BIOCHEMICAL and IMMUNOLOGICAL CHARACTERISATION OF PROTEINS OF THE CHROMAFFIN GRANULE MEMBRANE

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Aileme,

Saygi ve Sevgilerimle.

DECLARATION

This study was carried out under the guidance of Drs. David K. Apps, Jeff Haywood and John H. Phillips at the Department of Biochemistry, University of Edinburgh Medical School between September 1987 and December 1991.

The experimental work presented in this thesis is my own and this thesis has been composed by myself. Where contributions from others is discussed this is acknowledged in the text.

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ABSTRACT

One approach to studying the mechanism of exo- and endocytosis is to identify and characterise the proteins that present in the membranes of secretory vesicles that are not involved in the biosynthesis of their contents, and which display a wide tissue distribution.

The aim of this project was the characterisation of the membrane proteins of bovine adrenal chromaffin granules in order to determine their possible roles in either mediating exocytosis or in the organisation and intracellular movement of the granule. The characterisation was carried out by using mouse monoclonal antibodies that were raised against granule membrane-derived protein fractions. Such an approach was chosen to overcome the biochemical problems one would face if each antigen were to be individually purified and studied.

To obtain monoclonal antibodies against these components, antigen fractions enriched in them, but devoid of the abundant and immunogenic proteins of catecholamine biosynthesis (such as dopamine β-monooxygenase and cytochrome b_{561}) were required for immunising mice. These were generated first by using the phase separation property of the non-ionic detergent Triton X-114 to obtain a fraction that was enriched in membrane glycoproteins, and second by the liberation of tryptic peptides from chromaffin granule membranes that had been washed free of cytosolic and lumenal contaminants. Another method used was to obtain a fraction of proteins that bound to chromaffin granule membranes in the presence of elevated concentrations of free Ca^{2+}.

Identification of the mouse monoclonal antibodies required the development of rapid and sensitive methods of screening the hybridoma

supernatants. Procedures for immuno-dot and enzyme-linked immuno-sorbent assays were developed to identify and characterise the antibodies.

Using these approaches several monoclonal antibodies were obtained. One of these, *cgm80*, recognised a protein of molecular weight 78 - 94-kDa and pI 4 - 5, and was determined to be against the integral membrane protein glycoprotein II, the structure and function of which have not been characterised. Other monoclonal antibodies included *dbh1* and *cgm67* , which were respectively directed against dopamine β-monooxygenase and a protein of molecular weight 67-kDa and pI 5.4 - 6.2. Using these antibodies, the cgm67 antigen was studied in detail, and was found to be an integral chromaffin granule membrane protein. Its susceptibility to the enzymes endoglycosidase F and neuraminidase showed that it was N-glycosylated. Limited trypsin digestion of chromaffin granules and their membranes was used to determine its structure and transmembrane topology. Such treatments liberated a 39-kDa soluble fragment of the antigen which corresponded to the whole of its cytoplasmic domain. This domain was shown to contain both the epitope for the monoclonal antibody cgm67 and a calmodulin-binding site. The 39-kDa fragment was purified, the sequence of its N-terminal 20 amino acids determined and found to be identical to a sequence within the rat synaptic vesicle protein p65. The cgm67 antigen was therefore termed *bovine p65*. The structure and *in vitro* properties of bovine p65 has suggested that this protein performs an important function during exocytosis, possibly by mediating the docking of chromaffin granule to the plasma membrane.

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CHAPTER 1

CHAPTER 1

1.1 Introduction

It is well established that most proteins that are secreted from eukaryotic cells move by vesicular transport from the endoplasmic reticulum to the Golgi apparatus to the plasma membrane, in vesicles. The process of secretion, which depends on the the type of cell, can be categorised as either "*constitutive*" or "*regulated*" [Kelly, 1985; Burgess & Kelly, 1987]. The constitutive pathway is the general route in all cells for the transport of molecules that are constantly needed at the cell's exterior such as growth factors, extracellular components and enzymes. It is also used for the biogenesis of all the organelles along the secretory pathway including the plasma membrane. The secretory vesicle, the organelle involved in the final stages of secretion, transports these molecules from the trans-Golgi network to the cell surface at rates that keep pace with their synthesis. In contrast, regulated secretion occurs only in specialised secretory cells such as those found in endo- and exocrine cells, as well as in neurones. These cells contain specialised vesicles known as "*secretory granules*" which can selectively concentrate their contents many-fold and store them in close proximity to the plasma membrane until required. The release of the contents to the cell's exterior then occurs by exocytosis which is triggered by a specific external secretory stimulus in response to a change in the physiological state of the organism.

It is assumed that the regulated pathway has evolved from the constitutive pathway by the superimposition of specific control and targeting elements, since if these are perturbed, secretion occurs by default through the constitutive pathway [Burgess & Kelly, 1987]. Although much is known

about the contents of secretory granules and how these are accumulated, the biochemistry of granule movement and membrane fusion are not understood. It is assumed that these events must be regulated by proteins that are present in the membranes of the granules, although none of the few membrane proteins which have been identified so far have been assigned an exact role. Nevertheless, the proteins of the granule membrane provide a starting-point for the investigation of the molecular mechanism of exocytosis. The aim of this study was to characterise, by means of immunological techniques, the membranes of secretory vesicles from the adrenal medullary chromaffin cell ("*chromaffin granules*"), to further our understanding of the role of secretory vesicle membrane proteins in the mechanism of regulated secretion.

1.2 The adrenal chromaffin cell

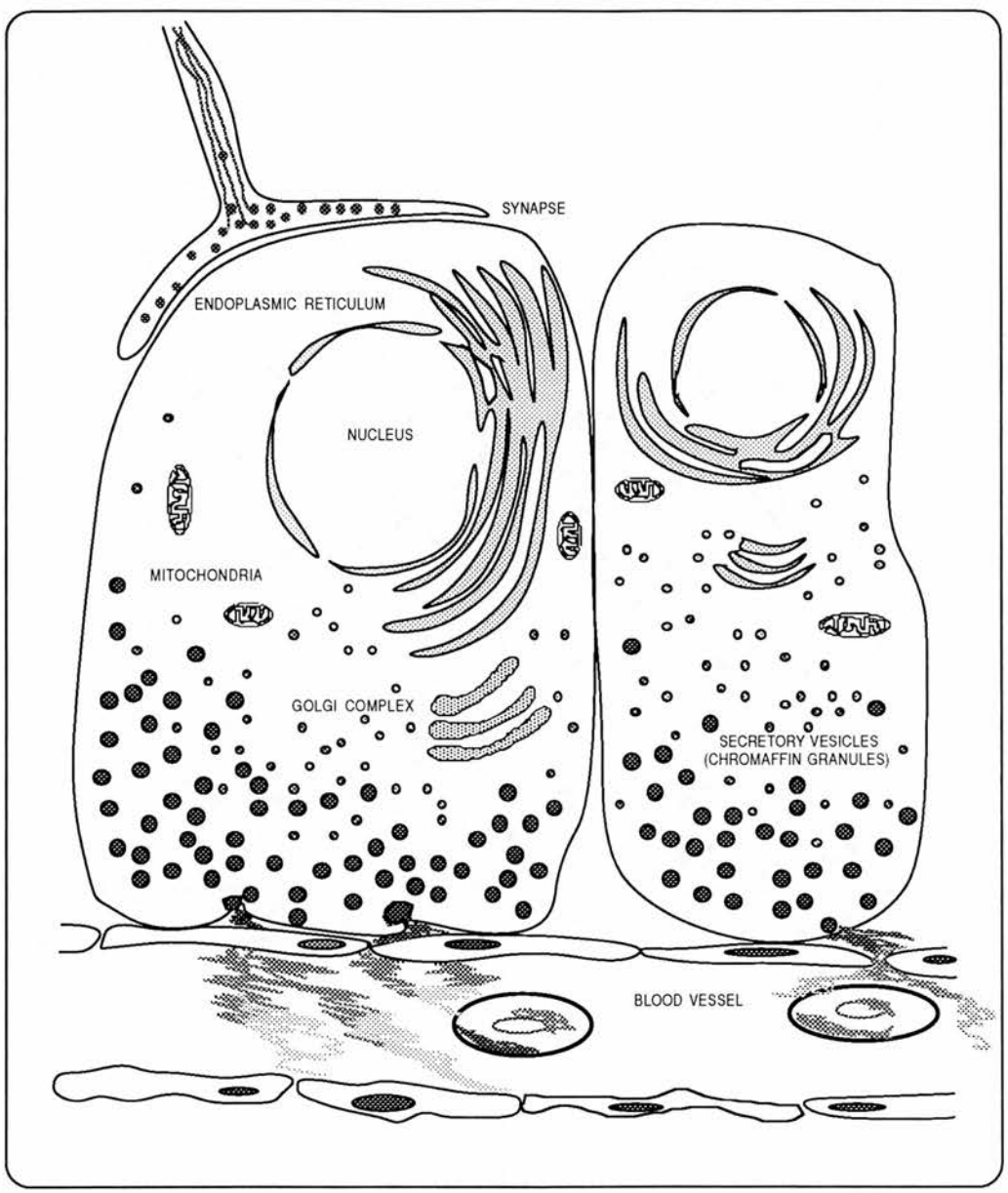
Chromaffin cells comprise the bulk of the adrenal medulla, a neuroendocrine tissue derived from the neural crest. They are regarded as modified post-ganglionic sympathetic neurones which secrete catecholamines, neuropeptides and proteins in response to neural stimulation by the pre-ganglionic cholinergic nerves that come from the spinal cord [Malmejac, 1964; Carmichael, 1986] (Figure 1.1). Early studies by Blashko and Welsh (1953) and Hillarp *et al.* (1953) established that these secretory products are sequestered in subcellular compartments, now known as chromaffin granules, and subsequent studies by Douglas showed that they are released to the cells' exterior by a mechanism analogous to stimulus-contraction in skeletal muscle [Douglas & Rubin, 1961*a*]. Consequently the chromaffin cell became an established model for the study of regulated secretion because of the ease with which its abundant and dense granules can be purified. Furthermore the availability of primary cultures [Fenwick *et al.*, 1978] and of a transformed rat cell line (PC12) [Greene & Tischler, 1976] has allowed the investigation of the molecular events occurring during biogenesis and exocytosis of the granules.

1.3 The chromaffin granule

Chromaffin granules, the secretory vesicles of chromaffin cells, are the re-usable shuttles for the molecules that are released from these cells by regulated secretion; a typical chromaffin cell contains up to 20,000 granules [Phillips, 1982; Phillips & Pryde, 1986]. Although regarded as a homogeneous population, it is important to stress that chromaffin granules vary both in

Figure 1.1

Schematic diagram of adrenal chromaffin cell [a]



[a] See Sections 1.2 & 1.3 for text.

terms of size and content [Coupland, 1968; Kryvi *et al.*, 1979; Terland *et al.*, 1979]. This is because the adrenal medulla is primarily composed of two different cell types - the adrenaline- and noradrenaline-storing chromaffin cells. These two types of cells are clearly distinguishable in electron micrographs, those secreting noradrenaline containing the denser-looking granules in comparison to those that secrete adrenaline [Coupland *et al.*, 1964; Coupland & Hopwood, 1966].

In this Chapter, chromaffin granules will be considered as a single population simply because there have not been many studies on the properties of the two types of granules. Therefore, the physical parameters of granules presented in Table 1.1 are only mean values, and serve to illustrate their magnitude.

[a] ***Contents of the chromaffin granule*** *:*

The matrix of the chromaffin granule is composed of a remarkably high concentration of secretory products. These are summarised in Table 1.2. The physiologically best-characterised and most abundant constituents of chromaffin granules are the catecholamines. Within the granule their total concentration is estimated to be around 550 mM; this is a typical feature of regulated secretory vesicles where active transport drives the accumulation of small molecules. The pathway of synthesis of catecholamines has long been established [Kirshner, 1975] and is initiated in the cytosol with the hydroxylation of L-tyrosine into L-dopa (Figure 1.2). This reaction, which is catalysed by the enzyme tyrosine hydroxylase (EC 1.14.16.2), is the rate-limiting step of catecholamine synthesis, and is regulated by substrate and co-factor availability as well as multi-site phosphorylation of the enzyme. The product dopa is then converted into dopamine by the cytosolic enzyme L-amino acid decarboxylase (EC 4.1.1.28). The synthesis of both noradrenaline

Table 1.1

Morphometric Analysis of Bovine Chromaffin Cells[a,b]

The adrenal chromaffin cell	
Diameter of a chromaffin cell	16 μm
Surface area of a spherical cell	800 μm^2
The adrenal chromaffin granule	
Average diameter of a chromaffin granule	0.28 μm
Surface area of a granule	0.25 μm^2
Approximate number of granules in a cell	30,000
Volume of cell occupied by granules	15 %
Total area of granule membrane in one cell	7,400 μm^2

a From Phillips & Pryde (1986), see also Phillips (1982).

b These are approximate values and they differ for adrenaline and noradrenaline-storing chromaffin cells e.g. average number of granules per adrenaline-storing cells is 23,000 and for noradrenaline-storing cell is 33,000.

Table 1.2

Matrix components of chromaffin granules

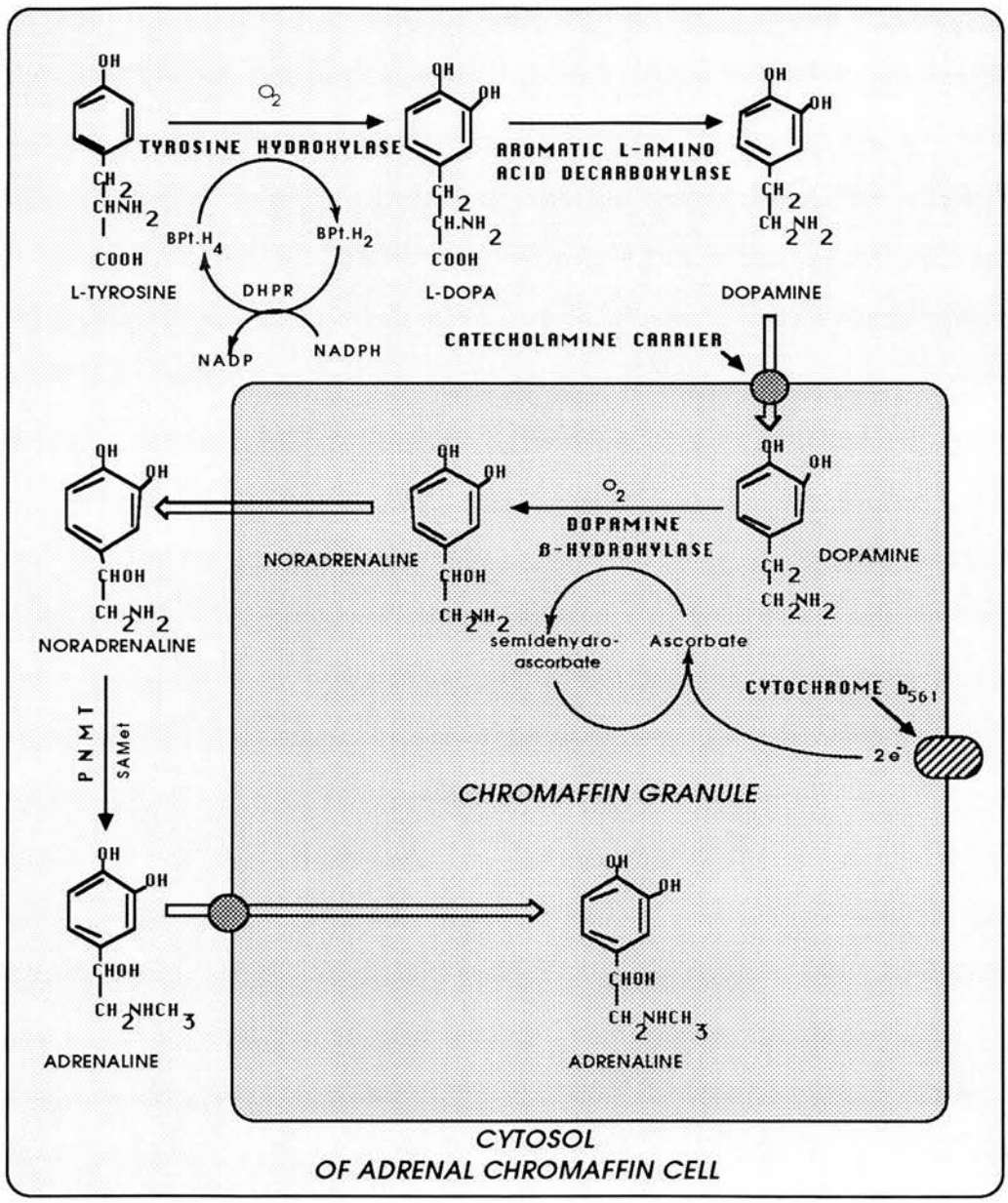
Matrix small molecules	**Approx no. of molecules per granule**[a]
Adrenaline	1.7×10^6
Noradrenaline	7.1×10^5
Dopamine	2.1×10^4
ATP	5.8×10^5
Other nucleotides	2.3×10^5
Ascorbic acid	1.0×10^5
Na^+	9.4×10^4
Ca^{2+}	4.1×10^4
Mg^{2+}	2.0×10^4
Enkephalins	7.5×10^3
Neuropeptide Y	5.0×10^2
Neurotensin	7.2×10^2
Substance P	3.0×10^2
Matrix Proteins and peptides	
Chromogranin A family	
Chromogranin B family	about 4 mg per mg of membrane
Proenkephalin family	protein (chromogranin A family
Prodynorphin family	composes ~50% of it).
Dopamine β-monooxygenase	
Acetylcholinesterase	
Proteases	
Matrix glycosaminoglycans	**μmol sugar/granule protein**[b]
Chondroitin sulphate	2.6
Heparin sulphate	0.1

[a] Mean values converted to number of molecules per granule from values taken from Winkler & Westhead (1980). *N.B.* Chromaffin granules are heterogeneous and adrenaline and noradrenaline granules differ [Terland *et al.* 1979; Bolstad *et al.* 1980]. Values for inorganic cations from Krieger-Brauer & Gratzl (1982). 1 mg total chromaffin granule protein is equivalent to 5.8×10^{11} vesicles [Schilling & Gratzl, 1988].

[b] Values from Geissler *et al.* (1977).

Figure 1.2

Catecholamine biosynthesis in adrenal chromaffin cells [a]



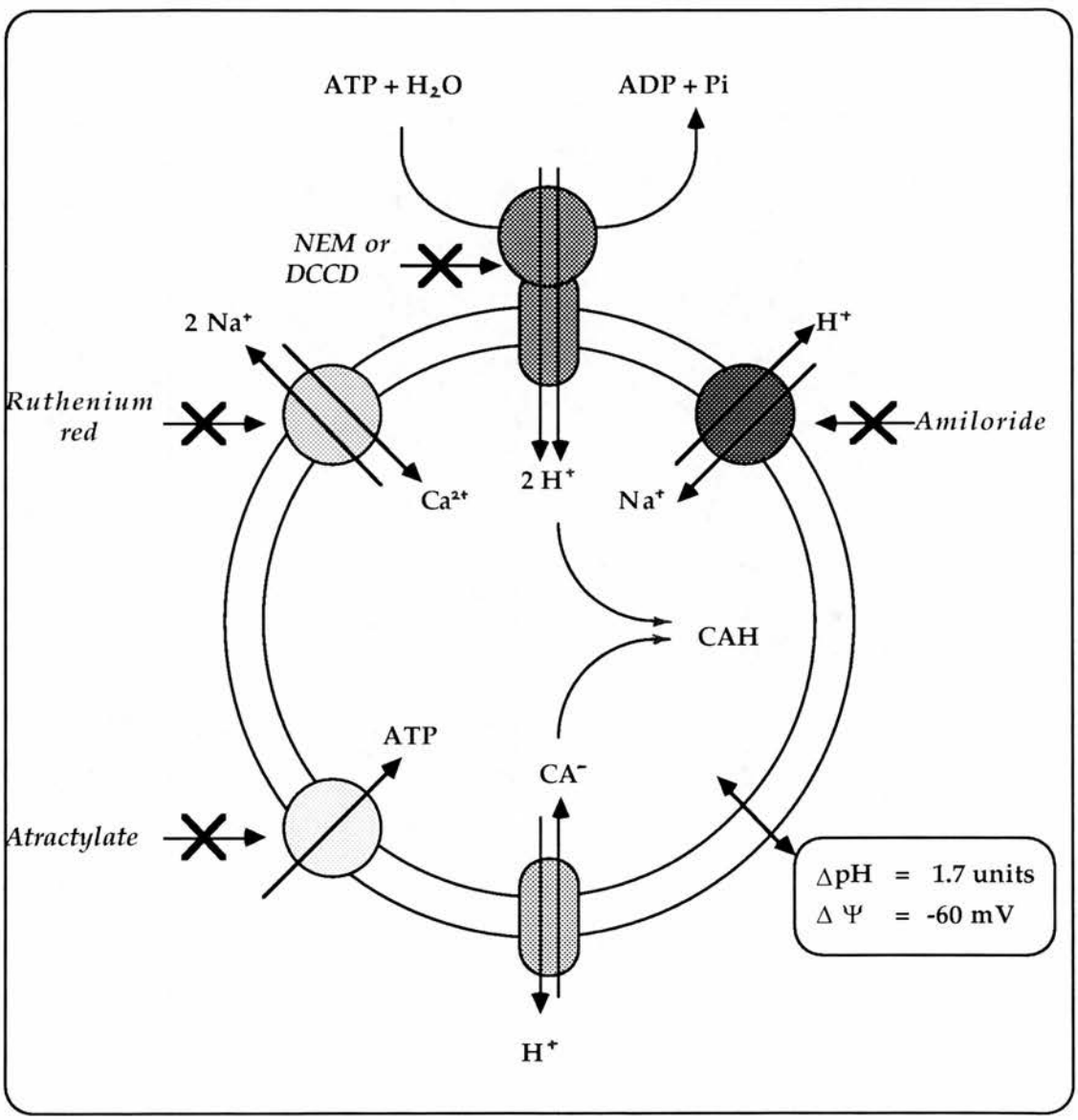
[a] See Section 1.3[a] for text.

KEY :

BPt : *biopterin,*
SAMet : *S-adenosy-L-methionine,*
PMNT : *ethanolamine-N-methyl transferase.*

Figure 1.3

Schematic representation of the ion and small molecule translocators present in the adrenal chromaffin granule membrane [a]



[a] See Section 1.3[a] for text.

and adrenaline requires the active uptake of cytosolic dopamine into the granules via an amine transporter and, once in the granule, the enzyme dopamineβ-monooxygenase catalyses its conversion into noradrenaline. This enzyme requires molecular oxygen and catalyses a redox reaction in which two electrons are transferred from ascorbic acid via the two copper ions of dopamine β-monooxygenase to dopamine. The ascorbic acid is regenerated from its oxidised form (semidehydro ascorbate) by non-enzymatic dismutation followed by reduction by electrons supplied from the transmembrane protein cytochrome b_{561} [Skotland *et al.*, 1980; Diliberto & Allen, 1980]. About three-quarters of the chromaffin cells of the bovine adrenal medulla contain adrenaline-storing granules and the other quarter contain noradrenaline-storing granules. In the cells with adrenaline-storing granules, noradrenaline leaves the granule and is converted into adrenaline by the cytosolic enzyme phenylethanolamine N-methyl transferase (PNMT, E.C.2.1.1.28). The adrenaline is then taken up into the granule via the amine carrier and stored until its release from the cell.

Another constituent of the chromaffin granule is ATP, which is present at around 150 mM. Although the actual reason for the presence of ATP in the granules is not known, it is thought to play an important role in reducing of the osmotic stress caused by the high concentration of catecholamines through ion pairing with Na^+ and molecular stacking with catecholamines [Kopell & Westhead, 1982]. Both ATP and a wide range of other nucleotides are taken up into the granule by an atractyloside-sensitive nucleotide-translocase, driven by the membrane potential [Aberer *et al.*, 1978; Weber & Winkler, 1981].

The chromaffin granule also contains secretory proteins and neuropeptides at concentrations of 180 mg/ml. The molecular mechanism by which these proteins are sorted to secretory granules is not clear, although

increasingly acidic condition and Ca^{2+} concentrations have been proposed to aggregate these proteins during the budding of the granules from the trans-Golgi network [Wallach & Schramm, 1971; Amsterdam & Jamieson, 1974; Palade, 1975; Huttner *et al.*, 1990]. Despite the high concentration of these intragranular proteins, there are a relatively few different types, the most prominent being chromogranin A. This protein, which constitutes about 7% of the total bovine adrenal medullary and 50% of the intragranular protein, belongs to a group of acidic glycoproteins that are homologous to the secretory proteins found in a wide range of endocrine secretory granules [Cohn *et al.*, 1982; O'Connor & Frigon, 1984; Kruggel *et al.*, 1985]. Structural studies of chromogranin A have revealed a high content of glutamate, extensive O-glycosylation and almost no defined tertiary structure [Smith & Winkler, 1967; Sharp & Richards, 1977; Gerdes *et al.*, 1989]. Electrophoretic studies show that chromogranin A which has an apparent molecular weight of 75-kDa (the actual polypeptide molecular weight being 48-kDa), is proteolysed into a family of peptides. These properties are also features of two related proteins, chromogranin B (secretogranin I) and chromogranin C (secretogranin II) which share homology particularly around the disulphide-bonded loops near the amino-terminus as well as at the carboxy-terminus [Smith & Winkler, 1967; Kiang *et al.*, 1982; Benedum *et al.*, 1986 & 1987]. The function of chromogranins is not known. They were initially thought to play a role in catecholamine storage but this seems unlikely in view of their wide distribution in secretory granules. More recently, Galindo *et al.* (1991) have suggested that chromogranins may be the precursor to biologically active peptides, since part of the primary sequence of chromogranin A is homologous to the peptide *pancreastatin* which regulates secretion from pancreatic β-cells [Tatemoto *et al.*, 1986]. Pancreastatin which inhibits secretion from pancreatic β-cells has been shown to have similar effects on exocytosis from cultured chromaffin cells.

Such peptides have not however been isolated from chromaffin cells but if they have a **physiological** existence, they may be part of a mechanism to switch off secretion following exocytosis.

Other secretory proteins present in chromaffin granules are the soluble forms of dopamine β-monooxygenase and glycoprotein III, and various neuropeptide precursors. Dopamine β-monooxygenase constitutes some 3 to 4% of the total soluble content of bovine chromaffin granules. Membrane-bound forms of dopamine β-monooxygenase and glycoprotein III are also present (see Section 1.4 for more detail). The enkephalin and dynorphin precursors are better characterised [Lemaire *et al.*, 1984]. The enkephalin precursors are proteolytically processed resulting in a family of peptides of up to 28-kDa [Patey *et al.*, 1984; Fischer-Colbrie & Frischenschlager, 1985]. The proteases that carry out the processing of these precursors are also present in the matrix of the granules, and to date three have been identified: a cobalt-stimulated carboxypeptidase ("H" or "E") and two trypsin-like endopeptidases [Fricker & Snyder, 1982; Hook & Eiden, 1984; Lindberg *et al.*, 1984]. Both bovine and rat carboxypeptidase H have been cloned and sequenced and shown to exist in two isoforms, identical with the previously-identified glycoproteins J and K [Gavine *et al.*, 1984; Fricker *et al.*, 1986; Rodriguez *et al.*, 1989]. Recent experiments with cultured chromaffin cells have demonstrated that some peptides derived from proenkephalin and chromogranin A may be processed extracellularly [Watkinson *et al.*, 1990].

Among the proteins reported to be present in the granule matrix in minor amounts is acetylcholinesterase; a membrane-bound form of this enzyme has also been reported [Gratzl *et al.*, 1981]. However, the location of this enzyme is controversial and its presence in the granules may be as a consequence of contaminating endoplasmic reticulum and plasma membrane, where this enzyme is present in high amounts [Gratzl, 1984;

Mizobe *et al.*, 1984; Burgun *et al.*, 1985]. Another less-well characterised minor constituent of the chromaffin granule matrix is the acidic 10-kDa copper-containing protein isolated by Grigoryan *et al.* (1981) and Mikaelyan *et al.* (1987). The structure and function of this protein is unknown, however, studies of its aminoacid composition and antigenicity have revealed that it is a member of the neurocuprein family of protein that have been identified in mammalian brain. The *in vivo* role of this protein in chromaffin granules is not clear, there have been reports that it binds catecholamine and, in its reduced form, participates in adrenochrome detoxification. The apo-form of this protein has also been reported to be a potent inhibitor of dopamine β-monooxygenase [Grigoryan *et al.*, 1981; Mikaelyan *et al.*,1987]. Resembling other proteins of the chromaffin granule matrix, this protein also appears to be present in both soluble and membrane-bound forms, its membrane anchor has been proposed to be an extended hydrophobic tail [Mikaelyan *et al.*,1987].

Some 3 - 4% of the chromaffin granule matrix consists of proteoglycans, which is typical of regulated secretory vesicles (Table 1.2). Their glycosaminoglycan components are thought to consist mainly of dermatan sulphate and chondroitin 4-6 sulphate [Kiang *et al.*, 1982; see Falkensammer *et al.*, 1985 for review]. The protein core of the proteoglycans were initially thought to be immunologically similar to that of chromogranin A, despite differences observed in their peptide maps [Banerjee & Margolis, 1982; Kilpatrick *et al.*, 1983]. These differences were attributed to the different glycosyl contents of the two proteins. However, the recent demonstration that the antibodies used in the comparison of the peptide maps were directed towards their glycosyl chains and not towards the polypeptides suggested that the protein core may indeed be dissimilar [Gowda *et al.*, 1990].

The final constituents of the chromaffin granule matrix are the ions. Among the cations are protons which are accumulated by the membrane-

bound ATPase I complex (Figure 1.3; see Section 1.4 for details of ATPase I). The accumulation of protons results in a lumenal pH of about 5.2 and generates a membrane potential of around -60 mV (inside positive). This membrane potential is the used for the uptake of small molecules and other ions as summarised in Figure 1.3. Other cations include Ca^{2+}. Although its total concentration in the granule is around 20 mM, only 10 μM is free, the rest being sequestered in chromogranin A/ATP complexes [Bulenda & Gratzl, 1985; Reiffen & Gratzl, 1986]. It is thought that accumulation of Ca^{2+} by the granules could be used as a mechanism for the long-term removal of Ca^{2+} from the cell. The uptake of Ca^{2+} into the granules is mediated through the ruthenium red-sensitive Na^+/Ca^{2+} antiporter. Although a link between this antiporter and the proton gradient was established by Haigh and Phillips (1990) via a H^+/Na^+ antiporter, the distribution of Ca^{2+} and Na^+ suggest that these anions do not equilibrate with protons. Magnesium ions are also present in the granules at around 5 mM, mostly complexed to ATP.

The anionic composition of the granules is less well defined: chloride ions are thought to cross the granule membrane passively driven by the membrane potential. It has been suggested that a mechanism analogous to the bicarbonate/chloride exchanger of erythrocytes may be present in chromaffin granule membranes to prevent the lysis of granules as a result of Cl^- uptake. It has also been suggested that Cl^- uptake into chromaffin granules may be mediated through a Cl^- channel analogous to that present in the membranes of coated vesicles and the Golgi apparatus [Xie *et al.*, 1989*a*; Barasach *et al.*, 1991].

[b] ***Lipid composition of the chromaffin granule membrane*** *:*

The membrane composition of the chromaffin granule has to be compatible with its function, primarily the accumulation and storage of its

Table 1.3

Lipid composition of chromaffin granule membranes[a]

Lipid	(μmol/mg membrane protein)
Cholesterol	1.44
Phosphatidylcholine	0.65
Lysophasphatidylcholine	0.41
Sphingomyelin	0.29
Phosphatidylethanolamine	0.84
Phosphatidylserine	0.06
Ganglioside GM_3	0.05
Fatty acids	0.10

[a] From Winkler (1976) and Dreyfus *et al.* (1977).

contents. Consequently this membrane is highly impermeable to ions and small molecules [Johnson & Scarpa, 1976]. Chromaffin granule membranes also have a high lipid to protein ratio (about 2 μmol phospholipid per mg of protein). The lipid composition is summarised in Table 1.3. Among the lipids, sphingomyelin and cholesterol are particularly abundant (e.g. 0.6 μmol cholesterol/μmol phospholipid) [Winkler, 1976]. Chromaffin granule membranes also have an usually high content of lysophosphatidylcholine (approximately 20% of the total phospholipid) [Da Prada *et al.*, 1972] which has been postulated to play an important role in the fusion between membranes [Howell & Lucy, 1969]. However only 10% of this phospholipid is in the outer leaflet of the lipid bilayer of the chromaffin granule membrane [Voyta *et al.*, 1978; de Oliveira-Filgueiras, 1979]. In contrast, phosphatidylethanolamine, together with phosphatidylinositol, is enriched in the outer leaflet [Buckland *et al.*, 1978]; the latter is in the correct orientation for the generation of inositol phosphates through the action of phospholipase C. It is interesting to note that phosphatidylinositol appears to be phosphorylated by a component of the chromaffin granule membrane [Phillips, 1973] and the product of this reaction, phosphatidylinositol 4,5-bisphosphate (PIP_2) is implicated in the regulation of actin filamentation by proteins such as villin and severin during exocytosis (see Section 1.5[c] and Matsudaira & Janmey, 1988 for review).

The other lipid component of the chromaffin granule membranes is the gangliosides which compose 2 - 3% of the phospholipids. Three different species of GM_3 comprises around 95% of the gangliosides [Dreyfus *et al.*, 1977; Serkine *et al.*, 1984]. As expected the oligosaccharide moieties of the gangliosides are exposed to the lumen of the granule [Westhead & Winkler, 1982].

[c] *Membrane proteins of chromaffin granules* :

About 20% of the proteins of chromaffin granules are membrane-bound. These proteins mediate many of the granules' functions, such as the biosynthesis and accumulation of catecholamines, its interaction with cytoplasmic factors and targeting to and from the plasma membrane. It is therefore not surprising to find that when preparations of chromaffin granule membranes are analysed by electrophoresis and Coomassie-blue staining, about 60 bands are detected [Abbs & Phillips, 1980]. Among these proteins, the two best-studied components of catecholamine biosynthesis, dopamine β-monooxygenase and cytochrome b_{561} constitute about half the protein content [see Winkler & Fischer-Colbrie, 1990]. The proton translocating ATPase I complex, which has a fundamental role in the bioenergetics of the granule, has also been characterised [Percy *et al.*, 1985; Cidon & Nelson, 1986]. Characterisation of the rest of the proteins has been complicated by not knowing whether many of the peripheral proteins found in the chromaffin granule membrane preparation are true components of it. Washing the membranes at pH 11 with sodium carbonate removes these adherent proteins, leaving about 40 polypeptides detectable on two-dimensional gels by Coomassie-blue staining. This is still an underestimate of the number of integral membrane proteins since about half of these are glycoproteins that are poorly stained. Lectin-binding provides a better means of visualising most but not all of these glycoproteins [Gavine *et al.*, 1984; Apps *et al.*, 1985; Pryde & Phillips, 1986]. The low abundance of some proteins means that they can only be visualised by specific antibodies (e.g. SV2, p65, p38), or else their presence is indicated by activities present in chromaffin granule membrane preparations (e.g. Na^+/H^+ antiporter). In short, the proteins of the chromaffin granule membrane can be categorised into four groups as summarised in Table 1.4.

Table 1.4

Chromaffin granule membrane proteins[a]

Identifiable[b] on gels and function known:	Not identifiable on gels and function known:
ATPase II	Nucleotide transporter
Cytochrome b_{561}	Phosphatidylinositol kinase
Catecholamine Transporter	Ca^{2+}/Na^{+}-antiporter
Dopamine β-monooxygenase	Na^{+}/H^{+}-antiporter
Glycoprotein J	
Glycoprotein K	
Glycoprotein H	
H^{+}-ATPase I subunits	

Identifiable on gels but function unknown:	Not identifiable on gels and function unknown:
Glycoprotein II	p65
Glycoprotein III	Synaptophysin (p38)
Glycoprotein IV	Synaptobrevin (p18)
Glycoprotein H	SV2

[a] Adapted from Winkler *et al.* (1986).

[b] Identifiable by Coomassie-blue staining.

An interesting, yet unresolved, feature of some of these membrane proteins is their dual distribution as both membrane-bound and soluble proteins. Within this group of proteins are dopamine β-monooxygenase, glycoprotein III and carboxypeptidase H. These proteins will be discussed in detail in Section 1.4.

1.4 Membrane proteins of the chromaffin granule

The proteins of the chromaffin granule membrane may be categorised into two groups depending on their abundance. The more abundant proteins such as dopamine β-monooxygenase, cytochrome b_{561} and carboxypeptidase H are needed for the biosynthesis and accumulation of the granules' "cargo", whereas the minor constituents, although their functions are not known, appear to be needed for the molecular organisation and intracellular transport of granules. The members of the former group are present only in those cells which store and secrete the products which they make and their abundance is consistent with the high concentration of these products (see Section 1.3[a] and Table 1.2). The low abundance and wider tissue distribution of the latter group, on the other hand, suggests that they a involved in a more general (housekeeping) function and may not be required in as many copies per granule as the biosynthetic proteins.

[a] *Major proteins of the chromaffin granule membrane* :

As described in Section 1.3[a], the major constituent of the chromaffin granule is catecholamines. Two chromaffin granule membrane proteins that are involved in their biosynthesis are dopamine β-monooxygenase and cytochrome b_{561}, which together constitute around 50% of the total membrane protein. Dopamine β-monooxygenase (EC 1.14.17.1), a copper-containing enzyme, catalyses the conversion of dopamine to noradrenaline in the chromaffin granule. It is a glycoprotein of about 300-kDa consisting of four subunits of 72- and 75-kDa, arranged as two pairs of disulphide-linked dimers [Hörtnagl *et al.*, 1972]. It is present in large dense core vesicles of sympathetic (noradrenergic) neurones as well as in chromaffin granules [Hartman, 1973;

Hökfelt *et al.*, 1975] but its presence in small dense-core vesicles is controversial (see Winkler & Fischer-Colbrie, 1990). The protein is N-glycosylated, each tetramer carrying about six oligosaccharides (two high-mannose and four complex biantennary) which show microheterogeneity [Margolis *et al.*, 1984] and it therefore binds both concanavalin A and wheat germ agglutinin [Gavine *et al.*, 1984].

The conversion of dopamine to noradrenaline by dopamine β-monooxygenase is a redox reaction requiring molecular oxygen and electrons (see Section 1.3[a]). Electrons for this reaction originate from the cytosol and are transported into the granule via the haem -containing integral membrane protein cytochrome b_{561} [Njus *et al.*, 1983], which accounts for around 20% of the total protein content of chromaffin granule membranes [Terland & Flatmark, 1980*a*; Apps *et al.*, 1984; Winkler, 1976]. This protein has been cloned and sequenced from cow [Perin *et al.*, 1988]; it is composed of 273 aminoacids and has an apparent molecular weight of 28-kDa on SDS-gels. It is predicted to cross the chromaffin granule membrane six times with both its N- and C- terminal domains exposed to the cytoplasm and is not glycosylated; both predictions are in agreement with earlier studies by Abbs & Phillips (1980). Recent studies have suggested that the N-terminus may be fatty acylated and thus be attached to the membrane, however the physiological significance of this is not clear [Kent & Fleming, 1990]. Cross-linking and redox titration studies have suggested that cytochrome b_{561} forms oligomers in the granule membrane [Apps *et al.*, 1984].

As a supplier of electrons for granules, cytochrome b_{561} is present in both large dense-core and small dense-core vesicles of sympathetic neurones [Asamer *et al.*, 1971], as well as in the secretory granules of anterior and posterior pituitary [Pruss & Shepard, 1987]. It is also present in the secretory granules of thyroid C-cells but is absent in the parathyroid and in the

endocrine cells of the pancreas [Weiler *et al.*, 1989]. Cytochrome b_{561} has also been reported to be present in heart muscle cells [Pruss & Shepard, 1987].

Among the other major proteins of the chromaffin granule membrane that are involved in the biosynthesis of intragranular contents is carboxypeptidase H. Previously known as carboxypeptidase E or enkephalin-convertase, carboxypeptidase H (EC 3.17.10) is involved in the biosynthesis of enkephalin. It removes C-terminal arginines and lysines from the partially-processed proenkephalin [Hook *et al.*, 1982; Fricker, 1986]. Carboxypeptidase H has been shown to be the same protein as the previously identified glycoproteins J and K of the chromaffin granule membrane [Wood *et al.*, 1984; Laslop *et al.*, 1986]. It displays a broad tissue distribution, and as well as in the adrenal medulla, it is present in pituitary, pancreas, endocrine cells of the gastrointestinal tract as well as in various regions of the brain [Lynch *et al.*, 1986,1987,1988; Hook *et al.*, 1985].

