]-sulphate pulse however, all the SgII was retained in the TGN (fractions 8-10).

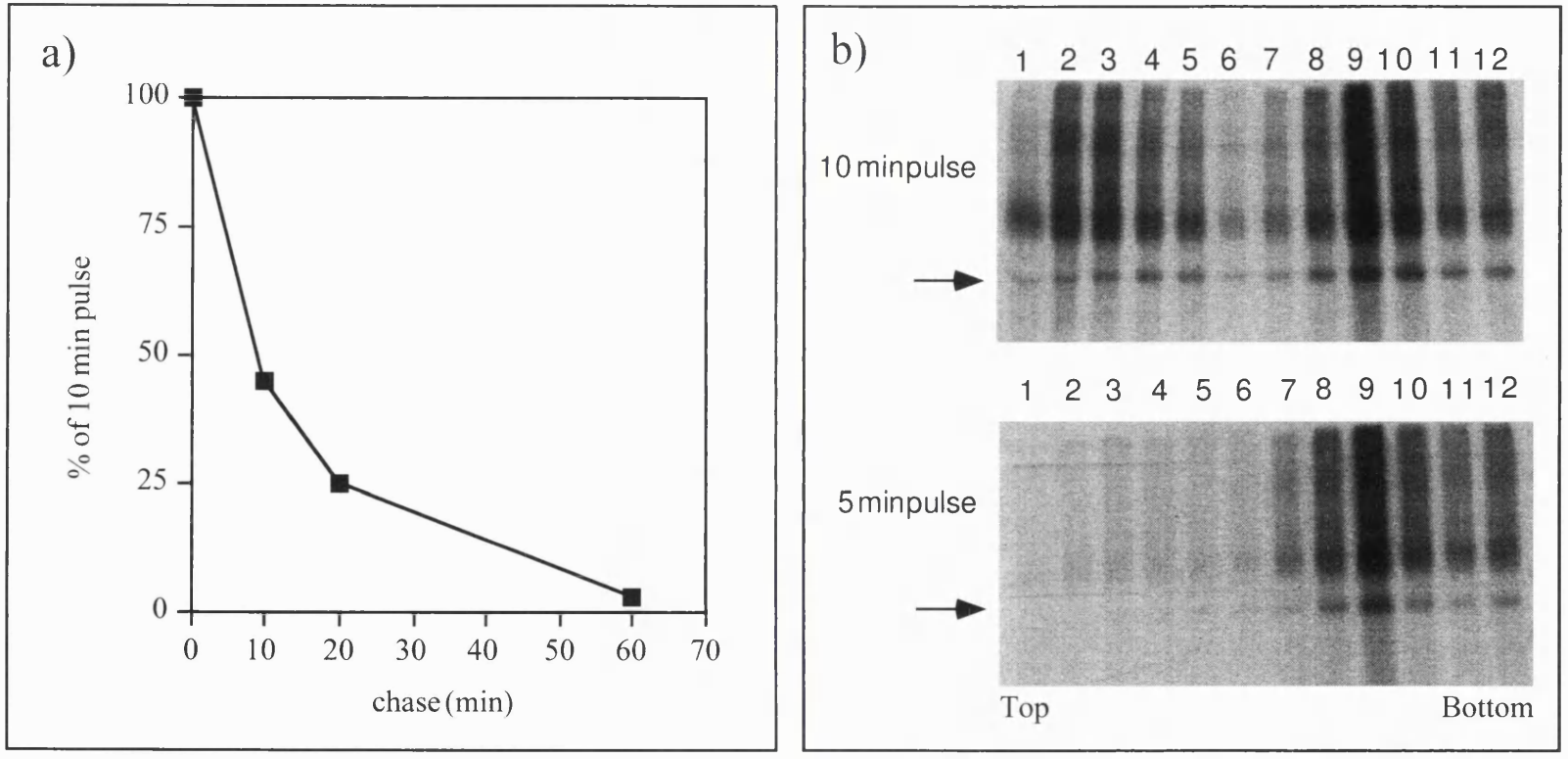


Figure 7/1: *ISGs rapidly loose their fusion competence after budding from the TGN*

(a) PC12 cells were pulse-labelled for 10 min with [^{35}S]-sulphate and chased for 0, 10, 20 or 60 min. A PNS was obtained for each condition and used in a standard fusion assay as described in Chapter 2. An aliquot (1/10) of the PNS was subjected to immunoprecipitation with Ab175 to determine the relative amount of p86 in the starting material. The relative fusion efficiency was calculated (p18x100/p86) and expressed as a percentage of the value obtained for the 10 min pulse condition.

(b) PC12 cells were pulse-labelled for 10 min or 5 min with [^{35}S]-sulphate and a PNS was obtained and subjected to velocity controlled sucrose centrifugation. Fractions of the gradient were analysed by 7.5% SDS-PAGE and fluorography. An arrow indicates SgII and the fraction numbers are indicated.

7.3 Fusion can be observed for a 5 min [^{35}S]-sulphate pulse but not after a 20°C block

PC12 cells were labelled by a 5 min [^{35}S]-sulphate pulse or by a 5 min [^{35}S]-sulphate pulse followed by a 5 min chase at 37°C or a 2 hrs chase at 20°C. The latter condition has been shown to block exit of secretory proteins from the TGN (Matlin and Simons, 1983). As a control, PC12 cells were labelled by a 10 min [^{35}S]-sulphate pulse as in Figure 7/1. A PNS was derived from each of these conditions, supplemented with PC2-ISGs, an ATP-regenerating system and fusion buffer and was incubated for 30 min at 37°C to allow fusion to take place. As described for Figure 7/1, the amount of p18 generated as a result of fusion and the relative amount of p86 in the starting material were measured by immunoprecipitation with Ab 69 and Ab 175 respectively, and the relative fusion efficiency was expressed as the ratio between p18 and p86. The result of such an experiment is shown in Figure 7/2. As expected, twice as much [^{35}S]-sulphate labelled SgII was immunoprecipitated from the PNS derived from cells labelled by a 10 min pulse, compared to cells labelled by a 5 min pulse (Figure 7/2a). The amount of p18 generated was very similar whether the 5 min [^{35}S]-sulphate pulse was followed by a 5 min chase or not and twice as large after a 10 min pulse (Figure 7/2b). In contrast, no significant amount of p18 was generated if the sulphate labelled cells were subjected to a 20°C block prior to homogenisation, indicating that the 20°C block compartment was not able to fuse with the ISGs. The relative fusion efficiency was slightly higher for a 5 min pulse followed by a 5 min chase then for a 5 min pulse and similar to a 10 min [^{35}S]-sulphate pulse.