One common feature of the biosynthetic enzymes such as dopamine β-monooxygenase and carboxypeptidase H is that they are present in both membrane-bound and soluble forms in approximately equal amounts. Another chromaffin granule protein, "*glycoprotein III*" with unknown function, is also present in two forms [Winkler & Fischer-Colbrie, 1990; Palmer & Christie, 1990]. The reasons for this dual distribution is not known; their genes have been cloned and sequenced [Lamouroux *et al.*, 1987 (DβH); Fricker *et al.*, 1986 & Rodriguez *et al.*, 1989 (CpH); Palmer & Christie, 1990 (GpIII)], and no apparent differences were found which could result in the two forms. It is possible that during evolution, the membrane-binding property of these proteins initially served as a sorting mechanism at the trans-Golgi network (such as that proposed for chromogranin A by Huttner *et al.*, 1990), and since then it evolved as a mechanism to preventing their loss from the cell during exocytosis. The membrane-bound forms of dopamine

β-monooxygenase and glycoprotein III have been shown to recycle into new granules after exocytosis [Phillips *et al.*, 1983; Patzak *et al.*, 1984; Hunter & Phillips, 1989].

The mode of attachment of these proteins to the chromaffin granule membrane is a controversial issue and three different models have been proposed: (1) uncleaved signal sequence, (2) phospholipid anchors, and (3) conformational change induced by the environment of the protein. Most studies have been carried out on dopamine β-monooxygenase - there is evidence for the presence of both an uncleaved signal sequence [Skotland *et al.*, 1977; Taljanidisz *et al.*, 1989] and affinity for phophatidylserine [Taylor & Fleming, 1989]. For glycoprotein III, membrane binding may be dependent on the lumenal milieu - this is suggested by the homology of this protein to glycoprotein 2 of rat sertoli cells which can aggregate red blood cells [Palmer & Christie, 1990; Jenne & Tschopp, 1989; Laine & Esser, 1989]. Whatever the mechanism of attachment, it is not clear how it results in an equal distribution of these proteins between the two forms. It must also be stressed that these proteins were not accessible to extragranular reagents [Abbs & Phillips, 1980].

The accumulation of the catecholamines and ions from the cytosol into chromaffin granules is dependent on the membrane potential and the pH gradient maintained by the ATPase I complex, a proton pump originally identified by Hillarp in 1962. ATPase I belongs to the vacuolar type of proton pumps which occurs in the membranes of intracellular acidic compartment, and is related to, but distinct from, the mitochondrial F-type ATP synthase [Pederson & Carafoli, 1987; Forgac, 1989; Moriyama & Nelson, 1989*a*,*b*]. The electrogenic translocation of protons into the granules is linked to cytosolic ATP hydrolysis and results in a pH gradient of 1.7 units and a membrane potential of about -60 mV (Figure 1.3). These gradients are used for the

accumulation of catecholamines as well as transport of Na^+ and Ca^{2+} ions into the granule.

ATPase I is a multisubunit complex of about 500-kDa. Although its exact subunit composition is controversial, it is generally regarded to be composed of six subunits of apparent molecular weights 120-, 72-, 57-, 40-, 33- and 16-kDa [Percy *et al.*, 1985; Cidon & Nelson, 1986; Pérez-Castiñiera & Apps, 1990]. In intact granules, the 120-, 72-, 57-, 40- and 33-kDa subunits are all accessible to treatment by impermeant reagents such as *N*-hydroxysuccinimidyl biotin, suggesting that they are exposed to the cytoplasm. Washing the membranes at pH 11 fails to remove the 120- and 16-kDa subunits, and these are the only subunits that are labelled with TID, a hydrophobic probe which reacts with those the regions of membrane proteins in contact with the phospholipid bilayer. Therefore the only membrane-spanning polypeptides of the complex are the 120- and 16-kDa subunits [Apps *et al.*, 1989]. The 120-kDa subunit is thought to be the only glycosylated polypeptide of the complex [Apps *et al.*, 1989; Gillespie *et al.*, 1991].

The 16-kDa subunit is thought to form a hexameric proton-translocating channel. Its sequence is highly homologous to other channel complex proteins such as the 15-kDa proteolipid of the mediatophore in *Torpedo* electric organs and the 16-kDa gap junction protein [Mandel *et al.*, 1988; Leitch & Finbow, 1990; Birman *et al.*, 1990]. Proteins of this nature are implicated in the formation of fusion pores that are necessary for fusion of lipid bilayers [Almers, 1990], but there is no evidence that the ATPase 16-kDa subunits perform such a function.

Most of the subunits of V-type ATPases have now been cloned and sequenced [Wang *et al.*, 1988; Manolson *et al.*, 1988]. The results, confirmed by biochemical studies, suggest that the subunit of 67-72-kDa contains the catalytic site [Percy & Apps, 1986], that the 57-kDa subunit has a regulatory

nucleotide binding site and the 16-kDa subunit comprises the proton channel. The functions of the other polypeptides, including the largest, trans-membrane subunit of 120-kDa remain unknown.

Although ATP is required for exocytosis, a direct role of the ATPase can be excluded because the ATPase can operate with a broad range of nucleotides whereas exocytosis is specific for ATP; and because the K_m for the ATPase is about 10-fold less that which is required for exocytosis. Furthermore, agents that inhibit the ATPase activity and those that dissipate the pH or potential gradients do not effect exocytosis [Holtz *et al.*, 1983; Knight & Baker, 1985].

The other ATPase activity present in chromaffin granule membrane as well as in bovine brain clathrin-coated vesicle membranes and secretory granules of the pituitary is due to a vanadate-sensitive ATPase II [Percy *et al.*, 1985; Xie *et al.*, 1989*b*; Russell, 1984]. In contrast to the ATPase I complex, ATPase II is a single polypeptide with an apparent molecular weight of 140-kDa by gel filtration and 120-kDa by SDS-gels [Percy *et al.*, 1985; Moriyama & Nelson, 1988*a*]. It is of unknown function, however Zachowski *et al.* (1989) have suggested that it may be involved in aminophospholipid translocation within the granule membrane.

Another major protein of the chromaffin granule membrane of unknown function is glycoprotein II. Glycoprotein II is a heterogeneous component of granule membranes with a pI of 4.2-4.7 and an apparent molecular weight of 80- to 100-kDa. [Pryde & Phillips, 1986; Winkler *et al.*, 1986]. It is extensively glycosylated with galactose, N-acetylglucosamine and sialic acid moieties and it has therefore been identified by lectin blotting [Gavine *et al.*, 1984]. It is present in both endocrine and exocrine secretory vesicles [Obendorf *et al.*, 1988] as well as in lysosomes [Weiler *et al.*, 1990] but its structure and function are not known. Its resistance to extraction by the

detergent lithium di-iodosalicylate has provided a rapid way of isolating it [Christie & Palmer, 1990]. Its N-terminal aminoacid sequence shows that it is enriched in cysteine and acidic aminoacids [Christie & Palmer, 1990]. Its orientation in the granule membrane is not known but it is accessible to extragranular reagents [Abbs & Phillips, 1980]. Unlike dopamine β-monooxygenase, glycoprotein III and carboxypeptidase H, it does not appear to be present in a soluble form in the matrix.

[b] ***Minor proteins of the chromaffin granule membranes that are also present in other secretory vesicle membranes*** :

An increasing number of secretory vesicle membrane proteins that are not involved the biosynthesis of vesicular contents and that display wide tissue distributions are being identified with the use of immunological techniques. These proteins appear to be present in both hormone-storing dense core vesicles (such as in the adrenal and pituitary glands), and in small synaptic vesicles. Their functions are not known but their wide tissue distribution, highly conserved structures and their *in vitro* activities strongly imply that they are involved in secretory vesicle organisation and trafficking. Although they appear to be abundant components of synaptic vesicles, their presence in chromaffin granules is masked by the abundance of the biosynthetic enzymes, secretory protein and peptides. As a consequence, in preparations of chromaffin granule membranes, they may only be visualised with the aid of specific antibodies. Among these proteins are SV2, p65, p38 (synaptophysin) and synaptobrevin; these will be reviewed in the following Sections.

(i) **p65 :**

As a consequence of raising and using monoclonal antibodies to characterise synapses at a molecular level, p65 was the first secretory vesicle membrane protein to be found to have wide tissue and species distribution and not to be involved in the biosynthesis of vesicular contents [Matthew *et al.*, 1981].

Using rat brain synaptic junctional complexes as an antigen to prepare monoclonal antibodies, Matthew *et al.* (1981) obtained two antibodies, *asv30* and *asv48*. Immunocytochemical analysis at the electron microscope level demonstrated that the epitope of the antigen recognised by these antibodies was present on synaptic vesicle membranes in all nerve terminals of the central nervous system, as well as on the secretory vesicle membranes of pituitary (anterior and posterior) and adrenal medulla [Matthew *et al.*, 1981; Fournier & Trifaró, 1988*a*]. Cell lines derived from the above tissue such as AtT20, GH3 and PC12 also possessed this antigen; the localisation of the antigen at higher resolution, as well as its accessibility to antibody-coated acrylamide beads, demonstrated that the epitope recognised by these antibodies was exposed to the cytoplasmic side of vesicles [Matthew *et al.*, 1981; Lowe *et al.*, 1988; Floor & Feist, 1989]. When the antigen was analysed by immunoblotting, its molecular weight was found to be 65-kDa in all vertebrate species tested and the antigen was hence termed "*p65*" [Matthew *et al.*, 1981]. The immunoreactivity of these monoclonal antibodies with p65 from different species, the similar molecular weights of different intact p65 and the correspondence of the proteolytic fragments suggested that p65 was a common, yet evolutionally- conserved protein of secretory vesicles in neural and endocrine tissue. However, p65 was not detected in exocrine tissue such as pancreas or in salivary glands and liver [Matthew *et al.*, 1981].

Although the wide tissue and species distribution of p65 suggests that

it carries out an important function, to date this function remains obscure. As with other secretory vesicle membrane proteins such as SV2, synaptophysin and synaptobrevin, the lack of *in vitro* assays which reconstitute exocytosis, and not knowing which stage of the exocytic pathway to study remain barriers to determining the function of p65. However, the discovery that p65 binds calmodulin, a protein which regulates the function of other proteins in response to the levels of intracellular Ca^{2+} concentrations, has been an important step in implicating p65 as a component of the secretory machinery of regulated secretory cells [Fournier & Trifaró, 1988*a*; Trifaró *et al.*, 1989]. Previous studies on calmodulin-binding proteins present in bovine chromaffin granule membranes had identified two proteins of 50/53- and 65/69-kDa which displayed Ca^{2+}-dependent calmodulin binding [Geisow *et al.*, 1982; Hikita *et al.*, 1984; Bader *et al.*, 1985]. It was subsequently shown by Fournier & Trifaró (1988*a*) that the 65/69-kDa chromaffin granule calmodulin-binding protein also bound *asv48*, the monoclonal antibody originally used to identify p65 [Matthew *et al.*, 1981], and that this protein was also present in chromaffin cell plasma membranes [Fournier & Trifaró, 1988*b*], confirming previous observations by Matthew *et al.* (1981). These findings suggested that p65 may be involved in the docking of secretory vesicles to the plasma membrane preceding membrane fusion and discharge of the vesicular contents.

The recent cloning and sequencing of the cDNA encoding p65 from rat, human and *Drosophila* has allowed Perin *et al.* and others to predict the structure of p65 [Perin *et al.*, 1990, 1991*a* &1991*b*]; these findings are supported by biochemical evidence [Tugal *et al.*, 1991; Perin *et al.*, 1991*b*]. According to these predictions, in both rat and human, p65 is an integral membrane glycoprotein of secretory vesicles, consisting of 421 aminoacids.

The protein spans the

vesicle membrane once and has a large cytoplasmic domain consisting of 343 aminoacids. The cytoplasmic domain appears to be important in the function of p65 as it is highly conserved - comparison of the human and rat sequences reveals that only two aminoacids are different in this domain (residues 190 and 375; see appendix I for sequences). Within the cytoplasmic domain, there are two regions in tandem showing 39% homology to the regulatory C2 domain of protein kinase C (PKC) [Perin *et al.*, 1990]. These two domains are only slightly more homologous to each other than they are to protein kinase C, and these differences are conserved across species, suggesting that they are not functionally equivalent. The cytoplasmic domain of p65 has been shown to bind acidic phospholipids with diacylglycerol backbones, and to agglutinate red blood cells [Perin *et al.*, 1990]. These functions have been assigned to the PKC-like domains since recombinant cytoplasmic domain constructs which contain only one such repeat retain phospholipid-binding but fail to agglutinate red blood cells [Perin *et al.*, 1991*b*].

As well as binding acidic phospholipids, it is suggested by Perin *et al* (1991*a*) that the PKC-like domains of p65 may also bind Ca^{2+}, because PKC isoenzymes that lack this domain do not require Ca^{2+} for activation [Nishizuka, 1989] and that Ca^{2+} binding may be physiologically important in the regulation of p65. Perin *et al* do not have direct evidence in support of this claim nor for their dismissal of the importance of Ca^{2+}-calmodulin binding to p65 [Südhof & Jahn, 1991]. Irrespective of whether p65 binds Ca^{2+} directly or is activated by Ca^{2+}-calmodulin, it appears that the function of p65 is regulated by changes in intracellular Ca^{2+} concentration; it has also been reported recently that the activity of p65 may be modulated by its phosphorylation state [Petrenko *et al.*, 1991] (see Section 1.6[b]).

The intralumenal (N-terminal) domain of p65 is the least conserved part of the protein - when rat and human sequences are compared, 6 out of 52

residues are different in this domain, as compared 2 out of 343 in the cytoplasmic domain. In *Drosophila*, this domain is 47 aminoacids longer than its mammalian counterpart [Perin *et al.*, 1991*a*]. Recently, an isoform of p65 has been cloned from mouse which is has the same overall structure, however it is expressed only in the certain regions of brain and not in neuroendocrine glands [Geppert *et al.*, 1991]. The intravesicular domain of this isoform is also divergent (46% homologous to the more widely distributed isoform). The only constant feature of the intravesicular domain is that it is N-glycosylated [Tugal *et al.*, 1991; Perin *et al.*, 1991*b*].

The p65 gene has been located in chromosome 12 (12cen-q21) in humans and chromosome 23B in *Drosophila* [Perin *et al.*, 1991*a*]. The 3' region of p65 mRNA appears to 87% conserved in rat and human with a region containing 22 consecutive copies of GTs, this region is not translated. The long untranslated 5' regions of p65 mRNA is also unusual - in *Drosophila*, this region contains five consecutive copies of CAGs [Perin *et al.*, 1991*a*]. These observations remain unexplained but illustrate that p65 must be involved in an important function.

One such function of p65 suggested by all investigators is in the docking of vesicles to the plasma membrane [Fournier & Trifaró, 1988; Perin *et al.*, 1991*b*; Südhof & Jahn, 1991]. There are three lines of evidence in support of this: first is that p65 is the only protein which is present in both secretory vesicle membranes and in plasma membrane [Fournier & Trifaró, 1988]; the second is that p65 has been suggested to form homooligomers [Perin *et al.*, 1991*b*;]; and thirdly, p65 can bind phospholipids in membranes [Perin *et al.*, 1990]. It may be that during exocytosis, p65 initially docks secretory vesicles to the plasma membrane with its phospholipid-binding activity and then forms an oligomeric fusion pore such as that suggested by Almers [Breckenridge & Almers, 1987; Almers, 1990]. This hypothesis remains to be

proved.

(ii) **Synaptophysin (p38) :**

Originally named synaptin, synaptophysin is a major constituent of synaptic vesicle membranes and has a wide distribution in the nervous system as well as in endocrine glands [see Winkler & Fischer-Colbrie, 1990, and references therein]. Synaptophysin is a glycoprotein of 38-kDa and has pI ranging from 4.5 to 5.8 [Rehm *et al.*, 1986; Johnston *et al.*, 1989; Obendorf *et al.*, 1988], but under non-reducing conditions it forms homooligomers and has an apparent molecular weight of 76-kDa on SDS-gels [Rehm *et al.*, 1986; Jahn & Maycox, 1988].

The primary structure of synaptophysin is known and, like p65, it is highly conserved [Buckley *et al.*, 1987; Leube *et al.*, 1987; Südhof *et al.*, 1987]. It consists of 307 aminoacids with membrane topology showing four hydrophobic transmembrane domains, its N-terminus as well as its larger C-terminal domain both being exposed to the cytoplasm. The primary sequence of synaptophysin shows homology to the connexons of gap junctions which form oligomeric cell to cell channels. If this homology is also functional, then together with its Ca^{2+}-binding activity [Rehm *et al.*, 1986], synaptophysin might be involved in the formation an exocytic fusion pore [Breckenridge & Almers, 1987; Almers, 1990]. The formation of such pores is supported by the *in vitro* observations that synaptophysin forms hexameric voltage- sensitive channels in lipid bilayers [Thomas *et al.*, 1988]. Recent identification of an oligomeric 36-kDa plasma membrane protein, "physophilin" (suggested to be the "receptor" of synaptophysin during exocytosis), has strengthened synaptophysin's putative role as the exocytic fusion pore [Thomas & Betz, 1990]. Synaptophysin has also been demonstrated to be a substrate for tyrosine kinase [Pang *et al.*, 1988], but as yet

the actual function of synaptophysin is unknown.

One obstacle to determining the function of synaptophysin has been the uncertainty of its subcellular localisation. In neurones, synaptophysin is clearly present in synaptic vesicles [Navone *et al.*, 1986; De Camilli & Navone, 1987], but its localisation in neuroendocrine cells has been controversial. The centre of this controversy is whether, in neuroendocrine cells, synaptophysin is present in membranes of large dense-core vesicles (such as chromaffin granules) or in small synaptic vesicle-like vesicles, or both. The presence of synaptophysin in small synaptic vesicle-like vesicles in endocrine cells has been taken to imply that there may be two parallel pathways of exocytosis in cell which display regulated secretion [De Camilli & Navone, 1987; De Camilli & Jahn, 1990]. However, careful biochemical analysis of the intracellular distribution of synaptophysin in chromaffin cells by Obendorf *et al.*, (1988) and Fournier *et al.*, (1989) has shown that synaptophysin is indeed present in chromaffin granules (see also Lowe *et al.*, 1988) as well as in a population of vesicles which band around 0.85 - 1.00 M sucrose. These vesicles have not been characterised but are probably the 50 nm synaptic vesicle-like vesicles observed by Navone *et al.*, (1986) and may hold the clue to the function of synaptophysin. Other membrane proteins such as SV2, p29 and synaptobrevin have also been reported to be enriched in these small synaptic vesicle-like vesicles [Baumert *et al.*, 1989].

The discrepancies about the localisation of synaptophysin appear to be as a result of the the low relative abundance of this protein in membranes of chromaffin granules. Schilling & Gratzl (1988) have determined that the copy number of synaptophysin per vesicle is around 20 in both chromaffin granule and synaptic vesicles. Since chromaffin granule membranes have a more complex composition than synaptic vesicles, the relative amount of synaptophysin in the former is around 100-times lower than in synaptic

vesicle membranes and is spread over 30-times the membrane surface area [Schilling & Gratzl, 1988; Obendorf *et al.*, 1988; Fournier *et al.*, 1989]. As a consequence, immunocytochemical methods used by Navone *et al.*, (1986) may not be sensitive enough to determine the presence of synaptophysin in chromaffin granules.

(iii) **SV2 :**

This glycoprotein, with an apparent molecular weight of 100-kDa, was initially identified by the use of monoclonal antibodies raised against cholinergic vesicles of *Torpedo californica* [Buckley & Kelly, 1985]. Its function and structure are not known but it has a wide distribution in large dense-core secretory vesicles of neural and endocrine cells, as well as in cell lines derived from the latter (PC12 and GH3). As well as being present in these vesicles, SV2 has also been identified in small synaptic vesicle-like vesicles of endocrine cells which are devoid of typical chromaffin granule marker proteins such as dopamine β-hydroxylase and cytochrome b_{561} but which contain synaptophysin [Floor & Feist, 1989; Winkler *et al.*, 1991]. It is thought to be a transmembrane protein since the epitope of the monoclonal antibody used for its identification is present on the cytoplasmic side of secretory vesicles and it is glycosylated [Buckley & Kelly, 1985].

(iv) **p29 & synaptobrevin (p18) :**

These recently-identified synaptic vesicle membrane proteins are also present in endocrine tissue such as adrenal medulla and the pituitary [Trimble *et al.*, 1988; Baumert *et al.*, 1989; Baumert *et al.*, 1990]. Immuno-cytochemically, these proteins have been located in the membranes of

synaptic vesicles in neurones and in the small synaptic vesicle-like vesicles of chromaffin cells, which also contain synaptophysin. These proteins have not been detected in the membranes of chromaffin granules using immunocytochemical techniques, but as with the subcellular distribution of synaptophysin discussed above (Section 1.4(b)(ii)), this may be because of their low relative abundance in the granule membrane.

The functions of these proteins are not known, although p29 is a substrate for tyrosine kinase [Baumert *et al.*, 1990], and synaptobrevin, like p65, is proposed to span the vesicle membrane once with the larger part of the protein exposed to the cytoplasm. Resembling p65, synaptobrevin is proposed to be present in isoforms [Elferink *et al.*, 1989].

(v) **svp25 :**

Svp25 is the latest synaptic vesicle membrane glycoprotein to be identified from the electric organ of *Torpedo californica* [Volknandt *et al.*, 1990]. Its function has not been determined, although it is phosphorylated *in vitro*, binds Ca^{2+} and forms hexameric oligomers suggesting a role either in ion transport into the synaptic vesicle or in the formation of an exocytic fusion pore. Unfortunately, the polyclonal antibodies raised against denatured svp25 did not cross-react with mammalian synaptic vesicle proteins despite the presence of a protein of the same molecular weight in the latter preparation. It therefore remains to be seen whether this protein is present in chromaffin granule membranes and what its function may be.

1.5 Proteins associated with chromaffin granules

Preparations of chromaffin granule membranes contain extrinsic proteins of extra-granular origin which co-purify with them. These proteins, which are summarised in Table 1.5, originate from both the lumen of the granule and from the cytosol. For some of these proteins, the nature of their association with the granule membrane is not known, but for others their binding is reversible and dependent on molecules such as Ca^{2+}, ATP and GTP which are intracellular messengers of exocytosis (see Section 1.6). This section will review the possible role of these proteins in exocytosis.

[a] ***Calcium-dependent granule-binding proteins*** *:*

Since the discovery that Ca^{2+} is an important factor for exocytosis [Douglas & Rubin, 1961*a*], there has been substantial research to identify proteins which can couple changes in the intracellular concentrations of Ca^{2+} to the exocytic machinery.

Calmodulin is a well-characterised 17-kDa acidic protein which regulates the function of many cellular proteins in response to changes in intracellular levels of Ca^{2+}, and therefore its role in exocytosis has been investigated. Calmodulin is present in chromaffin cells comprising around 0.01 to 0.04% of the total cellular protein [Kuo & Coffee, 1976; Hikita *et al.*, 1984]. Although its direct involvement in secretion is not yet proven, many of its properties suggest its role in secretion. For example, when chromaffin cells are challenged with secretagogues, around 25% of the cytosolic calmodulin becomes membrane-bound predominantly to the granules [Hikita *et al.*, 1984]. Furthermore, the inhibition of exocytosis by calmodulin antagonists (e.g. trifluoperazine) and by anti-calmodulin antibodies introduced into chromaffin cells present evidence that calmodulin is possibly

Table 1.5

Proteins associated with chromaffin granule membranes[a]

Protein and Origin		References
Ca^{2+}-dependent chromaffin granule-binding proteins	cytosolic *e.g. synexin, calpactin, caldesmon and other members of the "annexin" or "chromobindin" family of proteins. Also calmodulin and Protein kinase C.*	Creutz *et al.* (1978), Creutz *et al.* (1983), Geisow & Burgoyne (1982), Geisow & Burgoyne (1983), Geisow *et al.* (1982), Hikita *et al.* (1984), Martin *et al.* (1987). Terbush *et al.* (1988).
Cytoskeletal and cytoskeleton-binding and organising proteins	cytosolic *e.g. actin,tubulin and fodrin. α-actinin, gelsolin homologues and scinderin .*	Aunis & Perrin (1984), Bader & Aunis (1983), Bader *et al.* (1986), Jockusch *et al.* (1977), Fowler & Pollard (1982), Rodriguez Del Castillo *et al.* (1990), Sakurai *et al.* (1990 & 1991).
GTP-binding proteins	cytosolic - *Rab3A*	Matteoli *et al.* (1991).
Chromogranins (A, B and C)	lumen of chromaffin granules	Pryde & Phillips (1986).

[a] See Section 1.5 for text.

involved in the secretory process [Kenigsberg *et al.*, 1982; Brookes & Treml, 1983; Kenigsberg & Trifaró, 1985]. Similar biochemical evidence has been obtained with anterior pituitary cells, sea urchin oocytes and in the unicellular organism *Paramecium*. [Conn *et al.*, 1981; Steinhardt & Alderton, 1982; Momanyezi *et al.*, 1987]. However, there have also been reports that both calmodulin agonists and anti-calmodulin antibodies had no effects on saponin- and streptolysin-permeabilised chromaffin cells [Brook & Treml, 1984; Ahnert-Hilger *et al.*, 1989]. It must be pointed out that because calmodulin regulates many intracellular processes, it is difficult to distinguish between the general effects of the above reagents as opposed to their effects at the site of exocytosis.

A more direct way of demonstrating the role of calmodulin in secretion has been by studying its interaction at the chromaffin granule membrane. Chromaffin granules show Ca^{2+}-dependent calmodulin binding. The binding capacity of chromaffin granule membranes for calmodulin is low but highly specific: reported biding parameters are B_{max}= 3.3 pmol/mg, K_d=31 nM [Burgoyne & Geisow, 1981], and B_{max} = 30 pmol/mg, K_d = 13 nM [Hikita *et al.*,1984]. The granular receptors of calmodulin have been identified as two proteins of 50/53-kDa and 65/69-kDa, and binding to both of these is dependent Ca^{2+}. The 65/69-kDa protein has been identified as the integral membrane glycoprotein p65 which has a wide tissue distribution; the identity of the 50/53-kDa protein is not known but it could be a membrane-bound proteolytic product of p65 [Geisow & Burgoyne, 1983; Bader *et al.*, 1985; Tugal *et al.*, 1991]. Calmodulin also binds in a Ca^{2+}-independent manner to chromaffin granule membrane proteins of 21- and 24/25-kDa but these have not been characterised [Geisow *et al.*, 1982; Hikita *et al.*, 1984].

In the presence of elevated Ca^{2+} concentrations, calmodulin is able to promote the binding of a group of cytosolic proteins to the chromaffin

granule membrane [Geisow & Burgoyne, 1983] and among these is the actin- and phospholipid-binding protein, caldesmon, whose possible function in exocytosis is discussed below.

Other cytosolic proteins have also been shown to bind reversibly to chromaffin granule membranes in a Ca^{2+}-dependent manner and have therefore been implicated in the mechanism of exocytosis. These proteins have been collectively named as "annexins" or "chromobindins" [Creutz *et al.*, 1988; Geisow *et al.*, 1987; Burgoyne & Geisow, 1989]. Annexins are a group of seven Ca^{2+}- and lipid-binding proteins which contain either four or eight copies of a conserved 17-aminoacid sequence and a variable N-terminal region characteristic of each protein [Geisow *et al.*, 1987, Burgoyne & Geisow, 1989; Kretsinger & Creutz, 1986]. Synexin was the first annexin to be identified on the basis of its ability to aggregate and fuse chromaffin granules [Creutz *et al.*, 1978; Creutz, 1981; Creutz *et al.*, 1988; Burns *et al.*, 1989]. Compared to the other annexins, synexin has an extended N-terminal region [Burns *et al.*, 1989]. A model has been proposed for the fusogenic action of synexin in which it inserts itself into the membrane and forms a channel [Pollard *et al.*, 1988; Burns *et al.*, 1989]. However, these *in vitro* activities require a free Ca^{2+} concentration of around 10 - 100 μM, much higher than that suggested to be required in permeabilised cell experiments [Baker & Knight, 1981; Kao & Schneider, 1986; see also Burgoyne, 1984].

The best evidence for the role of annexins in exocytosis has been for the 36-kDa annexin, calpactin. Unlike other annexins, calpactin exists in association with an 11-kD polypeptide, the calpactin light chain, as $p36_2$-$p11_2$ heterotetramers [Gerke & Weber, 1984; Zokas & Glenney, 1987; Burgoyne & Morgan, 1990]. In the heterotetrameric form, calpactin has been shown to aggregate chromaffin granules at Ca^{2+} concentrations of around 1- 2 μM [Drust & Creutz, 1988; Burgoyne, 1988]. Furthermore, in the presence of arachidonic

acid (and 2 mM Mg^{2+}), calpactin can promote membrane fusion [Drust & Creutz, 1988], the significance of this activity is that arachidonic acid has been shown to be produced when bovine chromaffin cells are stimulated [Frye & Holz, 1984]. In the monomeric form, calpactin can also aggregate chromaffin granules but as with synexin, this requires Ca^{2+} concentration of around 10-100 μM, implying that the binding of the light chain lowers the Ca^{2+} dependence of calpactin in aggregating granules [Drust & Creutz, 1988]. The N-terminal "tail" of calpactin appears to be important in its function since the proteolytic peptide of calpactin without the first 27 aminoacids does not bind the light chain or lipid bilayers [Drust & Creutz, 1988; Ali *et al.*, 1989]. Heterotetramer formation by calpactin is regulated by both Ca^{2+} and by phosphorylation. In cultured chromaffin cells, phosphorylation of calpactin parallels nicotine stimulation. The kinase(s) which carry out this phosphorylation *in vivo* are not known, but calpactin heavy chain contains phosphorylation sites for $pp60^{c\text{-}src}$, protein kinase C, cAMP- and calmodulin-dependent protein kinases [Johnsonn *et al.*, 1986; Gerke, 1989]. *In vitro* results show that tetramer formation is inhibited by phosphorylation at $serine_{25}$ by protein kinase C, but not at $tyrosine_{23}$ by $pp60^{c\text{-}src}$ [Johnsonn *et al.*, 1986; Powell & Glenney, 1987]. Tyrosine phosphorylation appears only to inhibit the phospholipid-binding activity of calpactin [Powell & Glenney, 1987]. When phosphorylation of the heavy chain is blocked by the binding of antibodies against the N-terminal domain of calpactin in digitonin-permeabilised bovine chromaffin cells, a small increase in Ca^{2+}-dependent exocytosis is observed [Burgoyne & Morgan, 1990].

The most conclusive evidence for the role of calpactin (and other annexins) in exocytosis has come from experiments investigating the function of the consensus repeat sequence. When a peptide containing the consensus repeat sequence or antibodies against calpactin were introduced

into digitonin-permeabilised bovine chromaffin cells, exocytosis was inhibited [Ali *et al.*, 1989; Ali & Burgoyne, 1990]. Furthermore, addition of calpactin to digitonin-permeabilised cells from which cytosolic factors have leaked out (and consequently lost their ability to secrete) restored some of their Ca^{2+}-dependent exocytosis (Burgoyne & Morgan, personal communication; see Section 1.5[e]).

Calpactin (and other other annexins) have been localised just beneath the plasma membrane [Burgoyne *et al.*, 1986; Burgoyne & Cheek, 1987] and calpactin filaments which cross-link granules to the plasma membrane have been identified [Nakata *et al.*, 1990]. In view of the subcellular localisation and *in vitro* properties of calpactin, it has been suggested that it is involved in the final stages of exocytosis by mediating the binding and possibly the fusion of the granules with the plasma membrane [Burgoyne, 1991].

Caldesmon (or p70), another Ca^{2+}-calmodulin-dependent actin cross-linking protein [Burgoyne *et al.*, 1986]. Caldesmon-actin association is inhibited at micromolar Ca^{2+} concentrations, and it is suggested that this might lead to the opening up of the actin network in the periphery of the cell during exocytosis (see Section 1.5[c]). Caldesmon has a similar subcellular distribution as calpactin, just beneath the plasma membrane [Burgoyne *et al.*, 1986]. The possession of these properties suggests that caldesmon may also play an important role in the mechanism of exocytosis.

"Chromobindins" are a larger group of calcium-dependent granule-binding proteins which have been described by Creutz *et al.* (1983). Chromobindins not only include members of the annexins, but also proteins such as calmodulin, protein kinase C and a phosphatidylinositol-specific phospholipase [Summers & Creutz, 1985; Creutz *et al.*, 1985]. Seven polypeptides of the chromobindin family have been reported to form a

toroidal complex ("chromobindin A") on chromaffin granule membranes with ATPase activity but the exact role of this complex is unknown [Martin & Creutz, 1987].

[b] ***Secretory vesicle-binding phosphoproteins and kinases*** *:*

The requirement of ATP for exocytosis suggests that the exocytic machinery of cells could be regulated by phosphorylation (see also Section 1.6[b]). To date, the best evidence for the involvement of phosphoproteins in exocytosis relates to the cytosolic synaptic vesicle and cytoskeleton-binding proteins of the synapsin family (synapsin Ia, Ib, IIa, IIb) [see De Camilli & Navone, 1987; Burgoyne, 1990 and Südhof & Jahn, 1991 for reviews]. Synapsin I was first discovered as a major endogenous substrate for cAMP-dependent protein kinase in mammalian brain, but it is also phosphorylated by Ca^{2+}-calmodulin-dependent protein kinase II [Johnson *et al.*, 1972; Llinás *et al.*, 1985]. Phosphorylation of synapsin I by Ca^{2+}-calmodulin-dependent protein kinase II parallels neurotransmitter release and promotes its dissociation from both synaptic vesicles and from the cytoskeleton [De Camilli & Greengard, 1986; Schlieber *et al.*, 1986]. When dephospho-, but not phospho-synapsin I is microinjected into the preterminal digits of giant squid axon, neurotransmission is strongly inhibited [Llinás *et al.*, 1985]. This observation correlates with the actin-bundling properties of synapsins Ia/b [Petrucci & Morrow, 1987; Bähler & Greengard, 1987]. It is proposed that dephospho-synapsin cross-links synaptic vesicles to the cytoskeletal network in resting cells, and upon stimulation, it dissociates allowing the movement of vesicles towards the plasma membrane. The speed of synaptic release implies that this mechanism probably prepares the vesicles for exocytosis rather than regulating its final exocytic stage. Despite the presence of

phosphoproteins of the same size as synapsin, there is uncertainty whether synapsin is present in chromaffin granules [Burgoyne & Geisow, 1981 & 1982]. However, a related phosphoprotein (protein III, consisting of 55- and 74-kDa polypeptides) is present in chromaffin granule membranes and its phosphorylation increases upon stimulation [Burgoyne & Baines, 1987; Haycock *et al.*, 1988].

The discovery of tyrosine kinase $pp60^{c\text{-}src}$ as a component of chromaffin granule membranes in association with a 38-kDa protein has suggested a possible role of this kinase in the phosphorylation of calpactin upon its binding to chromaffin granule membranes, and therefore in the regulation of exocytosis (see Section 1.6[b]) [Parsons & Creutz, 1986; Grandori & Hanfusa, 1988]. Protein kinase C is another kinase which also binds to chromaffin granule membranes [Terbush *et al.*, 1988], its involvement in secretion will be discussed in Section 1.6[b].

[c] *Cytoskeletal and cytoskeleton-organising proteins* :

In resting chromaffin cells, granules are prevented from interacting with the plasma membrane by a dense cortical cytoskeletal network composed primarily of F-actin and fodrin. This cytoskeletal barrier is apparent in electron micrographs of resting chromaffin cells, as a distinct 400-nm zone devoid of granules just beneath the plasma membrane [Geisow & Burgoyne, 1982, 1986].

For exocytosis to take place, this network must be rearranged allowing granules access to the plasma membrane. The requirement for this rearrangement has been implied from experiment where the cytoskeletal network is perturbed by drugs or antibodies. For example, treatment of neutrophils or permeabilised chromaffin cells with drugs such as cytochalasin

B, which induce actin disassembly, decrease the Ca^{2+}-dependency of secretion, whereas phalloidin, which stabilises F-actin, inhibits secretion [Lelkes *et al.*, 1986]. Furthermore, following the stimulation of chromaffin cells, the sites of exocytosis appear to be devoid of F-actin [Vitale *et al.*, 1991].

There is increasing evidence that some of the proteins involved in the regulation of the cytoskeleton are peripheral components of chromaffin granules. As the action of these proteins appear to be under the control of factors or events similar to those implemented in the regulation exocytosis such as intracellular concentration of Ca^{2+}, inositol phospholipids and protein phosphorylation, they appear to be required for exocytosis [Matsudaira & Janmey, 1988; Vandekerckhove, 1990].

Among these cytoskeletal regulatory proteins present on chromaffin granules is α-actinin [Jockusch *et al.*, 1977; Bader & Aunis, 1983]. The non-muscle isoform of α-actinin is a 100-kDa Ca^{2+}-regulated actin cross-linking protein. In the absence of Ca^{2+}, α-actinin forms antiparallel homodimers which cross-link actin filaments. In the presence of micromolar Ca^{2+} concentration however, this complex dissociates. This behaviour of α-actinin is consistent with biochemical observations (see Burgoyne, 1984) and the hypothesis that the peripheral actin barrier must be disassembled during exocytosis [Cheek & Burgoyne, 1986].

Other granule-associated actin-binding cytosolic proteins are the *annexins* caldesmon and calpactin I (also known as calpactin and p36; see Section 1.5[a], Geisow *et al.*, 1987 and Burgoyne & Geisow, 1989). Caldesmon is a 70-kDa Ca^{2+}-calmodulin regulated protein which cross-links granules to F-actin [Burgoyne *et al.*, 1986; Vandekerckhove, 1990]. This interaction is inhibited at micromolar Ca^{2+} concentrations, thus allowing the dissociation of granules from the actin network in preparation for exocytosis. The physiological significance of actin-calpactin interaction is less obvious since

this requires millimolar Ca^{2+} concentrations.

Although not detected in chromaffin granule preparations, other actin-binding proteins such as villin and gelsolin may be crucial in the mechanism of exocytosis [Matsudaira & Janmey, 1988; Vandekerckhove, 1990]. Gelsolin-like proteins have been identified which cross-link F-actin at resting Ca^{2+} concentrations in chromaffin cells [Bader *et al.*, 1986, Sakurai *et al.*, 1990; Sakurai *et al.*, 1991]. Micromolar Ca^{2+} concentrations induce the actin-severing activity of villin and gelsolin which would allow granule movement to the plasma membrane. Furthermore, the actin-severing activity of these proteins is inhibited by phosphatidylinositol 4,5-bisphosphate (PIP_2, see Matsudaira & Janmey, 1988). Since this phospholipid is generated in the chromaffin granule membrane by phosphatidylinositol kinase [Phillips, 1973] this could provide a switch-off mechanism. The role of actin-severing proteins have also been implicated in exocytosis from sea urchin oocytes. Recently a Ca^{2+}-dependent actin severin protein, *scinderin*, which is different from gelsolin has been purified from bovine adrenal medulla [Rodriguez Del Castillo *et al.*, 1990]. The distribution of this protein as measured by immunofluorescence has suggested that scinderin has a role in the final stages of exocytosis [Vitale *et al.*, 1991].

Fodrin, the spectrin homologue present in chromaffin cells, has also been implied in the mechanism of exocytosis [Aunis & Perrin, 1984]. When anti-α-fodrin antibodies were introduced into permeabilised chromaffin cells, they resulted in the inhibition of secretion [Perrin *et al.*, 1987]. This effect is explained by previous observations that specific antibodies prevent Ca^{2+}-dependent proteolysis of fodrin which in turn would prevent the dissociation of the granules from the cytoskeletal network [Siman *et al.*, 1985]. In chromaffin cells, fodrin is present just beneath the plasma membrane; this subcellular localisation also supports a role for fodrin in the mechanism of

exocytosis [Perin & Aunis, 1985; Langley *et al.*, 1986].

Chromaffin granules have also been shown to bind tubulin with high affinity (K_d = 30 nM, B_{max} = 0.4 nmol/mg protein) but its significance is not clear [Bernier-Valentin *et al.*, 1983]. It has been shown recently that secretory granules are targeted towards the tips of neurone-like projections of endocrine cells using microtubules [Kreis *et al.*, 1989] and that microtubules are involved in the targeting of pre-lysosomal vesicles [Gruenberg *et al.*, 1989]. Since microtubules appear to organise the subcellular localisation of organelles, tubulin-binding by chromaffin granules could a part of this mechanism. However, microtubule-disrupting drugs apparently do not inhibit final stages of exocytosis in chromaffin cells, but inhibit the formation of new granules (see Burgoyne, 1984 and Burgess & Kelly, 1987).

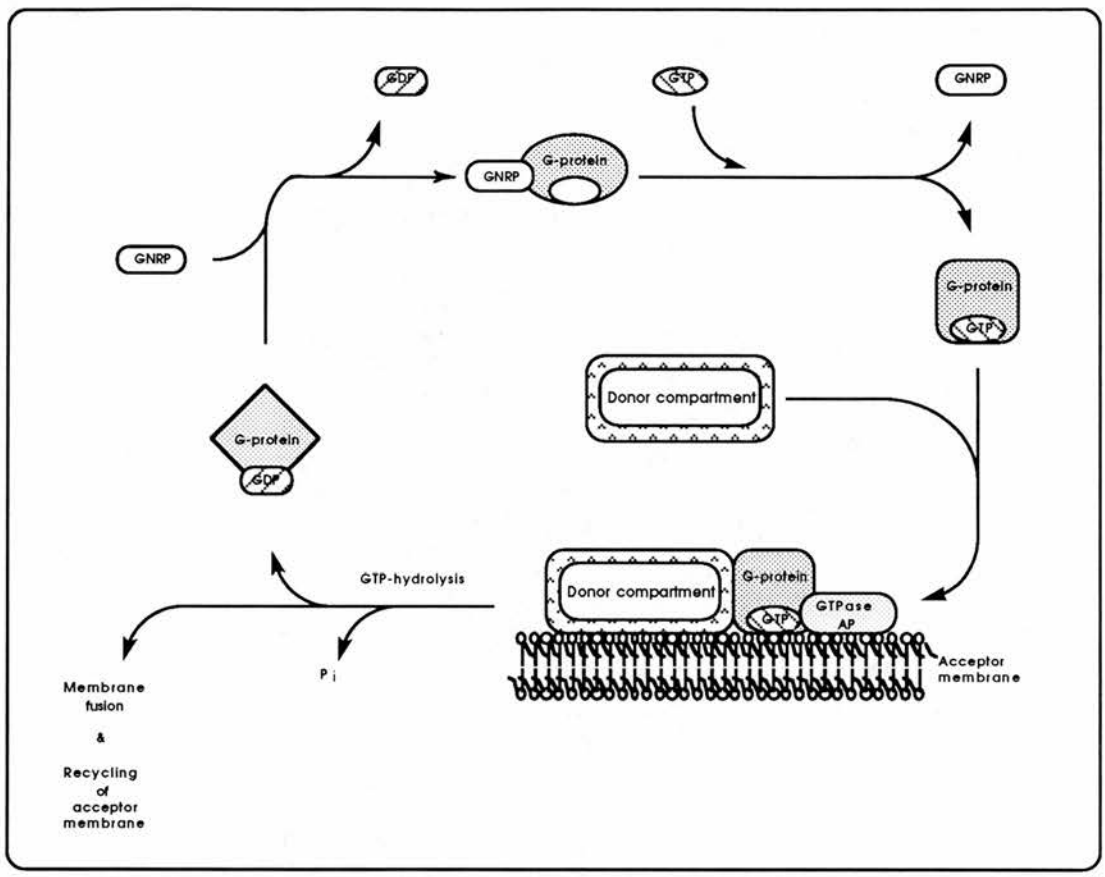
[d] ***GTP-binding proteins*** *:*

There is increasing evidence that GTP is required for the final stages of the exocytic process as well as for vesicular transport through the secretory pathway (see Section 1.6[d]; Balch,1989; Bourne *et al.*, 1990 and Balch, 1990]. This section will review some of the "G-proteins" that are implicated in the mechanism of exocytosis and that are components of chromaffin granule membranes.

From both biochemical and genetic evidence it has been established that low-molecular weight GTP-binding proteins of 20- to 25-kDa, related to the *ras* oncogene superfamily, are involved in the specific intracellular targeting of secretory vesicles. The common feature of these proteins is that GTP hydrolysis acts as a functional switch such that only the GTP-bound form of the protein associates with the secretory vesicle membrane. As shown in Figure 1.4, the membrane-bound form permits docking of the donor vesicle

Figure 1.4

Proposed mechanism of action for small molecular weight GTP-binding proteins involved in membrane targeting [a]



[a] See Sections 1.5[d] & 1.6[c] for text.

KEY:

GTP : *Guanine triphosphate*

GDP : *Guanine diphosphate*

Pi : *Inorganic phosphate*

GTPaseAP : *GTP-hydrolysis activating protein*

GNRP : *Guanine nucleotide release protein*

to the correct acceptor membrane. The interaction of the two correct compartments induces GTP hydrolysis, possibly by the interaction of a GTPase-activating protein, resulting in the return of the G-protein to the cytoplasm for another cycle [Plutner *et al.*, 1990]. The yeast Sec4 and Ypt1 proteins are examples of such proteins, their mammalian counterparts (*rab, rac, ral, rho and rap*) have also been identified

(see Balch, 1990 and Bourne *et al.*, 1990 for reviews). By analogy with the different localisation of Sec4 and Ypt1 in yeast cells (i.e. secretory vesicles and the Golgi apparatus respectively), the diversity of the mammalian counterparts suggests that these proteins also function at different stages of both constitutive and regulated secretory pathways. It has recently been shown that chromaffin granules and synaptic vesicles contain such proteins (Rab3A), and that their subcellular localisation appears to change during exocytosis [Burgoyne & Morgan, 1989; Fischer von Mollard *et al.*, 1991; Matteoli *et al.*, 1991].

Their close homology with *ras* suggests that these low-molecular weight GTP-binding proteins associate with vesicle membranes through a post-translationally added lipid-anchor, and that this is essential for their function [McIlhinney *et al.*, 1990; Walworth *et al.*, 1989; Pfanner *et al.*, 1990]. This has also been demonstrated by Molenaar *et al.* who showed that mutant forms of ras which ras lack their C-terminal cysteine to which the lipid anchor is attached, are non-functional forms of the protein.

Since GTP-hydrolysis is an obligatory step for the release of small G proteins from the donor/acceptor membrane complex prior to another round of vesicle targeting, treating cells with non-hydrolysable GTP analogues

inhibits secretion (see Section 1.6[d]). However, in neutrophils for example, such GTP analogues have been found to act synergistically with, and even to obviate the requirement for Ca^{2+} [Barrowman *et al.*, 1986; Cockcroft *et al.*, 1990]. This response, typical of the better-known 40- to 50-kDa heterotrimeric GTP-binding proteins [Casey & Gilman, 1988] led to the hypothesis that exocytosis could be under the control of such a G-protein, termed G_e [Gomperts, 1990], possibly linked to an as yet uncharacterised second messenger [Burgoyne, 1987]. The subcellular location of G_e is not clear but it could be an integral part of secretory vesicles [Gomperts, 1986].

[e] ***Other cytosolic vesicle-binding proteins*** *:*

The development of the permeabilised chromaffin cell assay has been an important step in studying the final stages of regulated exocytosis (see Section 1.8(a)). However, the loss of cytosolic factors from the cells into the bathing medium has been a limitation of the assay, resulting in "running-down" of the secretory responsiveness of the cells. Recently Morgan and Burgoyne have used this very limitation to isolate three cytosolic factors which appear to regulate exocytosis [Morgan & Burgoyne, personal communications]. These factors have been named EXO1, EXO2 (the two stimulatory factors) and SIP, the inhibitory factor. EXO1, the better characterised factor, has a native molecular weight of 70-kDa and runs at 29-kDa on SDS-gels. When EXO1 is reintroduced into permeabilised "ran-down" cells, it restores about 60% of the original secretory response of the cells, however, if EXO1 is incubated with tetanus toxin, it can no longer restore the secretory responsiveness of the cell. The inhibitory effect of tetanus toxin may be reversed if EXO1/tetanus toxin complex is run down a gel filtration column, suggesting that the interaction between these proteins is not covalent. The

neurotoxin from *Botulinum* does not appear to have any effect on EXO1. EXO1 isolated from cytosol treated with the phorbol ester PMA enhances the stimulatory effect of EXO1, and this effect is sensitive to staurosporine suggesting an involvement of protein kinase C in the regulation of EXO1.

EXO2, the 54-kDa stimulatory factor is less well characterised. The stimulatory effect of EXO2 is additive to that of EXO1, together they can restore nearly 100% of the secretory response of a run-down permeabilised chromaffin cell. Both trypsin- and NEM-treatment of cytosol abolishes the stimulatory effects of EXO1 and EXO2. The inhibitory factor SIP is not characterised. The exact functions of EXO1, EXO2 and SIP, and how it will fit into our current knowledge of the control of exocytosis remains to be established.

Studies using *in vitro* systems reconstituting the intracellular secretory pathway (other than the permeabilised chromaffin cell system) has identified three components that are required for the fusion of intra-Golgi vesicles. These are firstly, an N-ethylmaleimide-sensitive factor (*NSF*, the Sec18 protein) [Wilson *et al.*, 1989]; secondly, a set of proteins required for the binding of NSF to the donor membranes termed α-, β-, γ-*SNAPs* [Clary *et al.*, 1990]; and thirdly, a 25-kDa cytosolic protein that acts at a late prefusion step, termed *POP* [Wattenberg *et al.*, 1990]. In addition a late step in membrane fusion has been shown to be dependent on fatty acyl coenzyme A [Pfanner *et al.*, 1990]. These complexes have been shown to to function in other intracellular transport steps [Beckers *et al.*, 1989; Diaz *et al.*, 1989]. Although not yet identified in chromaffin cells, homologous proteins could be involved in the late stages of exocytosis.

Other proteins exhibiting reversible binding to secretory vesicles are the "coat proteins" involved in the membrane budding from both intracellular organelles and from the plasma membrane. The best

characterised coat protein is *clathrin* which associates with vesicles during membrane budding at the trans-Golgi network (TGN); during vesicle biogenesis and at the plasma membrane during endocytosis [Pierce & Robinson, 1990]. The clathrin-vesicle association is mediated through the *adaptins* HA1 or HA2, depending on which membrane the vesicle are budding from [Pierce & Robinson, 1990]. The other set of coat proteins are the *COP*s which only bind to inter-Golgi vesicles [Hurtley, 1991; Robinson, 1991]. Since the interaction between coat proteins and vesicles are stage-specific, there must be some component in vesicle membranes which directs this specificity. The nature of this specificity nor the components of the vesicle membrane involved in this interaction are established.

[f] ***Plasma membrane proteins which bind to chromaffin granules*** **:**

The final stages of exocytosis involves the docking of secretory vesicles to the plasma membrane and studies have been directed to determine if this interaction is mediated by proteins present in the plasma membranes. A 51-kDa protein has been identified through its *in vitro* ability to bind chromaffin granules [Meyer & Burger, 1989], and specific antibodies against this protein were shown to inhibit exocytosis in both chromaffin cells and in patch-clamped chromaffin cells indicating its possible involvement in secretion [Schweizer *et al.*, 1989]. However, the interaction of this protein with chromaffin granules was Ca^{2+}-independent. It has recently been shown that this protein is a member of the cytochrome P-450 family of proteins and therefore possibly not involved in exocytosis. Another plasma membrane protein of 36-kDa ("*physophilin*") from rat brain synaptosomes has been demonstrated to bind to the integral vesicle membrane protein, synaptophysin [Thomas & Betz, 1990]. This interaction was not influenced by

ATP, GTPγS, and like the 51-kDa plasma membrane protein, by Ca^{2+}. Since exocytosis requires these factors, further work is needed to establish the roles of these proteins in exocytosis.

The chromaffin granule membrane protein p65 has also been localised to the plasma membrane [Matthew *et al.*, 1981; Fournier & Trifaró, 1988*b*]. The self-aggregation property of this protein suggests that it may be involved in vesicle docking at the plasma membrane, and unlike the 51-kDa protein and physophilin, the action of p65 appears to be regulated by the concentration of Ca^{2+}(see Section 1.4[b](i)).

[g] ***Lumenal proteins*** *:*

Chromaffin granule membrane preparations washed at pH 11 with sodium carbonate liberate proteins that originate in the lumen of the granule. These include the chromogranin family and also dopamine β-mono-oxygenase. The significance of the affinity of the chromogranins for the chromaffin granule membrane is not clear but there is accumulating evidence to suggest that this membrane-binding ability may be an important factor in the mechanism by which these proteins are sorted and accumulated as they exit from the trans-Golgi network [Yoo *et al.*, 1990; Huttner *et al.*, 1990].

1.6 Factors regulating exocytosis

Exocytosis requires the coupling of an external stimuli to the intracellular machinery responsible for vesicular targeting and fusion. The combined use of "permeabilised" cells, *in vitro* systems, advanced Ca^{2+} imaging systems and genetic approaches have implicated factors such as Ca^{2+}, ATP, guanine nucleotides, inositol phosphates and arachidonic acid in stimulus-secretion coupling. The roles of these factors are not yet completely defined since they elicit different responses in different cells. This has raised the possibility that alternative pathways of exocytic control may have evolved, and that different cells do not utilise the same pathway. This section will review the evidence for the involvement of these factors in the regulation of exocytosis from bovine chromaffin cells.

[a] *Role of calcium ions in exocytosis* :

The essential requirement of calcium ions for exocytosis from regulated secretory cells such as the chromaffin cell has been known since the initial whole-gland perfusion studies of Douglas and Rubin (1961*a*, 1961*b*). These results have since been confirmed by more sophisticated experiments on populations of chromaffin cells which have been either permeabilised so that the cytoplasmic Ca^{2+} concentrations could be manipulated (Knight & Baker, 1982; Holz *et al.*, 1983; Dunn & Holz, 1983), or loaded with Ca^{2+}-sensitive fluorescent dyes to monitor intracellular fluxes of free Ca^{2+}. It is now agreed that the intracellular free Ca^{2+} concentration in resting cells is in the order of 0.05 - 0.1 μM, increasing to 10μM on stimulation (depending on the secretagogue) and that the Ca^{2+} dose-response for exocytosis is sigmoidal with half-maximal secretion occurring around 1 μM Ca^{2+}.

The advent of fluorescent dyes such as quin-2 and fura-2 that respond to free Ca^{2+} concentrations has been an important step in determining the role of Ca^{2+} in exocytosis from intact cell [Tsien *et al.*, 1982; Grynkiewicz *et al.*, 1985]. This technique has been further refined by the development of enhanced video-imaging systems which can continuously monitor the cytosolic Ca^{2+} levels within single cells. Single cell imaging has resolved discrepancies observed in the levels of Ca^{2+} that is required for secretion from cell populations [Burgoyne, 1984; Burgoyne & Cheek, 1985; Kao & Schneider, 1986], and has also given a great deal of information about the spatial distribution of the Ca^{2+} fluxes [O'Sullivan *et al.*, 1989; Cheek *et al.*, 1989]. It is now agreed that, in bovine chromaffin cells, the entry of *external* Ca^{2+} is an essential factor for exocytosis, an event which is initiated *in vivo* by the binding of acetylcholine to its nicotinic receptor. The stimulation of the nicotinic receptor results not only in the entry of Ca^{2+} through itself but also in membrane depolarisation, and consequent opening of voltage-gated Ca^{2+} channels [Holz *et al.*, 1982; Kilpatrick *et al.*, 1982]. Imaging experiments in which fibroblasts have been co-cultured with chromaffin cells to determine the latters' exocytic response [Cheek *et al.*, 1989] have shown that following nicotinic stimulation, although the increase in the cytoplasm Ca^{2+} concentration is eventually uniform, it is the initial subplasmalemmal ring of Ca^{2+} that results in exocytosis. This is consistent with the hypothesis that the final stages of exocytosis involve events in the periphery of the cell such as the breakdown of actin barrier and granule docking followed by the fusion of the granules with the plasma membrane. Furthermore the proteins that have been implemented in the control of these events, such as calpactin (see Section 1.5[a]), are all present in the subplasmalemmal region of the cell. Further evidence for this hypothesis is the inability of muscarinic agonists (which release Ca^{2+} from internal stores) to trigger significant levels of

exocytosis. Despite the importance of the subplasmalemmal Ca^{2+} in the exocytic response of bovine chromaffin cells, the initial free Ca^{2+} concentration in this region, as determined by fura-2 imaging, is only around 50 nM above the basal level, a value far below that required in permeabilised cells. This discrepancy is thought to be because of the limitations in the spatial and temporal resolution of the imaging system and the inability of fura-2 to measure Ca^{2+} concentration above 100 μM which is thought be reached immediately beneath the plasma membrane during exocytosis. This therefore raises the important question of what exactly is the "physiological" Ca^{2+} concentration which is required for exocytosis. The answer to this remains unsolved. Another unsolved problem is the identity of the intracellular receptors for Ca^{2+}. Although Ca^{2+} is certainly the main signal for exocytosis in all cell types, it seems likely that there is no single Ca^{2+}-receptor involved in the control of exocytosis and that Ca^{2+} mediates the action of both cytosolic proteins (annexins, α-actinin etc.) and secretory vesicle membrane proteins such as p65. The latter is a likely candidate for such a receptor in the secretory vesicle membrane since it has recently been suggested that its tandem protein kinase C like-domains may bind Ca^{2+} directly [Petrenko *et al.*, 1991]; this is in addition to the possible ability of p65 to bind Ca^{2+}-calmodulin [Fournier & Trifaró, 1988*a*].

[b] ***Protein phosphorylation and the role of ATP in exocytosis*** *:*

Many biochemical processes are controlled by reversible phosphorylation of key proteins, an event requiring the enzymatic transfer of the terminal phosphate of ATP to the regulated protein by kinases and its removal by phosphatases in response to changes in the cellular environment. Phosphorylation provides cells with a simple switching mechanism, and is a

process which has been predicted to be fast enough to control exocytosis [Zieseniss & Plattner, 1985; Almers, 1990]. It is therefore not surprising that the phosphorylation states of secretory cell proteins have been investigated. Upon stimulation, both cytosolic and chromaffin granule membrane proteins change their phosphorylation state [Côte *et al.*, 1986; Gutierrez *et al.*, 1988; Burgoyne & Geisow, 1981, 1982], however the relevance of these events in the control of exocytosis has not been established so far.

In digitonin-permeabilised bovine chromaffin cells, there is a small component of Ca^{2+}-dependent exocytosis in the absence of ATP and pretreatment of cells with metabolic inhibitors abolishes this response [Holz *et al.*, 1989]. Furthermore, the presence of millimolar ATP retards the rundown of secretory responsiveness of these cells [Morita *et al.*, 1988; Holz *et al.*, 1989; Ali & Burgoyne, 1990]. These observations suggest that ATP is not essential for the late stages of exocytosis but it is needed to maintain the cell in a primed state. Morphological studies on the intracellular distribution of chromaffin granules suggest that a sub-population which is in close proximity to the plasma membrane (and possibly docked to it), may be the primed granules that represent the ATP-independent component of exocytosis [Burgoyne *et al.*, 1982]. It should be pointed out that in sea urchin oocytes, in *Paramecium* and in alpha-toxin permeabilised PC12 cells where some granules are already docked to the plasma membrane, ATP is not required for exocytosis [Terbush *et al.*, 1988; Thastrup *et al.*, 1990]. Maintainance of this primed state may involve the turnover of the phosphorylation states of inositol phosphates and proteins, and in the case of proteins, dephosphorylation may be the key event which triggers exocytosis. Polyphosphoinositides have been implicated in the control of exocytosis, a role that is different from the generation of diacylglycerol and IP_3. Evidence for this is the inhibitory effect of inositol phosphate-specific phospholipase C

pretreatment of digitonin-permeabilised bovine chromaffin cells [Eberhand *et al.*, 1990], and the observation that there is a decrease in the polyphosphoinositide levels in cells that are incubated in the absence of ATP. Eberhand *et al.* suggest that polyphosphoinositides could be a substrate for a protein (such as calpactin) in attachment to membranes. The regeneration of phosphatidylinositol 4,5-bisphosphate (PIP_2) may also be important in priming further rounds of exocytosis by regulating the cytoskeleton; phosphatidylinositol kinase (E.C. 2.7.1.67), the enzyme involved in the formation of PIP_2 is present in the membranes of chromaffin granules [Phillips, 1973].

Another mechanism by which ATP may prime cells is as a substrate for protein-seryl phosphorylation, and this implies that dephosphorylation may be a key step in the mechanism of exocytosis. The evidence for this comes not from chromaffin cells, but from *Paramecium* in which the dephosphorylation of a cytosolic 65-kDa protein (parafusin) correlates with exocytosis [Zieseniss *et al.*, 1985]. This event has been postulated to be regulated by a Ca^{2+}-calmodulin dependent phosphatase related to calcineurin, because the microinjection of Ca^{2+}-calmodulin-calcineurin complex enhances exocytosis, whereas microinjection anti-calcineurin antibodies inhibits it [Momayezi *et al.*, 1987]. It has also been shown that dephosphorylation of parafusin in the presence of elevated Ca^{2+} levels changes its subcellular distribution from soluble to membrane-bound, a behaviour similar to that of calmodulin and protein kinase C [Satir *et al.*, 1989]. Further evidence that dephosphorylation may be involved in the final stages of exocytosis comes from the inhibitory effect of ATP on exocytosis from *Paramecium* "ghosts" despite its requirement in the intact organism [Vilmart-Seuwen *et al.*, 1986]. The yeast and mammalian homologues of parafusin have been identified, and its wide tissue distribution implies that it may mediate an important role in secretion [Satir *et al.*, 1989].

Very recently it has been shown that the secretory vesicle membrane protein p65 can be phosphorylated *in vitro* and that this is inhibited by the receptor for the black widow spider venom neurotoxin (α-latrotoxin) [Petrenko *et al.*, 1991]. Since the physiological effect of this toxin is to enhance neurotransmitter release, the authors concluded that exocytosis could be mediated by the dephosphorylated form of p65. However, these results should be treated with caution since the authors do not specify whether they are using toxin-receptor complex or just the receptor to inhibit the phosphorylation of p65 and the latter was phosphorylated by an unidentified endogenous kinase.

The priming effect of ATP could also be as a result of its "switch-off" effect on exocytosis. It has been established that following nicotinic stimulation of intact cells, calpactin is serine-phosphorylated at its N-terminal tail [Johnsson *et al.*, 1986; Creutz *et al.*, 1987; Gerke, 1989]. This induces p36 monomer formation, thus releasing it from both granule membranes and the cytoskeleton. Furthermore, phosphorylation of calpactin at its N-terminal tyrosine (reside 23) by $pp60^{c\text{-}src}$, a known component of chromaffin granule membranes, reduces the affinity of calpactin for phospholipids. Analogously to the cyclic role of low molecular weight GTP-binding proteins such as sec4 and rab3A (see Section 1.5[d] and Figure 1.4), the releasing step could be important in recycling calpactin for another round of granule exocytosis.

Although the activity of $pp60^{c\text{-}src}$ has been shown to decrease upon stimulation of cells, increase in the tyrosine phosphorylation of a number of proteins is detected under the same conditions [Ely et *al.*, 1990; Oddie et *al.*, 1989]. In particular, changes in the phosphorylation state of the protein pp42 precedes catecholamine release but the exact role of tyrosine or serine phosphorylation in the control of exocytosis remain unknown.

The direct activation of protein kinase C by Ca^{2+}, the requirement of

ATP in exocytosis, and the activation of exocytosis by phospholipase C treatment of permeabilised-chromaffin cells (resulting in diacylglycerol production which activates protein kinase C) has led to the investigation of the role of this kinase in the secretory response. The results obtained in such studies have not been conclusive, for example, although in intact bovine chromaffin cells the activation of protein kinase C by phorbol ester treatment has little effect on secretion [Brocklehurst *et al.*, 1985; Burgoyne & Norman, 1984; Knight & Baker, 1983], similar treatment reduces the requirement of Ca^{2+} for exocytosis in permeabilised cells [Knight & Baker, 1983; Pocotte *et al.*,1985]. Inhibition of protein kinase C by staurosporine or sphingosine, by long-term down-regulation or with its pseudosubstrate also has very little effect on Ca^{2+}-dependent exocytosis from digitonin-permeabilised bovine chromaffin cells [Morgan & Burgoyne, 1990; Burgoyne *et al.*, 1988; Terbush & Holz, 1990], but the treatment of cytosol with the phorbol ester PMA enhances the ability of EXO1 to activate exocytosis, and this is sensitive to staurosporine (see Section 1.5[e]). It therefore seems that protein kinase C does not have an essential, but only a regulatory role in the exocytosis from digitonin-permeabilised bovine chromaffin cells. However, the substantial stimulatory effect on exocytosis of protein kinase C activation in many other cells [Rink *et al.*, 1983; Wilson, 1990; Bittner & Holz, 1990], together with its ability to phosphorylate the proteins implicated in Ca^{2+}-dependent exocytosis [Lee & Holz, 1986] suggests that protein kinase C activation may represent a parallel pathway of exocytic regulation.

[c] ***Role of other second messengers in the control of exocytosis*** *:*

GTP is emerging as an important factor in the control of vesicular traffic. Its hydrolysis to GDP acts as an important "functional switch" and

ensures the correct interaction between intracellular compartments, including the interaction of secretory vesicles with the plasma membrane [Balch, 1990; Bourne *et al.*, 1990; Downward, 1990; Matteoli *et al.*, 1991]. The evidence for the role of GTP in the control of the last stages of exocytosis has come from the use of non-hydrolysable analogues of GTP such as GTPγS which acts as a functional block and allows the identification of the steps involved. Molecular cloning and sequencing of proteins involved in the secretory pathway, through the comparison of sequences, has also been important in identifying those steps regulated by GTP. Such studies have identified a family of low molecular weight GTP-binding proteins such as Sec4 (yeast) and Rab3A (neurones) which are homologous to the oncogene product $p21^{ras}$ (see Section 1.5[d] and above reviews). Studies in which the effects of GTPγS, GDP-β-S and GMP.PNP have been studied in permeabilised chromaffin cells has suggested the role of GTP in regulated secretion through mediating the functions of both low molecular weight GTP-binding proteins and a putative G-protein termed G_e [Gomperts, 1990; Burgoyne, 1991], the function of the latter protein, G_e , is appears to be independent of Ca^{2+}.

Cyclic nucleotides such as cAMP and cGMP have been implicated in the control of exocytosis since cholinergic and muscarinic stimulations (respectively) of chromaffin cells transiently increase their levels [Morita *et al.*, 1987; Schneider *et al.*, 1979]. Attempts to identify the role of cAMP in the control of exocytosis have produced conflicting results due to the difference in experimental parameters such as concentrations of forskolin and the length of time which cells have remained in culture [see Burgoyne, 1991 for review]. cGMP on the other hand has been reported to modulate nicotine-stimulated exocytosis, inhibiting exocytosis at high nicotine concentrations but stimulating it at low nicotine concentrations [Nakaki *et al.*, 1988; Derome *et al.*, 1981; O'Sullivan Burgoyne, 1990]. Increasing cGMP levels in intact cells by

agents such as atrial naturietic peptide, 8-bromo-cGMP or with nitroprusside increases the sensitivity of chromaffin cells to nicotinic stimulation [O'Sullivan & Burgoyne, 1990]. Nitroprusside is known to generate nitric oxide which is been regarded as another modulator of exocytosis since it can stimulate secretory response in perfused rat adrenals [Dohi *et al.*, 1983]. It has been reported that nitric oxide synthase, which catalyses the formation of nitric oxide, is present in nerve terminals innervating the adrenal medulla and its activity is stimulated in response to elevated Ca^{2+} levels [Bredt *et al.*, 1990]. This implies that co-release of acetylcholine and nitric oxide from the synapses will lead to the rise of intracellular Ca^{2+} and cGMP levels in chromaffin cells. However, cGMP has no effect on the intracellular concentration of Ca^{2+} in intact cells, it does not stimulate Ca^{2+}-dependent secretion from permeabilised chromaffin cells and its role in secretion is currently unclear [Knight & Baker, 1982; O'Sullivan & Burgoyne, 1990].

Arachidonic acid and other cis-unsaturated fatty acids have also been implicated in the regulation of exocytosis by the findings that they induced fusion of granules aggregated by synexin or calpactin in the presence of Ca^{2+} and are produced during stimulation of cells [Drust & Creutz, 1988; Frye & Holz, 1984; see Burgoyne & Morgan, 1990 and Burgoyne, 1991 for reviews].

1.7 Endocytosis and recycling of secretory vesicle membranes

With around 20,000 granules per cell, about 90% of the total membrane surface area of chromaffin cells is the chromaffin granule membrane (see Table 1.1). Upon stimulation, a release of 10% of the total cellular catecholamine content would result in doubling of the plasma membrane surface area, which unless rectified would clearly be lethal to the cell. Furthermore, in contrast to the rapid turnover of the soluble secretory components of chromaffin granules, there is little synthesis of the membrane components such as dopamine β-monooxygenase and cytochrome b_{561} [Winkler & Westhead, 1980; Falkensammer *et al.*, 1985]. A pathway of membrane retrieval must therefore exist not only to avoid enlargement of the plasma membrane but also to account for the slow turnover of the granule membrane proteins. Such a pathway would be consistent with hypothesis that secretory vesicles are reusable shuttles for the transportation of the secretory components, and that their membrane components are extensively reutilised after exocytosis [Winkler, 1977].

Following exocytotic stimulation, chromaffin granule membrane components such as dopamineβ-monooxygenase and glycoprotein III are exposed on, and subsequently retrieved from the cell surface by endocytosis [Phillips *et al.*, 1983; Patzak *et al.*, 1984; Hunter & Phillips, 1989]. This is supported by ultrastructural observations which show that membrane retrieval utilises coated vesicles that are also involved in the uptake of endocytic ligands such as transferrin and LDL [Nagasawa & Douglas, 1972; Patzak & Winkler, 1986; Patzak *et al.*, 1987].

The intracellular destination of endocytic cargo is initially to the endosome, an acidic compartment where endocytosed ligands dissociate from their receptors. The receptors then recycle to the plasma membrane via a

compartment in which they are co-localised with enzymes such as sialyltransferase, presumed to be the trans-Golgi network (TGN). Chromaffin granule membrane proteins are also thought to recycle through the endosomes to the TGN, where upon arrival, they are sorted into immature granules. Patzak and Winkler (1986) have shown this for glycoprotein III, which when immunolabeled at the cell surface, is subsequently localised in the newly formed chromaffin granules although some was also found in lysosomes. The exact route of recycling of chromaffin granule membrane proteins is not clearly established since this process appears to be effected by the length of time cells are cultured prior to experimentation [Patzak & Winkler, 1986; Patzak *et al.*, 1987], and through the lack of specific molecular probes for chromaffin granule membranes.

Cells efficiently concentrate the molecules destined to be endocytosed in clathrin-coated pits while excluding plasma membrane proteins from them. Studies on the control of this mechanism have shown the importance not only of the cytoplasmic domains of endocytosed proteins, but also of a group of cytosolic protein known as *adaptins* [Pearse & Robinson, 1990; Hurtley, 1990]. The selectivity of endocytosis of chromaffin granule membrane proteins therefore appears to be through the interaction of their cytoplasmic domains with adaptins. Integral membrane proteins of the chromaffin granule membrane such as p65, synaptophysin and synaptobrevin (see Section 1.4[b]) all have large cytoplasmic domains which may interact with adaptins, yet it is mysterious how proteins such as dopamine β-mono-oxygenase and glycoprotein III, which do not possess such domains (see Section 1.4[a]) are endocytosed and recycled. One possible explanation of this is that some integral membrane proteins of chromaffin granules may have roles in the organisation and targeting of such proteins. This is supported by observations that after exocytosis secretory vesicle antigens remain clustered

in the plasma membrane before being endocytosed [Burgoyne *et al.*, 1982; Phillips *et al.*, 1983]. Current research into the role of chromaffin granule membrane proteins is focussed on their function in exocytosis, but their possible role during endocytosis must not be ignored. It is therefore possible that proteins such as SV2, p65, synaptophysin, synaptobrevin and glycoprotein II may have a role in the recycling of secretory vesicle membrane proteins.

1.8 Approaches to the study of secretion from cells

[a] ***In vitro assays, cell-free systems and permeabilised cells*** **:**

An important strategy of scientific research is to isolate and simplify the event under investigation so that each of its components can be identified and studied. In the field of intracellular traffic, such a strategy has led to the development of *in vitro* systems or "cell-free" assays which can reconstitute various stages of the secretory and endocytic pathways [Balch *et al.*, 1984; Davey *et al.*, 1985]. Such systems have revealed factors that are required for intracellular transport such as ATP, GTP, Ca^{2+} and fatty acylation [Balch *et al.*, 1984; Melançon et *al.*, 1987; Beckers & Balch, 1989; Pfanner *et al.*, 1990], and also proteins such as *N*-ethylmaleimide sensitive factor (NSF), α-, β-, γ- *SNAP*s and *POP* which mediate it [Malhotra *et al.*, 1988; Wilson *et al.*, 1989; Clary *et al.*, 1990; Wattenberg *et al.*, 1990]. Studies in yeast genetics have shown that NSF is encoded by the *sec18* gene and that the GTP is required by the SEC4 gene product (see Balch, 1989 and 1990 for reviews).

A factor in the success of such *in vitro* systems has been the ease with which organelles can be isolated and the distinct modification proteins

undergo when they move from one compartment to another. In contrast, reconstitution of the late stages of intracellular traffic has been hampered by the difficulty of isolating pure plasma membrane fractions as "inside out" vesicles and by the absence of a biochemical event to monitor membrane fusion. Although a cell-free system has been developed [Nadin *et al.*, 1989], on the whole such attempts have been unsuccessful.

A different and highly successful approach has been the development of *permeabilised* chromaffin cells by Baker and Knight (1981, 1982). This technique utilises electrical discharge to perforate the plasma membrane permitting bypass of signal transduction at the plasma membrane level and allowing the cytoplasmic conditions to be manipulated. Furthermore the use of detergents such as digitonin to make larger holes in cells has allowed the introduction of antibodies and peptides into cells in order to study their effects. Such studies have shown the requirement of calpactin and fodrin in exocytosis [Perrin *et al.*, 1987; Drust Creutz, 1978; Ali *et al.*, 1989] and recent studies in introducing essential components which have leached out from permeabilised cells may identify more (see Section 1.5[e]).

[b] ***Monoclonal antibodies as tools*** *:*

Antibodies are products of the immune system of higher eukaryotes, their role being to tag foreign molecules and initiate their specific degradation by the phagocytic cells. Their specific affinity for almost any type of biological molecule has made them indispensable tools in biological research because they provide probes for the molecule of interest and allow its detection against a complex background of other molecules. The introduction of monoclonal antibody technology by Köhler and Milstein in 1975 has greatly accelerated the successful applications of antibodies in the investigation

biological of systems because monoclonal antibodies, unlike polyclonal antisera, can be raised against a complex mixture of molecules without the need to purify the individual components to homogeneity. This is especially important in the investigation of complex biological systems such as multiprotein complexes, cell surface antigens and intracellular organelles where many of the proteins present do not have a known functional assay that can be used in their isolation and study.

Since many of the functions of the secretory vesicles are carried out by proteins that are intrinsic to its membrane, a powerful technique in elucidating the mechanism of exocytosis has been to raise monoclonal antibodies against the proteins of secretory vesicle membranes in order to identify them. Such studies on synaptic vesicles from brain identified proteins such as SV2, p65, synaptophysin, p29 and synaptobrevin (see Table 1.6 and Section 1.4). Because of the more complicated composition of chromaffin granules, there have been no such attempts in chromaffin cells. It was the aim of this study to combine the advantages of both chromaffin cells (Section 1.2) and monoclonal antibody production to produce antibodies that recognise components of the chromaffin granule membrane so that they might be used to begin to resolve the mechanisms involved in its function.

Table 1.6

Application of monoclonal antibodies in the investigation of subcellular organelle membranes

Organelle	Antigen	Reference
Golgi complex	135-kDa Golgi antigen (Rat liver) 110-kDa antigen (Cultured Fibroblast) 103-108 & 180-kDa antigens (Rat autoimmune antibodies to acinar cells)	Burke *et al.* (1982), Lin & Queally (1982), Smith *et al.* (1984).
	Cytoplasmically oriented antigens	Chicheportiche *etal.* (1984)
Clathrin coated vesicles	Clathrin heavy chain antibodies (Pig brain)	Louvard *et al.* (1983).
Exocrine secretory vesicles	Insulin secretory vesicles 50, 80 &110-kDa antigens	Grimaldi *et al.* (1987).
Chromaffin granules	Cytochrome b_{561}antibodies	Pruss & Shepard (1987).
	Dopamine β-monooxygenase	This study.
	Bovine glycoprotein II	This study.
	Bovine p65	Tugal *et al.* (1991) & this study.
Synaptic vesicles	SV2	Buckley & Kelly (1985).
	p65	Matthew *et al.* (1981) , Obata *et al.* (1987).
	p38 (synaptophysin)	Buckley *et al.* (1985), Leube *et al.* (1987) Südhof *et al.* (1987)
	p29	Baumert *et al.* (1990).
	p18 (synaptobrevin)	Trimble *et al.* (1988).
Lysosome	lysosomal membrane antigens	Croze *et al.* (1984)
Plasma membrane	Chromaffin cells (54-kDa antigen)	Bohner *et al.* (1985)
	Hepatocytes	Hubbard *et al.* (1985)

CHAPTER 2

CHAPTER 2

Materials and Methods

2.1 Chemicals, biochemicals and other materials

[a] *Chemicals* :

All common laboratory chemicals were of highest grades, purchased either from Sigma Chemical Co. Ltd. (Poole, Dorset, U.K.) or BDH (Poole, Dorset, U.K.) unless specified.

Acetic, hydrochloric and ortho-phosphoric acids of AnalaR grade were purchased from BDH, Poole, Dorset, U.K.

DMSO and DMF were purchased from Sigma Chemical Co. Ltd., Poole, Dorset, U.K.

Methanol, ethanol, propan-1-ol and butan-2-ol were purchased from BDH, Poole, Dorset, U.K.

[b] *Biochemicals* :

Amberlite (XAD-2), ATP (di-sodium), BSA, benzamidine, 4-chloro-1-naphthol, deoxycholic acid (sodium salt), dithiothreitol, EDTA (di-sodium), Hepes, Mes, leupeptin, pepstatin, PMSF, poly L-lysine, SDS, TLCK, Trisma base, Triton X-100, Tween-20 were purchased from Sigma Chemical Co. Ltd., Poole, Dorset, U.K.

Acrylamide (Electran), bromophenol blue, N,N'methylene-bisacrylamide (Electran) and Nonidet P40, polyacrylamide (carboxy-modified) and TEMED were purchased from BDH, Poole, Dorset, U.K.

Ampholytes (pH 3-10 and 9-11) were purchased from BioRad-LKB

Instruments Ltd., South Croydon, Surrey, U.K.

Iodogen (Pierce) was purchased from ICN Flow, High Wycombe, Bucks, U.K.

Coomassie blue G-250, Ponceau S and urea were purchased from Serva, Heidelberg, Germany.

Enhanced chemiluminescence kit and Hyperfilm-MP autoradiography film was purchased from Amersham International, Amersham, Oxfordshire, U.K.

Triton X-114 was purchased from Fluka, Buchs, Switzerland.

Lectins were purchased from Sigma Chemical Co. Ltd., Poole, Dorset, U.K.

[c] ***Antibodies :***

Biotin-, horse radish peroxidase- and urease-linked anti-mouse IgG (whole molecule) and anti-mouse IgM (μ-chain specific) were purchased from Sigma Chemical Co. Ltd., Poole, Dorset, U.K.

Samples of the monoclonal antibody "*asv48*" were generously given by Dr. John L. Bixby (University of Miami, School of Medicine, Miami, USA.) and Prof. Robert D. Burgoyne (University of Liverpool, Dept. of Physiology, Liverpool, U.K.).

Samples of anti-cytochrome b_{561} polyclonal antibody was generously given by Dr. David K. Apps (Department of Biochemistry, University of Edinburgh Medical School, Edinburgh, U.K.) and samples of anti-glycoprotein II polyclonal antibody was generously given by Dr. R. Fischer-Colbrie (Department of Pharmacology, University of Innsbruck, Innsbruck, Austria).

[d] *Enzymes* :

Calmodulin-agarose, neuraminidase (EC 3.2.1.18, type X) and trypsin (EC 3.4.21.4) were purchased from Sigma, Poole, Dorset, U.K.

Endoglycosidase F (EC 3.2.1.96) and streptavidin were purchased from Boehringer, Mannheim, Germany.

[e] *Tissue culture materials* :

RPMI-1640 was purchased from Flow, High Wycombe, Bucks., U.K.

Aminopterin and hypoxanthine-thymidine media supplement were purchased from Sigma Chemical Co. Ltd., Poole, Dorset, U.K.

Myoclone foetal calf serum was purchased from Gibco BRL, Paisley, U.K.

Tissue culture plates (Falcon) and other plastics (Falcon) were purchased from Beveridge Ltd., Edinburgh, U.K.

Polyethylene glycol 1500 (46%, Hepes-buffered and sterile) was purchased from Boehringer, Mannheim, Germany.

[f] *Cell lines* :

NS-0 myeloma cell line was a generous gift of Dr. Jeff Haywood (Department of Biochemistry, University of Edinburgh Medical School, Edinburgh, U.K.).

[g] *Protein purification and sequencing equipment* :

Protein purification was performed using the Gilson FPLC system.

C-18 reverse phase and phenyl-TSK columns were purchased from Anachem, Luton, Bedfordshire, U.K.

High pressure liquid chromatography (HPLC) was performed on Applied Biosystems (ABI, model 130A) using an ABI-Aquapore RP-300 column (7μ,

30x2.1 mm).

Sequencing was performed on the ABI pulsed liquid sequencer model 477A using automated Edman degradation and the PTH-amino acids were separated by HPLC (ABI model 120A) using a C-18 reverse phase column (5μ, 220 x 2.1 mm).