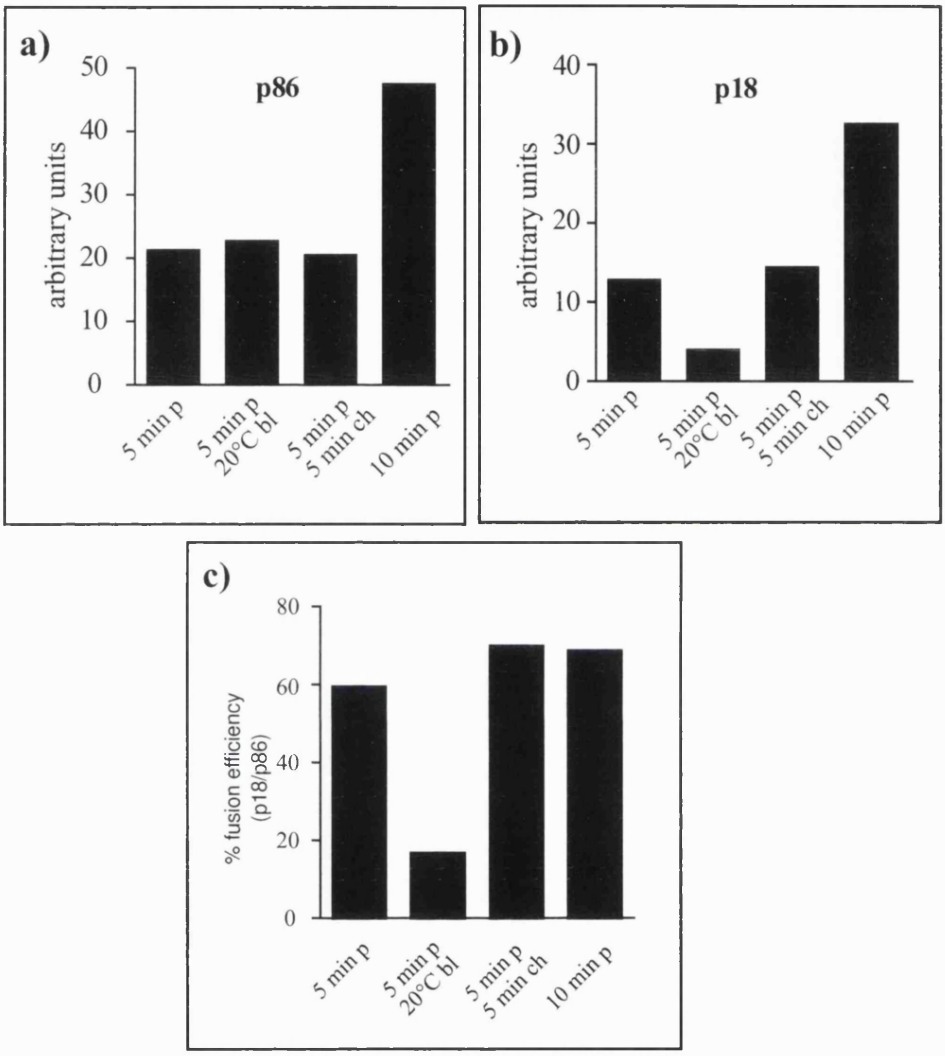


Figure 7/2: *Fusion can be observed for a 5 min [^{35}S]-sulphate pulse but not after a 20°C block*

PC12 cells were labelled by a 10 min [^{35}S]-sulphate pulse, or pulse-labelled for 5 min and chased at either 20°C for 2 hr, or 37°C for 5 or 10 min. A PNS was obtained for each condition and an aliquot (1/10) was subjected to immunoprecipitation with Ab175 to determine the relative amount of p86 in the starting material **(a)**. The rest of the PNS was used in a standard fusion assay as described in Chapter 2 and the amount of p18 generated was determined by immunoprecipitation with Ab69 **(b)**. The relative fusion efficiency was expressed as p18x100/p86 **(c)**.

7.4 ISGs bud with high efficiency from the TGN during the fusion incubation

It has been shown in Chapter 6 that TGN-membranes isolated from PC12/PC2 cells were not able to fuse in the cell-free assay. However the results in Figure 7/2 suggest that fusion is observed if all the [^{35}S]-sulphate labelled SgII is contained in the TGN at the beginning of the fusion assay. How do these two results fit together? It has previously been shown by others that formation of ISGs from the TGN in PC12 cells can be reconstituted in a cell-free budding assay with an efficiency of about 35% after an hour incubation and under slightly different conditions than those of the fusion assay presented here (Tooze and Huttner, 1990; Tooze and Huttner, 1992). It was therefore possible that the fusion signal observed for a PNS derived from PC12 cells labelled by a 5 min [^{35}S]-sulphate pulse was the result of the fusion of ISGs that had been formed during the course of the fusion incubation. In order to see whether budding of ISGs did take place during the fusion assay, a PNS was derived from PC12 cells labelled by a 7 min [^{35}S]-sulphate pulse (a 7 min pulse allows one to increase the signal while retaining virtually all the labelled SgII in the TGN), supplemented with an ATP-regeneration system and fusion buffer, and incubated either at 4°C or at 37°C for 30 min. The PNS was then layered carefully on top of a linear sucrose gradient and subjected to velocity controlled centrifugation in order to separate TGN and ISGs. Figure 7/3 shows that ISGs are indeed formed during the fusion incubation: After incubation at 37°C approximately 66% of the total [^{35}S]-sulphate labelled SgII is found on top (fractions 1-6) of the gradient, while more or less all of the SgII is retained in the TGN (fractions 9-11) at 4°C. The budding-efficiency was typically in the range of 55% which was comparable to the efficiency observed by others in a cell-free budding assay (Tooze and Huttner, 1990).

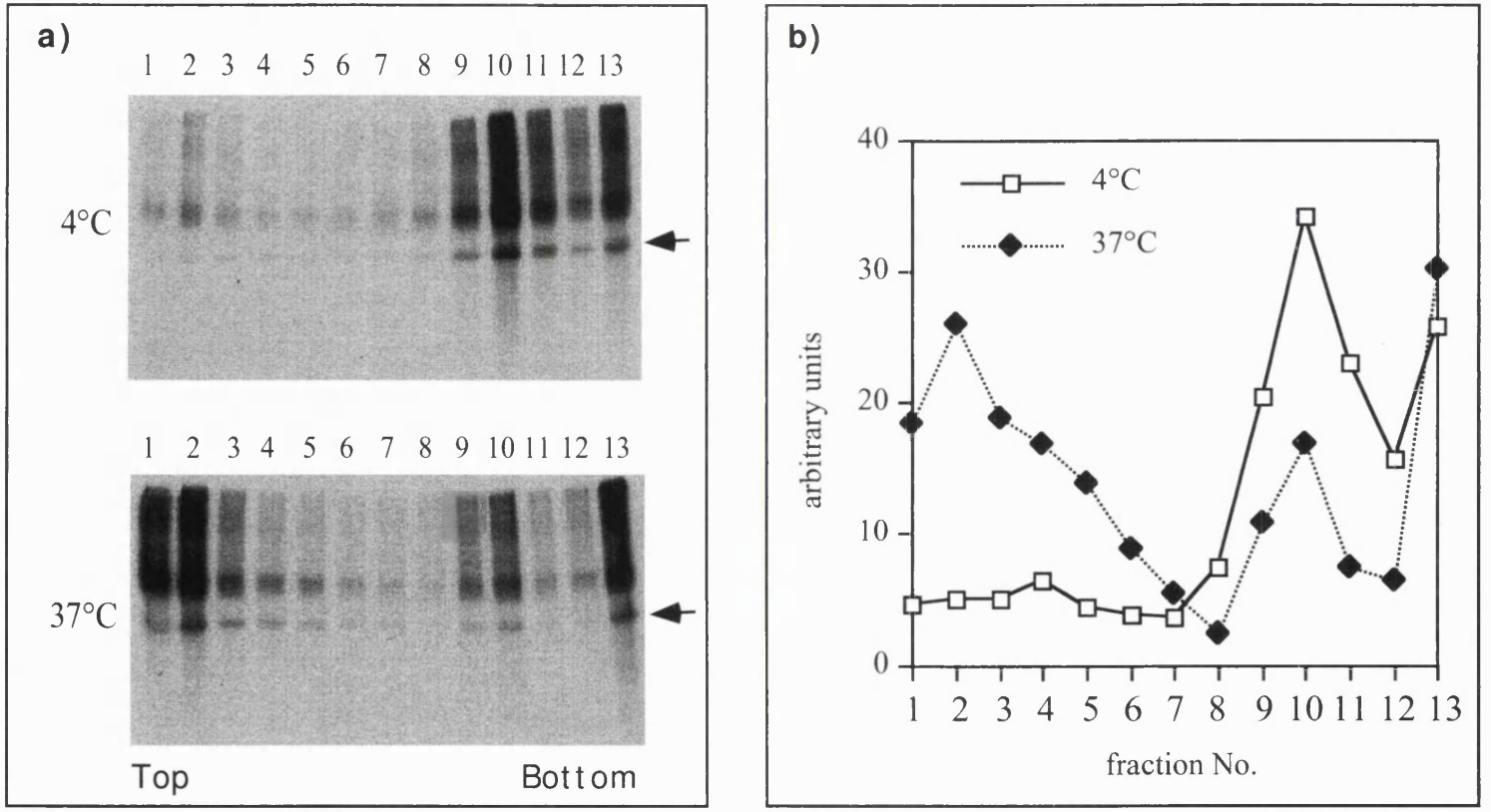


Figure 7/3: *Cell-free budding of ISGs*

A PNS was derived from PC12 cells labelled by a 7 min [^{35}S]-sulphate pulse, supplemented with an ATP-regeneration system and fusion buffer, and incubated either at 4°C or at 37°C for 30 min. The PNS was then subjected to velocity controlled sucrose gradient centrifugation and fractions were analysed by 7.5% SDS-PAGE and fluorography **(a)**. The arrow indicates the position of SgII. The amount of SgII (p86) was quantitated by densitometry **(b)**.