[h] ***Other materials*** *:*

Dialysis tubing (Gallenkamp) was purchased from Fisons, Loughborough, Leicestershire, U.K.

ELISA plates (Falcon) were purchased from Beveridge Ltd., Edinburgh, U.K.

Nitrocellulose (Schleicher & Schuell, 0.45μm) was purchased from Anderman & Co., Kingston upon-Thames, Surrey, U.K.

Methods

2.2 Preparation of subcellular fractions and organelles

[a] *Adrenal medullary homogenate* :

Fresh bovine adrenal glands were obtained from the local slaughterhouse. The medullae dissected from 40 glands were placed in 400 ml of 0.3 M sucrose containing 10 mM Hepes-NaOH (pH 7.2) and when required 2 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 1 mM benzamidine at 0°C. The dissected glands were passed through a stainless steel mincer followed by homogenisation by five passes of a motor-driven Teflon/glass homogenizer.

[b] *Crude chromaffin granules and the mitochondrial fraction* :

The homogenate was centrifuged at 1,500 g_{av} (Beckman Instruments, JA-14 rotor) for 5 minutes at 4°C to remove intact cells, nuclei and debris. The resultant post-nuclear supernatant was centrifuged at 19,000 g_{av} in the same rotor for 30 minutes at 4°C to pellet the granules from the cytosol and microsomes. Crude granules were then resuspended in 150 ml of the same buffer and washed free of soluble contaminants by centrifugation at 18,000 g_{av} (Beckman Instruments, JA-20 rotor) for 20 minutes at 4°C.

The pellets, consisting of a pink inner ring of chromaffin granules and an upper fluffy layer of mitochondria, were resuspended by gentle homogenisation to 120 ml in the homogenisation buffer and were layered over six 50 ml "cushions" of 1.6 M sucrose containing 10 mM Hepes-NaOH (pH 7.2) and centrifuged at 161,000 g_{av} (Beckman Instruments, Ti-45 rotor) for

60 minutes at 4°C. By this procedure, the mitochondria were trapped at the 0.3/1.6 M sucrose interface while the dense granules pelleted.

[c] ***Chromaffin granule membranes*** *:*

The pellet of granules was lysed by homogenisation in 10 mM Hepes-NaOH (pH 7.2), 0.1 mM PMSF and 1 mM benzamidine at 0°C followed by dilution to 210 ml. The membranes were pelleted by centrifugation at 161,000 g_{av} for 20 minutes and further purified by resuspending them to 40 ml with the lysing buffer and layering them over three 50 ml cushions of 1.0 M sucrose in 10 mM Hepes-NaOH (pH 7.2) followed by centrifugation at 161,000 g_{av} for 30 minutes at 4°C. The pure chromaffin granule membranes were collected from the 0.3/1.0 M sucrose interface and washed free of sucrose by centrifugation at 161,000 g_{av} for 20 minutes at 4°C and finally resuspended in 10 mM Hepes-NaOH (pH 7.2), 1 mM DTT to a protein concentration of 10 mg/ml and stored at -70°C.

[d] ***Adrenal medullary mitochondrial membranes*** *:*

Mitochondria from the 0.3/1.6 M sucrose interface (Section 2.2[b]) were lysed by homogenisation in 10 mM Hepes-NaOH (pH 7.2), 0.1 mM PMSF and 1 mM benzamidine at 0°C followed by dilution to 210 ml. The membranes were pelleted by centrifugation at 161,000 g_{av} (Beckman Instruments, Ti-45 rotor) for 20 minutes and further purified using six continuous 0.5-1.6 M sucrose gradients by centrifugation at 207,000 g_{av}. (Beckman Instruments, SW-41 rotor) for 16 hours. The membranes were washed free of sucrose by pelleting them at 412,000 g_{av} (Beckman Instruments,

TL-100.3 rotor) for 10 minutes at 2°C, resuspended in 10 mM Hepes-NaOH (pH 7.2) and stored at -70°C.

[e] ***Adrenal medullary microsomal membranes*** *:*

The supernatant from the 19,000 g_{av} centrifugation of the homogenate (Section 2.2[b]) was further cleared of granules and mitochondria by centrifugation as above for 20 minutes. The resultant supernatant was then layered onto 20 ml cushions of 1.4 M sucrose in 10 mM Hepes-NaOH (pH 7.2) and centrifuged at 161,000 g_{av} (Beckman Instruments, Ti-45 rotor) for 60 minutes at 4°C. The microsomes were collected from the 0.3/1.4 M sucrose interface. This method not only prevented the aggregation of the vesicular structures, but also enabled the residual chromaffin granules and mitochondria to be cleared from the microsomes. The microsomes were stored at -70°C.

[f] ***Adrenal medullary cytosol*** *:*

Cytosol was generally prepared for the isolation of Ca^{2+}-dependent chromaffin granule-binding proteins that are implicated in the mechanism of exocytosis, the subcellular distribution of which depends on the levels of intracellular Ca^{2+} concentrations (see Chapter 1). Therefore to minimise the loss of these proteins into the pellet fraction, cytosol was prepared in the presence of 5 mM EDTA; otherwise the both the addition of EDTA and the ammonium sulphate precipitation step described below were omitted.

The adrenal medullae were dissected and processed as in the preparation of crude granules. The supernatant from the 19,000 g_{av}

centrifugation of the homogenate was further cleared of granules, mitochondria and microsomes by centrifugation at 161,000 g_{av} (Beckman Instruments, Ti-45 rotor) for 60 minutes at 4°C. The cytosolic fraction was fractionated by addition of solid ammonium sulphate. The protein fraction that precipitated between 55 and 90% saturation was redissolved in 10 mM Hepes-NaOH (pH 7.2). To remove ammonium sulphate and other ions and molecules from this preparation, it was dialysed against 100-volumes of 10 mM Hepes-NaOH (pH 7.2) over 24 hours with 5 changes of buffer and stored frozen at -70°C.

[g] ***Preparation of calcium-dependent chromaffin granule-binding protein*** **:**

Chromaffin granule membranes were incubated at 1 mg protein per ml of cytosolic fraction (both prepared as described above) in the presence of 20 μM $CaCl_2$, 1 mM MgATP, 0.2 mM PMSF and 1 mM benzamidine at 37°C for 30 minutes. The chromaffin granule membranes were then pelleted by centrifugation at 161,000 g_{av} (Beckman Instruments, Ti-45 rotor) for 20 minutes at 4°C and washed twice by resuspension to one-tenth original incubation volume in 10 mM Hepes-NaOH (pH 7.2) containing 10 μM $CaCl_2$ and 1 mM MgATP and centrifuged at 207,000 g_{av} (Beckman Instruments, Ti-70.1 rotor) for 15 minutes at 4°C. The final pellet was resuspended to the same volume in 10 mM Hepes-NaOH (pH 7.2) containing 5 mM EGTA and centrifuged as described above. The resultant supernatant was dialysed against two changes of 100-volumes of 1mM Hepes-NaOH (pH 7.2) containing the above protease inhibitors over 2 hours and stored at -70°C.

[h] ***Preparation of synaptosome membranes from rat fore-brain*** :

Rat cerebral cortex (approximately 1 g wet weight) was homogenised in 10 ml of 0.32 M sucrose in 10 mM Hepes-NaOH (pH 7.2) at 4°C and centrifuged at 4000 g_{av} (Beckman Instruments, JA-20 rotor) for 10 minutes at 4°C. The supernatant was removed and layered onto a 4 ml cushion of 1.2 M sucrose in 10 mM Hepes-NaOH (pH 7.2) and centrifuged at 160,000 g_{av} (Beckman Instruments, Ti-70.1 rotor) for 15 minutes at 4°C. Partially purified synaptosomes were collected from the 0.32/1.2 M sucrose interface and layered onto 4 ml cushions of 0.8 M sucrose in 10 mM Hepes-NaOH (pH 7.2) and centrifuged at the same conditions as the previous spin. The pellet consisting of purified synaptosomes was harvested in 10 mM Hepes-NaOH (pH 7.2) and lysed in 20 ml of the same buffer by homogenisation to obtain broken membranes. The membrane pellet was harvested following centrifugation at 160,000 g_{av}, resuspended in 10 mM Hepes-NaOH (pH 7.2) and stored at -70°C.

2.3 Treatment of Subcellular Organelle Membranes with Reagents

[a] ***Washing membranes at pH 11*** :

Membranes were resuspended at a protein concentration of 1 mg/ml by gentle homogenisation in 0.1 M Na_2CO_3 (pH 11) containing 1 mM EDTA and 0.2 mM PMSF and incubated on ice for 30 minutes with occasional agitation (Higgins, 1984; Howell & Palade, 1982). The membranes were then recovered from the solution by centrifugation at 412,000 g_{av} (Beckman Instruments, TL-100.3 rotor) for 15 minutes at 2°C and resuspended in 10 mM

Hepes (pH 7.2), at a protein concentration of about 5 mg/ml. The supernatant from the above centrifugation step was recentrifuged at the same conditions. Both the membranes and the supernatant were then dialysed twice against 1000-volumes of 10 mM Hepes (pH 7.2) containing 0.1 mM PMSF over 24 hours.

[b] ***Fractionation of membrane proteins with Triton X-114*** *:*

The Triton X-114, pre-condensed by the method of Bordier (1981) was made up as a 10% (w/v) solution for this procedure. Membranes in Tris-buffered saline (TBS, pH 7.4) were resuspended to a protein concentration of 4 mg/ml by gentle homogenisation in the presence of 2% (w/v) cold Triton X-114 at 0°C and incubated on ice for further 5 minutes. The solubilised membranes were then centrifuged at 412,000 g_{av} (Beckman Instruments, TL-100.3 rotor) for 15 minutes at 2°C to remove the insoluble precipitate formed by the hydrophobic proteins, termed "*P1*". The P1 pellet was washed twice at 0°C by resuspension to its original volume in the presence of 2% (w/v) Triton X-114 in TBS, then resuspended in one-fifth original volume of TBS.

The supernatant left after the removal of P1 was layered over 1 ml cushions of 0.25M sucrose/TBS/0.06% (w/v) Triton X-114 in conical glass centrifuge tubes. The tubes were incubated at 30°C for 5 minutes to separate the detergent phase followed by centrifugation at 2,500 g_{av} (swing-out bench centrifuge, top speed) for 5 minutes at room temperature. The supernatant ("*aqueous phase*") was removed from above the cushion. The cushion was then carefully removed, the "*detergent-rich*" phase recovered and resuspended to its original volume in ice-cold TBS.

To remove cross-contaminants from these phases, the above

procedure was repeated twice with intermediate incubations on ice for 5 minutes. Finally, the detergent phase was resuspended to one-fifth original volume in ice-cold TBS for further use.

The residual detergent in the aqueous phase was removed by dialysis against 100-volumes of TBS containing 1% (w/v) Amberlite XAD-2, 0.2 mM PMSF, 1 mM benzamidine at 4°C for 5 days with two changes of buffer. The dialysed faction was diluted with 3-volumes of TBS and centrifuged at 412,000 g_{av} (Beckman Instruments, TL-100.3 rotor) for 15 minutes at 2°C. The pellet (the "*glycoprotein-rich*" fraction) was resuspended by homogenisation in 10mM Hepes-NaOH (pH 7.2) with 0.1% (v/v) Triton X-100. All fractions were stored at -70°C.

[c] *Labelling proteins with biotin* :

Membrane proteins were resuspended in 100 mM bicarbonate buffer (pH 9.5) at a protein concentration of about 2 mg/ml. Soluble proteins were either dialysed into the same buffer or were centrifuged through a Biogel P6-DG mini-column equilibrated with the same buffer. Fresh biotin-N-hydroxysuccinimide (NHS-Biotin) ester stock in DMF was added to a final protein:NHS-biotin ratio of 2:1 (w/w) for up to 1 hour at room temperature while gently stirring. The reaction was terminated by the addition of Tris-HCl (pH 7.0) to final concentration of 200 mM. Membrane proteins were washed free of NHS-Biotin by centrifugation and resuspension in 10 mM Hepes-NaOH (pH 7.2); soluble proteins were centrifuged through a Biogel P6-DG mini-column equilibrated with 10 mM Hepes-NaOH (pH 7.2) or dialysed against the same buffer.

[d] ***Deglycosylation of membrane glycoproteins*** *:*

Deglycosylation was carried out using endoglycosidase F (EC 3.2.1.96, Boehringer) and desialylation with neuraminidase (EC 3.2.1.18, Sigma, type VI). For deglycosylation with endoglycosidase F, 100 µg of the Triton X-114 aqueous fraction of chromaffin granule membranes were lyophilised and resuspended with the following reagents to give a final protein concentration of 1.6 mg/ml: 50 mM Mes-NaOH (pH 6.5), 0.5% (w/v) deoxycholate, 1% (v/v) Triton X-100 and 33 units/ml endoglycosidase F. The digestion was carried out at 37°C for 6 hours in the presence of 5 mM benzamidine, 200 µM PMSF, 5 µg/ml leupeptin, 5 µg/ml pepstatin, 50 µM TLCK and 15 mM EDTA (pH 6.5). The samples were lyophilised, resuspended in [x1] SDS-sample buffer and analysed by SDS-polyacrylamide gel electrophoresis followed by immuno-blotting.

For endoglycosidase treatments that were to be analysed by two-dimensional electrophoresis, the aqueous fraction of chromaffin granule membranes was dialysed for 16 hours against two changes of 1000-volumes of 5 mM Mes-NaOH pH 6.5 containing 0.1% (v/v) Triton X-100, 100 µM PMSF and 1 mM benzamidine at 4°C. The reaction was carried out as above and the samples were dialysed for 2 hours against two changes of 600-volumes of 1 mM Hepes-NaOH (pH 7.2) containing 0.1% (v/v) Triton X-100 and the above protease inhibitors at 4°C using a BioRad micro-dialyser. The dialysed samples were lyophilised, resuspended in two-dimensional electrophoresis sample buffer and analysed by immunoblotting.

For digestion with neuraminidase, 100 µg of the Triton X-114 aqueous phase were lyophilised and resuspended to a final concentration of 1.6 mg/ml in 50 mM Mes-NaOH pH 5.5 with 0.2% (v/v) Triton X-100 and the above protease inhibitors. Neuraminidase (Sigma type VI, dissolved in the

reaction buffer to a concentration of 10 units/ml) was added to a final concentration of 1 unit/ml and the reaction mixture was incubated at 37°C for 5 hours. The samples were lyophilised, resuspended in [x1] SDS-sample buffer and analysed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting.

For neuraminidase-treated samples that were to be analysed by two-dimensional electrophoresis, 0.1% (v/v) Triton X-100, 100 μM PMSF and 1 mM benzamidine were added to the aqueous phase and dialysed for 16 hours against two changes of 1000-volumes of 5 mM Mes-NaOH pH 5.5 containing 0.1% (v/v) Triton X-100, 100 μM PMSF and 1 mM benzamidine at 4°C. Aliquots of 50 μg of the dialysed aqueous phase were lyophilised and resuspended to a protein concentration of 0.5mg/ml as above. Neuraminidase (Sigma type X, resuspended in the reaction buffer to a concentration of 40 units/ml) was added to a concentration of 10 unit/ml and incubated as above. The reaction was terminated by dialysis for 2 hours against two changes of 600-volumes of 1 mM Hepes-NaOH (pH 7.2) containing 0.1% (v/v) Triton X-100 and the above protease inhibitors at 4°C using a BioRad micro-dialyser. The dialysed samples were lyophilised, resuspended in two-dimensional electrophoresis sample buffer and analysed by immunoblotting.

[e] ***Protease digestion of intact chromaffin granules and membranes*** *:*

The crude granule fraction and chromaffin granule membranes resuspended in 10 mM Hepes-NaOH (pH 7.2) to 1 mg of protein per ml with were treated with trypsin, papain or pronase at various concentrations at 20°C for up to 60 minutes. The digestions were stopped with the addition of the following protease inhibitors: 50 μM TLCK, 5 mM benzamidine, 5 μg/ml

leupeptin, 5 μg/ml pepstatin, 20 mM EDTA and 200 μM PMSF. In the case of purified chromaffin granule membranes, the soluble peptides were separated from the membrane-bound peptides by centrifugation at 412,000 g_{av} (Beckman Instruments, TL-100.3 rotor) for 15 minutes at 2°C. The resulting pellet was washed twice by resuspension and centrifugation in 10 mM Hepes-NaOH (pH 7.2) containing the same protease inhibitors. The supernatant fraction was recentrifuged as above to remove any residual membrane-bound proteins. With crude intact granules, the digestion was terminated as above. The reaction mixture was then placed on top of a 2 ml cushion of 1.6 M sucrose, 10 mM Hepes-NaOH (pH 7.2) and centrifuged at 412,000 g_{av} for 15 minutes at 2°C. The upper layer (0.3 M sucrose) containing the soluble peptides was removed and recentrifuged to pellet any membranes and mitochondria. The granule pellets were resuspended in 3 ml of 10 mM Hepes-NaOH pH 7.2 containing the protease inhibitors, and the membranes were then pelleted and washed twice. The resultant membrane fractions were then denatured by the addition of hot [x4] acidic SDS-sample buffer (0.2 M potassium biphthalate, 0.2 mM EDTA, 20% (w/v) SDS, 40% (v/v) glycerol, 0.015% (w/v) bromophenol blue, pH 4.0) in the presence of 10 mM DTT. The soluble fractions were lyophilised and taken up in one-tenth original volume of [x1] SDS-sample buffer (pH 4.0, as above). The samples were then analysed by immunoblotting.

[f] *Calmodulin-affinity chromatography of protein fractions obtained from chromaffin granule membranes* :

Triton X-100 (0.1% (v/v) final), 0.2 mM PMSF and 1 mM benzamidine were added to the Triton X-114 aqueous phase of chromaffin granule

membranes which was dialysed against three changes of 1000-volumes of 1mM Hepes-NaOH (pH 7.2), 0.05% (v/v) Triton X-100, 0.2 mM PMSF and 1 mM benzamidine over 18 hours. Tryptic supernatant of chromaffin granule membranes was dialysed without Triton X-100 under the same conditions. Both preparations were concentrated five-fold by rotary evaporation. The Triton X-114 aqueous fraction at a protein concentration of 5 mg/ml or tryptic supernatant at a concentration of 1 mg/ml in 10 mM Hepes-NaOH pH7.2 were incubated with one-tenth final volume of packed calmodulin-agarose in the presence of 100 μM $CaCl_2$ for 20 minutes at room temperature on a rotating platform. The incubation was terminated by pelleting the calmodulin-agarose in a microcentrifuge for 1 minute. The pellet was then washed three times with 10 mM Hepes-NaOH (pH 7.2) containing 100 μM $CaCl_2$. Bound proteins were eluted by resuspending the final pellet in 10 mM Hepes-NaOH, 5 mM EGTA (pH 7.2) followed by centrifugation as above. The supernatant was then lyophilised and taken up either in urea-containing sample buffer for isoelectric focusing or in [x1] SDS-sample buffer for analysis by immunoblotting.

2.4 Electrophoretic Methods

[a] *SDS-polyacrylamide slab gel electrophoresis:*

Proteins were analysed on 10% acrylamide gels, unless otherwise stated, in the presence of SDS (Douglas & Butow, 1973) and with the discontinuous buffer system of Laemmli (1970). The dimensions of the gels were either 135 x 125 x 1.5 mm or 85 x 90 x 0.75 mm.

(i) ***Separating gel :*** Acrylamide stock for the gels were from a 30:0.8% mixture of acrylamide/N,N' bis-acrylamide. The gel mix contained the required percentage of acrylamide/N,N' bis-acrylamide mixture in 375 mM Tris HCl (pH 8.8), 0.1% (w/v) SDS, and 0.5% (w/v) polyacrylamide (M_r 250,000) added to improve the mechanical strength of the gel. Gels were chemically polymerised with 0.05% (v/v) TEMED and 0.1% (w/v) ammonium persulphate. Butan-2-ol (water-saturated) was layered onto the unpolymerised gel solution to ensure a flat top and was washed away after the gel polymerised.

(ii) ***Stacking gel :*** A stacking gel containing 4.5% (w/v) of the acrylamide/N,N' bis-acrylamide mixture in 125 mM Tris-HCl (pH 6.8), 0.1% (w/v) SDS and 0.5% (w/v) polyacrylamide (M_r 250,000) was poured on top of the polymerised separating gel. The pH difference between the two gels and electrode buffer produced stacking of the proteins.

(iii) ***Electrode buffer :*** The electrode buffer contained 50 mM Tris base, 380 mM glycine and 0.1% (w/v) SDS. The buffer was stored in a five-fold concentrated form.

(iv) ***Sample preparation for SDS-polyacrylamide gel electrophoresis:*** Samples were prepared by different methods depending on whether the gel was to be stained or to be used for immunoblotting. In some cases, samples were desalted by dialysis against two changes of 1000-volumes of 2 mM

Hepes-NaOH (pH 7.2) at 4°C for 2-8 hours before to sample preparation.

SDS-sample buffer: Samples were loaded onto SDS-polyacrylamide gels in 50 mM Tris-HCl (pH 6.8), 5% (w/v) SDS, 0.2 mM EDTA, 10% (v/v) glycerol and 0.001% (w/v) bromophenol blue in the presence of 10 mM DTT. The SDS-sample buffer was stored in a four-fold ([x4]) concentrated form without DTT.

For stained gels: Membranes derived from subcellular organelles were diluted up to 3 ml with distilled water and pelleted by centrifugation at 412,000 g_{av} (Beckman Instruments, TL-100.3 rotor) for 15 minutes at 2°C. The supernatants were discarded and the pellets were taken up in [x1] SDS-sample buffer. Samples requiring delipidation were treated with approximately 100-volumes of acetone:ethanol (1:1) at -20°C for 60 minutes followed by centrifugation at 15,000 g_{av} (Beckman Instruments, JA-20 rotor, in Corex glass centrifugation tubes with rubber adapters) for 20 minutes at 4°C. The supernatants were discarded and the pellets were taken up in [x1] SDS-sample buffer.

For immunoblotted gels: Subcellular organelle membranes were generally resuspended in 1 mM Hepes-NaOH (pH 7.2) to protein concentrations above 4 mg/ml and directly dissolved in the [x1] SDS-sample buffer to give the required protein concentration. In some cases, the samples were lyophilised. The lyophilised samples were dissolved in one-fourth final volume of 7 M urea, followed by one half-final volume of [x2] SDS-sample buffer (with 20 mM DTT) and finally one-fourth final volume of distilled water. This method particularly suited preparations of the more hydrophobic proteins such as bovine chromaffin granule p65.

(v) ***Running conditions :*** The larger dimension gels were run at 60-80 V constant voltage for up to 16 hours; the mini-gels were run at 20 mA

constant current for up to 2.5 hours.

(vi) ***Fixing and staining gels :*** Proteins were fixed in the separating gel with 10% (v/v) acetic acid and 20% (v/v) methanol for 30 minutes and stained with 0.25% (w/v) Coomassie Brilliant Blue R250 in 50% (v/v) methanol, 7.5% (v/v) acetic acid for 5 minutes on a shaking platform. Gels were destained in 10% (v/v) methanol, 7% (v/v) acetic acid for 2-10 hours; polyurethane foam pieces were placed in the destaining solution to aid the destaining process.

Gels requiring sensitive detection were silver-stained using the method of Wray *et al.* (1981) after fixation and three one-hour washes in 50% (v/v) methanol with 5 minute intermediate washes in distilled water.

All fixation, washing and staining steps were carried out at room temperature on an orbital mixer.

[b] ***Two-dimensional electrophoresis*** *:*

Two-dimensional electrophoresis was carried out essentially by the method of O'Farrell (1975).

(i) ***Isoelectric focusing :*** The isoelectric focusing gel mixture was composed of 4.5% (w/v) acrylamide, 0.06% (w/v) bis-acrylamide, 9.5 M urea, 2% Nonidet P-40 and 1.8% (v/v) ampholytes pH 3-10. The mixture was poured into glass tubes (130 x 2 mm internal diameter) to a height of 110 mm and was polymerised with 0.04% (w/v) ammonium persulphate.

Samples of soluble proteins were generally desalted by dialysis against two changes of 1000-volumes of 2 mM Hepes-NaOH (pH 7.2) at 4°C for 2-8 hours. Membrane protein preparations were generally resuspended in 2 mM Hepes-NaOH (pH 7.2) after ultracentrifugation. Both types of sample were lyophilised and solubilised in the gel mix without acrylamide/

bis-acrylamide but containing 0.5% (v/v) mercaptoethanol and 0.001% (w/v) bromophenol blue.

The samples were loaded from the cathodic (basic) end of the gel. The cathode buffer was 1% (v/v) ethanolamine and the anode buffer was 0.5% (v/v) orthophosphoric acid. The gels were focused initially by increasing the voltage across the tubes while maintaining a current of 0.2 mA per tube, then run at 400 V for 16 hours. Finally the proteins were focused at 800 V for 1 hour before the gels were removed from the tubes. The isoelectric focusing gels were incubated in soaking buffer (50 mM Tris-HCl (pH 6.5), 3% (w/v) SDS, 10% (v/v) glycerol and 0.001% (w/v) bromophenol blue) for 20 minutes before loading onto the second dimension.

(ii) ***Second dimension :*** The focusing gels were attached on top of a SDS-polyacrylamide gel (135x125x1.5 mm) using 1% (w/v) agarose dissolved in the soaking buffer and the gel was run as described earlier. Gels requiring staining in Coomassie blue were fixed for 3 hours with three changes of the fix solution followed by an overnight incubation in two-dimensional gel fixer (25% (v/v) iso-propanol, 10% (v/v) acetic acid) to remove ampholytes from the gel.

2.5 Binding of Proteins onto Nitrocellulose Membranes

[a] *Electrophoretic transfer of protein* :

The SDS-polyacrylamide gel containing separated proteins and a sheet of nitrocellulose (0.45 μm) of similar size were sandwiched in tight contact and placed in buffer containing 20 mM Na_2HPO_4, 0.02% (w/v) SDS and 20% (v/v) methanol with the nitrocellulose oriented towards the anode. Proteins were transferred with a current of 0.8 A over 2 hours.

[b] *Dot-blots* :

Non-denatured proteins were applied onto a sheet of nitrocellulose previously incubated in distilled water for 5 minutes, using a BioRad Bio-Dot® microfiltration apparatus by passive filtration to allow for quantitative binding of proteins. For screening of monoclonal antibodies, up to 30 μg of protein were loaded onto each dot at a protein concentration of 0.2 mg/ml in TBS. Samples of intracellular organelle membranes at a protein concentration of 0.2 mg/ml were solubilised with 0.05% (v/v) Triton X-100 before application onto the nitrocellulose. For samples of unknown protein concentrations such as column fractions, up to 250 μl could be applied per dot. After passive filtration excess solvent was removed by briefly application of vacuum.

2.6 Detection of Proteins Bound to Nitrocellulose with Antibodies and Lectins

After protein transfer, nitrocellulose sheets were washed briefly with three changes of distilled water and stained for 5 minutes with Ponceau S (0.4% (w/v) in 2% (w/v) TCA) to assess the efficiency of transfer. The nitrocellulose was destained initially with distilled water, then with TBS for 10 minutes. Non-specific sites on the nitrocellulose were blocked by incubation in 0.5% (v/v) Tween-20 in TBS for 60 minutes on an orbital mixer.

After binding of the samples, dot-blots were washed briefly with three changes of TBS and the non-specific sites were blocked as above.

[a] ***Immunoblots*** *:*

For detection of proteins with monoclonal antibodies, the sheet of nitrocellulose containing the bound samples was incubated with culture supernatant at 1:2 to 1:20 dilutions in 1 mg/ml (w/v) BSA in TBS with 0.1% (w/v) sodium azide for up to 16 hours on a rocking platform at room temperature. Blots were incubated with polyclonal antibodies at 1:100 to 1:1000 dilutions in 1 mg/ml BSA in TBS with 0.1% sodium azide for 2 hours as above.

Non-specifically bound monoclonal antibodies were washed from the nitrocellulose with two 5-minute washes in TBS, two in 0.1% (v/v) Tween-20 in TBS and one in TBS over 25 minutes. The blots were then incubated in 1:500 to 1:1000 dilution of biotinylated secondary antibody in TBS containing 1mg/ml BSA for 2 hours followed by the above wash protocol. The secondary antibody used was compatible with the class of primary immunoglobulin used and the species in which it was raised. Finally the

blots were incubated in ^{125}I-labelled streptavidin (10 Bq/µl) for 1 hour. The blots were washed as above and dried in a 50°C oven. The antigen-antibody complexes were visualised following autoradiography at -70°C between intensifying screens. In some cases, the blots were visualised using "enhanced chemiluminescence" (ECL). For this procedure the blots were incubated with horseradish peroxidase-linked second antibodies (diluted 1 in 2000 - 10000 in azide-free TBS containing 1mg/ml BSA) followed by the same wash protocol as above. Finally the blots were washed with 10 ml of 1:1 mixture of the two ECL reagents and the chemiluminescence was detected using autoradiography film.

[b] *Lectin blots* :

Lectin overlays of proteins were carried out essentially as described for immunoblots. The blots were incubated with either ^{125}I-labelled lectin (at 10 Bq/µl) or unlabelled lectin (10 µg/ml) for 16 hours. For the procedure using concanavalin A, see Clegg (1982). The wash protocols for the lectin blots were the same as immunoblots and the complexes were visualised following autoradiography at -70°C between intensifying screens.

2.7 Screening Procedures for Monoclonal Antibodies

[a] *Enzyme-linked immunosorbent assay* :

ELISA plates (Falcon) were pre-coated with 100 µl of poly L-lysine (17 kDa) at 20 µg/ml in 10 mM Mes-Tris buffer (pH 6.5) for 1 hour at room temperature. The poly L-lysine was replaced with the antigen, diluted in 10mM Mes-Tris buffer, pH 6.5 (see Chapter 3, Table 4.2 for concentrations). The wells were coated with the antigen for 90 minutes at room temperature. To block the non-specific sites of the wells and to remove excess antigen, the wells were incubated with veronal buffer (50 mM sodium barbitone, 85 mM NaCl, 5 mg/ml BSA and 0.1 mg/ml sodium azide, pH 8.0) for 60 minutes and washed with the same buffer twice.

Culture supernatants (with 0.1% (w/v) azide) were placed in the wells and incubated for at least 3 hours at room temperature. Unbound immunoglobulins were washed away with 3 changes of PBS over 3 minutes. To detect bound immunoglobulins, the wells were incubated with urease-linked anti-mouse IgG and IgM antibodies at a dilution of 1:750 in 1 mg/ml BSA in PBS for 2 hours. The wells were washed once with PBS and twice with distilled water and incubated with the urease substrate (0.1% (w/v) urea, 0.008% (w/v) bromocresol purple, 0.2 mM EDTA adjusted to pH 4.8) pre-equilibrated to 37°C for up to 30 minutes while constantly agitating the plate. The bound urease activity was assayed spectrophotometrically at 570nm using a Dynatech ELISA plate reader.

[b] *Dot-blotting assay* :

Nitrocellulose sheets were set up in 96-well format as described in Section 2.5[b] and after dotting the antigen, the non-specific sites were blocked

with 0.5% (v/v) Tween-20 in TBS for 1 hour. The nitrocellulose membrane was placed back into the dot-blotting apparatus with the position of the dots corresponding to the wells. The apparatus was sealed to the atmosphere and the wells were incubated with the culture supernatants for up to 5 hours and processed as described for immunoblotting (Section 2.6[a]).

[b] ***Western blotting*** *:*

Samples for analysis were separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes as described in Sections 2.4[a] and 2.5[a] except that the nitrocellulose membranes were cut into 7.5 mm strips before transfer and placed in contact with the gel as usual. The strips with the transferred proteins were placed in a BioRad incubation tray, blocked as above and incubated with a minimum of 2 ml of the culture supernatants. Culture supernatants of smaller volume were incubated with the nitrocellulose sheet in a Biometra multiblotting apparatus.

2.8 Generation and Maintainance of Hybridoma Cell Lines

[a] *Immunisation protocol* :

Mice (6 to 10 weeks old) were injected subcutaneously with 100μg of the antigen at a protein concentration of 1 mg/ml in Freund's complete adjuvant. Three weeks after the first immunisation, a similar dose of the antigen was injected intraperitoneally and this was repeated up to twice more at three-weekly intervals. Three days before the fusion, one of the mice was immunised once more with 50 μg of the antigen intravenously.

[b] *Media and conditions for culturing myelomas and hybridomas* :

Myeloma and hybridoma cell lines were cultured in RPMI-1640 media with 25 mM Hepes-NaOH (pH 7.2) and supplemented with 0.1 mM hypoxanthine, 16 μM thymidine, 50 units/ml penicillin, 50 μg/ml streptomycin, 10 mM $NaHCO_3$, 0.1 mM β-mercaptoethanol and 15% (v/v) myoclone foetal calf serum (RPMI/HT/FCS). This media was further supplemented with 0.5 μM aminopterin for the culture and selection of hybridomas for at least 1 week after the fusion.

Both myeloma and hybridoma cell lines were cultured at 37°C in a sterile incubator containing 5 % (v/v) CO_2, 100% humidity.

[c] *Fusion and cell culture protocols* :

The spleen of an immunised mouse was removed under sterile conditions and disrupted into a cell suspension in serum-free RPMI/HT medium (see Section 2.8[b]) equilibrated to 37°C. Cells were washed twice by

centrifugation at 500 g_{av} for 5 minutes at room temperature and finally resuspended in 15 ml of the same medium. Myeloma cells (NS-0) were cultured in RPMI/HT media containing 15% foetal calf serum and passaged into two 75 cm^2 flasks approximately 40 hours before the fusion. NS-0 cells were harvested in log growth and washed as above and resuspended in 5 ml serum-free RPMI/HT medium equilibrated to 37°C. The lymphocyte and NS-0 cell concentrations were determined using a haemocytometer. The NS-0 culture supernatant was retained and mixed with a suspension of thymus (feeder T-cells) cells obtained from a 6 to 8 week old non-immune mouse.

The NS-0 and the spleen cells were mixed in a ratio of 1:5 and the mixture centrifuged at 500 g_{av} for 5 minutes at room temperature. The supernatant was removed and the cells were resuspended in 1 ml sterile 46 % polyethylene glycol (Hepes buffered PEG-1500, equilibrated to 37°C) over 30 seconds whilst gently agitating. The PEG was diluted using serum-free RPMI/HT medium 90 seconds after the start of the fusion, initially at a rate of 0.5 ml over 1 minute, followed by 2 ml over 2 minutes and finally 10 ml over 2 minutes. The fusion mixture was centrifuged at 500 g_{av} for 5 minutes at room temperature and the cells were resuspended in 60 ml of the T-cells in RPMI/HT media containing 15% foetal calf serum and 0.5 μM aminopterin at 37°C.

The fusion mixture was dispensed into six 96-well culture plates. The cells were fed with RPMI/HT media containing 15% foetal calf serum and 0.5 μM aminopterin at 3 days after the fusion and cultured for a further 4-6 days before the hybridoma cell density was sufficient to remove the culture supernatants for immunoassays.

The cells in the wells showing positive immunoreactivity were

transferred into 24-well plates at about 50 to 80% confluency and the culture supernatants were assayed again. The immunoreactive cells were then transferred into 25 cm^2 flasks. The cells were harvested for freeze-down in log growth and the remainder of the cells were allowed to grow to confluency. The supernatants of the confluent cells were harvested and stored at 4°C in the presence of 0.1% (w/v) sodium azide.

[d] ***Cloning of hybridoma cell lines*** **:**

Hybridoma cell lines producing antibodies of the required specificity were cloned by limiting dilution. To aid cell growth in the wells with low number of cells, 100 µl of mouse thymus (feeder) cells in RPMI/HT media containing 15% foetal calf serum and 1µM aminopterin were placed into the wells of sterile 96-well tissue culture plates; usually three plates were prepared for each cell line. Into column "1" wells of each cloning plate, 100 µl of hybridoma cells, at a density of 10000/ml were dispensed, mixed and 100 µl of the mixture was transferred into the wells in column "2". Such 1 in 2 dilutions were made across the plate.

After three days in culture, 50 µl of fresh RPMI/HT media containing 15% foetal calf serum was placed into each well without disturbing the cell colonies. The number of colonies in each well was determined after a further two days in culture days; wells with up to 3 colonies were chosen to be screened for antibody production. Cells originating from a single colony and producing the desired antibodies were expanded to flasks and were harvested at log growth to be frozen down for long-term storage in liquid nitrogen. The remainder of these cells were taken through another round of cloning using the above procedure.

[e] *Long-term storage of hybridoma and myeloma cell lines :*

(i) Freezing down cells : Exponentially-growing cells were shaken off from culture flasks and centrifuged at 500 g_{av} for 5 minutes at room temperature. The pellet of cells was resuspended in 1ml ice cold foetal calf serum with 10% (v/v) DMSO and transferred into 2 ml sterile screw-top vials. The vials were kept on ice until they were wrapped up in a polystyrene box and placed at -70°C. Within 1 week, the vials were transferred into liquid nitrogen where they were kept until required.

(ii) Thawing-out cells : The vials were removed from liquid nitrogen, rapidly thawed out at 37°C and transferred into a sterile tube containing 10 ml RPMI/HT media with 15% foetal calf serum. The cells were washed by centrifugation 500 g_{av} for 5 minutes at room temperature and resuspension in the same medium. The resuspended cells were placed in a 25cm^2 flask containing a lawn of mouse thymus cells and cultured as usual.

[e] *Storage of hybridoma and myeloma supernatants :*

Hybridoma cell line supernatants were kept with the addition of 0.1% (w/v) sodium azide at either 4°C for immediate use or frozen at -20°C.

2.9 Other methods

[a] *Protein concentration determination* :

Protein concentration estimations were performed according to the method of Bradford (1976) using BSA as the standard.

[b] *Dialysis tubing* :

Dialysis tubing was boiled in distilled water/EDTA for 1 hour prior to use. The tubing was washed with distilled water and stored in 50% (v/v) ethanol at 4°C until required.

[c] *^{125}I-labelling proteins* :

Streptavidin was labelled with ^{125}I using the iodogen method of Franker & Speck (1978) to a specific activity of 25 MBq/mg.

CHAPTER 3

CHAPTER 3

The generation of polypeptides from chromaffin granule membranes for the production of monoclonal antibodies

3.1 Introduction

One of the approaches used to elucidate the mechanism of exocytosis has been to search for integral components of secretory vesicle membranes that are involved in the secretory process. However, progress has been slow because of our limited knowledge of the composition of secretory vesicle membranes as well as not knowing what kinds of molecules to look for.

One way of tackling such a problem has been to raise monoclonal antibodies against the components of secretory vesicle membranes. Such an approach, which overcomes the need to purify each component, has been successfully applied to the study of synaptic vesicle membrane proteins (see Section 1.8(b)). Probably as a consequence of their more complicated composition, there have not been reports of such attempts with chromaffin granules. It was the aim of this study to apply to the well-studied chromaffin cell the advantages of monoclonal antibody production (Sections 1.2 & 1.8(b)) to produce antibodies that recognise components of the chromaffin granule membrane so that these antibodies might be used to investigate the functions of the membrane proteins.

Despite the advantages offered by monoclonal antibodies in the study of individual components of a complex mixture, the success of obtaining the *desired* antibodies against chromaffin granule membranes depends on two key points:

(1) Maximisation of the chances of the immune system of the immunised animal recognising and responding to the proteins of interest;

(2) Establishing sensitive and efficient methods of screening for the antibody-producing hybridomas.

A particular difficulty in producing monoclonal antibodies against the components of the chromaffin granule membrane is that about half of the total protein content of the these membranes is accounted for by just two proteins; dopamine β-monooxygenase and cytochrome b_{561}, both of which are involved in the well-studied pathways of catecholamine biosynthesis [Winkler & Westhead, 1980; Terland & Flatmark, 1980]. Furthermore, preparations of these membranes also include a substantial amount of "contaminants" both from within the granule and from the cytoplasm (see Chapter 1, Section 1.5 and Table 1.5 for a list of these proteins). The intragranular component chromogranin A, together with the major membrane proteins dopamine β-monooxygenase and cytochrome b_{561}, are highly immunogenic. Consequently even when mice are immunised with whole chromaffin cells, antibodies are raised against these proteins [Pruss & Shepard, 1987].

The chances of raising monoclonal antibodies against the minor components of the chromaffin granule membrane which might be involved in vesicle organisation and trafficking would be maximised by techniques that allow their partial isolation and enrichment. One such technique, used in this study, has been the fractionation of membrane proteins by phase separation in the detergent Triton X-114 [Bordier, 1981; Pryde, 1986]. Another method has been the isolation of tryptic peptides from chromaffin granule membranes previously washed with EDTA and sodium carbonate (pH 11). The specific membrane-binding properties of some of the cytosolic proteins

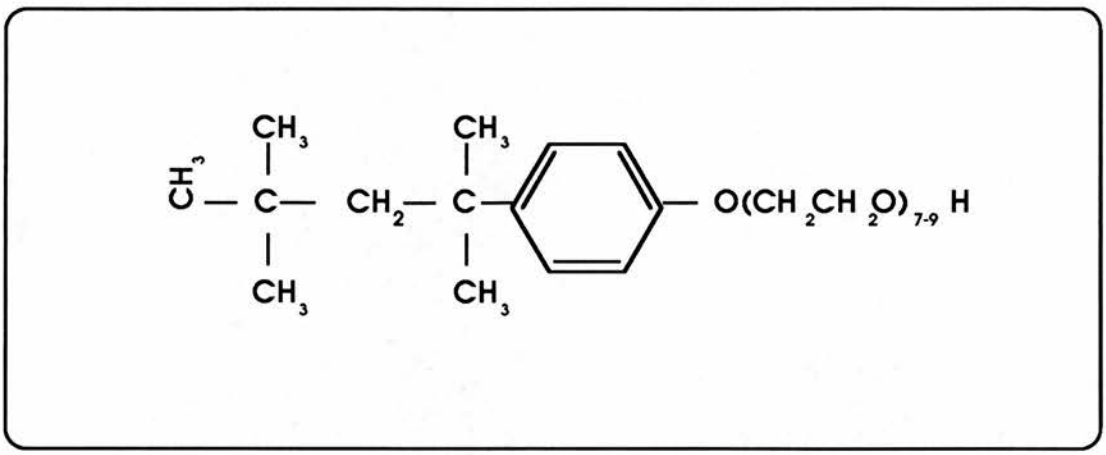
that "contaminate" chromaffin granule preparations have also been exploited in the preparation of proteins for raising monoclonal antibodies [Geisow & Burgoyne, 1982; Creutz et *al.*, 1983].

The fractionation of chromaffin granule membrane proteins with the non-ionic detergent Triton X-114 has been an important technique in providing material for both the production and characterisation of monoclonal antibodies. Detergents of the Triton series are amphipathic molecules composed of an aromatic, hydrophobic head and a repetitive hydrophilic polyoxyethylene tail which determines the physical properties of the molecule in solution (Figure 3.1). For example Triton X-100, which has an average repeat of nine polyoxyethylene groups, forms micelles composed of about 140 molecules at 0°C when at or above its critical micellar concentration (cmc). Increasing the temperature of such a solution has no visible effect until the characteristic "cloud-point" is reached when the micelles aggregate and separate from the solution in a separate phase. For Triton X-100, this occurs at 64°C, but the more hydrophobic detergent Triton X-114, which has on average around seven polyoxyethylene groups, has its cloud point at the more useful temperature of 20°C [Bordier, 1981; Pryde, 1986].

In 1981 Bordier, using Triton X-114, showed that a mixture of proteins could be resolved into distinct groups depending on their hydrophobicities and he applied this principle to extract the membrane proteins from human erythrocytes. Biological membranes are composed of phospholipid bilayers containing integral membrane proteins anchored by hydrophobic domains and when they are treated with Triton X-114 at 0°C, micelles containing detergent, phospholipids and membrane proteins are formed. At the cloud-point these mixed micelles aggregate and precipitate, forming a detergent-rich

Figure 3.1

Polyoxyethylene p-t octyl phenol: The Triton X-114 molecule.



The average number of ethylene oxide units for Triton X-114 is between 7 and 9.

phase enriched in intrinsic membrane proteins, distinct from an aqueous phase containing extrinsic and soluble proteins.

The ease with which Triton X-114 resolves membrane proteins while retaining their biological function has led to its application to other biological membrane preparations, including chromaffin granule membranes [Clementson *et al.*, 1984; Maher & Singer, 1985; Pryde & Phillips, 1986]. It soon became apparent that, because of their extensive hydrophilic domains, some membrane-bound glycoproteins partitioned anomalously, in that they remained in solution after phase separation at 30°C [Pryde, 1986]. However, if the Triton X-114 concentration of the aqueous phase (~700 μM), which is about three times higher than the cmc, is reduced below the cmc by exhaustive dialysis using Amberlite XAD-2 beads, the membrane glycoproteins form insoluble aggregates and precipitate, leaving the truly soluble proteins in solution. In the present study, this behaviour has been exploited in producing a fraction enriched in membrane glycoproteins that could be used to immunise mice for the production of monoclonal antibodies.

3.2 Results & Discussion

[a] *Generation of a membrane glycoprotein-rich fraction* :

Figure 3.2(a) is a two-dimensional gel showing the protein composition of chromaffin granule membranes; about half of these proteins are glycoproteins which stain poorly with Coomassie blue and are better visualised by lectin blotting (Figures 3.2(b) and (c); see also Pryde & Phillips, 1986). Among the chromaffin granule membrane glycoproteins which bind the lectins concanavalin A and wheat germ agglutinin are the biosynthetic enzyme dopamine β-monooxygenase, glycoproteins J, H and K, as well as glycoproteins II, III, IV and V. However, it is not possible to visualise some other chromaffin granule membrane glycoproteins such as p65 with either Coomassie blue-staining or with the above lectins.

Upon treatment with the non-ionic detergent Triton X-114 according to the scheme shown in Figure 3.3(a), the proteins of the chromaffin granule membrane are fractionated into distinct groups (Figure 3.3(b)). The first of these fractions is the "*phospholipid-rich*" pellet which contains the subunits of the H^+-translocating ATPase complex of the chromaffin granule as well as other hydrophobic proteins (Figure 3.3(b), track 3) [Percy *et al.*, 1985; Pérez-Castiñiera & Apps, 1990]. The bulk of the Triton X-114 remains in the supernatant but when the solution is warmed up to 30°C, the detergent separates out into a distinct "*detergent-rich*" phase which contains several intrinsic proteins such as cytochrome b_{561}, ATPase II and the membrane-associated form of the enzyme dopamine β-monooxygenase (Figure 3.3(b), track 4) [Percy *et al.*, 1985; Pryde & Phillips, 1986]. Most intrinsic glycoproteins of the chromaffin granule membrane remain in the aqueous phase (Figure 3.3(b), track 5 and Figure 3.4(a)), but can be separated from soluble secretory

Figure 3.2

Composition of adrenal chromaffin granule membranes.

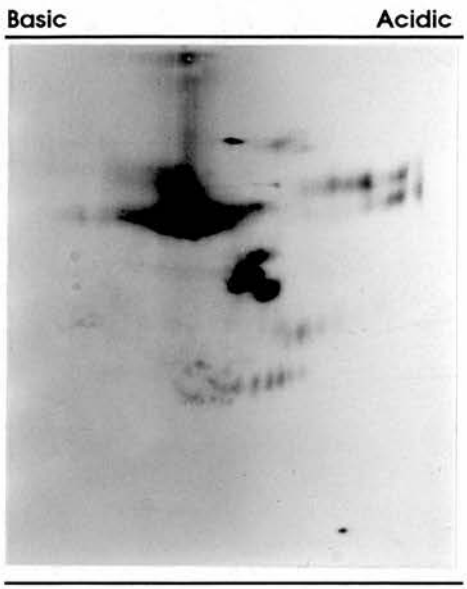
(a) Coomassie-blue stained two-dimensional gel of chromaffin granule membrane proteins:

300 µg of chromaffin granule membranes were separated by two-dimensional electrophoresis, gels were either Coomassie-blue stained (**(a)**), or the proteins were transferred onto nitrocellulose and were visualised by lectin blotting (**(b)** & **(c)**).

Figure 3.2

Composition of adrenal chromaffin granule membranes.

(b) Two-dimensional blot showing concanavalin A-binding proteins of the chromaffin granule membrane:

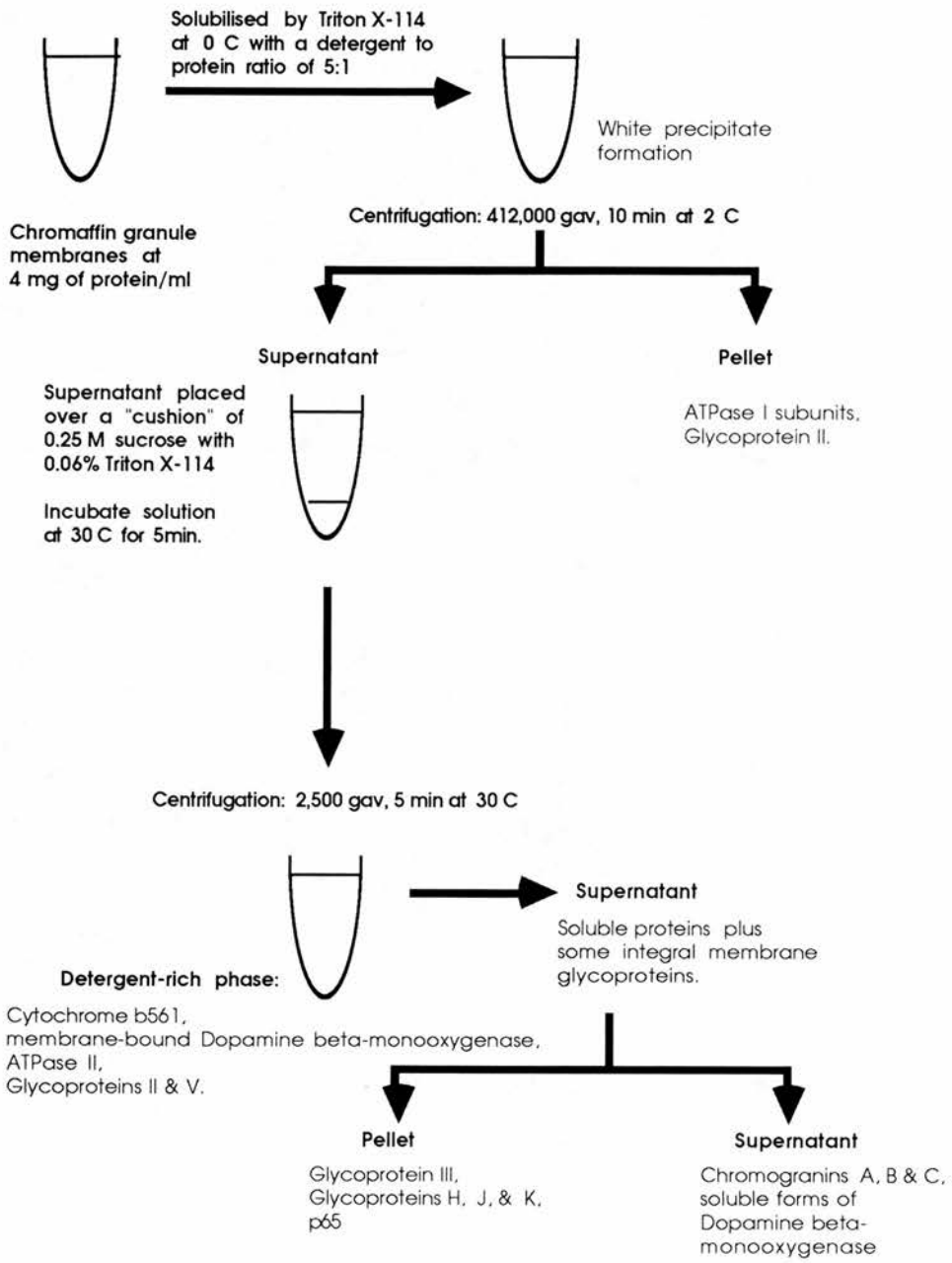


(c) Two-dimensional blot showing wheat germ agglutinin-binding proteins of the chromaffin granule membrane:

Figure 3.3

Triton X-114 fractionation of adrenal chromaffin granule membrane proteins.

(a) Scheme for the fractionation of chromaffin granule membranes using the non-ionic detergent Triton X-114:[a]

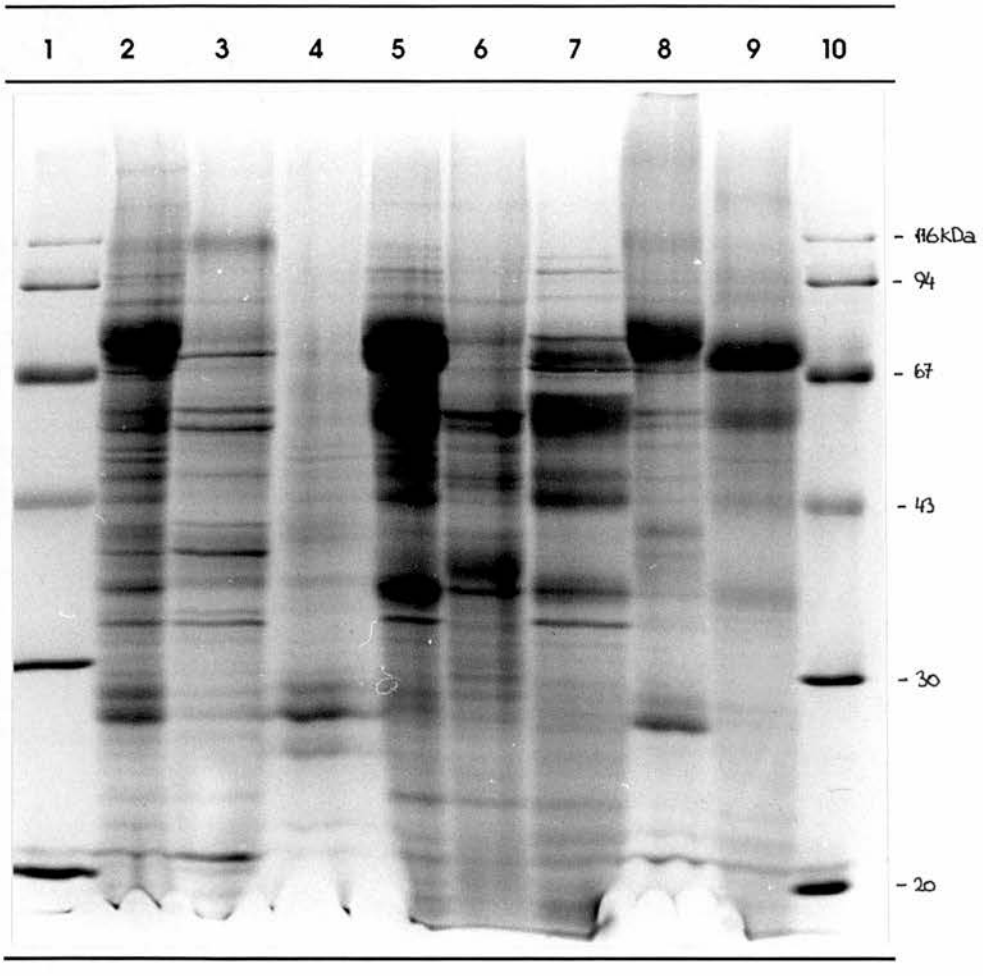


[a] Adapted from Pryde (1986).

Figure 3.3

Triton X-114 fractionation of adrenal chromaffin granule membrane proteins.

(b) Protein composition of Triton X-114 fractions derived from chromaffin granule membranes:



Track 1: Molecular weight markers; **Track 2**: chromaffin granule membranes; **Track 3**: phospholipid-rich pellet; **Track 4**: detergent-rich pellet; **Track 5**: aqueous supernatant; **Track 6**: glycoprotein-rich pellet; **Track 7**: soluble and extrinsic proteins of chromaffin granules; **Track 8**: Na_2CO_3-washed chromaffin granule membranes; **Track 9**: proteins released from Na_2CO_3-washed chromaffin granule membranes; **Track 10:** Molecular weight markers. 90 µg protein was loaded per lane of a large format gel.

proteins such as chromogranins and most of the soluble dopamine β-monooxygenase (Figure 3.3(b), track 7) by the removal of the residual detergent by exhaustive dialysis (Figure 3.3(b), track 6). Figure 3.4(b) is a two-dimensional gel showing the composition of this "glycoprotein-rich" fraction which was used to immunise mice.

[b] *Generation of soluble peptides from integral membrane proteins* :

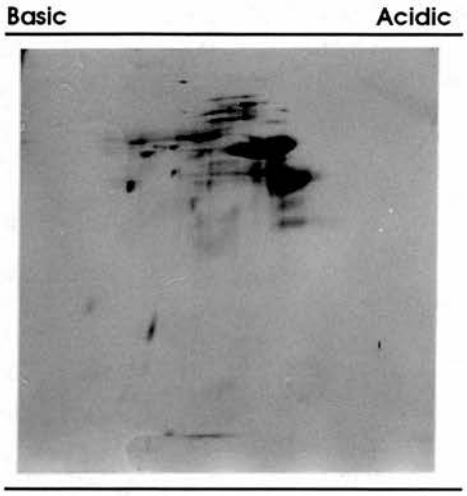
The second approach to the production of suitable antigens has been to generate tryptic peptides from integral membrane proteins, an approach developed after experiences with the intrinsic membrane protein p65 (see Chapter 5).

To ensure that antibodies were generated against only the integral components of chromaffin granules membranes, the membranes were washed free of peripheral and membrane-associated proteins with 100 mM Na_2CO_3 /10 mM EDTA at pH 11 as described in Chapter 2 [Howell & Palade, 1982; Higgins, 1984]. This procedure breaks open vesicular structures and removes adherent proteins from them. Figure 3.3 (b) (tracks 8 & 9) and Figures 3.5(a) & (b) show the composition of the membranes after this treatment and the polypeptides removed from them. The majority of the proteins removed from chromaffin granule membranes by this procedure are the lumenal chromogranins, enkephalin precursors and their proteolytic products (see Chapter 1, Section 1.3[a]). The composition of this fraction is therefore very similar to the proteins released from intact granules by hyperosmotic lysis [Apps *et al.*, 1985]. This procedure also removes cytosolic proteins which adhere to chromaffin granule membrane preparations but, which because of the abundance of the lumenal proteins, are not easily

Figure 3.4

Protein composition of fractions obtained by Triton X-114 phase-separation of adrenal chromaffin granule membranes.

(a) Composition of the "aqueous-supernatant" derived from Triton X-114 fractionated chromaffin granule membranes by Coomassie-blue staining (300 µg):



(b) Composition of the "glycoprotein-rich" pellet derived from Triton X-114 fractionated chromaffin granule membranes by Coomassie-blue staining (300 µg):

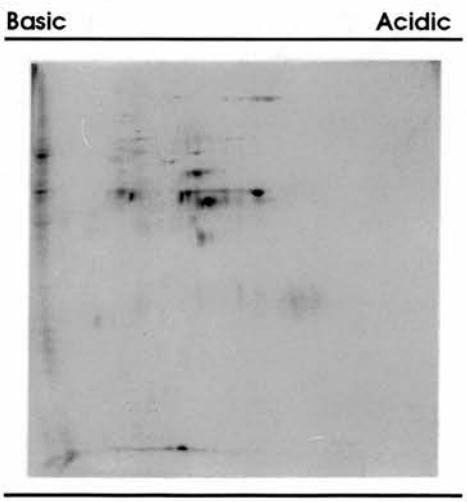
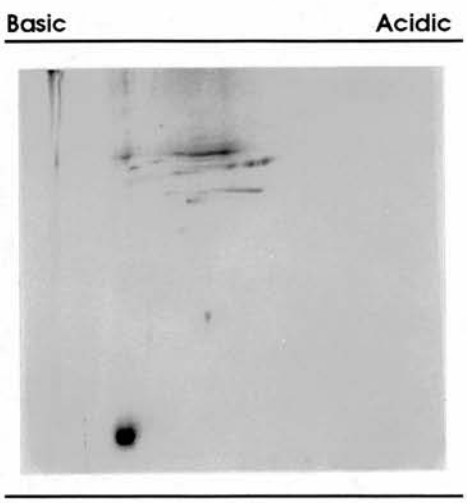


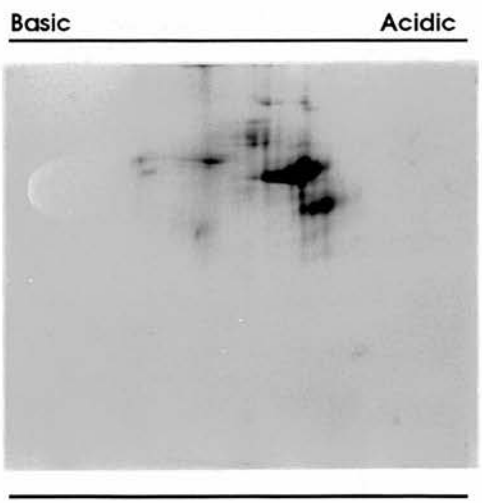
Figure 3.5

Intrinsic and extrinsic proteins of adrenal chromaffin granule membranes.

(a) Composition of Na_2CO_3 (pH 11)-washed chromaffin granule membranes showing the intrinsic membrane components (300 µg):



(b) Composition of the proteins removed from chromaffin granule membranes by Na_2CO_3 (pH 11)-washing (300 µg):



identified by Coomassie blue-staining.

The washed membranes obtained by the above procedure were then treated separately with three trypsin concentrations (0.1, 1.0 and 20.0 μg trypsin per mg of membranes) to obtain a range of soluble peptides which were separated from residual membranes and used to immunise mice. The generation of tryptic peptides as an immunogen minimised the chances of obtaining antibodies against the protease-resistant biosynthetic enzyme dopamine β-monooxygenase (Figure 5.4(f)). The composition of this mixture can be seen in Figure 3.7 (tracks 3, 4, 5); it is not possible to identify any of these peptides without specific antibodies.

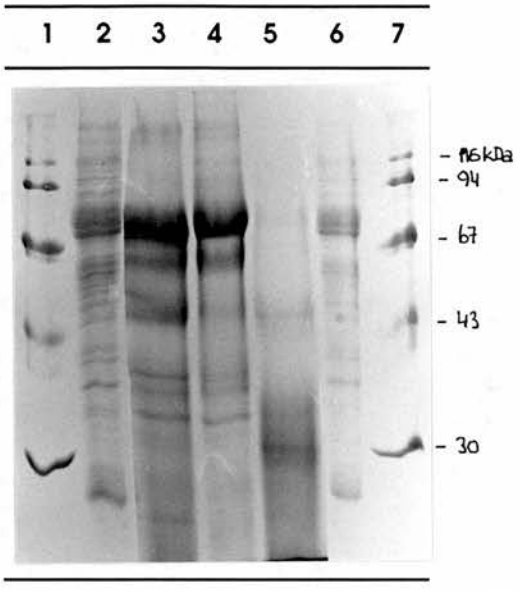
[c] *Isolation of chromaffin granule membrane-binding cytosolic proteins* :

As reviewed in Chapter 1, Section 1.5, many of the peripheral proteins of chromaffin granules membranes are of cytosolic origin but reversibly associate with granules under defined conditions. Among these are the *annexins* or *chromobindins* which bind to chromaffin granule membranes in the presence of elevated Ca^{2+} levels and are thus implicated in the mechanism of exocytosis [Geisow & Burgoyne, 1982; Creutz *et al.*, 1983; Drust *et al.*, 1988]. A third approach to this study has been to use chromaffin granule membranes as an affinity matrix, in order to isolate such families of proteins for the production of monoclonal antibodies.

A fraction enriched in Ca^{2+}-dependent chromaffin granule membrane-binding proteins was prepared by incubating chromaffin granule membranes with a cytosolic fraction in the presence of 10 μM $CaCl_2$ and 1 mM MgATP as described in Chapter 2. Figure 3.7 shows the polypeptides obtained during a typical purification - although some 15 distinct polypeptides were eluted from

Figure 3.6

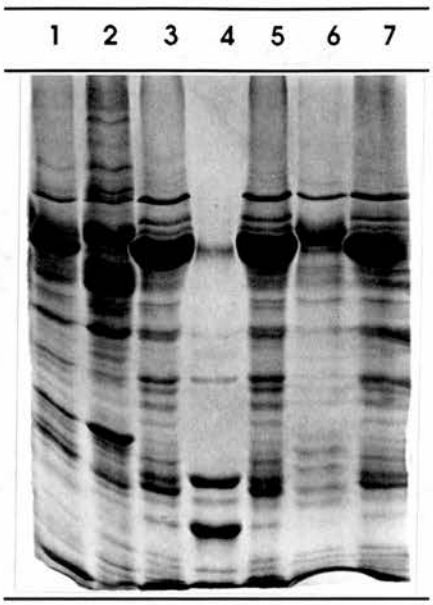
Preparation of tryptic peptides from integral membrane protein of adrenal chromaffin granules.



Track 1 & 7: molecular weight markers; **Tracks 2 & 6**: Na_2CO_3-washed chromaffin granule membranes; **Tracks 3, 4, 5**: tryptic peptides liberated from Na_2CO_3-washed chromaffin granule membranes with 0.1, 1.0 and 20.0 µg/ml trypsin respectively.

Figure 3.7

Preparation of Ca^{2+}-dependent adrenal chromaffin granule membrane-binding proteins.



Track 1: Adrenal medullary cytosol; **Track 2**: cytosolic proteins precipitated with 55% ammonium sulphate; **Track 3**: cytosolic proteins remaining in solution in 55% ammonium sulphate; **Track 4**: cytosolic proteins remaining in solution in 90% ammonium sulphate; **Track 5**: cytosolic proteins precipitated between 55 - 90% ammonium sulphate; **Track 6**: Ca^{2+}-dependent chromaffin granule membrane-binding proteins; **Track 7**: proteins remaining in solution after one round of incubation with chromaffin granule membranes to remove Ca^{2+}-dependent chromaffin granule membrane-binding proteins.

chromaffin granule membranes by this procedure (Figure 3.6, Track 6), the yield was only around 20 μg protein from 1 mg of membranes. The low yield appeared to be a result of the low binding-capacity of the membranes since no difference in the polypeptide composition of the cytosolic fraction was observed by Coomassie blue-staining of gels after the incubation. Increasing the concentration of chromaffin granule membranes to increase the yield appeared to increase non-specific binding of soluble proteins. Therefore to provide enough material for immunisation and screening, repetitive rounds of incubations of a large amount of cytosol were performed. The Ca^{2+}-dependent chromaffin granule membrane binding proteins obtained in this manner were concentrated by lyophilisation followed by resuspension in a small volume for immunisation of mice.

3.3 Conclusions

An important approach to the identification of the proteins involved in regulated secretion is to use antibodies directed against those proteins found in secretory vesicle membranes. Many of these proteins are present in low amounts and the generation of monoclonal antibodies allows the characterisation of such proteins that are otherwise inaccessible by standard biochemical methods.

Despite the advantages of monoclonal antibodies, the chances of obtaining them would be greatly enhanced if partially-purified preparations of chromaffin granule membranes could be used as immunogens. In this Chapter, I have described three different approaches that were used to prepare such immunogens in order to raise mouse monoclonal antibodies. These methods are:

(a) the fractionation of chromaffin granule membranes using phase separation in the non-ionic detergent Triton X-114 to prepare a fraction enriched in membrane glycoproteins.

(b) the preparation of soluble tryptic peptides from integral membrane proteins of chromaffin granule.

(c) the preparation of cytosolic proteins that bind specifically to chromaffin granule membranes in the presence of elevated Ca^{2+}-concentrations.

All of the above fractions were used to immunise mice and their spleens were used in the generation of hybridoma cell lines which were subsequently screened for the production of specific monoclonal antibodies.

CHAPTER 4

Chapter 4

Screening for hybridomas secreting monoclonal antibodies against chromaffin granule membrane-specific and associated antigens.

4.1 Introduction

The production of monoclonal antibodies against a mixture of antigens such as the chromaffin granule membrane is possible *only* if the lymphocyte clones that are amplified by the immune system in response to individual antigens are extracted for culturing and identified. The extraction of the lymphocytes, their fusion to myeloma cells and their continuous culture are standard procedures which have been perfected over the years, but the methods used for identifying the hybridomas of interest depend on the nature of the antigen and this is the most important step in obtaining monoclonal antibodies. An important criterion for a method designed to screen for monoclonal antibodies raised against a mixture of antigens is that it must be sensitive enough to detect antibodies against any component of the immunisation mixture. This means that the greater the number of components in the immunising mixture, the more sensitive the screening procedure has to be. This applies in particular to detecting monoclonal antibodies against those components of the chromaffin granule membrane which constitute only 0.5 - 5 % of the total membrane protein. This Chapter will outline methods developed to identify monoclonal antibodies against such antigens and shows how they have been applied to screen hybridomas obtained from three different fusions.

Among the many available screening methods, the easiest to perform

and the most reproducible are various antibody capture assays, in which excess antigen is attached to a solid support. Table 4.1 outlines the assays which have been used in this study. In all cases, the antibodies were allowed to bind to the immobilised antigen mixture and the immune complexes were then detected using either enzyme-linked or radioactively-labelled reporter molecules. This was usually done using secondary antibodies raised against the immunoglobulins of the species in which antibodies to the antigens were made. Other possible methods use the immunoglobulin (IgG)-binding ability of protein A or protein G. The secondary reagents used in most assays are conjugated to enzymes such as horseradish peroxidase, alkaline phosphatase or urease for which there are simple colorimetric assays. Colorimetric assays which give soluble reaction products, such as those used for ELISAs, may be quantified by spectrophotometry; insoluble products are used for blots and are less easily quantified. Secondary reagents may also be radiolabelled with ^{125}I which can be detected by autoradiography or by γ-counting. Biotinylated secondary antibodies can also be used, the highly specific interaction between biotin and labelled-streptavidin being used to detect immune complexes. Multiple biotinylation of the secondary antibody results in the amplification of the signal and increases the sensitivity of detection. The sensitivity can be further increased if ^{125}I-streptavidin is used. For this reason, this was the method used to detect antibodies against minor components of the chromaffin granule membrane by dot- and immunoblots. However the biotin-streptavidin system was not successful in ELISAs or with the more sensitive enhanced chemiluminescence detection (see later).

Another way of increasing the sensitivity of detection is to screen with preparations that are enriched in the minor constituents of chromaffin granule membranes, although this requires prior knowledge of the antigen

Table 4.1

Methods of screening for antibody production

Method	Solid support	Detection	Advantages	Disadvantages
ELISA	PVC plates	Enzyme-linked colour production.	Large number of samples may be screened. Requires as little as 100µl culture supernatant.	Low antigen bindingcapacity; Requires ELISA plate reader for quantitation of permanent records. Not possible to identify immunoreactive species. Conditions of assay must be optimised.
Dot blots	Nitrocellulose	Enzyme-linked colour production or enhanced chemiluminescence (ECL).	Large munber of samples may be screened. HIgh sensitivity if ECL used. High antigen-binding capacity. Requires as little as 50µl culture supernatant. Permanent record of results.	Commercially made dot-blotting apparatus required. Not possible to identify immunoreactive species.
Western Blotting	Nitrocellulose	Enzyme-linked colour production or enhanced chemiluminescence (ECL).	Possible to identify immuno-reactive species. Permanent record of results.	Requires at least 2ml culture supernatant. To test less amount of culture supernatant, "multiblotter" is required.
Immunohistology	(fixed cells)	Enzyme-linked colour production or fluorescence.	Possible to identify subcellular target of antibody. Requires as little as 50µl culture supernatant.	Quantitation not possible. Not sensitive enough for scarce antigens. Fluorescence prone to photobleaching.

itself. However in methods in which a large quantity of cruder antigen is bound, there will be sufficient minor components available to bind antibodies. This is possible with dot blots, using nitrocellulose to bind antigen. The protein-binding capacity of nitrocellulose is about 500 $\mu g/cm^2$ or around 50 μg per dot [Harlow & Lane, 1988]. On the other hand, polyvinylchloride, used for ELISA plates, binds approximately 1000-fold less but the method can be as sensitive as dot blots if the conditions of the assay are optimised. The optimisation of this assay is discussed in detail in the Result & Discussion section of this Chapter.

Other important factors to be considered in the choice of an immunoassay for the detection of monoclonal antibodies are its speed and capacity. Hybridoma supernatants are available for screening approximately 8 to 14 days after the fusion and the cells producing the required antibodies must be identified and expanded within the next 24 hours while still in late log growth. Failure to do so will result in the cells entering stationary phase, making them increasingly difficult to culture. Successful fusion or cloning plates can generate hundreds of cell colonies, so the primary screening procedure must have the capacity as well as the speed to cope with such numbers of samples. All the methods listed in Table 4.1 are capable of identifying positive supernatants within 8 to 10 hours but only ELISAs and dot blots also have the capacity to screen large number of samples. The latter are also applicable to 100 μl or less of culture supernatant which is the amount available from 96-well culture dishes. Despite its advantage in defining the specificity of antibodies, immunoblotting of SDS-polyacrylamide gels was not used as a primary screening procedure because it requires at least 2 ml of culture supernatant. It detects only those antibodies whose epitopes are resistant to denaturation by SDS, and therefore is not suitable when

native antigens are used for immunisation as they were in this study. This technique and its two-dimensional counterpart were used only as a final screen of hybridomas which were positive with ELISAs and dot-blots. Thus the different methods listed in Table 4.1 have been used in combination to prevent the possible limitations of one method from misidentifying supernatants.

The recent development of enhanced chemiluminescence (ECL) kits was an important breakthrough in the sensitivity of immunoassays. With this system, the enzymatic activity of conventional enzyme-linked second antibodies is converted to light emission which is detected using autoradiography film. This system requires about ten-fold less antigen for a given dilution of primary antibody, and also ten-fold less secondary antibody. Furthermore it does not require the overnight autoradiography which was used to detect ^{125}I-streptavidin-labelled immune complexes, and because it is a non-radioactive method it is safer.

Advances made in equipment have also made screening easier and more efficient, for example, the BioRad Bio-Dot® system allows not only uniform application of the antigen but also the incubation of 96 different hybridoma supernatants in an ELISA-plate format. The combination of the high antigen-binding capacity of dot blots, the advantages of the BioRad Bio-Dot® system and the high sensitivity of ECL were important in screening for monoclonal antibodies. Another advance has been the multiblotter from Immunetics (Biometra). This apparatus only requires 200 µl of each culture supernatant and can screen up to 25 samples by immunoblotting. The combination of this and the ECL system allows the characterisation of supernatants from as early as the 96-well stage.

The Results and Discussion section of this Chapter will present how the above screening procedures were adapted and used to identify monoclonal antibodies from three different fusions. The first of these fusions was for antibodies that were raised by immunising mice with a fraction enriched in proteins that bind onto chromaffin granules in the presence of elevated Ca^{2+} concentrations; this was initially screened using optimised ELISAs. A second fusion was for antibodies that were raised against the glycoprotein-rich fraction of chromaffin granule membranes and screened using a combination of ELISAs and dot blots against two different antigens. The final fusion which was to generate monoclonal antibodies against the integral proteins of the chromaffin granules was screened using the combination of dot-blots and ECL.

4.2 Results & Discussion

[a] *Development of ELISAs and Dot blots:*

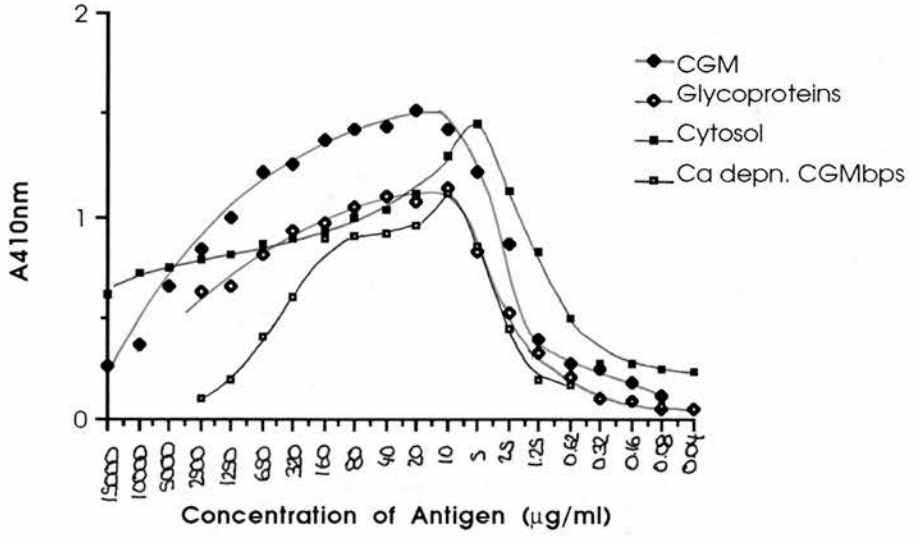
(i) *ELISAs*

The successful application of enzyme-linked immunosorbent assays (ELISAs) depends primarily on the concentration at which the antigen is immobilised and the choice of the detection system. However, the problem facing attempts to set up any immunoassay to detect antibodies against uncharacterised proteins is that it is not possible to know how it will perform without the antibodies, yet the assay is required to obtain them.

A useful way of optimising immunoassays without the use of antibodies is to non-specifically label the antigen with biotin and use the specific interaction between biotin and streptavidin to assess the conditions. To investigate the optimal conditions for ELISAs to screen monoclonal antibodies, antigens were therefore biotinylated and incubated at a range of concentrations in ELISA plates precoated with poly-L-lysine as described in Chapter 2. The plates were processed by blocking the non-specific binding sites for 1 hour followed by a 2 hour incubation in culture medium. At the end of this incubation, the wells were washed three times with PBS/0.1% Tween-20 and incubated for a further 1 hour with a 1:2000 dilution of streptavidin-horseradish peroxidase (-HRP) to mimic antibody binding. The plates were then washed as before and the bound antigen was measured by monitoring the enzymatic conversion of the substrate ABTS into a chromogenic product at 410nm. Figure 4.1 shows that the binding of streptavidin-HRP to immobilised antigen was bell-shaped, indicating that the detection signal was dependent on the concentration at which the antigen

Figure 4.1

Optimisation of ELISAs to screen for monoclonal antibodies raised against antigens derived from adrenal medulla: The effect of antigen-binding concentrations on signal strength.



CGM = chromaffin granule membranes;

Ca depn. CGMbps = Ca^{2+}-dependent chromaffin granule membrane-binding proteins.

Table 4.2

The optimal concentration of antigen for coating ELISA plates for the detection of monoclonal antibodies.

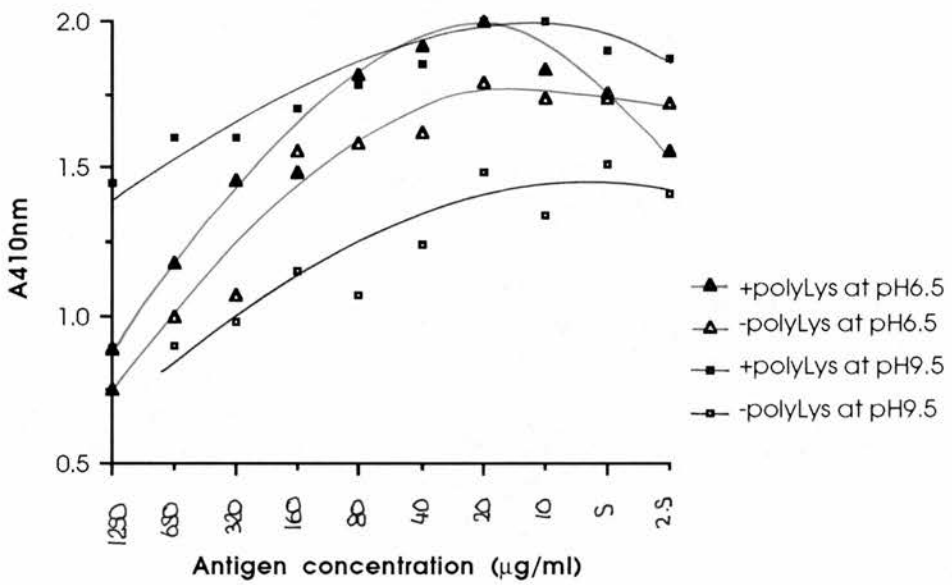
Antigen	Coating Concentration (µg/ml)
Chromaffin granule membranes	20
Glycoprotein-rich fraction	10
Cytosol	5
Ca^{2+}-dependent granule-binding proteins	10

was attached to the ELISA plates. A possible explanation of this behaviour is that if antigen is bound at concentrations above the capacity of the plastic matrix, then during incubations it will leach off the walls of the wells and bind to the streptavidin in solution. This will reduce the amount of streptavidin available to bind to immobilised antigen and therefore reduce the signal. Such would also be the case when hybridoma culture supernatants are incubated. The above procedure was carried out for each of the fraction that were to be used for the screening of monoclonal antibodies in this study and the optimal concentration of each antigen is shown in Table 4.2.

The antigen-binding conditions were adapted from Grimaldi *et al.* (1987) who coated ELISA plates with insulinoma granule membranes using 10 mM Mes-Tris at pH 6.5. Another method uses 100 mM carbonate/ bicarbonate at pH 9.5 [Harlow & Lane, 1988]. Poly-L-Lysine-precoating is also a recommended method of binding antigens to ELISA plates, and is known to increase the binding of some antigens. When binding of solubilised chromaffin granule membranes were investigated using these different conditions, it was found that poly-L-lysine precoating increased the detection signal of ELISAs by about 10% at pH 6.5 and around 40% at pH 9.5. There was no significant difference between the detection signal strengths when antigen-binding to poly-L-lysine precoated plates were compared at pH 6.5 and 9.5, although it appeared that at pH 9.5 maximal signal could be obtained with antigen concentrations half that at pH 6.5 (i.e. 10μg/ml of chromaffin granule membranes at pH 9.5, as compared to 20 μg/ml at pH 6.5). As a consequence of these experiments, poly-L-lysine-precoated ELISA plates were coated at optimal antigen concentrations shown in Table 4.2 using 10 mM Mes-Tris at pH 6.5. The more neutral pH was preferred to avoid possible anomalous

Figure 4.2

Optimisation of ELISAs to screen for monoclonal antibodies raised against antigens derived from adrenal medulla: The effects of poly-L-lysine precoating plates and antigen-binding pH.