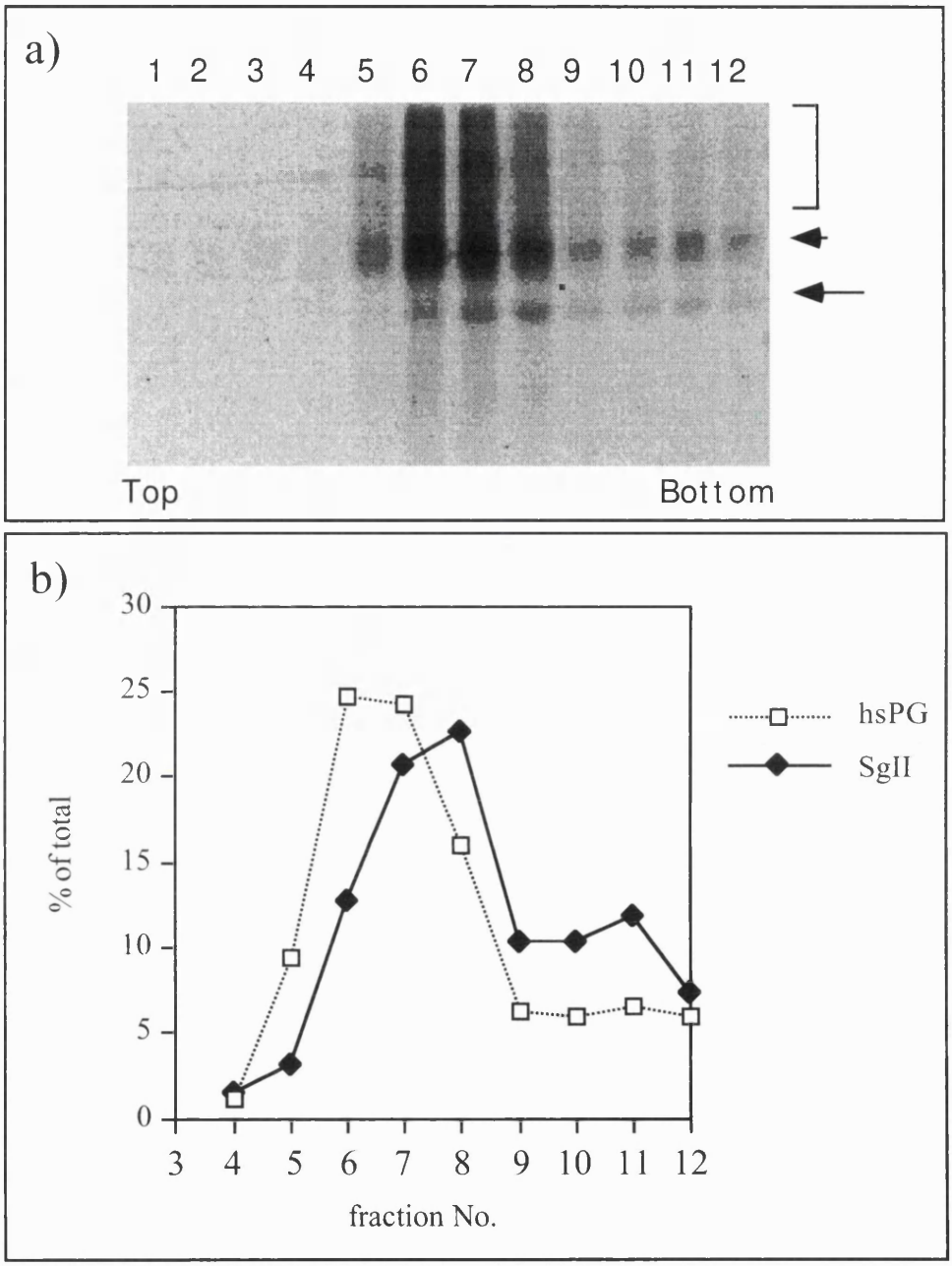


Figure 7/4: *Sorting of SgII from the heparin sulphated proteoglycan (hsPG)*

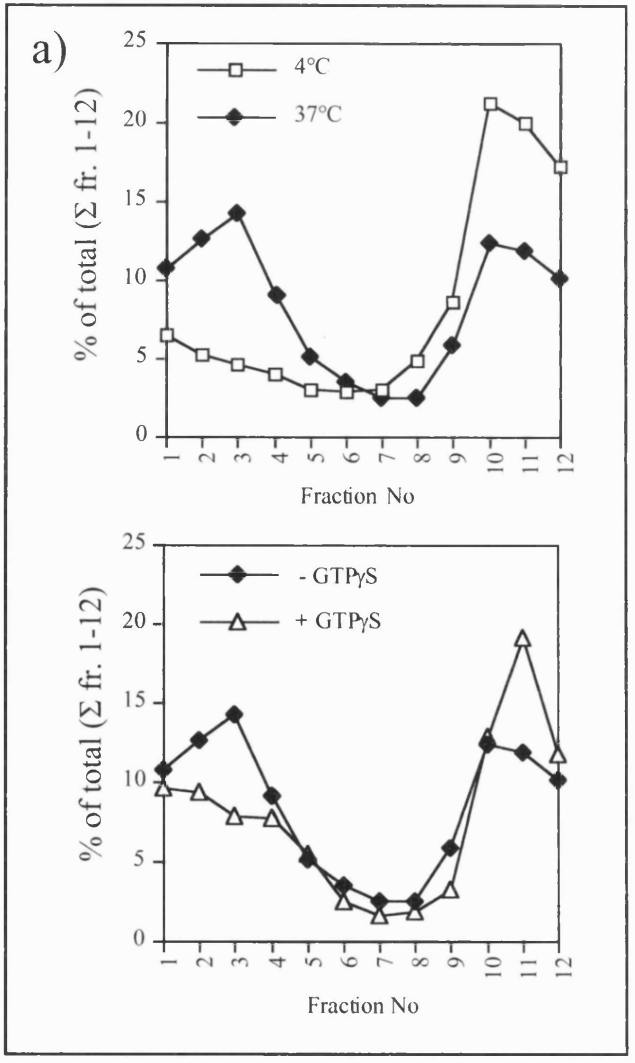
Fractions 2 to 5 of the velocity gradient shown in Figure 7/3 (bottom gel) were subjected to equilibrium sucrose gradient centrifugation and fractions were analysed by 7.5% SDS-PAGE and fluorography. **(a)** The fraction numbers and the positions of the following proteins are indicated: SgII (arrow), CgB (arrowhead) and hsPG (bracket). **(b)** The total amount of SgII and of hsPG on the gradient (sum of fractions 1-12) was determined and the distribution of each protein is shown as % of total in each fraction.

7.5 SgII is correctly sorted from the heparin sulphated proteoglycan under conditions of the fusion assay

The appearance of [^{35}S]-sulphate labelled SgII on top of the sucrose gradient could also result from fragmentation of the TGN during the fusion assay, rather than from budding. To discriminate between these two possibilities, fractions 2 to 5 from the top of the linear velocity gradient were pooled and subjected to a second sucrose density equilibrium centrifugation that has been described for the isolation of PC2-ISGs in Chapter 3 (section 3.2). If the [^{35}S]-sulphate labelled SgII is sorted correctly into ISGs during the assay, then the peak of the [^{35}S]-sulphate labelled SgII should resolve from the peak of [^{35}S]-sulphate labelled hsPG that is sorted into constitutive secretory vesicles (Tooze and Huttner, 1990). If however the [^{35}S]-sulphate labelled SgII is still contained in TGN-fragments, no sorting of the two markers should be observed. Figure 7/4 shows that SgII is indeed sorted into ISGs (fractions 7 and 8) that are denser than the constitutive secretory vesicles containing the bulk of the hsPG (fractions 6 and 7).

7.6 Budding of ISGs is inhibited by GTPγS

GTPγS inhibits budding of ISGs from the TGN *in vitro* (Tooze, *et al.*, 1990b) and it was shown that the formation of ISGs from the TGN is regulated by G-proteins both of the family of small ras-like GTPases (Barr and Huttner, 1996; Chen and Shields, 1996) and by heterotrimeric G-proteins (Barr, *et al.*, 1992; Barr, *et al.*, 1991a). If the fusion signal measured for a fusion assay based on a 7 min [35S]-sulphate pulse protocol is indeed due to fusion of newly budded ISGs, then the addition of a drug like GTPγS that inhibits budding should also inhibit fusion. A PNS was derived as in section 7.3 from PC12 cells labelled by a 7 min [^{35}S]-sulphate pulse, supplemented with an ATP-regenerating system and fusion buffer and incubated in the presence or absence of PC2 ISGs and in the presence or absence of GTPγS for 30 min



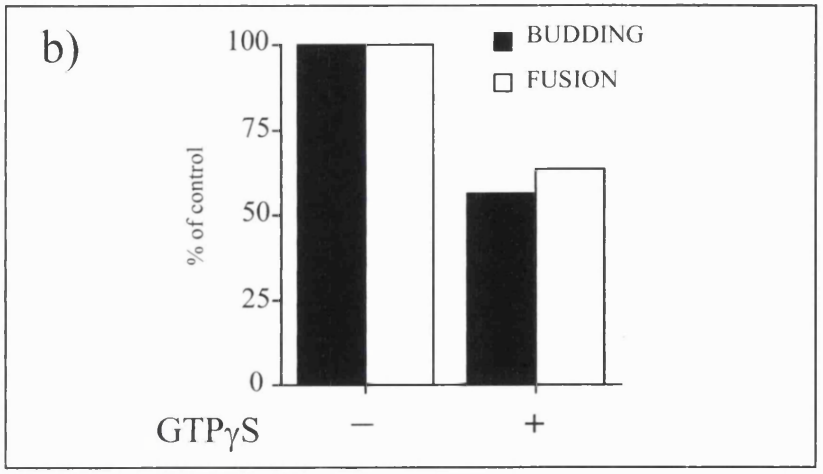


Figure 7/5: *GTPγS inhibits cell-free budding of ISGs*

A PNS was derived from PC12 cells labelled by a 7 min [^{35}S]-sulphate pulse, supplemented with an ATP-regenerating system and fusion buffer and incubated at 37°C either in the presence (+GTPγS) or absence of 100 μM GTPγS (-GTPγS=37°C) or at 4°C in the absence of GTPγS (4°C) for 30 min. (a) A sample of the PNS was analysed by velocity controlled centrifugation, fractions were analysed by 7.5% SDS-PAGE and fluorography the distribution of SgII (p86) is indicated as % of the sum of SgII in fractions 1-12). (b) Another sample of the PNS was used for standard fusion reactions in the presence or absence of GTPγS (100 μM) and analysed as described in Chapter 2. The results are expressed as % of the control incubation (+GTPγS) after subtraction of the PC2-independent signal. The percentage of budding was calculated from (a) as SgII (fractions 1-6)/total SgII and expressed as % of the control reaction (-GTPγS, 37°C).

at 4 or 37°C. A sample of the PNS was then analysed by velocity controlled centrifugation to monitor budding, while other samples were further incubated for processing to assay for fusion. Figure 7/5 shows that budding was indeed inhibited by 50% in the presence of GTPγS in agreement with previous observations (Tooze, *et al.*, 1990b). Fusion of ISGs was also inhibited by approximately 40% in the presence of GTPγS. In contrast, it was shown in Chapter 5 that GTPγS had only a small effect on fusion if the acceptor was derived from PC12 cells labelled by a 30 min [^{35}S]-sulphate pulse in which case most of the sulphate-labelled SgII has left the TGN (Figure 5/5b). It is therefore likely that the inhibition seen in Figure 7/5 is indeed a consequence of the decrease in budding. Thus, this result suggests that budding of ISGs is required for fusion to occur, if all the sulphate labelled SgII is in the TGN at the start of the incubation.

7.7 Identification of the fused ISGs by subcellular fractionation

It has been demonstrated in Chapter 5 that the p18 generated as a result of fusion is contained within a membrane bound compartment. The experiment shown in Figure 7/6 demonstrates that this compartment behaves similarly to secretory granules on a linear sucrose gradient. PC12 cells were labelled by a 7 min [^{35}S]-sulphate pulse and a PNS was obtained. To this PNS an ATP-regenerating system and fusion buffer were added and the samples were incubated at 37°C in the presence or absence of PC2-ISGs for 30 min or left on ice (4°C). After the incubation, samples were cooled down on ice and subjected to velocity controlled sucrose centrifugation on a linear (0.3M to 1.2M) sucrose gradient. Fractions were collected from the gradients and a small aliquot of each fraction was analysed by 7.5% SDS-PAGE (Figure 7/6b, only the gradients for two conditions, 4°C and 37°C +PC2-ISGs, are shown). The remaining part of the fractions was then diluted with 10 mM HEPES, pH 7.2 to the same sucrose density (0.34 M), and the membranes were pelleted by centrifugation at 100,000 g for 1 hr.

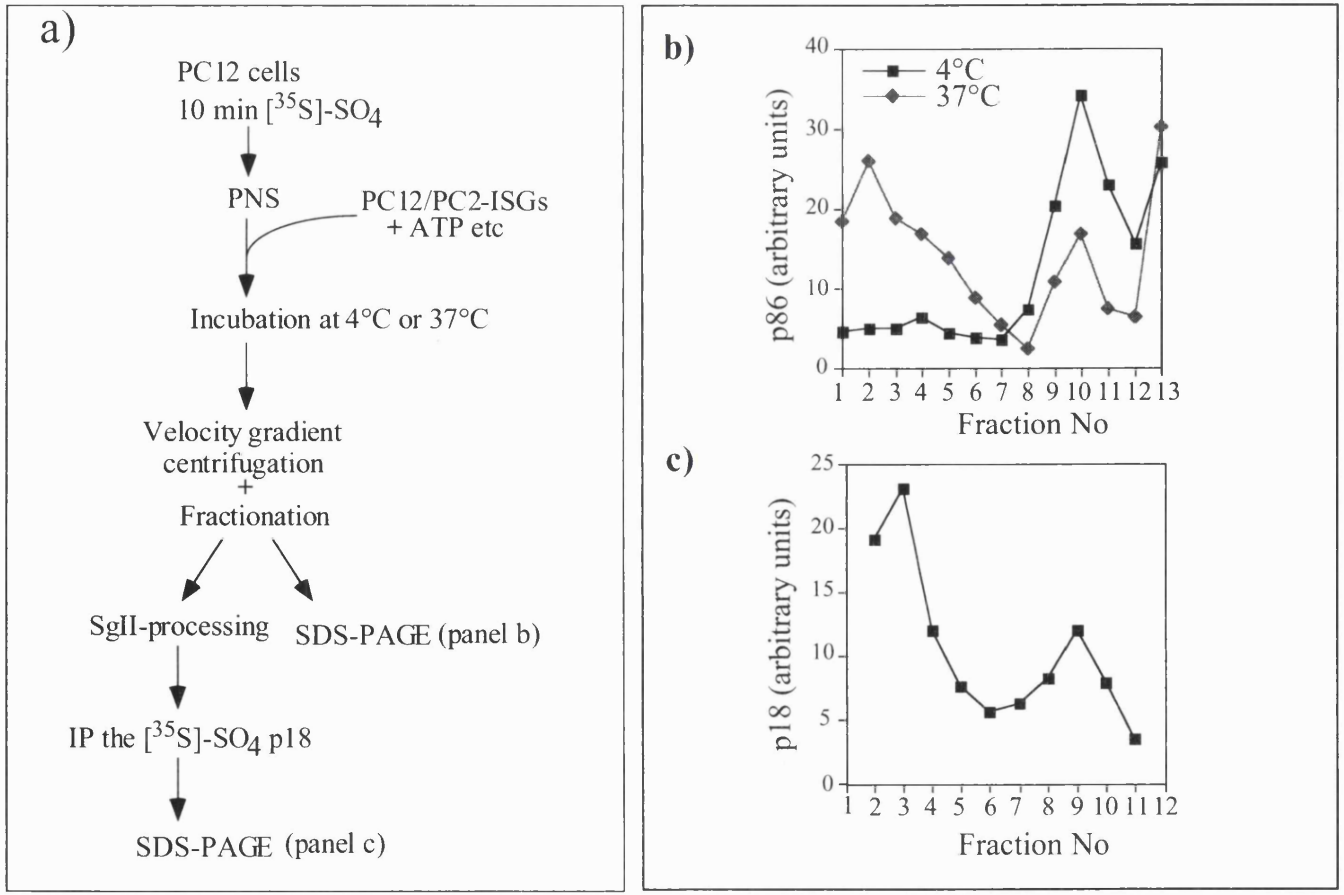


Figure 7/6: *Identification of the fused ISGs by subcellular fractionation*

The membranes were resuspended in 0.3 M sucrose buffered with 50 mM MES pH 5.5 and incubated for 30 min on ice followed by 90 min at 37°C. The samples were lysed and used for immunoprecipitation with Ab 69 as usual. The p18 signal generated in the absence of PC2-ISGs was barely detectable and was subtracted from the signal obtained in the presence of PC2-ISGs. Figure 7/6c shows the distribution of the PC2-dependent p18 signal over the gradient. The peak of the p18 was found on top of the gradient in fractions 2 and 3 and only little p18 was detected at the bottom of the gradient.