Optimisation of ELISAs for the screening of monoclonal antibodies:

Figure 4.1: The effect of the concentration at which ELISA plates were coated with various antigens on the detection signal: Biotinylated antigen fractions derived from adrenal medullary chromaffin cells were incubated at the concentrations shown as described in Chapter 2, Section 2.7[a]. Antigen binding was detected using the enzymatic activity of horseradish peroxidase-conjugated streptavidin, and monitored at 410nm.

Figure 4.2: The effect of poly-L-lysine precoating of ELISA plates, and the pH of antigen-coating was investigated by incubating biotinylated solubilised chromaffin granule membranes at the concentrations shown and signal strengths were determined by using the enzymatic activity of horseradish peroxidase-conjugated streptavidin monitored at 410nm.

behaviour of some antigens at extreme pH.

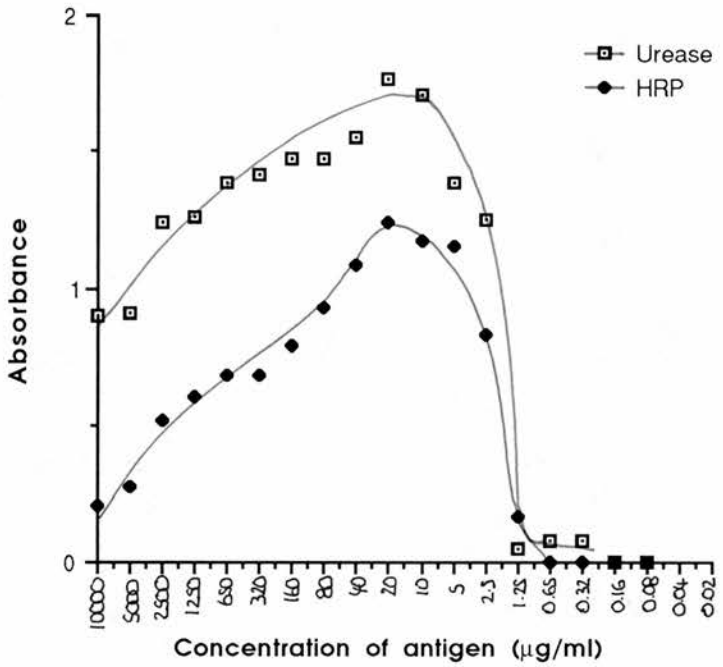
It was also found that the detection system used in ELISA drastically effected the signal strength. This was not apparent until the monoclonal antibody against dopamine β-monooxygenase (dbh1) was raised and used to validate the results obtained by the biotinylated antigen approach. Figure 4.3 shows the results when the biotin-streptavidin system was compared with urease-conjugated second antibodies. Surprisingly, the signal strength of the biotin-streptavidin system (which should give signal amplification) was less than that of urease-conjugated second antibodies. This was probably because the extra incubation and wash steps required by the biotin-streptavidin system led to loss of immune complexes from the wells thus reducing the signal. Therefore the urease system was adopted as the standard ELISA detection system. This had the added advantage that the reaction product, ammonia, changed the colour of the substrate solution from yellowish-amber to deep purple, making the qualitative visualisation of the results easier. One drawback of the system was that because the colour change was due to the change of pH of the unbuffered substrate solution, the wells had to be washed free of PBS and care had to be taken so that ammonia from one well did not affect neighbouring wells by incubating the plates on an orbital shaker.

(ii) *Dot- and immunoblots*

Immunoassays in which excess antigen is attached to nitrocellulose can give a linear response [Harlow & Lane, 1988]. Useful antibodies are generally revealed by as little as 1 - 5 µg of chromaffin granule membranes loaded onto 0.75 mm thick minigels when the biotin-^{125}I-streptavidin detection system is used. In order to screen by dot-blotting for antibodies

Figure 4.3

Optimisation of ELISAs to screen for monoclonal antibodies raised against antigens derived from adrenal medulla: Comparison of the immunoreactivity of the monoclonal antibody *dbh1* detected using either the urease- or biotin-streptavidin-horseradish peroxidase-detection systems.



The immunoreactivity of the monoclonal antibody *dbh1* against chromaffin granule membranes attached to ELISA plates at a range of concentrations was determined using either urease-conjugated or biotinylated secondary antibodies. Biotinylated second antibodies were detected using streptavidin-horseradish peroxidase conjugate.

which could be useful for immunoblotting, up to 30 μg of chromaffin granule membranes were loaded per dot. This value is higher than that required for immunoblots but within the binding capacity of nitrocellulose and was used to increase the sensitivity of the detection. Despite the extra sensitivity of the ECL system, to be able to detect the antibodies against the minor components of chromaffin granule membranes, the same amount of antigen was loaded.

When immunoblots were used to screen culture supernatants, up to 1500 μg of antigen was loaded as a single sample onto large-format gels (0.5 mm thick) or 300 μg onto 0.75 mm thick minigels. After electrophoresis, the antigens were transferred onto nitrocellulose sheets which were then cut into strips for incubation in separate supernatants; the multiblotter from Immunetics did not require strips.

In all screening methods, a mixture of anti-mouse-IgG and -IgM secondary antibodies was used to detect immunoglobulins belonging to these classes.

[b] *Screening of fusions* :

(i) ***Monoclonal antibodies against calcium-dependent chromaffin granule binding proteins***

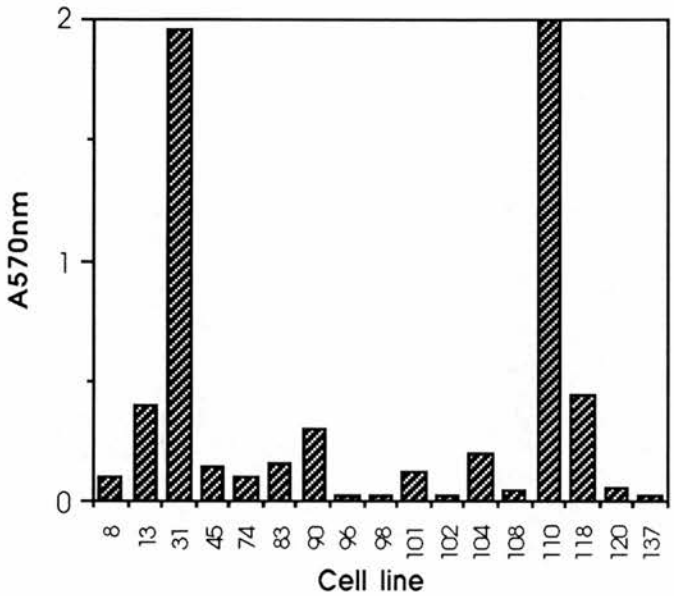
A fraction enriched in cytosolic proteins that show Ca^{2+}-dependent binding to chromaffin granule membrane (Chapter 3) was used to immunise mice and their spleens were fused with NS-0 myeloma cells. After approximately 8 days, 179 aminopterin-resistant colonies were tested by ELISA using the same fraction that was used for the immunisation. A summary of the positive colonies is given in Figure 4.4(a). These were expanded initially in 24-well plates, then in flasks to obtain supernatants for immunoblotting against cytosol. As seen in Figure 4.4(b), the immunoreactivities varied both in terms of strength and specificity. Since at this stage none of the cell lines were cloned, it was not surprising to find a range of polypeptides reacting with the different supernatants. However with cell lines 13 and 83, there were stronger immunoreactivities against proteins of ~55- and ~85-kDa and these were therefore selected for cloning by limiting dilution; all other cell lines were frozen uncloned for storage in liquid nitrogen.

When the clones 13.10 and 83.6 were tested, there was no enhancement of the 55- and 85-kDa bands. To investigate the specificity of the immunoreactivities against the weaker bands, a dilution assay was performed. As shown in Figure 4.4(c), diluting the antibody did not reduce its immunoreactivity against the minor bands. One explanation could be that an epitope which generates a monoclonal antibody may consist of only a few aminoacids and consequently may be present in several proteins (see clone 13.10). Another explanation is that the different bands may be proteolytic products of one protein. In such a case, as seen for clone 83.6 in Figure 4.4(c),

Figure 4.4

The immunoreactivity of culture supernatants from hybridomas generated against Ca^{2+}-dependent adrenal chromaffin granule membrane-binding proteins.

(a) by ELISA:



(b) by immunoblotting:

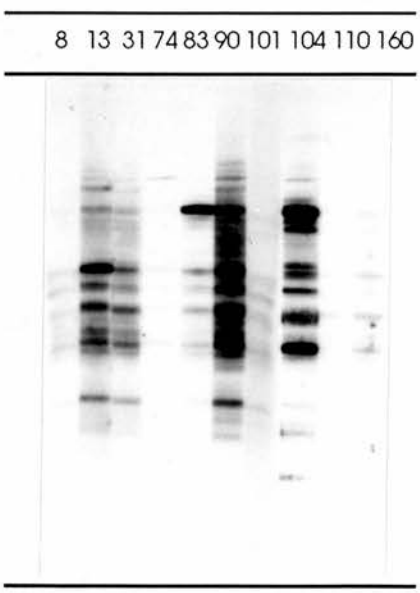
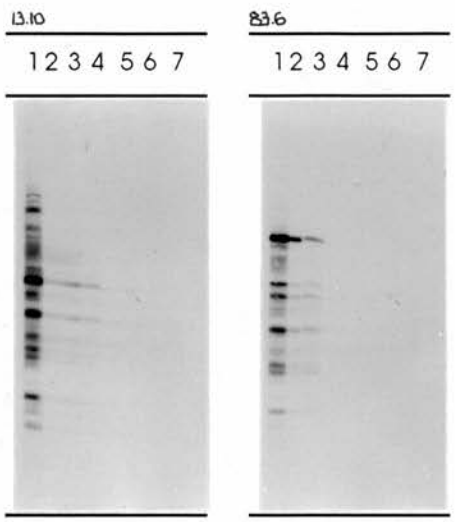


Figure 4.4

The immunoreactivity of culture supernatants of hybridomas generated against Ca^{2+}-dependent chromaffin granule membrane-binding proteins.

(c) Specificity of monoclonal antibodies 13.10 & 83.6: Antibody dilution assay.



The immunoreactivity of culture supernatants of hybridomas generated against Ca^{2+}-dependent chromaffin granule membrane-binding proteins.

Figure 4.4(a): Immunoreactivities of culture supernatants from hybridomas generated by immunising mice with Ca^{2+}-dependent chromaffin granule membrane-binding proteins (numbers indicated) were assayed using ELISA against Ca^{2+}-dependent chromaffin granule membrane-binding proteins. Immunoreactivities were quantitated by the activity of urease-conjugated anti-mouse-IgG and -IgM secondary antibodies at 510nm.

Figure 4.4(b): Immunoblot showing the immunoreactivities of culture supernatants from hybridomas generated by immunising mice with Ca^{2+}-dependent chromaffin granule membrane-binding proteins (numbers indicated) against adrenal medullary cytosol.

Figure 4.4(c): The specificity of the immunoreactivities of culture supernatants from clonal hybridomas 13.10 and 83.6 were investigated by serial dilution of culture supernatant: **Tracks 1**: undiluted culture supernatant; **Tracks 2**: 1 in 5 dilution; **Tracks 3**: 1 in 10 dilution; **Tracks 4**: 1 in 25 dilution; **Tracks 5**: 1 in 50 dilution; **Tracks 6**: 1 in 100 dilution; **Tracks 7**: 1 in 200 dilution; **Tracks 8**: 1 in 400 dilution.

the major immunoreactive band should be the one of highest molecular weight. Because of the success of other fusions, these antibodies were not investigated any further.

(ii) ***Monoclonal antibodies against glycoproteins of the chromaffin granule membrane***

As a strategy for obtaining monoclonal antibodies against the minor membrane components of chromaffin granule membranes, a glycoprotein-rich fraction of these membranes (see Chapter 3) was used to immunise mice. Hybridomas were generated and their supernatants were tested using both ELISAs and dot-blots. For ELISAs, plates were either coated with solubilised chromaffin granule membranes or with the glycoprotein-rich fraction. To overcome the possibility that some antigens were not able to attach to ELISA plates and therefore were excluded from the assay, supernatants were also tested against chromaffin granule membranes by dot-blotting. Urease-conjugated second antibodies were used to detect mouse antibodies in ELISAs and the biotin-^{125}I-streptavidin system was used for dot-blots.

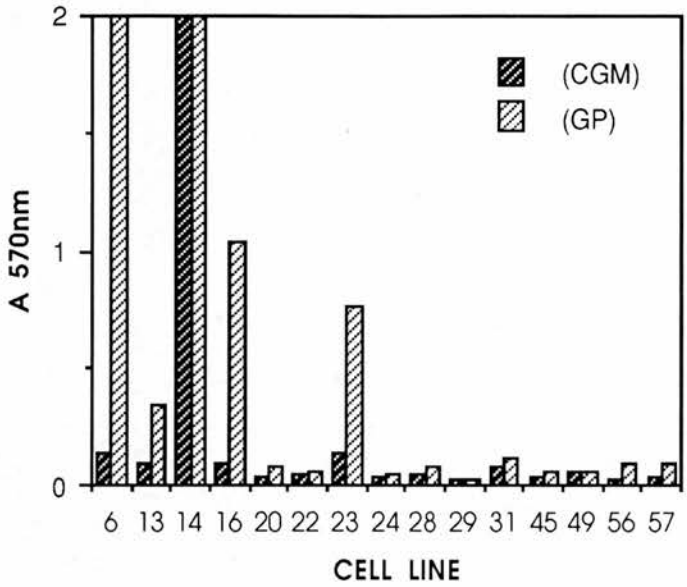
Figure 4.5(a) shows the results from ELISAs when hybridomas generated from this fusion were tested. Using the glycoprotein-rich fraction in ELISAs substantially increased the sensitivity of the assay as compared to the reactivities against chromaffin granule membranes. The results obtained from dot-blots were identical to those obtained by ELISAs but the reactivities against chromaffin granule membranes of cell lines 6, 14 and 23 were much more pronounced, probably because more antigen is available in dot-blots.

The cell lines that were positive were expanded into flasks and their supernatants were tested by immunoblotting. Figure 4.5(b) shows the

Figure 4.5

The immunoreactivities of culture supernatants from hybridomas generated against adrenal chromaffin granule membrane glycoproteins.

(a) by ELISAs:



(b) by immunoblotting:

Figure 4.5

The immunoreactivities of culture supernatants from hybridomas generated against adrenal chromaffin granule membrane glycoproteins.

(a) Immunoreactivities of culture supernatants from hybridomas generated by immunising mice with a membrane glycoprotein-rich fraction derived from chromaffin granule membranes by Triton X-114 fractionation using ELISAs against either solubilised chromaffin granule membranes (CGM) or against glycoproteins (GP) derived from them. Immunoreactivities were quantitated by the activity of urease-conjugated anti-mouse-IgG and -IgM antibodies at 510nm.

(b) Immunoblot showing the immunoreactivities of culture supernatants from hybridomas generated by immunising mice with a membrane glycoprotein-rich fraction derived from chromaffin granule membranes by Triton X-114 fractionation against chromaffin granule membrane proteins.

immunoreactivities of these supernatants: only cell lines 6 and 14 were reactive. These reacted against 67- and 75-kDa proteins respectively and the reactivity of no. 14 was substantially more than that of no. 6. Since these antibodies recognised both native and denatured epitopes, they were chosen for cloning.

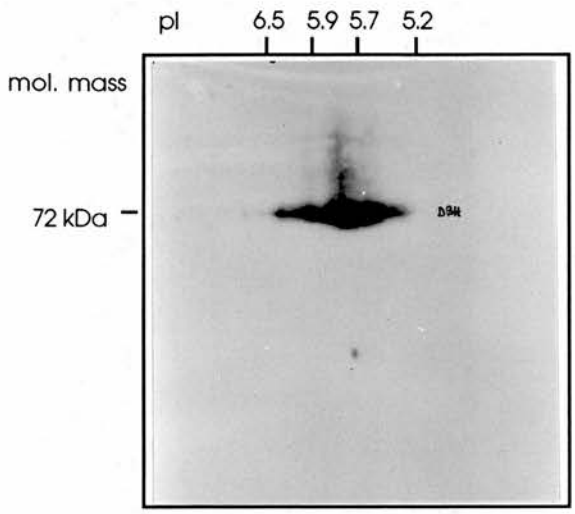
To further characterise supernatants 6 and 14, two-dimensional immunoblots were carried out using chromaffin granule membranes. As seen in Figure 4.6(a), the immunoreactivity from cell line 14 was characteristic of the catecholamine biosynthetic enzyme dopamine β-monooxygenase and the antibody was termed *dbh1*. The immunoreactivity from cell line 6 was less obvious (Figure 4.6(b)). It reacted with a protein of apparent molecular weight 67-kDa and pI 5.4 - 6.2, and was therefore named *cgm67*. The characterisation of the cgm67-reactive protein (p65) is the subject of Chapter 5. Both cell lines were cloned by limiting dilution and used to generate ascites tumours in mice.

The antibodies dbh1 and cgm67 were identified as IgG and IgM type mouse immunoglobulins respectively. This was carried out by ELISA in which secondary antibodies against either mouse IgG or IgM were used to measure the immunoreactivity of dbh1 or cgm67 (Figure 4.7(a)). The monoclonal antibody dbh1 was clearly an IgG-class immunoglobulin and cgm67 appeared to be an IgM but the lower titer of this antibody prevented firm conclusions being drawn. In order to establish if cgm67 was indeed an IgM, the molecular mass of cgm67-immunoreactivity was determined by gel filtration under non-reducing conditions using a TSK-G3000 SWXL column with molecular weight cut off at 500-kDa. The molecular weight of IgM being around 10^6-Da, its immunoreactivity would be expected in the void volume of the column whereas IgG-immunoreactivity would appear in later

Figure 4.6

Two-dimensional immunoblots showing the immunoreactivities of the monoclonal antibodies *dbh1* and *cgm67* against adrenal chromaffin granule membrane proteins.

(a) *dbh1:*



(b) *cgm67:*

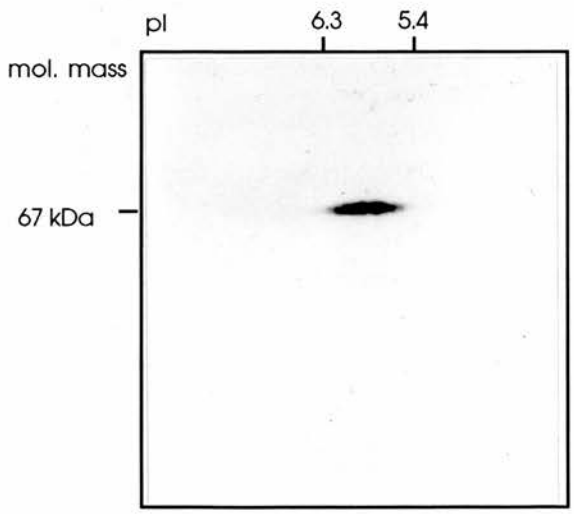
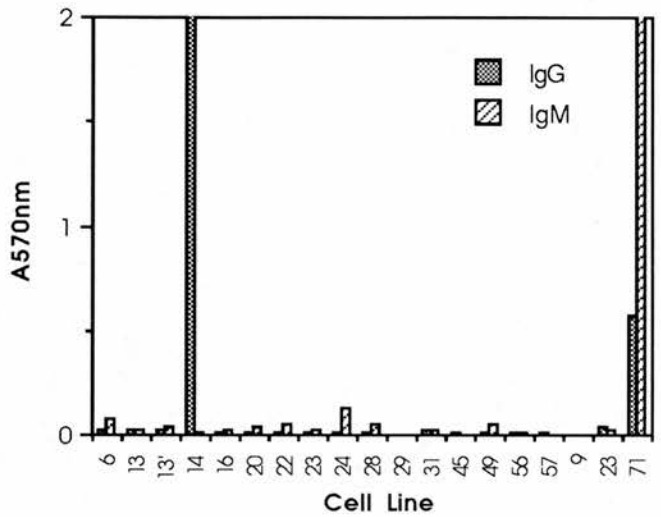


Figure 4.7

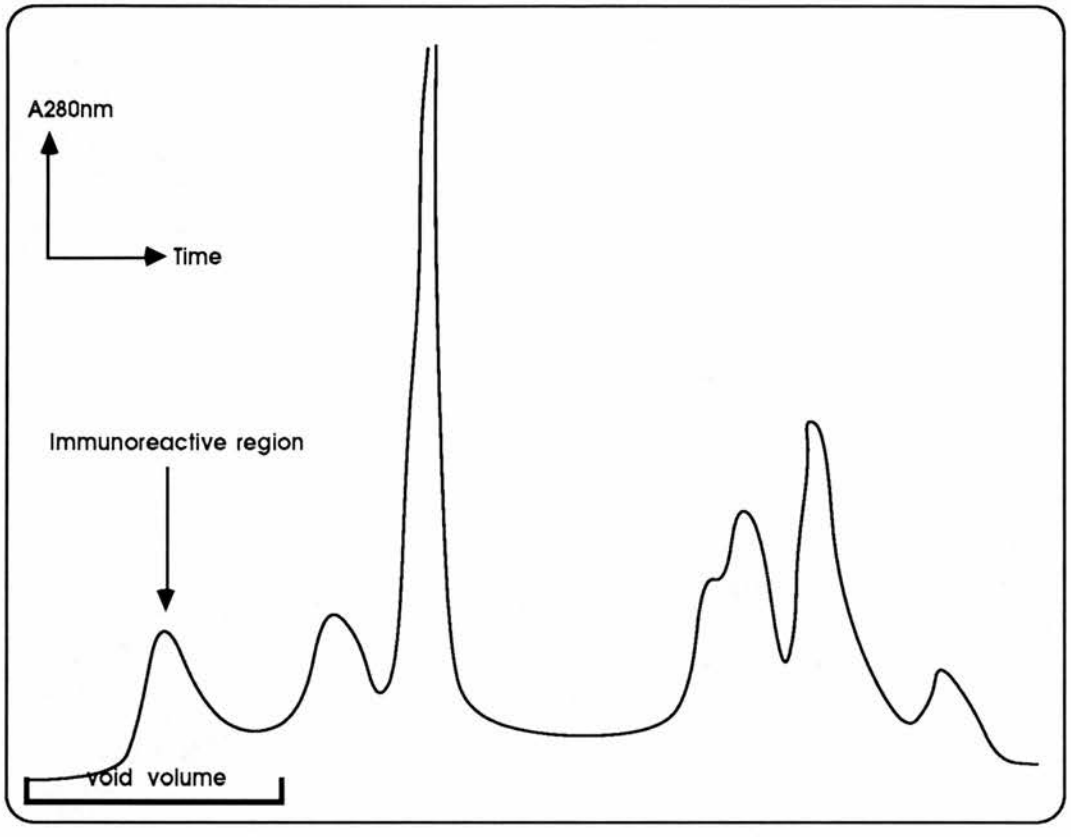
Immuno-typing of monoclonal antibodies raised against adrenal chromaffin granule membrane glycoproteins.

(a) by ELISA:



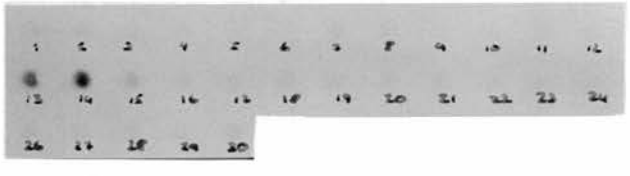
(a) To determine whether the monoclonal antibodies raised against chromaffin granule membrane glycoproteins were IgGs or IgMs, the immunoreactivities of the culture supernatants against the Triton X-114 "glycoprotein" fraction was determined using ELISAs with either anti-mouse IgG or anti-mouse IgM secondary antibodies conjugated to urease. Immunoreactivities were quantitated by monitoring the activity of urease at 570nm, as described in Chapter 2, Section 2.7[a].
Cell line 6 secretes *cgm67*, Cell line 14 secretes *dbh1*.
(b) To establish that cgm67 (the monoclonal antibody secreted from cell line 6) was indeed an IgM, culture supernatant of cell line 6 was fractionated by gel filtration using a TSK-G3000 SWXL column with molecular weight cut off at 500-kDa. Immunoreactivity of each fraction was determined by dot-blotting the column fractions against chromaffin granule membranes, followed by probing the dot-blot with ^{125}I-labelled anti-mouse IgM secondary antibodies. Immunoreactivity was visualised by autoradiography. Fractions 1 -18 are the void volume of the column; fraction numbers are indicated on the dot-blot.

(b) Gel filtration profile of culture supernatant from cell line 6 (*cgm67*):



Void volume is collected in fractions 1- 18.

Immunoreactivity of fractions derived from cell line 6 by gel filtration using a TSK-G3000 SWXL column:



fractions. As seen in Figure 4.7(b), the cgm67-immunoreactivity eluted in the void volume, confirming that cgm67 was indeed an IgM.

Although the aim of this project was to obtain monoclonal antibodies against the uncharacterised proteins of the chromaffin granule membrane, the anti-dopamine β-monooxygenase antibody from cell line 14 (*dbh1*) proved to be very useful as a positive control in the characterisation of other monoclonal antibodies.

(iii) ***Monoclonal antibodies against intrinsic proteins of the chromaffin granule membrane***

As described in Chapter 3, one of the approaches to obtaining monoclonal antibodies against only the intrinsic proteins of the chromaffin granule membrane was to liberate tryptic peptides from membranes that had been washed with 100 mM sodium carbonate (pH 11). The washing step ensured that chromaffin granule membranes were free of extrinsic proteins and mild trypsin treatment reduced the chances of obtaining antibodies against the protease-resistant dopamine β-monooxygenase. The mixture of tryptic peptides was used to immunise mice and hybridomas were prepared as usual. The hybridomas were screened against chromaffin granule membranes using dot-blots, bound mouse antibodies being detected by enhanced chemiluminescence.

Figure 4.8(a) shows the primary screen results of the hybridomas from this fusion. The hybridomas secreting chromaffin granule membrane-specific antibodies were expanded and their supernatants were tested against chromaffin granule membranes by immunoblotting. Figure 4.8(b) shows that

8 out of the 13 hybridomas tested secreted antibodies against an antigen of ~80-kDa. The immunoreactivities of the others varied but the most striking feature of this fusion was that it was biased towards the larger antigens of the chromaffin granule membrane.

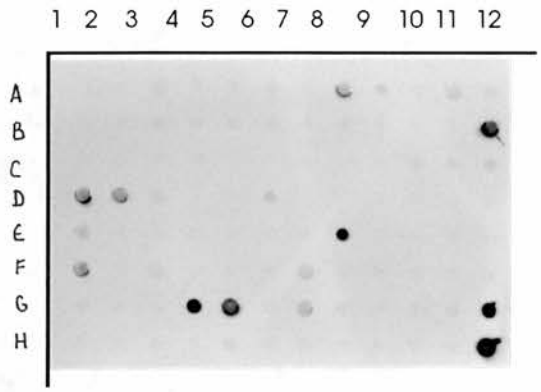
The antibody secreted by cell lines 9 (*cgm80*) was subsequently tested by two-dimensional immunoblotting against chromaffin granule membranes (Figure 4.8(c)). This antibody detected an antigen of 78 - 94-kDa with distinct isoforms which had pI values around 4 - 5. The cgm80 cell line was cloned and used to generate ascites tumours; the other cell lines were stored uncloned in liquid nitrogen.

The molecular weight and the distinctive acidic pI suggested that cgm80 could be against the previously identified, but biochemically uncharacterised, glycoprotein II - a protein with a wide distribution in secretory vesicles of both endo- and exocrine glands as well as in kidney lysosomes [Obendorf *et al.*, 1988; Weiler *et al.*, 1990]. To establish whether cgm80 was indeed against glycoprotein II, the immunoreactivities of cgm80 and a polyclonal antiserum against this protein (obtained from Dr. Fischer-Colbrie, Innsbruck, Austria) were compared by two-dimensional immunoblotting. To determine the precise position of the immunoreactive spots of the two antibodies, the immunoreactivity of dbh1 (a monoclonal antibody against dopamine β-monooxygenase) was used as an internal marker of molecular weight and pI. As seen in Figure 4.9(a) and (b), the mono- and polyclonal antibodies stained the same regions of the immunoblot suggesting that the two antibodies recognised the same protein. To further prove that these antibodies recognised the same protein, the anti-glycoprotein II antiserum/dbh1 blot was reincubated with cgm80. This resulted in the intensification of the spots that were originally immuno-stained by the

Figure 4.8

The immunoreactivities of culture supernatants from hybridomas generated against tryptic peptides derived from integral proteins of adrenal chromaffin granule membrane.

(a) by dot-blots:



(b) by immunoblots:

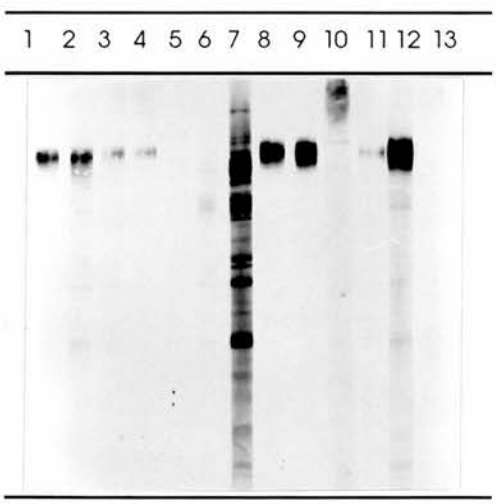
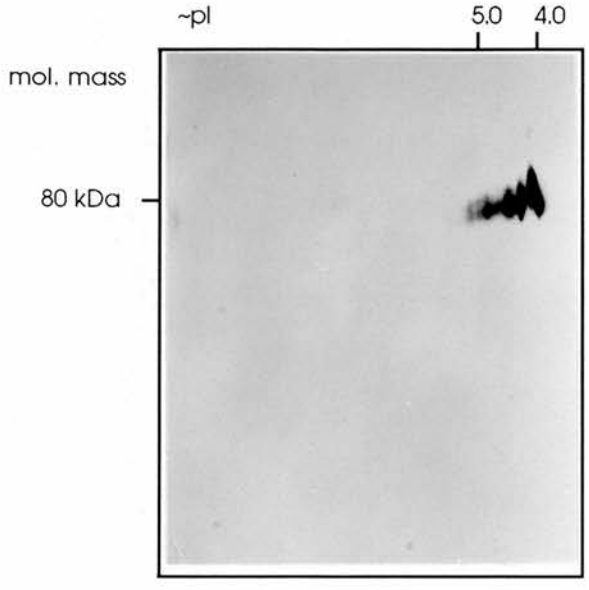


Figure 4.8

The immunoreactivities of culture supernatants from hybridomas generated against tryptic peptides derived from integral proteins of adrenal chromaffin granule membrane.

(c) Two-dimensional immunoblot showing the immunoreactivity of the monoclonal antibody *cgm80:*



(a) Immunoreactivities of culture supernatants from hybridomas generated by immunising mice with soluble tryptic peptides derived from integral membrane proteins of chromaffin granule membranes using dot-blots against solubilised chromaffin granule membranes. Immuno-reactivities were detected by using enhanced chemi-luminescence followed by exposure to autoradiography film.

(b) Immunoblot showing the immunoreactivities of culture supernatants from hybridomas generated by immunising mice with soluble tryptic peptides derived from integral membrane proteins of chromaffin granule membranes against chromaffin granule membrane proteins. Immunoreactivities were detected by using enhanced chemi-luminescence followed by exposure to autoradiography film.

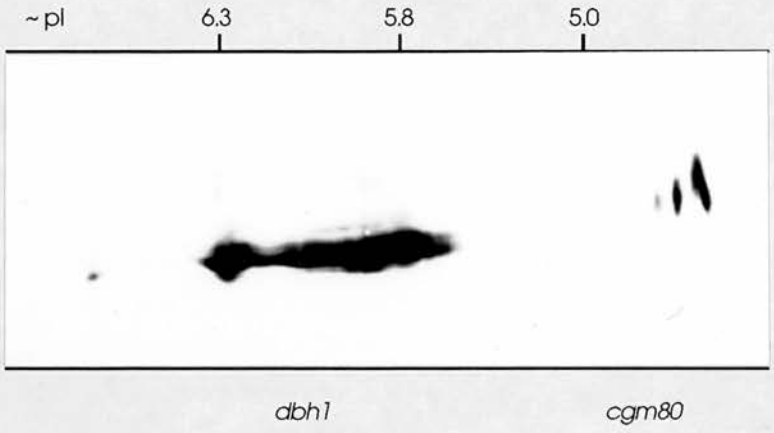
(c) Two-dimensional immunoblots showing the immunoreactivity of the monoclonal antibody *cgm80* (secreted by cell line 9) against chromaffin granule membrane proteins.

Figure 4.9

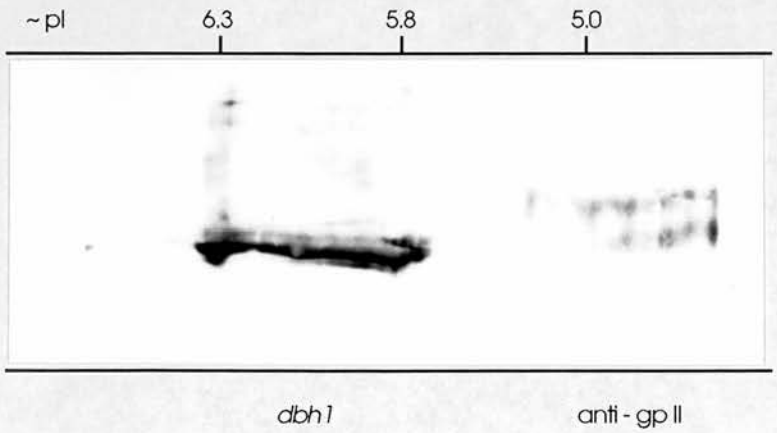
Identification of the cgm80-antigen as glycoprotein II .

Comparison of the immunoreactivities of the monoclonal antibody *cgm80* and an anti-glycoprotein II polyclonal antibody using two-dimensional immunoblotting:

(a) Two-dimensional immunoblots showing the position of the cgm80-reactive protein relative to dopamine β-monooxygenase:



(b) Two-dimensional immunoblots showing the position of glycoprotein II relative to dopamine β-monooxygenase:



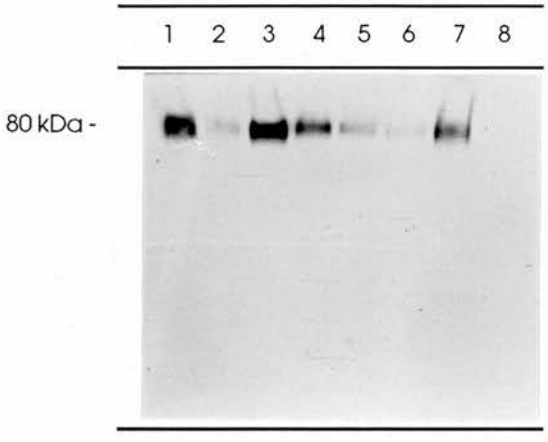
anti-glycoprotein II antiserum. Furthermore, upon longer exposures of the immunoblots, common degradation products were observed. An independent approach taken to identify the cgm80-antigen was to compare its fractionation using the detergent Triton X-114 with that of glycoprotein II. Using concanavalin A-binding property of glycoprotein II, Pryde & Phillips (1986) have shown that glycoprotein II fractionates predominantly into the detergent-rich phase - this was confirmed using anti-glycoprotein II antisera (Figure 4.9(c)). As seen in Figure 4.9(c), the cgm80-antigen also fractionated predominantly into the detergent-rich phase. Taken together, these results therefore show that cgm80 is an anti-glycoprotein II monoclonal antibody.

Cgm80 immunoreactivity was detected only if anti-mouse IgG secondary antibodies were used, implying that it is a mouse IgG-class antibody.

Figure 4.9

Identification of the cgm80-antigen as glycoprotein II .

(c) Immunoblot showing the distribution of *cgm80* immunoreactivity on fractionation of chromaffin granule membranes with the non-ionic detergent Triton X-114:



Track 1: chromaffin granule membranes; **Track 2**: phospholipid-rich pellet; **Track 3**: detergent-rich pellet; **Track 4**: aqueous supernatant; **Track 5**: glycoprotein-rich pellet; **Track 6**: soluble and extrinsic proteins of chromaffin granules; **Track 7**: Na_2CO_3-washed chromaffin granule membranes; **Track 8**: proteins released from Na_2CO_3-washed chromaffin granule membranes. 20 µg of each fraction was loaded per lane of a minigel.

4.3 Conclusions

The most important part of producing monoclonal antibodies against components of a mixture of antigens is the availability of screening procedures that can identify hybridomas producing antibodies against the individual components.

In this Chapter, I have outlined how immunological techniques for the detection of monoclonal antibodies were set up and shown how these have been used. Monoclonal antibodies 13.10 and 83.6 were raised against a fraction enriched in cytosolic proteins that bind to chromaffin granules in the presence of elevated Ca^{2+} concentrations. Three other monoclonal antibodies against chromaffin granule membranes (cgm67, cgm80 and dbh1) were also identified and characterised: they are directed against p65, glycoprotein II and dopamine β-monooxygenase respectively. These monoclonal antibodies will be used initially in the characterisation of the proteins with which they react, and ultimately to help to explore the molecular mechanisms that govern the organisation and trafficking of the regulated secretory vesicle.

CHAPTER 5

CHAPTER 5

Characterisation of bovine chromaffin granule p65 using the monoclonal antibody cgm67

5.1 Introduction

Among integral components of secretory vesicle membranes must be those proteins that are responsible for its organisation and exocytic function, but their study has been complicated by the complex composition of such membranes. Production of monoclonal antibodies against components of these membranes provides one way of overcoming this problem and initiating such investigations. Using the chromaffin granules as a model system, a membrane-derived glycoprotein-rich fraction was produced and used to raise several monoclonal antibodies, among them *dbh1* and *cgm67* (see Chapters 3 & 4). These monoclonal antibodies recognised the biosynthetic enzyme dopamine β-monooxygenase and a 67-kDa protein respectively. The latter protein appeared to be novel and its characterisation is the subject of this Chapter.

In view of the many extrinsic components present in preparations of chromaffin granule membranes, the initial aim in the characterisation of the cgm67-antigen was to define its subcellular localisation, by subcellular fractionation of adrenal medulla and immunoblotting the fractions using cgm67. The partitioning of the cgm67-reactive antigen during Triton X-114 fractionation of chromaffin granule membranes and its susceptibility to oligosaccharide-cleaving enzymes were also explored to further analyse the nature of this antigen.

Studies on the position of the cgm67-epitope were performed by partial trypsin digestion of chromaffin granules and their membranes. This led to the isolation of a 39-kDa tryptic peptide containing the cgm67-epitope, which was purified and partially sequenced.

5.2 Results

[a] *Localisation of cgm67-immunoreactivity* :

The subcellular localisation of cgm67-immunoreactivity was established by fractionation of bovine adrenal medulla by differential centrifugation, and by continuous sucrose density gradients (as described in *Methods*, Chapter 2), followed by immunoblotting. Figure 5.1(a) shows that when the post-nuclear supernatant was fractionated on a continuous sucrose gradient, cgm67-immunoreactivity was found in the same region of the gradient as dopamine β-monooxygenase, an established marker enzyme of chromaffin granules, between 1.8 and 1.95 M sucrose. It has been shown for some integral membrane proteins of secretory vesicles, such as synaptophysin, that as well as being present in chromaffin granules, they are also present in a vesicle population devoid of the typical granule markers which band around 1.2 M sucrose [Obendorf *et al.*, 1988]. In comparison with the immunoreactivity in the granule region of the sucrose gradient, no appreciable amount of cgm67-immunoreactivity was present in any other region of the gradient. However, after longer periods of autoradiography, weak cgm67-immunoreactivity was also observed in the 0.85 M sucrose region of the gradient where broken chromaffin granule membranes are present. As a consequence of the low sensitivity of the ^{125}I-labelled second antibody system which was used in this experiment (to avoid a problem inherent in the more sensitive biotin-streptavidin system, namely detection of naturally-occurring biotinyl proteins), firm conclusions about the apparent dual distribution of the cgm67-antigen could not be drawn.

The cgm67-immunoreactivity was located in the membranes of broken chromaffin granules by more conventional fractionation methods as

Figure 5.1

Characterisation of the cgm67-antigen.