7.8 Identification of the fusion competent compartment in the PNS by subcellular fractionation.

In order to identify the fusion competent compartment, the PNS was subjected to subcellular fractionation prior to the fusion incubation. A PNS was prepared from PC12 cells labelled by a 10 min [^{35}S]-sulphate pulse and subjected to velocity controlled sucrose gradient centrifugation. Fractions were collected from the gradient and a sample of each fraction was used for immunoprecipitation of p86 with Ab 175 to measure the distribution of the [^{35}S]-sulphate labelled SgII in the starting material. Each fraction of the gradient was diluted to 0.34 M sucrose each and the membranes were pelleted by centrifugation at 100,000 g for 1 hr. The membrane pellets were resuspended in buffer or in PC12 cytosol and supplemented with an ATP-regenerating system and fusion buffer. The samples were then incubated in the absence or presence of PC2-ISGs for 30 min at 37°C and the fusion assay was developed as usual. Figure 7/7a shows the distribution of p86 and p18 (top panel). The PC2-independent signal for p18 was barely detectable and was subtracted from the PC2-dependent signal. The distribution of p86 was slightly shifted in this experiment such that only a small amount of [^{35}S]-sulphate labelled SgII was found on top of the gradient. The largest amount of p18 was generated in incubations containing fractions 5 to 8 (Figure 7/7a, top panel)

and the highest ratio of p18 generated to [^{35}S]-sulphate labelled p86 was found for fractions 5 to 7 (Figure 7/7a, bottom panel).

When velocity gradient fractions were prepared in the same way from PC12 cells labelled by a 30 min [^{35}S]-sulphate pulse, the [^{35}S]-sulphate labelled SgII was distributed over two peaks, one at the top of the gradient and one at the bottom of the gradient (Figure 7/7b, top panel). These fractions were pelleted and resuspended in cytosol. An ATP-regenerating system and fusion buffer were added and the samples were incubated in the presence or absence of PC2-ISGs for 30 min at 37°C. After the subsequent processing incubation, the amount of [^{35}S]-sulphate labelled p18 that had been generated was determined by immunoprecipitation for each fraction. Figure 7/7b shows that under these conditions the membranes which generated the largest amount of p18 migrated on top of the gradient (middle panel). Although a large proportion of the p18 was also generated in an PC2-independent way, the PC2-dependent signal for p18 was clearly higher for each fraction of the gradient, indicating that each fraction was able to fuse to some extent. When the distributions of p18 and p86 were compared, the ratio of p18 to p86 was highest in the middle part of the gradient, indicating that the most fusogenic ISGs were found in the middle of the gradient (fractions 6 and 7) agreement with Figure 7/7a (bottom panel). This experiment shows that it is possible to physically separate a fusogenic ISG-population from the TGN and observe PC2-dependent p18 accumulation in the absence of newly budded ISGs.

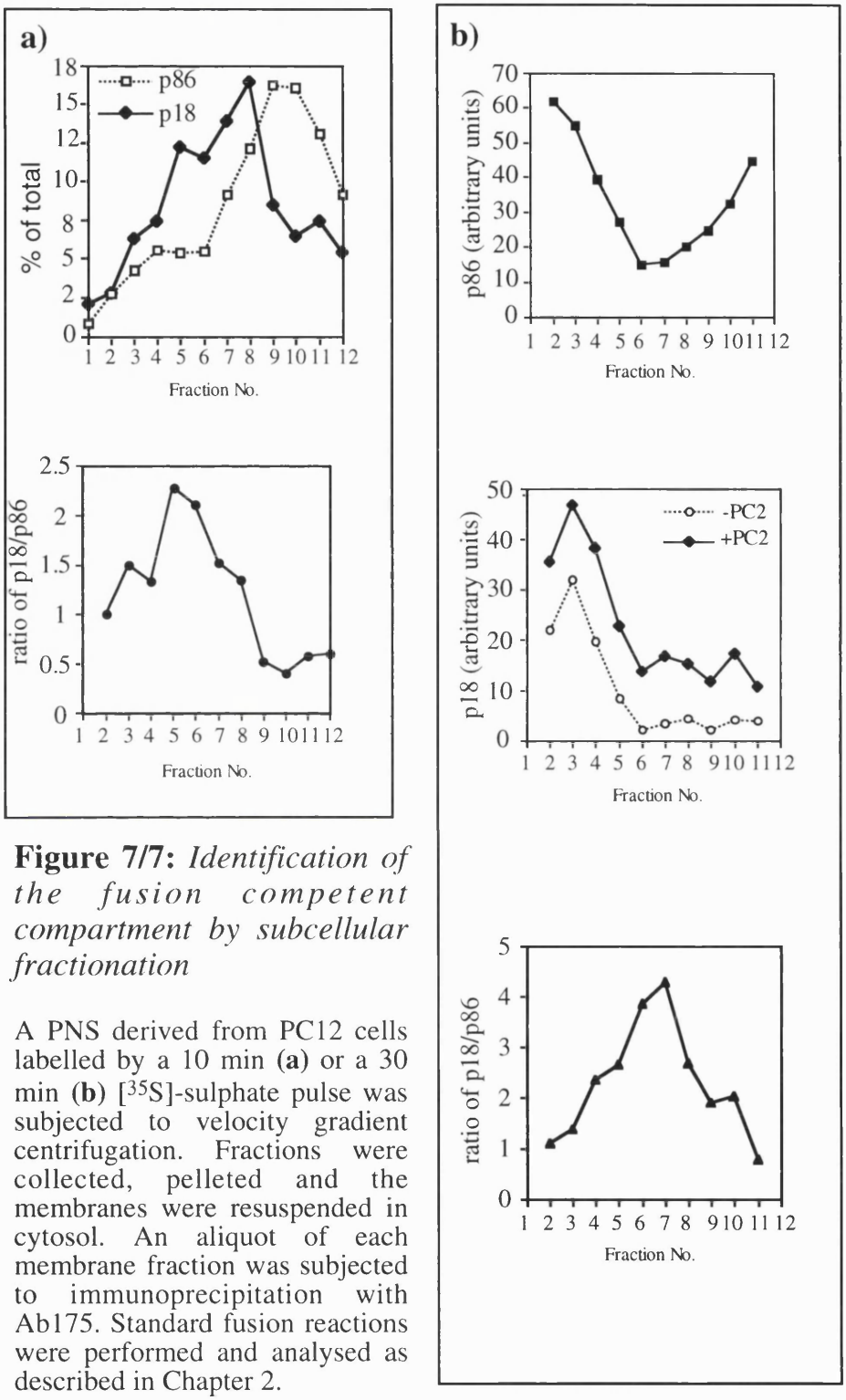


Figure 7/7: *Identification of the fusion competent compartment by subcellular fractionation*

A PNS derived from PC12 cells labelled by a 10 min **(a)** or a 30 min **(b)** [^{35}S]-sulphate pulse was subjected to velocity gradient centrifugation. Fractions were collected, pelleted and the membranes were resuspended in cytosol. An aliquot of each membrane fraction was subjected to immunoprecipitation with Ab175. Standard fusion reactions were performed and analysed as described in Chapter 2.

7.9 Discussion

7.9.1 What is the identity of the fusion competent ISG ?

The specificity of the present fusion assay is determined by the two following facts:

1) the "donor" population of membranes is defined by the presence of the enzyme PC2 which is sorted very efficiently into the regulated secretory pathway (Dittié and Tooze, 1995). Furthermore it has been shown in Chapter 3 that PC2 cleaves SgII only once incorporated into ISGs. Therefore active PC2 (with respect to SgII-cleavage) is only found in ISGs and MSGs. The PC2-ISGs are semi-purified and separated from TGN and MSGs by subcellular fractionation (Tooze, *et al.*, 1991).