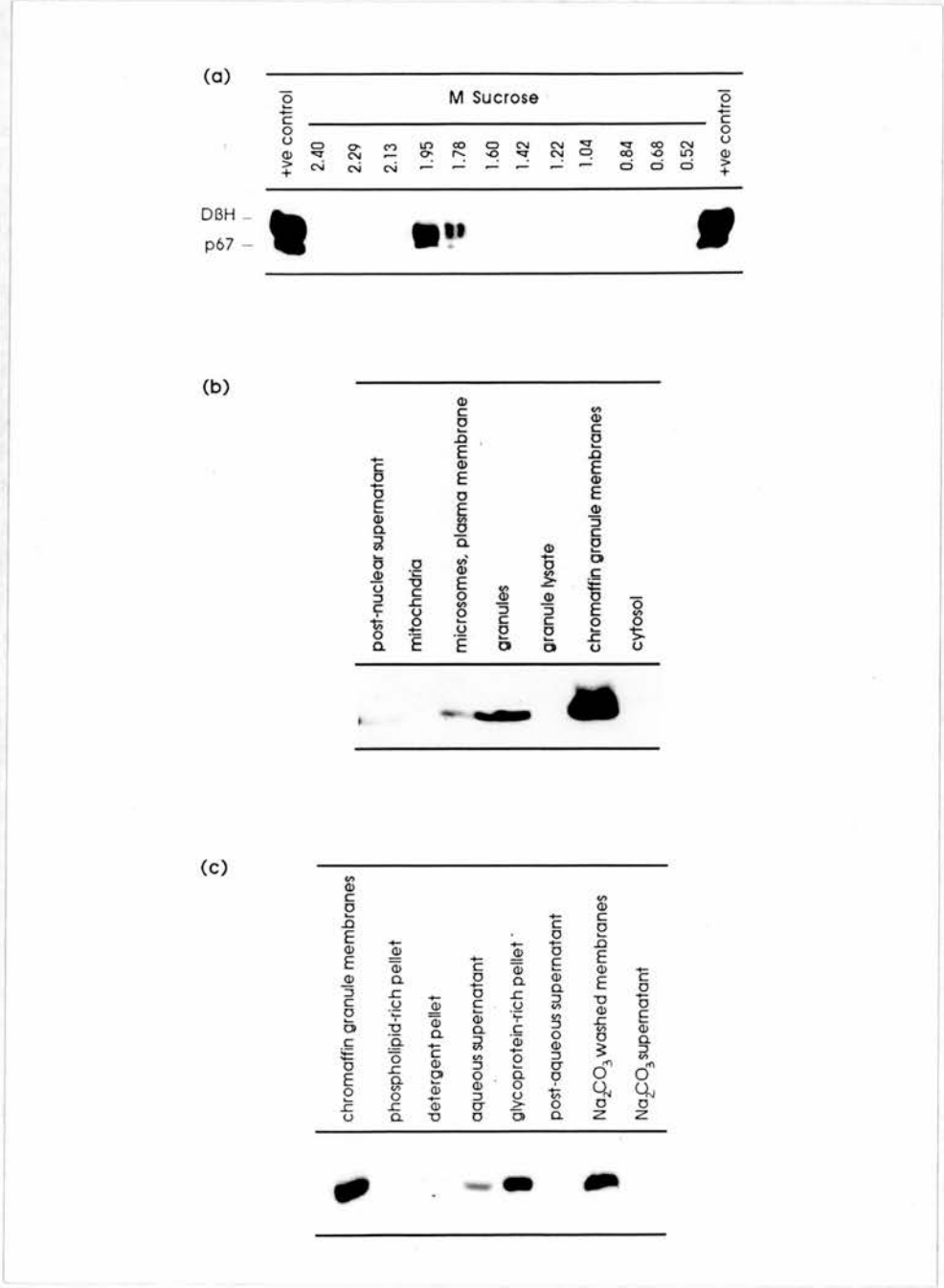


Figure 5.1

Characterisation of the cgm67-antigen.

(a) Subcellular distribution of the cgm67-reactive antigen: Postnuclear supernatant of adrenal medulla was separated by using a sucrose density gradient (0.5 to 2.4 M) and fractionated (12 x 1 ml). Equal volumes of each fraction (40 µl) were analysed by immunoblotting using first *cgm67*, followed by *dbh1* on the same sheet of nitrocellulose in order to compare the distribution of the *cgm67*-reactive antigen with that of dopamine β-monooxygenase.

(b) Subcellular distribution of the cgm67-reactive antigen: Adrenal medullae were fractionated by differential centrifugation and equal protein (100 µg; large format gel) from each subcellular fraction was analysed by immunoblotting using the monoclonal antibody *cgm67*.

(c) Fractionation of chromaffin granule membranes by Triton X-114: Immunoblot showing the distribution of *cgm67* immunoreactivity on fractionation of chromaffin granule membranes with the non-ionic detergent Triton X-114. 20µg of each fraction was loaded per lane of a minigel.

seen in Figure 5.1(b). Despite the large population of secretory granules in chromaffin cells (Chapter 1, Section 1.3), the weak cgm67-immunoreactivity in the post-nuclear supernatant suggested a much lower abundance of the cgm67-antigen than of dopamine β-monooxygenase. When chromaffin granule membranes were extensively washed with Na_2CO_3 at pH 11 to remove extrinsic proteins, cgm67-antigen remained in the membrane fraction, suggesting that it is an integral membrane protein (Figure 5.1(c)). Further fractionation of membranes with the non-ionic detergent Triton X-114 suggested that the cgm67-antigen was a membrane-bound glycoprotein which is present in the "*aqueous*" phase but which precipitates out into the "*glycoprotein-rich*" fraction once the residual Triton X-114 is removed by dialysis (Figure 5.1(c)).

[b] ***Identification of cgm67-antigen as bovine p65*** *:*

A 65-kDa antigen of synaptic vesicles termed "p65" has also been shown to be the major calmodulin-binding protein of chromaffin granule membranes [Matthew *et al.*, 1981; Fournier & Trifaró, 1988] (see Chapter 1, Section 1.4(b)(i)). The apparent molecular weight of the cgm67-antigen was similar to that of p65. To determine whether the protein recognised by cgm67 was p65, a calmodulin-binding fraction from solubilised chromaffin granule membranes was prepared by calmodulin-agarose affinity chromatography (see *Methods*, Chapter 2). The polypeptides in this fraction were separated by two-dimensional electrophoresis, and blotted with both cgm67 and asv48, the monoclonal antibody originally used in characterising p65 (Matthew *et al.*, 1981). A trace of dopamine β-monooxygenase was added to the material loaded onto the gel, and was used as an internal marker of pI and molecular

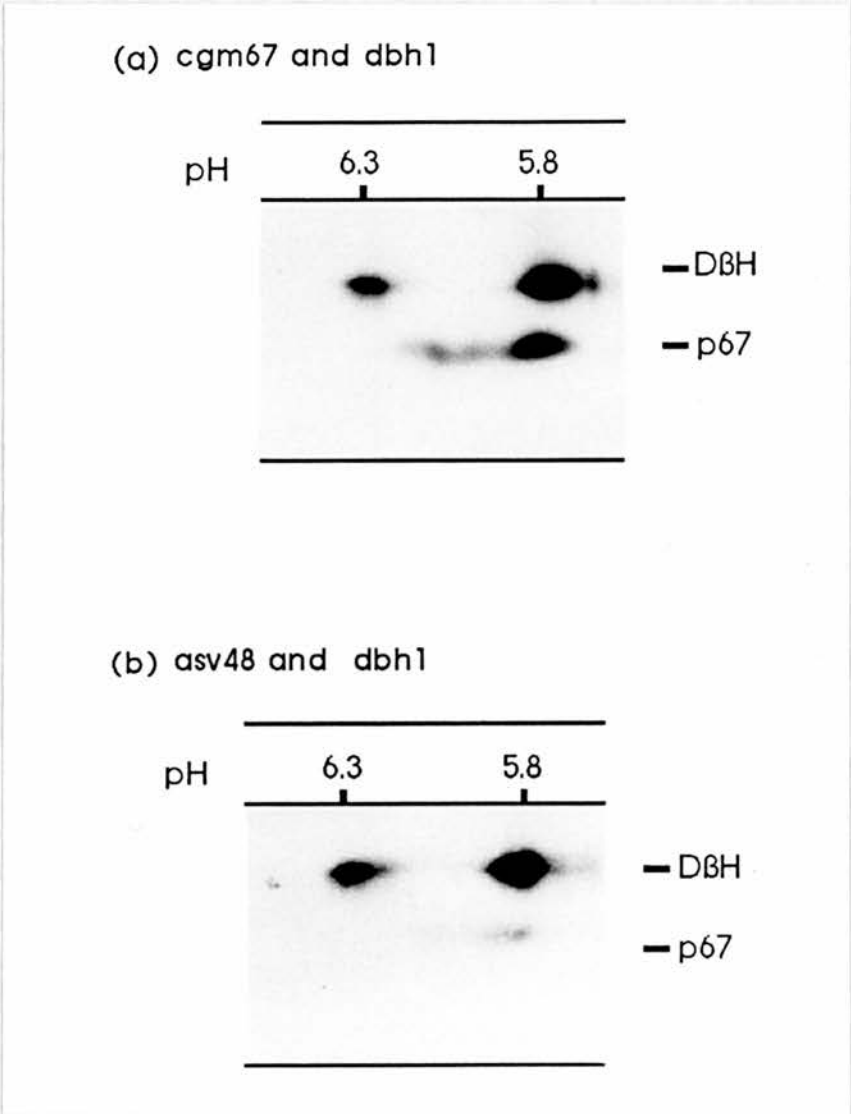
weight. It was detected with dbh1, an IgG-class anti-dopamine β-monooxygenase monoclonal antibody. The positions of the asv48- and cgm67-immunoreactive spots were coincident, although that due to cgm67 was much stronger than that due to asv48 (Figures 5.2(a) & 5.2(b)). As a further proof of similarity, the asv48/dbh1 blot was then incubated with cgm67, which resulted in the intensification the spot previously stained by asv48 (cf. Figures 5.2(a)). As p65 has been shown to bind both asv48 and calmodulin and to be present in bovine chromaffin granule membranes [Fournier & Trifaró, 1988*a*], it was concluded that cgm67 bound to an epitope on that protein, although its apparent molecular weight was 67-kDa, rather than 65-kDa as has been reported in the rat brain [Matthew *et al.*, 1981].

Another approach taken to establish whether the cgm67-antigen was the same protein as p65 was to compare the immunoreactivities of tryptic peptides derived from the cgm67-antigen using the monoclonal antibodies asv48 and cgm67. Intact granules were treated (0, 2.5 and 600 μg/ml trypsin for 60 minutes at 37°C) and the supernatant and residual membrane fractions analysed separately. Figure 5.2(c) is an immunoblot showing the soluble and membrane-bound asv48-reactive tryptic peptides. Comparison of these peptides with those that are reactive with cgm67 (Figure 5.5) shows that the two antibodies recognised the same tryptic peptides and were therefore almost certainly directed against the same protein.

The cgm67-antigen was therefore the bovine homologue of the previously-discovered rat synaptic vesicle protein, and is termed *bovine chromaffin granule p65.*

Figure 5.2

Comparison of the immunoreactivities of the monoclonal antibodies *cgm67* and *asv48* :



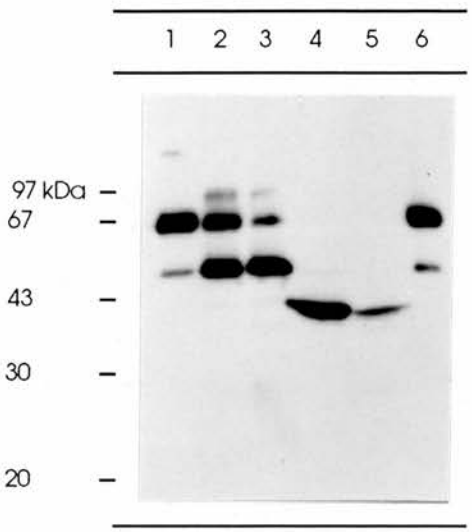
(a) Two-dimensional immunoblots showing the position of the *cgm67*-reactive protein relative to dopamine β-monooxygenase.

(b) Two-dimensional immunoblots showing the position of the *asv48*-reactive protein relative to dopamine β-monooxygenase.

Figure 5.2

Comparison of the immunoreactivities of the monoclonal antibodies *cgm67* and *asv48* :

(c) *asv48*-reactive tryptic peptides derived from chromaffin granule membranes (refer to Figure 5.6(b) & (d)):



Pure chromaffin granules were incubated with a range of trypsin concentrations (**Track 1**: 0 μg/ml; **Track 2**: 2.5 μg/ml; **Track 3**: 600 μg/ml; **Track 4**: 2.5 μg/ml; **Track 5**: 67 μg/ml) and the soluble and insoluble fractions were examined separately (**Tracks 1, 2, 3**: insoluble peptides (membrane-bound); **Tracks 4,5**: soluble peptides). **Track 6** is a control without trypsin, containing chromaffin granule membrane proteins.

[c] *Glycosylation of bovine chromaffin granule p65* :

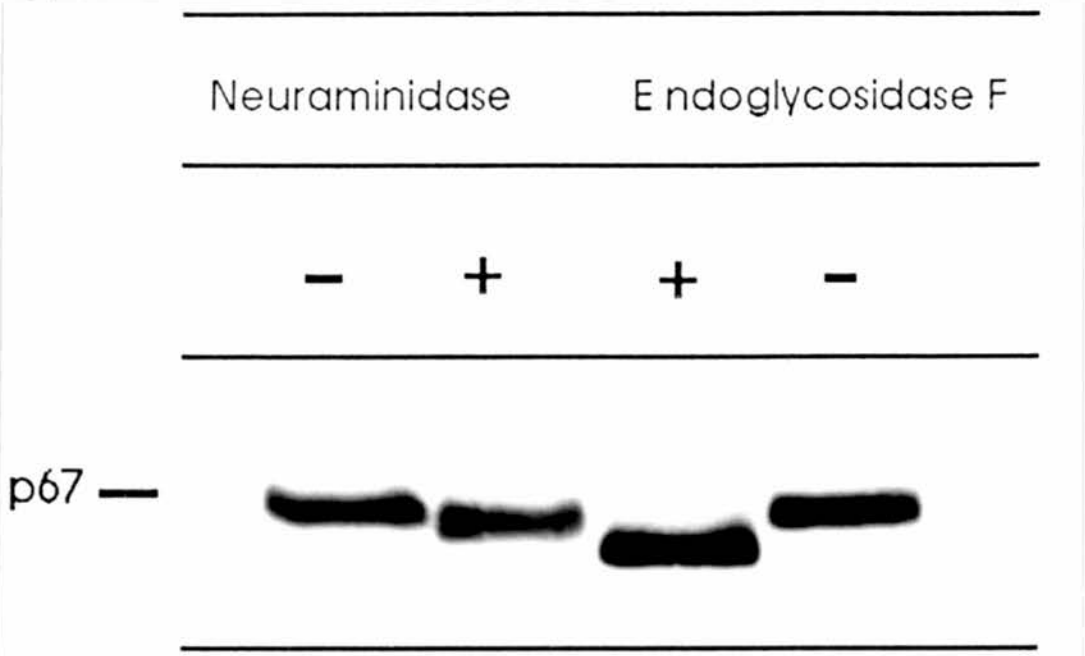
Bovine chromaffin granule membrane p65 behaved like several other intrinsic membrane glycoproteins when fractionated in the non-ionic detergent Triton X-114. To confirm that bovine p65 is indeed glycosylated, the aqueous fraction from Triton X-114-treated chromaffin granule membranes in which membrane glycoproteins were still in solution was treated with the oligosaccharide-cleaving enzymes neuraminidase or endoglycosidase F, in the presence of protease inhibitors as described in Chapter 2. The digestion products were separated and immunoblotted with cgm67. Neuraminidase, which removes terminal sialic acid residues, reduced the apparent molecular weight of the protein by approximately 1-kDa (Figure 5.3(a)). On the other hand, treatment with endoglycosidase F, which removes N-glycans of the high-mannose and complex types from core polypeptides, reduced the molecular weight by approximately 5-kDa. Together these results indicate that bovine p65 contains one or more N-linked oligosaccharide chains, terminating in sialic acid and galactose. The oligosaccharide moiety of bovine p65 does not bind concanavalin A or wheat germ agglutinin (see Chapter 3, Figures 3.2(b) & (c)) but does bind peanut agglutinin after neuraminidase treatment (Figure 5.3(b)).

Recent publication of the cDNA sequence of the rat p65 [Perin *et al.*, 1990] has revealed only one putative N-linked glycosylation site in the intragranular region of the protein. At this stage it is not possible to predict the number of glycosylation sites on the bovine p65. Recently the rat p65 has also been shown to be N-glycosylated [Perin *et al.*, 1991*b*].

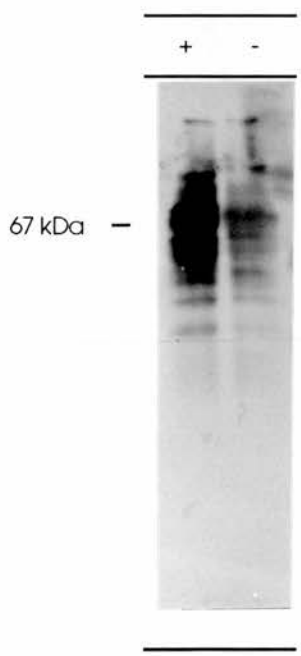
Figure 5.3

Glycosylation of bovine chromaffin granule p65.

(a) Enzymatic degradation of the oligosaccharide chains of p65 using neuraminidase and endoglycosidase F.



(b) Peanut agglutinin-binding glycoproteins of chromaffin granule membranes before (−) and after (+) digestion with neuraminidase:



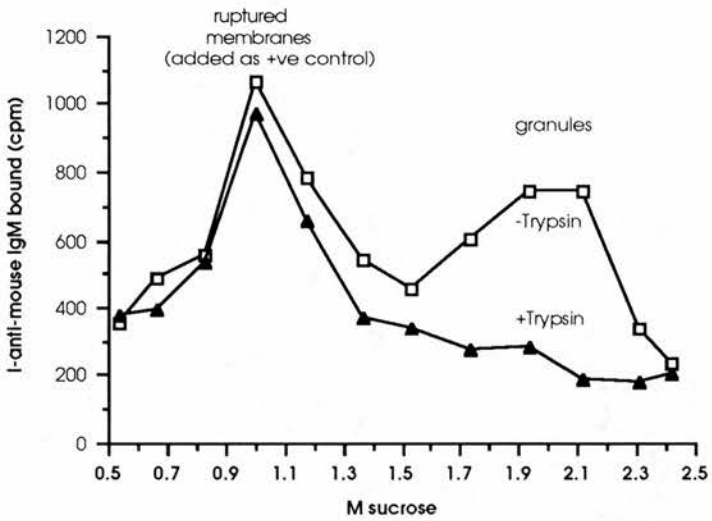
[d] ***Subcellular orientation of the cgm67 epitope*** :

To establish the orientation of the cgm67 epitope in the chromaffin granule membrane, crude intact granules were mixed with purified chromaffin granule membranes and the mixture was incubated with cgm67 for 10 minutes at room temperature. The organelle/antibody mixture was then loaded onto a continuous sucrose gradient (0.5 to 2.4 M) and centrifuged to equilibrium. Since only bound monoclonal antibodies would enter the gradient, if the cgm67 epitope was on the cytoplasmic side of the granules bound antibody would be detected in the granule region (1.80 to 1.95 M sucrose) as well as in the broken membrane region (around 0.85 M sucrose); in the case of a lumenal epitope, cgm67 would only be present in the broken membrane region. After centrifugation to equilibrium, the gradient was fractionated and the distribution of cgm67 in the gradient was assayed by dot-blotting the fractions onto a nitrocellulose membrane and probing them with ^{125}I-anti-mouse IgM antibodies. The ^{125}I bound to each dot was subsequently quantitated by γ-counting. Figure 5.4(a) shows that cgm67 was present in both intact granule and in the broken granule membrane region of the gradient. In a parallel experiment intact granules were first treated with 0.1 mg/ml trypsin to establish if the cgm67 epitope in intact granules was accessible to proteolysis and hence cytoplasmically oriented. After 1 hour, the trypsin was inhibited with TLCK, PMSF and trypsin inhibitor. The unruptured trypsin-treated granules were then mixed with broken chromaffin granule membranes, incubated with cgm67, and processed as above. The added broken chromaffin granule membranes served as a positive control to check that cgm67 was not affected by any residual uninhibited trypsin. Treatment of the granules with trypsin abolished the binding of cgm67 to them but not to the untreated broken granule membranes, implying that the epitope is on the

Figure 5.4

Subcellular orientation of the *cgm67*-epitope.

(a) Distribution of *cgm67* bound to subcellular organelles derived from adrenal medulla:



(b) Distribution of *dbh1* bound to subcellular organelles derived from adrenal medulla:

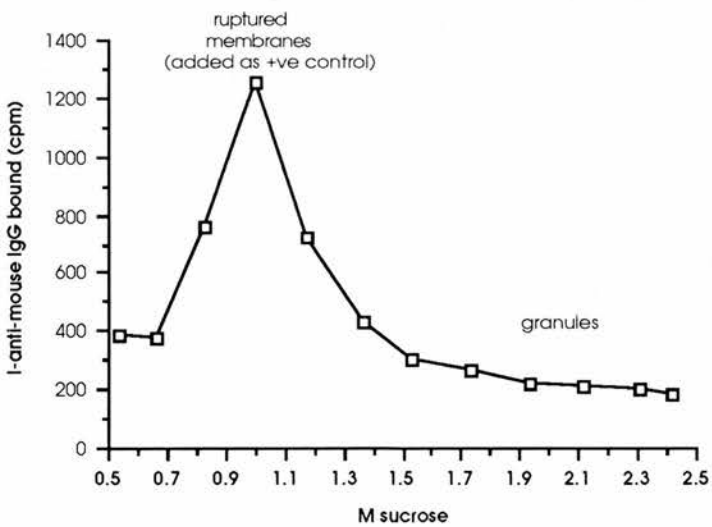


Figure 5.4

Subcellular orientation of the *cgm67*-epitope.

I. Antibody/organelle sucrose gradients:

(a) To determine the subcellular orientation of the epitope of *cgm67*, crude chromaffin granule fraction derived from the adrenal medulla (~150 µl; 5 mg) was mixed with ~0.5 mg purified chromaffin granule membranes in 0.3 M sucrose/10 mM Hepes (pH 7.2). To this mixture, 250 µl of *cgm67* culture supernatant was added and the mixture was incubated at room temperature for 5 minutes. The organelle/monoclonal antibody mixture was then loaded onto a continuous sucrose gradient (0.5 to 2.4 M) which was centrifuged to equilibrium. After centrifugation to equilibrium, the gradient was fractionated and the distribution of *cgm67* in the gradient was assayed by dot-blotting the fractions onto a nitrocellulose membrane and probing them with ^{125}I-anti-mouse IgM antibodies. The ^{125}I bound to each dot was subsequently quantitated by γ-counting. In a parallel experiment trypsin-treated chromaffin granules (see Chapter 2, Section 2.3[e]) were loaded onto the gradient.

(b) The procedure described above was carried out using the monoclonal antibody *dbh1* directed against dopamine β-monooxygenase.

II. The effect of protease treatment of intact granules:

The effect of papain-, pronase- and trypsin-treatment of intact granule on bovine p65 (**(c)**), dopamine β-monooxygenase (**(d)** & **(f)**) and cytochrome b_{561} (**(e)**) were investigated by purifying membranes from protease-treated intact chromaffin granules and analysing them by immunoblotting using the monoclonal antibodies *cgm67*, *dbh1* and a polyclonal antibody against cytochrome b_{561} obtained from Dr. D.K. Apps (Dept. of Biochemistry, Univ. of Edinburgh). **Tracks 1**: granule incubated without protease for 60 minutes at room temperature; **Track s 2**: papain-treated granules; **Tracks 3**; pronase-treated granules; **Tracks 4**: trypsin-treated granules; **Tracks 5**: granules incubated for 60 minutes at 0°C; **Track 6** (Fig. 5.4(f) only): intact granule incubated without trypsin for 60 minutes at room temperature; **Track 7** (Fig. 5.4(f) only): intact granule incubated with 0.1 µg/ml trypsin for 60 minutes at room temperature with the addition of 0.5 % Triton X-100; **Track 8** (Fig. 5.4(f) only): as track 7, but incubated with 1 µg/ml trypsin with the addition of 0.5 % Triton X-100; **Track 9** (Fig. 5.4(f) only): as track 7, but incubated with 20 µg/ml trypsin with the addition of 0.5 % Triton X-100; **Track 10** (Fig. 5.4(f) only): as track 7, but incubated with 100 µg/ml trypsin with the addition of 0.5 % Triton X-100; **Track 11** (Fig. 5.4(f) only): intact granules incubated with 100 µg/ml trypsin for 60 minutes at room temperature without the addition of 0.5 % Triton X-100.

cytoplasmic side of the granule membranes. In a similar experiment, the monoclonal antibody against dopamine β-monooxygenase (dbh1) was only detected in the broken membrane region showing that as expected its epitope was lumenal (Figure 5.4(b)).

Another approach to investigating the orientation of the cgm67 epitope was to determine its susceptibility to protease treatment. Intact chromaffin granules were therefore treated with 0.1 mg/ml trypsin, papain or pronase for 60 minutes at room temperature, and membranes were purified from them. These membranes were immunoblotted with cgm67 to establish the fate of its epitope following such treatment. As with the organelle/ antibody gradients described above, protease treatment resulted in the complete loss of the cgm67 epitope from the granules (Figure 5.4(c)). In contrast, dopamine β-monooxygenase, which is not exposed to the cytoplasm, was unaffected by these extragranular reagents (Figure 5.4(d)). Cytochrome b_{561}, a transmembrane protein with extragranular N- and C- termini [Perin *et al.*, 1988] was found, as expected, to be protease-sensitive (Figure 5.4(e)). The addition of 0.5 % Triton X-100 to the granules to lyse them before protease treatment made dopamineβ-monooxygenase protease-sensitive (Figure 5.4(f)). These experiments confirm that the cgm67 epitope is extragranular and that the granules remain unruptured during the protease treatments.

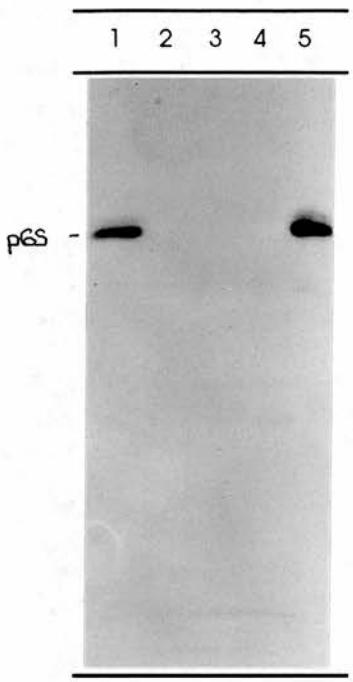
[e] ***Epitope mapping and transmembrane topology of bovine p65*** *:*

To establish the membrane topology of bovine p65, intact granules or their broken membranes were treated with a range of trypsin concentrations. This resulted in the concentration-dependent release of the cgm67-reactive peptides (Figure 5.5). A major soluble cgm67-reactive peptide of 39-kDa was

Figure 5.4

Subcellular orientation of the *cgm67*-epitope.

(c) The effect of protease treatment of intact granules on the epitope of *cgm67*:



(d) The effect of protease treatment of intact granules on the epitope of *dbh1*:

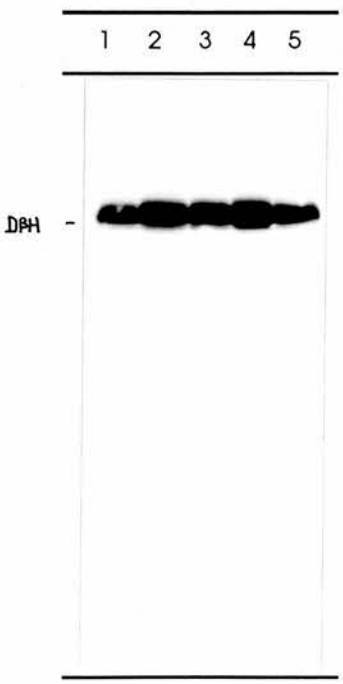
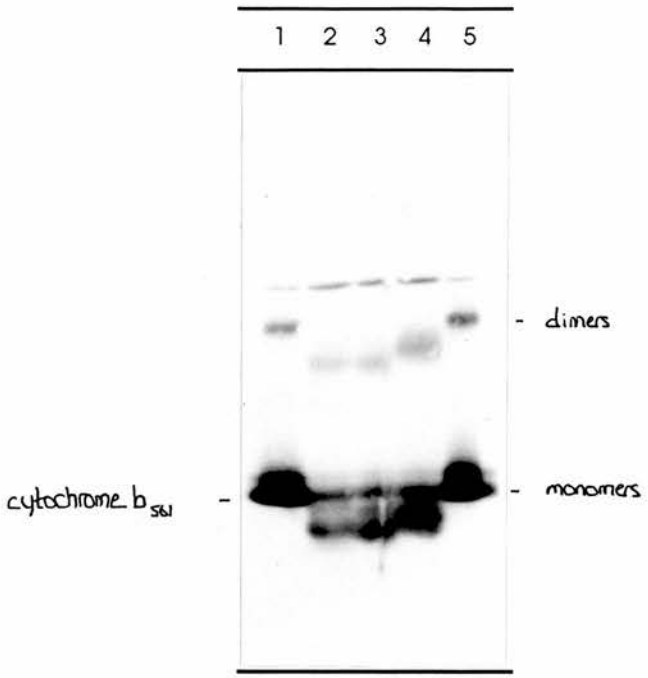


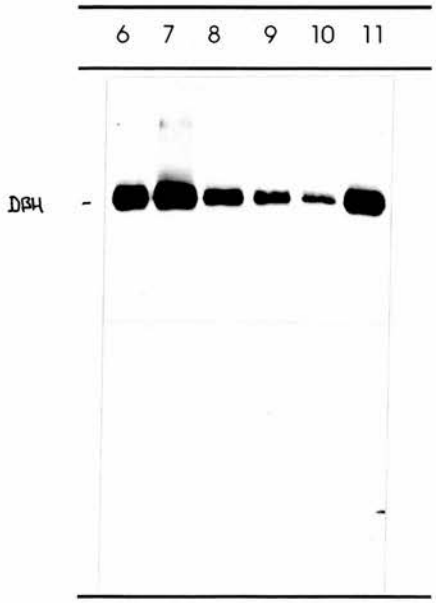
Figure 5.4

Subcellular orientation of the *cgm67*-epitope.

(e) The effect of protease treatment of intact granules on cytochrome b_{561}:



(f) The effect of protease- and detergent-treatment of intact granules on dopamine β-monooxygenase:



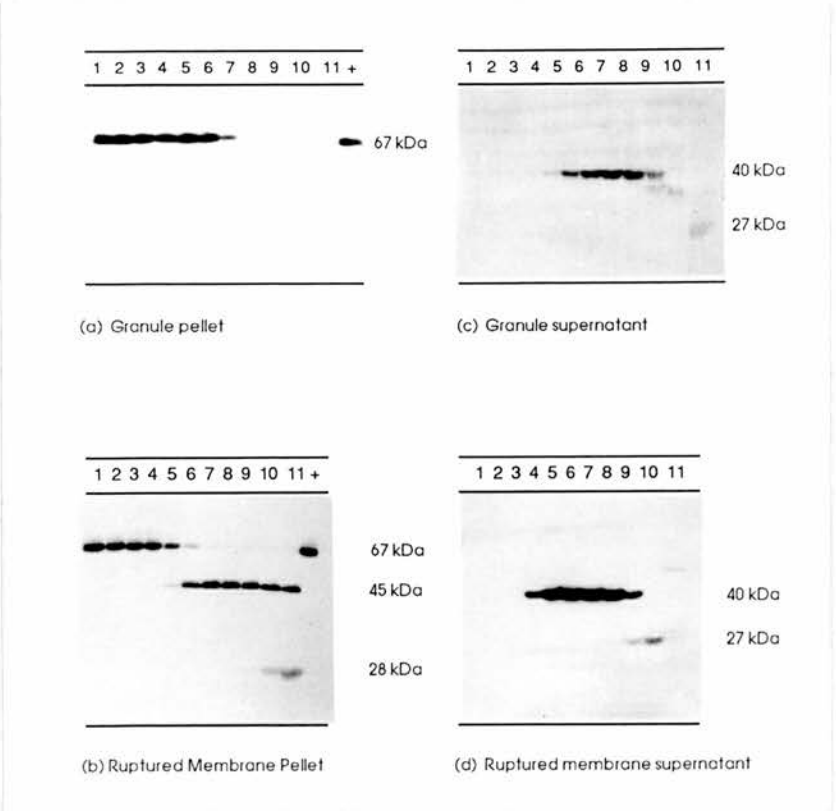
released from both intact granules and their broken membranes with as little as 0.01 μg/ml trypsin apparently by cleavage at a trypsin-hypersensitive site. As the trypsin concentration was increased from 0.01 to 25 μg/ml more of the 39-kDa fragment was released (Figures 5.5(c) & (d), tracks 4-10), but at higher concentrations (>25 μg/ml) this fragment began to be degraded to a 27-kDa soluble fragment (tracks 9-11). As the detection of the fragments depends upon their immunoreactivities, p65-derived polypeptides that do not contain the cgm67 epitope are not seen in these blots.

As well as these soluble products, trypsin treatment of broken membranes also generated membrane-bound cgm67-reactive peptides (Figures 5.5(b)). As with intact granules, on trypsin treatment of broken membranes the amount of residual (intact) p65 decreased as the trypsin concentration was increased from 0.01 to 2.5 μg/ml (Figures 5.5(b) tracks 1-6), and a new membrane-bound form of apparent molecular weight 45-kDa appeared. This 45-kDa species was *not* seen when intact granules were incubated with trypsin (Figure 5.5(a)), nor was any other membrane-bound cgm67-immunoreactive product. At higher trypsin concentrations, the 45-kDa peptide was converted to a 28-kDa form (Figure 5.5(b), tracks 8-10).

These results are consistent with the release of a large (39-kDa, immunoreactive) domain of p65 into the soluble fraction from both broken membranes and intact granules, and the release of a smaller, lumenal domain (22-kDa, and non-immunoreactive) from broken membranes only. The cgm67 epitope is present on the larger domain which is cytoplasmically oriented, as it is released from intact chromaffin granules by protease treatment, and no membrane-bound degradation product can then be detected (Figures 5.6(a)). The 39-kDa cytoplasmically oriented tryptic peptide has a single isoelectric point of 6.4 (Figure 5.6).

Figure 5.5

Trypsin degradation of bovine p65:



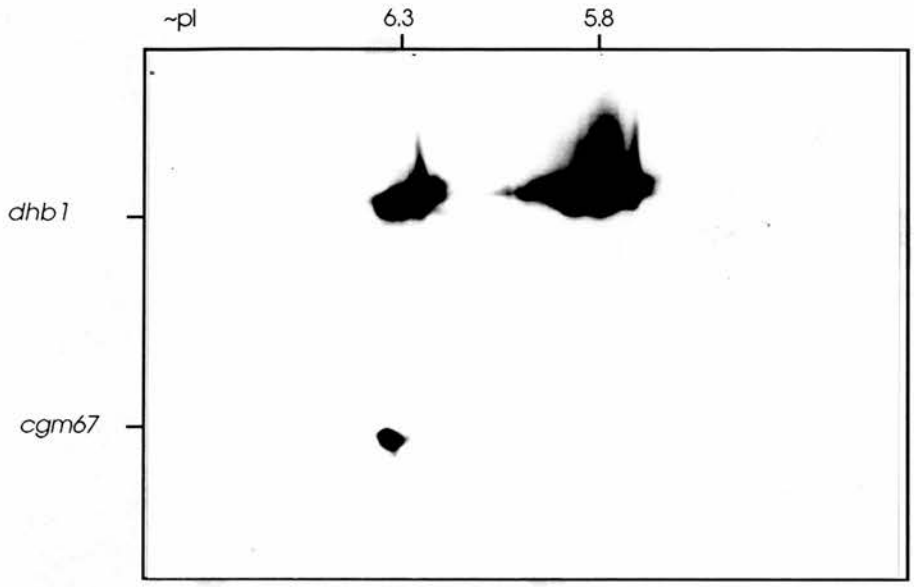
Intact chromaffin granules or chromaffin granule membranes were incubated with a range of trypsin concentrations (**Tracks 1**: 0.01 μg/ml: **Tracks 2**: 0.03 μg/ml; **Tracks 3**: 0.09 μg/ml; **Tracks 4**: 0.27 μg/ml; **Tracks 5**: 0.81 μg/ml; **Tracks 6**: 2.5 μg/ml; **Tracks 7**: 7.4 μg/ml; **Tracks 8**: 22 μg/ml; **Tracks 9**: 67 μg/ml; **Tracks 10**: 200 μg/ml; **Tracks 11**: 600 μg/ml) and the soluble and insoluble (membrane-bound) fractions were examined separately by immunoblotting with the monoclonal antibody *cgm67*. **Tracks** "**+**" are controls without trypsin.

(a) & **(b)** show the insoluble (membrane-bound) tryptic peptides generated;

(c) & **(d)** show the soluble tryptic peptides generated.

Figure 5.6

The 39-kDa cytoplasmic domain of bovine p65.



Soluble peptides derived from limited trypsin treatment of chromaffin granule membranes (1 µg trypsin per mg/ml of membranes) were separated by two-dimensional electrophoresis and immunoblotted using the monoclonal antibody *cgm67*. The *cgm67* immunoblot was reincubated with the monoclonal antibody *dbh1* to establish the isoelectric point of the 39-kDa tryptic peptide of bovine p65. *dbh1*-immunoreactivity is resistant to trypsin treatment at 1 µg trypsin per mg/ml of membranes (see Figure 5.4**(d)**). The 39-kDa tryptic peptide of bovine p65 has a single isoelectric point of 6.4.

[f] *Purification and partial aminoacid sequence of the cytoplasmic domain of bovine p65* :

The presence of the large cytoplasmic domain of bovine p65 suggested that this domain may be important in its *in vivo* function. Since this domain could be solubilised by mild trypsin treatment of chromaffin granule membranes, its purification was undertaken so that its biochemical characterisation and studies of its function could be initiated. The purification of this domain would also allow a partial aminoacid sequence to be obtained which would permit oligonucleotide primers to be made for use in the polymerase chain reaction, so that the corresponding DNA sequence could be obtained from a bovine adrenal cDNA library.

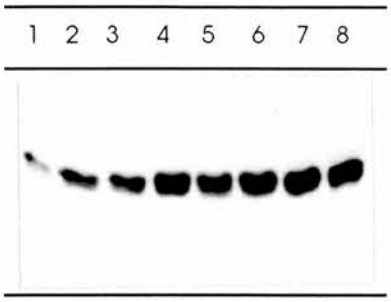
The results in Figure 5.5(d) show that the optimal amount of trypsin required to solubilise the 39-kDa cytoplasmic domain of bovine p65 was around 1 μg in a 1mg/ml solution of chromaffin granule membranes. Using these conditions, the yield of the cytoplasmic domain from chromaffin granule membranes was maximal between 40 and 60 minutes of incubation at 37°C at which times little residual 39-kDa fragment remained in the membranes (Figures 5.7(a) & (b)). At the end of the incubation, the trypsin treatment was inhibited with 50 μM TLCK and 200μM PMSF. The soluble tryptic peptides were separated from the membranes by centrifugation and the supernatant was adjusted to 1.25 M ammonium sulphate with ice cold 3 M ammonium sulphate in 10 mM phosphate (pH 7.0) and loaded on a phenyl-5PW-TSK hydrophobic interaction column.

Under these conditions the 39-kDa fragment bound to phenyl-5PW, whereas most other polypeptides did not. It was eluted from the column at a concentration of ammonium sulphate of 700 mM (Figure 5.8(a)). The presence of this polypeptide was assayed by dot-blotting the fractions and

Figure 5.7

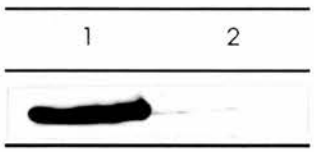
Generation of the cytoplasmic domain of bovine chromaffin granule p65.

(a) Time-course of p65 cytoplasmic domain generation:



Chromaffin granule membranes were incubated with 1 μg/ml trypsin as described in Chapter 2, Section 2.3[e] and the soluble peptides generated were analysed by immunoblotting using the monoclonal antibody *cgm67*. **Track 1**: 0 min.; **Track 2**: 10 min.; **Track 3**: 20 min.; **Track 4**: 30 min.; **Track 5**: 40 min.; **Track 6**: 50 min.; **Track 7**: 60 min.; **Track 8**: 120 min.