2) the "acceptor" population is determined by the [^{35}S]-sulphate labelling protocol. The sulphotransferase that modifies SgII and other proteins is only present in the TGN and sulphation seems to be the last modification prior to the incorporation of SgII into ISGs (Huttner, 1982; Lee and Huttner, 1983). In Chapters 5 and 6 the acceptor (PC12 cells) population was defined by a continuous 30 min [^{35}S]-sulphate pulse, resulting in [^{35}S]-sulphate labelled SgII in the TGN and in a wide range of ISGs at different stages of maturation. It has been shown in Chapter 5, that neither a donor (PC2-containing) fraction enriched in TGN-membranes, nor in MSG-membranes were able to fuse with the acceptor population defined by a 30 min [^{35}S]-sulphate pulse indicating that the assay indeed measures fusion of semi-purified PC2-ISGs with PC12-ISGs.

In order to obtain a higher resolution as to which subcompartment in the acceptor (PC12) PNS undergoes fusion with the PC2-ISGs, a pulse-chase [^{35}S]-sulphate labelling protocol was used. The fusion competence of these time-defined ISG-populations decreased rapidly with the chase time and was highest if the PC12 cells were pulse-labelled only and not chased (Figure 7/1). The amount of p18 generated by fusion of the donor with an acceptor population labelled by a 10 min

[^{35}S]-sulphate pulse was twice as high as the amount generated either by fusion with an acceptor population labelled by a 5 min [^{35}S]-sulphate pulse or by a 5 min [^{35}S]-sulphate pulse followed by a 5 min chase (Figure 7/2). This indicates that both the [^{35}S]-sulphate labelled SgII residing in the TGN and the [^{35}S]-sulphated SgII that has already left the TGN are both able to contribute to the fusion signal.

The interpretation of the pulse-chase data is complicated by the fact that this fusion assay also supports budding, suggesting that ISGs may form and fuse during the course of the fusion assay. While it cannot be excluded that the TGN itself is also able to fuse with the PC2-ISGs, the following points speak against this. 1) Fusion was not observed when the [^{35}S]-sulphate labelled SgII was accumulated in the TGN by a 20°C block (Figure 7/2). 2) If all the sulphate-labelled SgII was contained in the TGN at the beginning of the incubation, inhibition of budding by GTPγS also resulted in an inhibition of fusion (Figure 7/5). 3) As shown in Chapter 3, in contrast to the 20°C block compartment (Figure 3/10), the TGN itself does not support SgII processing (Figure 3/8). It remains possible that PC2-ISGs fuse with the acceptor TGN followed by formation of new ISGs that then contain both PC2 and [^{35}S]-sulphate labelled SgII, however this scenario is considered less likely.

7.9.2 ISGs loose fusion competence rapidly after budding from the TGN.

The kinetics of the loss of fusion competence indicate a half time of approximately 10 min, whilst no fusion could be detected after one hour of chase (Figure 7/1). A half-time of 45 min has been previously calculated for maturation of ISGs to MSGs based on the correlation of chase-time and the density shift of the sulphate-labelled material that can be observed by sucrose gradient centrifugation (Tooze, *et al.*, 1991). The rate with which ISGs become refractory to fusion is thus greater than the rate of maturation as determined by density shift. This observation

suggests that the most fusogenic membranes are in effect younger than the originally characterised ISG-population that can be identified by a 5 min pulse and a 15 min chase protocol. After one hour of chase time, this population of "pre-ISGs" has presumably been consumed by fusion (*in vivo*) with each other or with ISGs. It also indicates that the major fusion event measured in the cell-free assay might not be the rate-limiting step in the maturation process. It does however not exclude that ISGs can fuse with each other (see also Figure 5/2a), but the efficiency of this fusion event might be too low to be detected in the assay. It is also possible that the conditions of the cell-free assay are suboptimal for this homotypic fusion event. For example, "older" ISGs might be more fragile, or more prone to be inactivated *in vitro* than pre-ISGs, while remaining fully fusion competent *in vivo*.

It is proposed here that the ISG defined by Tooze et al. using a 5 min [^{35}S]-sulphate pulse and a 15 min chase (Tooze, *et al.*, 1991) and isolated in the same way as has been used in this work to isolate the PC2-ISGs can fuse with younger, newly formed secretory granules. These pre-ISGs might be very heterogeneous in size and engaged in a dynamic equilibrium of fusion and fission. This is suggested by two observations. 1) Whilst all the SgII labelled by a 5 min [^{35}S]-sulphate pulse is present in one peak (two fractions) at the bottom of the velocity gradient, during the following chase, the [^{35}S]-sulphate labelling gradually shifts to the top of the gradient until after 15 min of chase, all the [^{35}S]-sulphate label is found on top of the gradient (A. Dittié, unpublished results). While this is in part due to the lack of synchrony in budding, it might also indicate the presence of intermediate structures of heterogeneous size. 2) The most fusogenic compartment was found in fractions of the gradient that neither coincided with the TGN nor with the ISGs and maybe equivalent to a pre-ISG.

7.9.3 Why do ISGs loose their fusion competence ?

In the model originally proposed by Tooze et al. (Tooze and Stinchcombe, 1992) and illustrated in Figure 1/3, formation of an MSG relies on two separate but possibly linked processes: First, a series of fusion events (or multiple fusions) and second, sorting of proteins to be removed from the granule by budding of clathrin coated vesicles containing these proteins. The loss of fusion competence during maturation might be a direct result of either or both of those two processes. First, there might be a limited number of fusion sites on each ISG that can not be recycled or reactivated. Fusion would then run down after a certain number of fusion events. If the number of fusion sites is a constant parameter than such a scenario could contribute to the homogenous size-distribution of the MSG. Endosomes derived from macrophages have been shown to undergo multiple fusion events (Diaz, *et al.*, 1989b). Fusion of endosomes was exhausted after a limited number of fusion events as preincubation of the endosomes in separate tubes ran down the assay (Diaz, *et al.*, 1989b). The resulting compartment was of the same density as the starting endosomes but of a larger size. A rundown of ISG-fusion after preincubation has been shown in Figure 6/5a, although it is possible that this rundown results from the inactivation of some cytosolic factors. Second, a loss of fusion competence could also be caused by the removal of proteins that are involved in fusion by clathrin coated vesicle formation. It has been suggested that ISG-fusion is paralleled by sorting of non-granule proteins into clathrin coated buds which form clathrin coated vesicles that are either targeted to the endosome, the TGN or the plasma-membrane (Tooze and Stinchcombe, 1992; Urbé, *et al.*, 1997). It is possible that each round of fusion is accompanied or followed by a fission event (or coated vesicle formation) resulting in the removal of proteins that are required for fusion but no longer needed once the secretory granule has matured.

As described in Chapter 6, one family of proteins that are strongly implicated in intracellular fusion events are the SNAREs. After a heterotypic fusion event it is

necessary that the v-SNARE is recycled to the donor compartment to be used in another round of vesicle docking and fusion. Given that these proteins are tightly membrane-associated, recycling will necessarily involve another vesicular transport step. It is also thought that these recycling SNAREs have to remain in some inactive conformation and that reactivation (possibly catalysed by rab proteins) will only occur once the v-SNARE is incorporated into a new vesicle. It is therefore conceivable that SNAREs involved in ISG-fusion are simply inactivated after the fusion event and that recycling only takes place after fusion with the plasma-membrane. It is conceivable that such putative ISG SNAREs constitute a constant and limited number of docking/fusion sites per granule and fusion would thus cease as soon as they have all been used. Alternatively, SNAREs involved in ISG fusion, whether inactivated or not, might be removed from the ISG during maturation and recycled back to the TGN to be incorporated into a newly forming ISG.

7.9.4 What is the fusion product ?

Preliminary experiments described in this chapter suggest that the fused ISGs behave similarly to the non-fused ISG as the p18 generated only in the fused ISGs migrates on top of the velocity gradient. A small amount of p18 is also generated in membranes with a similar sedimentation behaviour as the TGN. As SgII-processing has not been observed *in vitro* in isolated TGN-membranes (Figure 3/8), it is not clear what these membranes represent. Immunoisolation of TGN with TGN-specific markers might provide a clue as to whether this p18 is generated in the TGN or in different membranes of a similar density.

In an assay measuring fusion of endosomes derived from macrophages, Diaz and co-workers found that the endosome resulting from multiple fusion events as shown both by morphological and biochemical means, was larger but remained the same density as the starting material (Diaz, *et al.*, 1989b). It would be interesting to

analyse the fused ISGs both by differential centrifugation (to determine size) and by equilibrium gradient centrifugation to analyse their density in relation to MSGs. When ISGs were formed *in vitro* from the TGN and analysed first by velocity controlled and then by equilibrium centrifugation, a small population of the ISGs seemed to have acquired a density that was similar to the MSGs (Figure 7/6, fraction 11). Analysis of the distribution of p18 generated as a result of fusion on equilibrium gradients will be required to determine whether this population corresponds to the ISGs that have fused with each other or with "older" ISGs in the PNS.

Chapter 8: Conclusion

8.1 Processing of SgII by PC2 and the pH of the ISG

Using a cell-line called PC12/PC2, derived from PC12 cells that are stably transfected with the endopeptidase PC2, it has been shown in Chapter 3 that processing of secretogranin II (SgII) can be observed in isolated immature secretory granules (ISG) in a temperature- and ATP-dependent manner. The stimulatory effect of ATP on processing could be attributed to the activation of the vacuolar H^+-ATPase and a concomitant decrease in intragranular pH. The ISG therefore provides an adequate environment for correct processing of SgII by PC2.

The rate of SgII processing in isolated ISGs was strongly dependent on the intragranular pH, suggesting that processing of SgII could be used as a pH indicator for the granule interior. A standard curve was prepared using SgII processing in ISGs equilibrated at a range of pH values. The extent of processing in ISGs incubated in the presence of ATP at physiological pH was compared with the standard curve and the intragranular pH was determined. From these observations, an intragranular pH of 6.3+/- 0.1 for ISGs in a physiological buffer in the presence of ATP was predicted. Hence, the pH of ISGs seems to be similar to the pH of the *trans*-Golgi network (TGN) and is clearly higher than the pH of mature secretory granules (MSGs, pH 5.0-5.5). It is proposed here that further acidification of the secretory granule to a pH below 6.3 requires membrane remodelling events such as ISG-ISG fusion and membrane retrieval. Such membrane remodelling events could for example lead to an enrichment of vacuolar H^+-ATPases over other proteins which might restrain acidification (e.g. Na^+/K^+- ATPase).

Interestingly, no processing of SgII could be observed in a membrane fraction that was highly enriched in TGN-membranes under conditions for which processing was readily obtained in isolated ISGs, suggesting that factors other than pH might be important for correct processing of SgII by PC2. Accordingly processing of SgII pulse-labelled for 5 min with [^{35}S]-sulphate and chased for 15 min into ISGs could be observed in semi-intact cells equilibrated at acidic pH, whereas SgII pulse-labelled for 5 min in the TGN was not processed. However, under identical conditions processing of SgII could be observed if cells were chased *in vivo* for a minimum of 30 min at 20°C following the 5 min [^{35}S]-sulphate pulse and prior to permeabilisation. It is proposed that the conditions within the TGN after a 20°C block become similar to conditions within the ISG. These conditions favour processing possibly by creating microdomains in which the local concentration of PC2 and SgII are higher than in untreated TGN operationally defined by the 5 min [^{35}S]-sulphate pulse.

8.2 Cell-free fusion of ISGs

Based on studies outlined above on SgII processing by PC2 in isolated ISGs, a cell-free assay was established in which the fusion of immature secretory granules was reconstituted and characterised. The assay is based on content mixing of two ISG populations of which one, derived from wild-type PC12 cells, contains [^{35}S]-sulphate-labelled SgII and the other, derived from PC12/PC2 cells, contains the endopeptidase PC2. Fusion was monitored by detecting the [^{35}S]-sulphate labelled SgII-processing end-product p18 that is generated in a second incubation stage under conditions which favour processing (processing incubation).

The choice of SgII processing as a read-out for fusion was based on the following considerations: First, endoproteolytic processing is a good measure for content mixing of the ISGs as processing is a granule based activity. It has been shown in Chapter 3 that PC2 is only active once packaged into secretory granules and is fully

active in the ISG. Second, SgII is a good substrate for PC2 and is processed in PC12/PC2 cells to a similar degree as in chromaffin cells (Chapter 3). Third, SgII is sulphated in the TGN and is very efficiently sorted into the secretory pathway. Using radioactive pulse-chase experiments with [^{35}S]-sulphate, the fusion characteristics of operationally defined subpopulations of ISGs can be selectively analysed (Chapter 7). It should be noted that the use of radioactive sulphate was crucial for this study as the PC12/PC2 cells are in all respects, except for expression of PC2, identical to the PC12 cells and that there is no PC12 cell specific substrate that is not also processed in PC2-ISGs.

To quantitate the extent of SgII processing, antibodies specific for the processing product p18 were generated and characterised (Chapter 4). In the basic form of the fusion assay PC12/PC2-cell derived ISGs were added to a PNS of PC12 cells labelled for 30 min with [^{35}S]-sulphate. Fusion was shown to be temperature- and time-dependent and require ATP-hydrolysis. No fusion signal was obtained if the PC2-ISGs were added after the fusion incubation, demonstrating that fusion is not supported in the processing incubation. Minor amounts of endogenous processing of SgII were observed in the absence of PC2-ISGs in the form of a p18 background signal, most likely as a consequence of the activation of an unknown endogenous endopeptidase. Titration of PC2-ISGs showed that fusion was saturable. Furthermore the addition of unlabelled PC12-cell derived ISGs to the assay resulted in the competition of the fusion signal. Fusion was specific to ISGs as addition of MSGs or TGN-membranes derived from PC12/PC2 cells did not result in a fusion signal.

8.3 Molecules involved in the fusion of ISGs

Trypsin treatment of the PC2-ISGs completely abolished the fusion signal, demonstrating that membrane associated proteins are required on both fusing partners.

Neither botulinum neurotoxins A, C or D had an effect on fusion indicating that the so-called "terminal SNARES" are not involved in this fusion event.

In a variation from the original protocol, a membrane pellet was prepared from a PNS derived from [^{35}S]-sulphate labelled PC12 cells and fusion was shown to be strictly cytosol dependent. Fusion did not require any factors specific to the regulated secretory pathway as cytosol derived from HeLa cells was able to support the assay. NEM-treatment of the cytosol did not effect the fusion signal whereas NEM-treatment of both membranes and cytosol completely abolished fusion of ISGs. This NEM-inhibition was overcome by addition of fresh, untreated cytosol, suggesting that an NEM-sensitive factor is present on the membranes and can be recruited from cytosol. Fusion could be partially restored by addition of recombinant His-tagged NSF and α-SNAP to NEM-treated samples. However NSF and α-SNAP had an inhibitory effect on fusion if added to untreated cytosol. It is therefore unclear at present whether NSF and α-SNAP are the only NEM-sensitive factors involved in ISG fusion. Another candidate protein, p97, is very abundant both in PC12 cytosol and on the ISG membrane and further work is required to probe a possible involvement of p97 in ISG fusion.

GDI-mediated removal of the small GTPase rab11 had a small but reproducible inhibitory effect on fusion. These data suggest that ISG fusion does not depend on the recruitment of rab11 from the cytosol but do not exclude a role for membrane bound rab11 in this fusion event. GTPγS had an inhibitory effect on fusion if all the [^{35}S]-sulphate labelled SgII was contained in the TGN prior to the incubation (Chapter 7), while much less of an effect was observed in experiments based on a 30 min [^{35}S]-sulphate pulse (Chapter 5). This result is consistent with the previously reported GTPγS inhibition of ISG formation from the TGN (Tooze, *et al.*, 1990b).

The cell-free assay can now be used to identify the cytosolic proteins involved in ISG fusion. Of particular interest will be the identification of the cytosolic component which is not sensitive to NEM treatment but required for fusion.

8.4 Homotypic versus heterotypic fusion

Homotypic fusion is defined as fusion of like with like. While heterotypic fusion is a well-understood process underlying vectorial membrane transport and is necessary for the maintenance of the identity of subcellular compartments, the function of homotypic fusion is much less well understood. The discrimination between homotypic and heterotypic fusion is a theoretical one and it is now clear that the known fusion mechanisms, whether NSF- or p97-dependent, do not segregate according to this categorical definition. Indeed homotypic fusion between yeast vacuoles and fusion between early endosomes is dependent on NSF (Haas and Wickner, 1996, Diaz, *et al.*, 1989a) while homotypic fusion between Golgi cisternae requires both p97 and NSF (Acharya, *et al.*, 1995; Rabouille, *et al.*, 1995).

Fusion between ISGs is here referred to as homotypic to contrast this fusion event with heterotypic fusion between either ISGs or MSGs and the plasma-membrane. It has been shown in Chapter 5 that fusion can be observed between PC2-ISGs and PC12 ISGs labelled by a 10 min pulse and a 15 min chase indicating that homotypic fusion between ISGs does indeed take place. The most efficient fusion event measured was however between earlier ISG-stages (pre-ISGs) and the ISG. Complicating matters is the fact that only one of the ISG-populations was purified while the other one, although operationally defined by [^{35}S]-sulphate labelling, was used in a crude fraction. Attempts to obtain a fusion signal from PC12 ISGs isolated in the same way as the PC2-ISGs by two consecutive gradients have so far not been successful. It is possible that this is due to the loss of an essential factor that is required on one of the two fusing membranes and is not contained in the cytosol. Enrichment in the ISGs, and separation from the TGN, by a single velocity controlled centrifugation step did not inactivate the ISGs (Chapter 7). Future experiments to investigate fusion in the absence of budding could be designed using this single gradient separation procedure.

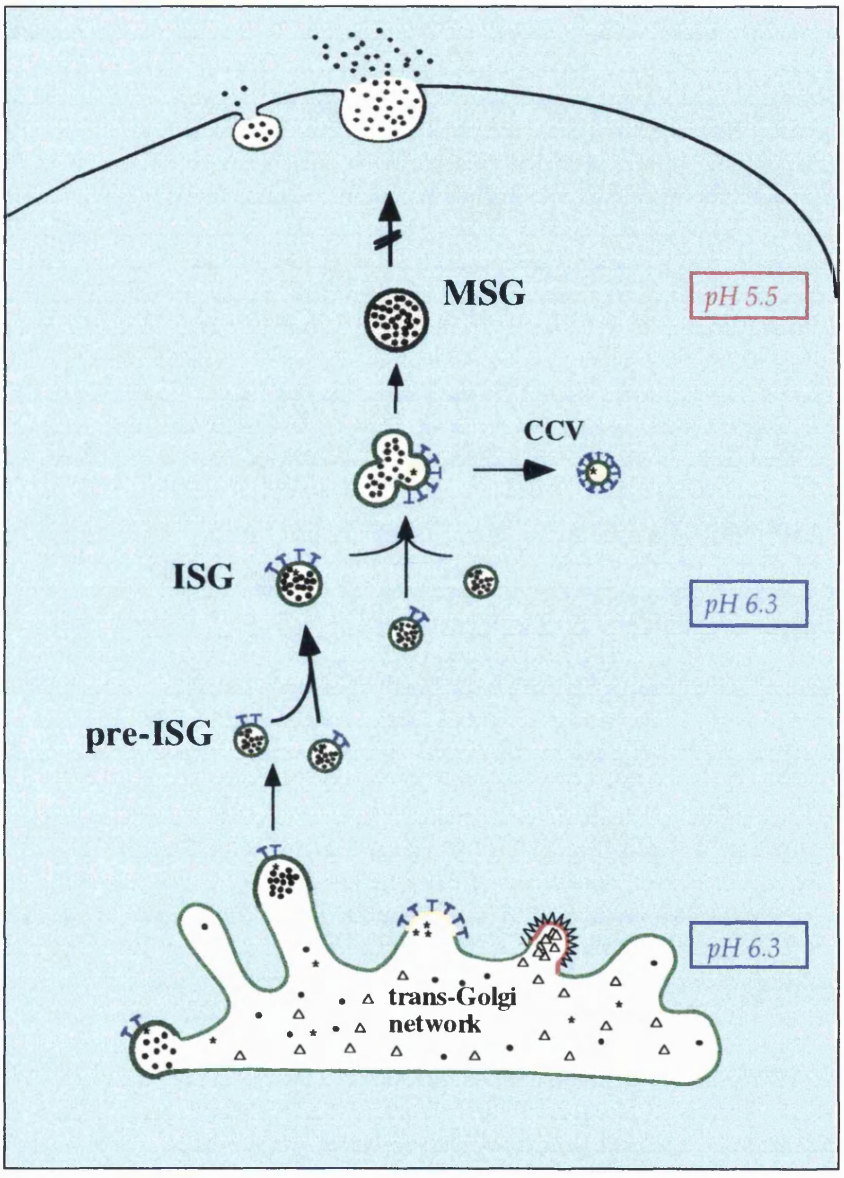


Figure 8/1: *Secretory granule biogenesis*

ISG, immature secretory granule; MSG, mature secretory granule; CCV, clathrin coated vesicle

Legend:
- T clathrin triskelion
- ★ lysosomal enzyme
- △ constitutive secretory protein
- • regulated secretory protein

8.5 Homotypic fusion and maturation.

The [^{35}S]-sulphate labelled fusogenic compartment was further characterised using different pulse-chase protocols to determine the time window within which ISGs are fusion competent (Chapter 7). Fusion competence, determined as the amount of p18 obtained from the [^{35}S]-sulphate labelled full-length SgII (p86), was shown to decrease rapidly with prolonged chase-times. Fusion was found to be most efficient if the [^{35}S]-sulphate labelled SgII was partially within the TGN prior to the fusion assay but could still be observed if the [^{35}S]-sulphate labelling period was followed by a 20 min chase. This chase period results in the transfer of all of the [^{35}S]-sulphate labelled SgII to the ISG. Using sucrose gradient centrifugation it was shown that ISGs are formed efficiently from the TGN under the conditions of the assay. It was therefore suggested that ISGs fuse rapidly after budding from the TGN.

Indeed it was shown that ISGs rapidly become refractory to fusion after budding from the TGN such that the fusion signal was decreased by half if the [^{35}S]-sulphate had been chased for 10 min following a 10 min pulse. The decrease in fusion competence with time is reminiscent of the fusion characteristics in the endocytic pathway. Data from several independent fusion assays showed that fusion competence of early endosomes decreases rapidly with internalisation time (Diaz, *et al.*, 1988; Gruenberg and Howell, 1987). Gruenberg and Howell reported a half time for the loss of fusion competence of less than 3 min (Gruenberg and Howell, 1987). The most fusogenic compartment in the regulated secretory pathway appeared to be distinct from the TGN and ISGs and might correspond to an earlier intermediate in secretory granule maturation here referred to as a pre-ISG (see Figure 8/1).

What then is the function of homotypic fusion of ISGs in neuroendocrine cells? Fusion might be a prerequisite for post-TGN sorting of proteins from the ISG. First, it is conceivable that the small clathrin-patch on the ISG does not comprise a large enough number of coat proteins to generate a clathrin coated vesicle from the ISG. Second,

there might not be enough excess membrane available in one ISG to form a clathrin coated vesicle. Fusion of several ISGs would make more membrane available to form a vesicle and would also increase the number of adaptor/clathrin binding sites on the larger immature secretory granule.

The partial clathrin coat most likely reflects the presence of proteins that are sorted into the ISG either by mistake (mannose-6-phosphate receptor and associated lysosomal enzymes) or on purpose (putative sorting receptors) and have to be removed from the secretory granule during maturation. If one assumes that only 20% of the ISG membrane (surface area: $\Pi \times (40)^2 = 5026.5\ nm^2$) is covered with clathrin and if this patch is the only membrane that can be retrieved (20% of $5026.5\ nm^2 = 1005.3\ nm^2$), it can be calculated that a minimum of 2 ISGs have to fuse to accumulate enough clathrin coated membrane to form one clathrin coated vesicle of 50 nm diameter (surface area: $\Pi \times (25)^2 = 1963.5\ nm^2$). Furthermore if one vesicle of 50 nm diameter (surface area: $1963.5\ nm^2$) was to form from one single ISG, this would consume 40% of the ISG-membrane (surface area: $5026.5\ nm^2$). Fusion of 3 ISGs ($3 \times 5{,}026.5 = 15{,}079.5\ nm^2$) to form one MSG (surface area: $\Pi \times (60)^2 = 11{,}309.7\ nm^2$) would generate enough excess membrane for the formation of 2 clathrin-coated vesicles ([15,079.5 - 11,309.7] / 1963.5 = 1.92) while fusion of 5 ISGs (5 x 5,026.5 = 25,132.5) could provide enough membrane for 7 clathrin coated vesicles ([25,132.5 - 11,309.7] / 1963.5 = 7.04).

It is possible that the size of the ISG is dictated at the level of the TGN by the biophysical properties of the matrix content and is smaller than the optimal quantum size for exocytosis. Homotypic fusion could resolve this problem by allowing remodelling of the granule dimensions during maturation. The homogenous size distribution of MSGs suggests that the homotypic fusion of ISGs has to be a highly regulated process. The fusion assay characterised in this thesis can now be used to study the regulation of this crucial event in secretory granule maturation.

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