(b) Generation of p65 cytoplasmic domain by multiple rounds of trypsin-treatment:

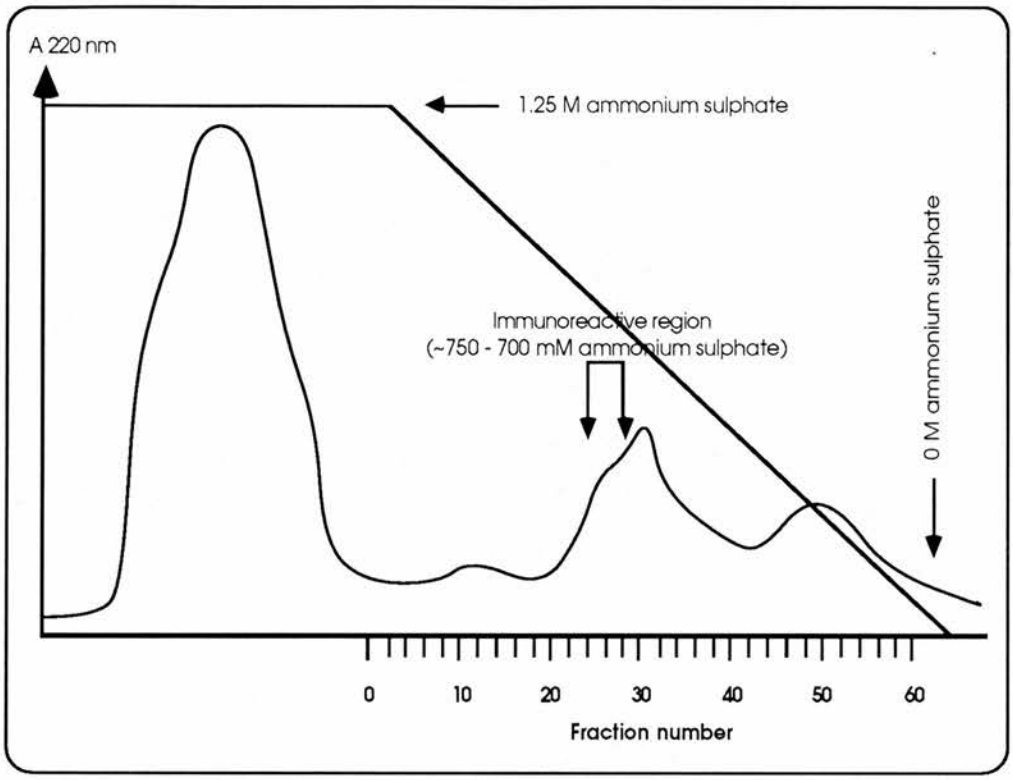


Chromaffin granule membranes were incubated with 1 μg/ml trypsin for 60 minutes at 37°C. The digestion was terminated by incubation on ice and the soluble tryptic peptides were separated from the chromaffin granule membranes by centrifugation. The membranes were then reincubated with 1 μg/ml fresh trypsin for 60 more minutes at 37°C. The generation of the cytoplasmic domain of bovine p65 was analysed by immunoblotting using the monoclonal antibody *cgm67*. **Track 1**: p65 cytoplasmic domain generated after one round of trypsin-treatment; **Track 2**: p65 cytoplasmic domain generated after a second round of trypsin-treatment.

Figure 5.8

Purification of the cytoplasmic domain of bovine p65.

(a) Hydrophobic interaction chromatography profile:



(b) Immuno-dot assay of the fractions:

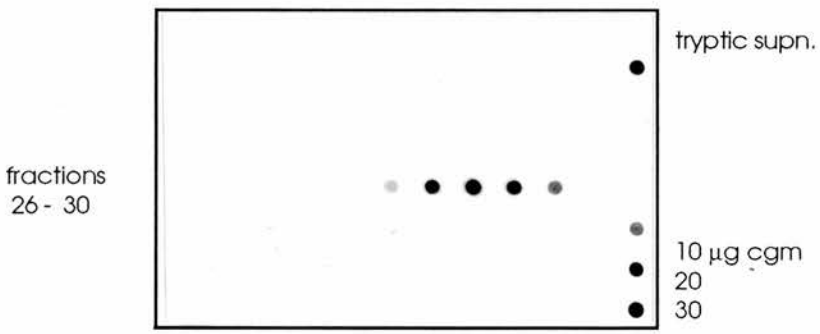
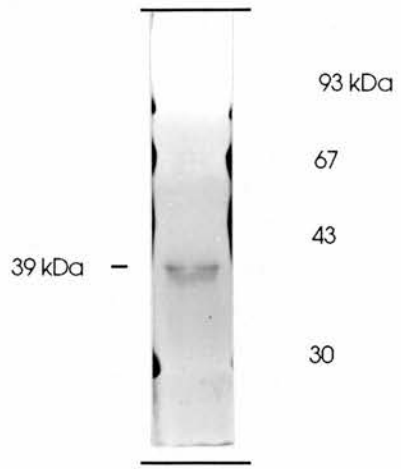


Figure 5.8

Purification of the cytoplasmic domain of bovine p65.

(c) Polypeptide composition of cgm67-reactive fraction:



Purification of the cytoplasmic domain of bovine p65:

(a) Soluble tryptic peptides obtained from bovine chromaffin granule membranes were applied to a phenyl-5PW-TSK hydrophobic interaction chromatography column at an ammonium sulphate concentration of 1.25 M (in 50 mM phosphate buffer, pH 7.0). Unbound peptides were washed off the column with 1.25 M ammonium sulphate/ 50 mM phosphate buffer (pH 7.0) and the bound peptides were eluted with a 30 ml linear gradient of ammonium sulphate. The protein concentration of the column eluate was monitored at 220 nm.

(b) The eluted peptides were assayed for the *cgm67* epitope by immuno-dot-blotting. The *cgm67*-reactive peptides eluted from the column at around 750 - 700 mM ammonium sulphate.

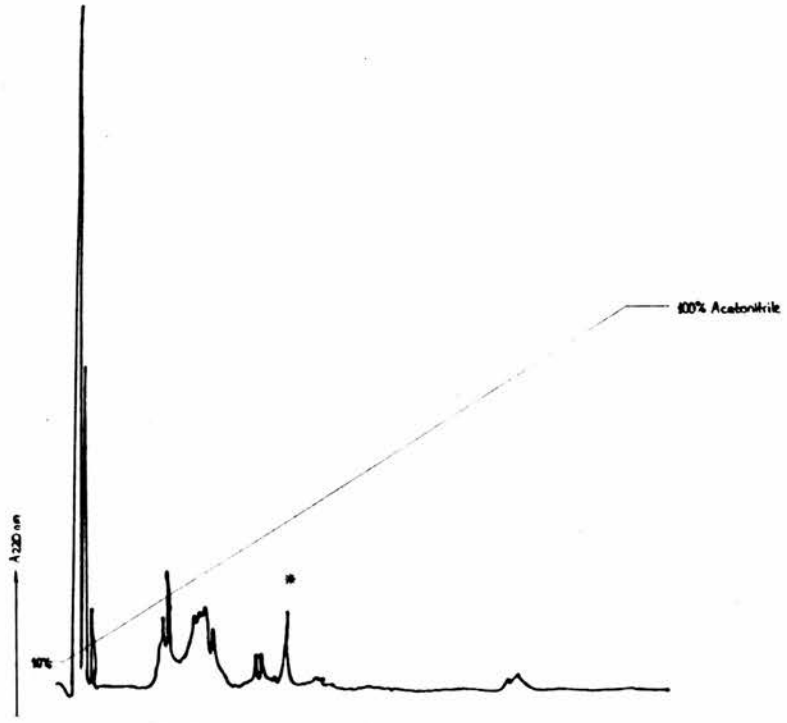
(c) The cgm67-reactive fractions were pooled and extensively dialysed to remove ammonium sulphate, concentrated by rotary evaporation for further purification using a C18 reverse-phase HPLC column. The polypeptide composition of the cgm67-reactive fractions were determined by SDS-PAGE.

(d) The pooled cgm67-reactive fractions were further fractionated using a C18 reverse-phase HPLC column and the composition of each protein peak was determined by SDS-PAGE (**(e)**); *marks *cgm67*-reactive peak; "+" marks pooled active fraction from **(c)**.

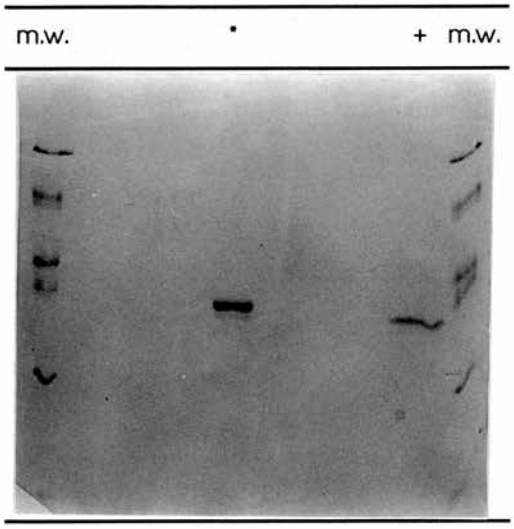
Figure 5.8

Purification of the cytoplasmic domain of bovine p65.

(d) C 18 reverse-phase HPLC profile:



(d) Polypeptide composition of the fractions obtained from C 18 reverse-phase HPLC:



probing with the monoclonal antibody cgm67 (Figure 5.8(b)).

The cgm67-reactive fractions were pooled and extensively dialysed against 0.1 mM phosphate buffer (pH 7.0) to remove ammonium sulphate. Figure 5.8(c) shows that the 39-kDa cytoplasmic domain was free of most other polypeptides at this stage and was immunoreactive. The dialysed fraction was concentrated by rotary evaporation and bound to a C18 reverse-phase column in 10% acetonitrile/0.1% trifluoroacetic acid in water. In contrast to the composition shown on gels, the HPLC profile of the pooled fraction suggested a complex mixture of peptides (Figure 5.8(d)). The 39-kDa polypeptide eluted from the column as a single peak at 45% acetonitrile. When this fraction was checked for purity by SDS-PAGE, it appeared as a doublet (Figure 5.8(e)). Both polypeptides were immunoreactive with cgm67 and were therefore sequenced together by automated Edman degradation. Table 5.1 shows the N-terminal aminoacid sequence of the 39-kDa polypeptide.

[g] ***Calmodulin binding site of bovine p65 is cytoplasmically oriented*** **:**

The putative role of calmodulin in secretion has been discussed in Chapter 1. It has been shown that calmodulin binds to chromaffin granule membrane proteins of apparent molecular weights 50-53 and 65-69-kDa [Burgoyne & Geisow, 1981; Hikita *et al.*, 1984]. Although the latter protein has been identified as p65 by immunological methods, the techniques used by these authors to determine calmodulin-binding were not able to resolve the orientation of the calmodulin binding site, nor determine whether the calmodulin receptors were integral membrane components. If calmodulin is indeed involved in exocytosis, it must bind specifically only to the cytoplasmic domains of the above proteins.

Table 5.1

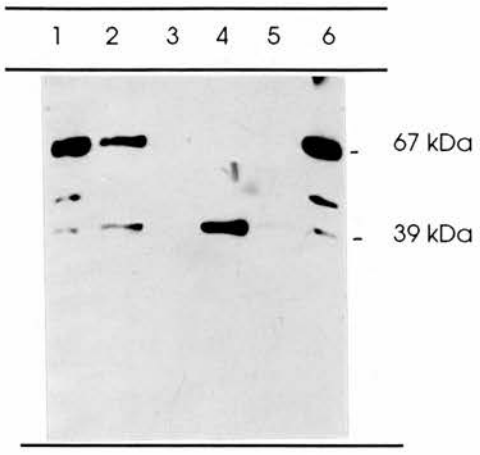
N-terminal aminoacid sequence of the 39-kDa tryptic peptide of bovine p65.

Cycle	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Aminoacid	**T**	**M**	**K**	**D**	**Q**	**A**	**L**	**K**	**D**	**D**	**D**	**A**	**E**	**T**	**G**	**L**	**T**	**D?**	**G**	**E**
Yield (pmol)	28	19	17	14	28	26	28	11	8	10	6	17	9	9	15	7	5	2	7	8
Rat p65	T	M	K	D	Q	A	L	K	D	D	D	A	E	T	G	L	T	D	G	E

About 32 pmol of peptide was loaded on to the sequencer, and the aminoacid recoveries are corrected for background. In each case the aminoacid quoted is that with the highest recovery in that cycle. The sequence of rat p65 (residues 112 - 131) is shown for comparison (see also Appendix I).

Figure 5.9

Binding of bovine p65 and its cytoplasmic domain to calmodulin.



The aqueous phase derived from Triton X-114 fractionated chromaffin granule membranes (**Tracks 1** and **6**) was incubated with immobilised calmodulin in the presence of 100 μM (total) Ca^{2+} and eluted with 5 mM EGTA. **Track 2**: eluate; **Track 3**: eluate when when 2 mM EGTA was included during the incubation. **Track 4** and **Track 5** show a similar experiment in which the cytoplasmic domain of p65, liberated by treatment of chromaffin granule membranes with trypsin (see Figures 5.5 & 5.6) was incubated with immobilised calmodulin. Proteins eluted were visualised by immunoblotting with the monoclonal antibody *cgm67*.

In previous sections of this Chapter it was shown that the cytoplasmic domain of bovine p65 could be solubilised by protease treatment, and that cgm67 recognises an epitope on this domain. To investigate the position of the calmodulin-binding site of p65, both the "aqueous" fraction of Triton X-114-treated chromaffin granule membranes (containing intact p65) and trypsin-solubilised peptides from these membranes were applied to calmodulin-agarose in the presence of 100 μM (total) Ca^{2+}. Following extensive washing with 10 mM Hepes-NaOH (pH 7.2) containing the same total calcium ion concentration, the bound proteins were eluted with 5 mM EGTA in 10 mM Hepes-NaOH (pH 7.2). Analysis of the EGTA-eluate by immunoblotting with cgm67 (Figure 5.9) showed that both p65 and its 39-kDa cytoplasmic domain bound to immobilised calmodulin. The binding of both polypeptides was abolished if 2 mM EGTA was included in the binding buffer. A 40-kDa cgm67-immunoreactive peptide present in the aqueous fraction also bound to immobilised calmodulin but that of ~50-kDa did not.

[h] ***p65 from sources other than bovine chromaffin granule membranes*** *:*

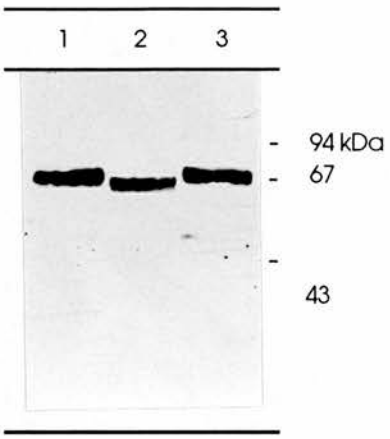
The monoclonal antibody cgm67 reacted with p65 from both human and rat sources. Figure 5.10 shows an immunoblot with cgm67 against secretory vesicle membranes prepared from bovine adrenal medulla, human phaeochromocytomas and synaptosomes from rat forebrain. The human and bovine p65 migrated with an apparent molecular weight of 67-kDa in contrast to rat p65 which was at 65-kDa, in agreement with previous reports [Matthew *et al.*, 1981].

When secretory vesicle membranes were fractionated with the non-ionic detergent Triton X-114 at 30°C, human p65 fractionated

Figure 5.10

p65 from sources other than the bovine adrenal medulla.

Comparison of the molecular weights of p65:



Preparations of bovine chromaffin granule (**Track 1**), rat synaptic vesicles (**Track 2**) and chromaffin granule membranes from human phaeochromocytoma (**Track 3**) were separated by SDS-PAGE and immunoblotted with the monoclonal antibody *cgm67*.

predominantly into the "detergent-rich" phase in contrast to bovine p65 which fractionated in the "aqueous" phase (Figure 5.11(a)). The endogenous 50-kDa cgm67-reactive peptide derived from human p65 also fractionated like the intact protein but an endogenous 40-kDa cgm67-reactive peptide (presumably the cytoplasmic domain) behaved as a soluble protein and fractionated into the aqueous phase. When rat forebrain synaptosomes were fractionated with the detergent Triton X-114 using the same protocol as that for chromaffin granule membranes, p65 appeared in all fractions (Figure 5.11(b)). This result and the high protein content of the "phospholipid-rich" fraction suggested that fractionation was incomplete under the conditions used. Further studies to optimise the conditions for Triton X-114 fractionation of synaptosomes were not done.

The partitioning pattern of human p65 in Triton X-114 implies that it is less hydrophilic than its bovine counterpart. This behaviour and the molecular weight difference between the species could be because of sequence divergence or different glycosylation of the intralumenal domain since the aminoacid sequence of the cytoplasmic domain of p65 is highly conserved [Perin *et al.*, 1991*a*] (see later).

Figure 5.11

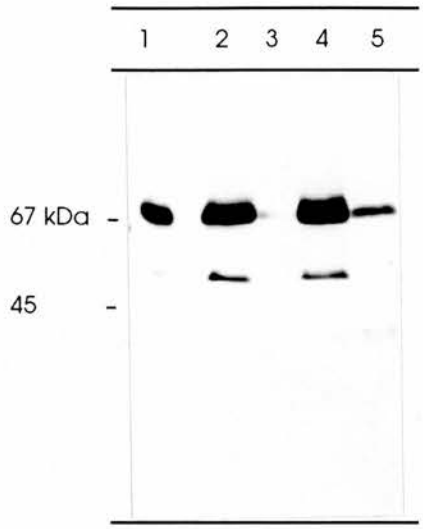
Fractionation of p65 from sources other than the bovine adrenal medulla by Triton X-114.

Immunoblots showing the distribution of *cgm67* immunoreactivity (p65) on fractionation of chromaffin granule membranes from human phaeochromocytomas **(a)** or synaptic vesicles from rat fore-brain **(b)** with the non-ionic detergent Triton X-114. 20μg of each fraction was loaded per lane of a minigel. **Track 1**: bovine chromaffin granule p65; **Track 2**: human chromaffin granule p65; **Track 3**: P1 fraction from Triton X-114 treated human chromaffin granule p65; **Track 4**: detergent phase from Triton X-114 treated human chromaffin granule p65; **Track 6**: aqueous phase from Triton X-114 treated human chromaffin granule p65; **Track 7** & **12**: bovine p65; **Track 8**: rat synaptic vesicles; **Track 9**: P1 from Triton X-114 treated rat synaptic vesicles; **Track 10**: detergent phase from Triton X-114 treated rat synaptic vesicles; **Track 11**: aqueous phase from Triton X-114 treated rat synaptic vesicles.

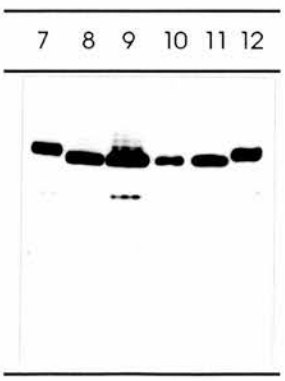
Figure 5.11

Fractionation of p65 from sources other than the bovine adrenal medulla by Triton X-114.

(a) p65 from human phaeochromocytomas:



(b) p65 form rat synaptic vesicles:



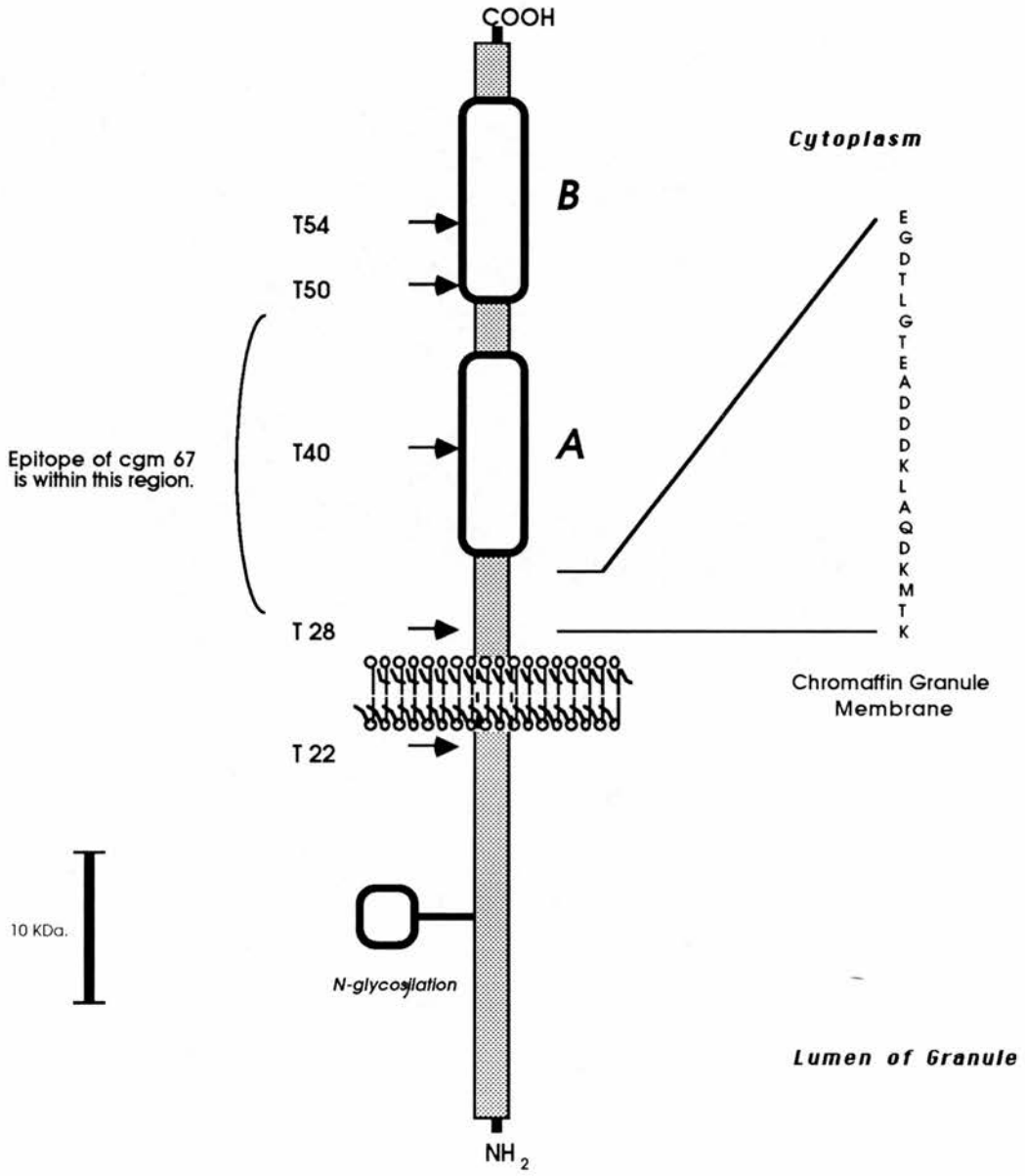
5.3 Discussion & Conclusions

Experiments with the monoclonal antibody cgm67 have revealed a 67-kDa transmembrane glycoprotein of chromaffin granules which binds calmodulin. This protein appears to be homologous to p65, the protein from rat synaptic vesicles described by Matthew *et al.* (1981). The epitope recognised by cgm67 was localised to the cytoplasmic domain of the protein both by protease treatment and subcellular fractionation of intact granules on granule/antibody gradients.

The structure of bovine p65 was analysed by limited trypsin digestion of both intact granules and their ruptured membranes followed by the analysis of the fragments which continued to bind cgm67. These results are summarised in Figure 5.12 which shows a schematic structure of bovine chromaffin granule membrane p65. The trypsin cleavage sites shown in this figure are numbered according to their apparent distance (in kDa) from the N-terminus. The membrane-bound 45-kDa fragment is produced by cleavage of p65 at T22; that of 28-kDa by a further cleavage at T50. The soluble 39-kDa fragment is produced by cleavage at T28, and the soluble 27-kDa fragment is produced from this by removal of a 12-kDa fragment, either from its N-terminus (cleavage at T40) or its C-terminus (cleavage at T55), assuming it is generated by a single cut. The hypersensitivity of sites T22 and T28 to trypsin means that no membrane-bound tryptic fragment of molecular weight greater that 45-kDa is detected by cgm67. The 39-kDa fragment includes most of the cytoplasmic domain of p65 and contains the cgm67 epitope between T28 and T50; more precise location is not possible without further fragmentation and sequencing. The epitope recognised by asv48, the monoclonal antibody used in the original identification of p65, was also

Figure 5.12

Schematic diagram of bovine chromaffin granule p65:



T22, T28, T40, T50 and T54 are trypsin cleavage sites; boxes A and B indicate regions which in rat p65 contain sequence similarity (39 %) to the C2 regulatory domain of protein kinase C. This diagram has been constructed from the data shown in Figure 5.5.

analysed in a similar manner. This antibody was able to bind to the same cgm67-immunoreactive tryptic peptides, however, asv48 did not react as strongly with the 27-kDa soluble and the 28-kDa membrane-bound tryptic peptides. This implies that the epitopes recognised by the two antibodies are not the same, but are localised to a common tryptic peptide. The structure predicted from the data above is consistent with the recently published cDNA sequence of rat brain p65 [Perin *et al.*, 1990], and is identical to the independent work of Perin *et al.* (1991*b*).

The biological function of p65 is unknown but it presumably involves its large cytoplasmic domain. Previous studies have shown that p65 binds calmodulin, an ubiquitous Ca^{2+}-binding protein which modulates the function of many proteins in response to the intracellular level of Ca^{2+}, and which has been implicated in the mechanism of exocytosis. In this Chapter, using the monoclonal antibody cgm67, it was confirmed that bovine p65 binds to immobilised-calmodulin. It was also shown that the calmodulin-binding site is located on the cytoplasmic of domain p65, and therefore accessible to calmodulin *in vivo*. It was found that an endogenous 40-kDa peptide of p65 also binds to immobilised calmodulin, but that one of ~50-kDa does not. The identity of the 40- and 50-kDa peptides are not established but the Triton X-114 fractionation of the 40-kDa peptide and its molecular weight are both consistent with its being the cytoplasmic domain of p65, probably generated by proteolytic cleavage at the hypersensitive site at T28. The ~50-kDa fragment partitions as a membrane-bound polypeptide and may be generated by proteolytic cleavage near T55, within the cytoplasmic domain of p65. If this is so, it implies that the calmodulin-binding site may be at the C-terminus of p65; however, using only cgm67 and without further fragmentation of the cytoplasmic domain of p65, it is not possible to

determine the exact location of this calmodulin-binding site. Using "^{125}I-calmodulin overlay" of SDS-gels, Trifaró and his co-workers were unable to find calmodulin-binding peptides of p65. This could either be because the calmodulin-binding site of p65 might be denatured and no longer able to bind ^{125}I-calmodulin when chromaffin granule membrane proteins are separated on SDS-gels and also because there may simply be not enough proteolytic peptides of p65 in their preparations.

The significance of the calmodulin binding is presumably to activate a specific, but as yet unknown, function of p65. One of the properties of rat p65 is the ability of its cytoplasmic domain to bind acidic phospho- and sphingolipids, and to agglutinate rabbit erythrocytes [Perin *et al.*, 1990]. The cDNA sequence of this domain has shown the presence of a tandem repeat homologous to the C2 regulatory domain of protein kinase C [Perin *et al.*, 1990]. The function of these domains are not clear, nor is whether or how these activities are linked to calmodulin-binding.

To study the cytoplasmic domain of p65, it was purified to homogeneity by hydrophobic interaction and reverse-phase chromatography. The N-terminal aminoacid sequence of this 39-kDa domain was determined by automated Edman degradation. The sequence of the first 20 aminoacids, which is shown in Table 5.2, was identical with residues 112 - 131 of the rat p65 sequence, following the potential trypsin cleavage site at lysine-111. This sequence lies between the proposed transmembrane span and the first region with homology to the C2 regulatory domain of protein kinase C (Figure 5.13). A minor peptide of molecular weight slightly less than 39-kDa which co-purified with the 39-kDa peptide was also sequenced - its sequence was identical but 3 aminoacids shorter at the N-terminus than that of the 39-kDa peptide and was probably generated by cleavage of p65 at the next lysine, *i.e.* 3

residues closer to the C-terminus. The aminoacid sequence data thus provided direct confirmation of the membrane orientation and the identity of the cgm67-reactive protein.

This aminoacid sequence identity between part of bovine and rat p65 and the fact that the monoclonal antibodies cgm67 and asv48 recognise p65 from different species suggests this protein is highly conserved. That this indeed is the case is shown in the recently-published p65 cDNA sequences from human and *Drosophila* [Perin *et al.*, 1991*a*]. When human and rat sequences are compared, within the 343 aminoacid cytoplasmic domain, only two residues are different. Sequence diversity in p65 is found only in the intragranular domain, which probably explains the differences in molecular weight and Triton X-114 fractionation patterns observed. The molecular weight of bovine and human p65 was determined as 67-kDa, and that of rat as 65-kDa by immunoblotting with the monoclonal antibody cgm67 - these values are much larger than the polypeptide molecular weight calculated from the rat sequence, which is 47,576 Da [Perin *et al.*, 1990].

The sequence homology of p65 from different sources as diverse as human and *Drosophila*, its calmodulin- and phospholipid-binding properties, its ability to agglutinate red blood cells and the fact that the cytoplasmic domain may be phosphorylated *in vitro* all suggest that p65 performs an essential function *in vivo* [Perin *et al.*, 1990, 1991*a*, 1991*b*; Petrenko *et al.*, 1991; Tugal *et al.*, 1991]. Ultimately, it is hoped that the characterisation of p65 in this study, together with the use of the monoclonal antibody cgm67, will lead to the discovery of this function.

CHAPTER 6

Chapter 6

Summary, conclusions and perspectives

In cells such as those present in the adrenal medulla, the pituitary or in the nervous system which export their products through the regulated secretory pathway, these products are taken up, concentrated and stored in organelles called "*secretory granules*" (see Chapter 1). The secretory products remain within these granules until an external stimulus triggers their release out of the cell. The pharmacology of secretion and the intracellular events that lead up to exocytosis have been the subject of many studies and are reasonably well understood, however, the mechanisms by which these signals are translated into the movement of secretory granules to the plasma membrane, and by which the granules dock and fuse with it are not understood. Our lack of understanding of these events is because of our limited knowledge about the proteins that are involved in these processes. It is assumed that at least part of this "*exocytic machinery*", together with other proteins involved in the organisation and trafficking of secretory vesicles are integral components of their membranes. Within the last decade, such an hypothesis has led to an increasing number of studies being carried out on secretory vesicle membrane proteins, including studies of their phosphorylation states, their ability to bind either Ca^{2+} or Ca^{2+}-calmodulin and their ability to interact with intracellular components such as cytosolic proteins, the cytoskeleton and other organelles (see Chapter 1). Another, and particularly successful approach, has been the immunological characterisation of secretory vesicle membrane proteins, in particular those present in synaptic vesicle membranes. The success of these studies has greatly depended on the development of the hybridoma technology by Köhler & Milstein (1975), because it allows the generation of specific monoclonal antibodies against

components of secretory vesicles which are otherwise inaccessible by standard biochemical techniques.

The aim of the present study was the immunological characterisation of the membranes of *chromaffin granules*, the secretory vesicles of adrenal chromaffin cells, in order to identify proteins which might mediate the process of exocytosis or organisation of the granule.

The immunological characterisation was initiated by raising monoclonal antibodies against the membrane proteins of bovine chromaffin granules, and in particular, against those components which are not obviously involved in the biosynthesis of secretory material. As stressed in Chapters 3 and 4, the success of this approach depended on the combination of using membrane-derived fractions capable of generating useful antibodies, and on the development of sensitive immunoassays which could identify them.

The two fractions successfully used as immunogens in raising monoclonal antibodies against chromaffin granule membrane proteins were first, a fraction enriched in membrane glycoproteins derived from chromaffin granule membranes by fractionating them with the non-ionic detergent Triton X-114, and second, a fraction of soluble tryptic peptides derived only from integral membrane components (see Chapter 3). Hybridomas generated as a result of immunising mice with these antigens were screened using immunoassays developed to be sensitive enough to identify any antibody raised against a component of the immunisation mixture. These immunoassays were in the form of ELISAs and dot-blots (see Chapter 4).

As a result of these approaches three monoclonal antibodies (*cgm67*, *cgm80* and *dbh1*) were obtained. These monoclonal antibodies were directed against the integral membrane proteins p65, glycoprotein II and against the biosynthetic enzyme involved in the synthesis of catecholamines, dopamine

β-monooxygenase (Chapter 4). As well as these, the monoclonal antibodies 13.10 and 83.6 were raised by using a fraction enriched in a group of cytosolic proteins that bound to chromaffin granule membranes in the presence of elevated Ca^{2+} concentrations.

The cgm67-reactive antigen was characterised in detail (Chapter 5). It was established that cgm67 bound to an integral membrane protein of molecular weight 67-kDa and pI 5.4 - 6.2 present in adrenal chromaffin granule membranes of both cow and humans. The cgm67-antigen was also present in rat synaptic vesicle membranes. It was also established that cgm67-antigen was the bovine homologue of the rat synaptic vesicle protein p65 which had been identified previously by Matthew *et al.* (1981).

The structure of bovine p65 was analysed by using proteases and oligosaccharide-cleaving enzymes. The results showed that p65 was an integral membrane glycoprotein of chromaffin granules which spanned the membrane once with its C-terminal two-thirds exposed to the cytoplasm (see Figure 5.13). It was found that the cytoplasmic domain of bovine p65 could be solubilised by mild trypsin digestion as a 39-kDa peptide, and that this domain bound both cgm67 and Ca^{2+}-calmodulin (Chapter 5). This domain was purified and its N-terminus was partially sequenced by automated Edman degradation; the 20-aminoacid stretch was found to be identical to the corresponding region of rat p65 as determined from its cDNA sequence [Perin *et al.*, 1990].

Recently, rat p65 has also been characterised by Perin *et al.* (1991*b*). Its structure is identical to that of bovine p65, and furthermore its sequence is 97% identical to human p65 [Perin *et al.*, 1991*a*]. The high degree of sequence homology of p65, particularly in the cytoplasmic domain, suggests that it mediates an important function mediated by this domain which is conserved across species, even down to *Drosophila* [Perin *et al.*, 1991*a*]. One striking

feature of the cytoplasmic domain of p65 is that it contains two regions in tandem showing 39% homology to the C2 regulatory domain of protein kinase C [Perin *et al.*, 1990]. This region has been shown been shown to bind acidic phospholipids [Perin *et al.*, 1991*b*]. Other *in vitro* activities of the cytoplasmic domain of p65 are its ability to bind Ca^{2+}-calmodulin [Tugal *et al.*, 1991] and to form homodimers [Perin *et al.*, 1991*b*]. It has also been suggested that this domain could bind Ca^{2+} directly, however no evidence has been presented for this claim [Perin *et al.*, 1991*b*].

Although the physiological relevance of Ca^{2+}-calmodulin binding by p65 has been dismissed by Südhof & Jahn (1991) without direct evidence, the fact that p65 binds Ca^{2+} (either directly, or indirectly as Ca^{2+}-calmodulin) implies that its activity could be responsive to changes in the intracellular levels of Ca^{2+}.

It has recently been shown that p65 is phosphorylated *in vitro*, and that this event is inhibited by the receptor for the black widow spider venom neurotoxin, α-latrotoxin [Petrenko *et al.*, 1991]. Since the physiological effect of this toxin is to enhance neurotransmitter release, Petrenko *et al.* have concluded that exocytosis could be mediated by the dephosphorylated form of p65. Such observations fit in with the requirement of ATP as a "primer" of exocytosis and the importance of dephosphorylation as a step preceding exocytosis (see Chapter 1, Section 1.6[b]). If p65 does have an *in vivo* role in mediating exocytosis, this function whatever it may be, must be regulated such that p65 is only functional under conditions which trigger exocytosis. The combination of the *in vitro* activities displayed by p65 suggests that it may indeed have a role in exocytosis and it has been suggested by Fournier & Trifaró (1988*b*) and Südhof & Jahn (1991) that this role could be in the docking of secretory vesicles to the plasma membrane (see Section 1.4[b](a)).

The ultimate aim in the characterisation of p65 is to establish whether

p65 is involved in exocytosis, and for this its biochemistry must be studied using a functional assay which reconstitutes exocytosis. A suitable and a well-characterised assay to study the role of p65 in exocytosis is the permeabilised chromaffin cell system (see Section 1.8[a]). Since the function of p65 appears to be through its cytoplasmic domain, in such a system, this domain is accessible to manipulation. Such studies should proceed along the following routes:

(1) Using the N-terminal partial aminoacid sequence of the cytoplasmic domain of p65, oligonucleotide probes should be designed so that the corresponding cDNA may be isolated from a bovine adrenal cDNA library using the polymerase chain reaction. The isolated cDNA for the cytoplasmic domain of p65 could then be cloned into a vector so as to express a fusion protein (e.g. a protein A/p65 chimera) which would allow the purification of the this domain. Similarly, the cDNA for the cytoplasmic domain could be used to dissect this domain into "functional" units which could also be expressed and purified. These peptides could then be introduced into permeabilised chromaffin cells either as fusion proteins, or after proteolytic cleavage from the chimeras to determine their effects on Ca^{2+}-dependent exocytosis. Inhibition of exocytosis would mean that the recombinant cytoplasmic domain is competing with the endogenous p65 and by using the smaller recombinant portions of this domain, the important sections of p65 may be established.

(2) Another approach to determine the role of p65 in exocytosis could be through making polyclonal antibodies against both the recombinant cytoplasmic domain of p65 and its peptides and determining their effects (and of cgm67) on Ca^{2+}-dependent exocytosis in the permeabilised chromaffin cell system.

(3) Whether p65 is phosphorylated *in vivo*, and whether such an event

controls its function must be determined. These studies could be carried out on ^{32}P-labelled intact chromaffin cells. By using mild trypsin digestion followed by immunoprecipitation, the phosphorylation state of the cytoplasmic domain of endogenous p65 could be analysed using one- and two-dimensional gels in cells before and after stimulation.

(4) To determine whether phosphorylation controls the function of p65, the effect of introducing recombinant phospho- and dephospho-p65 cytoplasmic domains into permeabilised chromaffin cells could be studied by determining whether they alter the Ca^{2+}-dependence of secretion.

(5) Finally the kinase(s) and phosphatase(s) which control the phosphorylation state of p65 *in vivo* should be established in order to determine the how exocytic signals are transmitted to the exocytic machinery of the cell. Initially this could be tackled by fractionating cytosol and assaying for its ability to phosphorylate the recombinant cytoplasmic domain of p65 or smaller recombinant portions of this domain. To establish the physiological relevance of the phosphorylation, the phospho-cytoplasmic domains could be assayed for their effect on Ca^{2+}-dependency of exocytosis as suggested in point (4) above. Establishing the residues that are phosphorylated would also determine whether p65 is serine- or tyrosine-phosphorylated.

The studies on the structure and *in vitro* activities of p65 in this study and by others have implicated its possible role in exocytosis. It is hoped that using this knowledge, the above aims can be investigated so that the possible role of p65 in mediating exocytosis can be discovered. It is also hoped that using the monoclonal antibody cgm80, the role of glycoprotein II can also be discovered.

APPENDICES

Appendix I

Aminoacid sequence of p65 from Human, Rat and *Drosophila* [a]

[a] Sequences from Perin *et al.* (1990) and Perin *et al.* (1991*a*)

Appendix II

Aminoacid codes and data

Amino Acid	Abbreviations		m.w.	Property
Alanine	Ala	**A**	89	Alliphatic
Arginine	Arg	**R**	174	Basic
Asparagine	Asn	**N**	132	Amide
Aspartate	Asp	**D**	133	Acid
Cysteine	Cys	**C**	121	Thiol
Glutamine	Gln	**Q**	146	Amide
Glutamate	Glu	**E**	147	Acidic
Glycine	Gly	**G**	75	Alliphatic
Histidine	His	**H**	155	Basic
Isoleucine	Ile	**I**	131	Alliphatic
Leucine	Leu	**L**	131	Alliphatic
Lysine	Lys	**K**	146	Basic
Methionine	Met	**M**	165	Thiol
Phenylalanine	Phe	**F**	165	Aromatic
Proline	Pro	**P**	115	Amide
Serine	Ser	**S**	105	Alliphatic alcohol
Threonine	Thr	**T**	119	Alliphatic alcohol
Tryptophan	Trp	**W**	204	Aromatic
Tyrosine	Tyr	**Y**	181	Aromatic alcohol
Valine	Val	**V**	117	Alliphatic

Appendix III

Published papers.

The work presented in this thesis has been published in the following papers:

Gillespie J., Ozanne S., Tugal H.B., Percy J.M., Warren M., Haywood J. & Apps D.K. (1991) The vacuolar H^+-translocating ATPase of renal tubules contains a 115-kD glycosylated subunit. *FEBS Lett.* **282**: 69-72.

Tugal H.B., van Leeuwen F., Apps D.K., Haywood J. & Phillips J.H. (1991) Glycosylation and transmembrane topography of bovine chromaffin granule p65. *Biochem. J.* **279**: 699-703.

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Ali S.M. & Burgoyne R.D. (1990) The stimulatory effect of calpactin (annexin II) on calcium-dependent exocytosis in chromaffin cells: Requirement of both the N-terminal and core domains of p36 and ATP. *Cell Signal.* **2**: 265-276.

Ali S.M., Geisow M.J. & Burgoyne R.D. (1989) A role for calpactin in calcium-dependent exocytosis in adrenal chromaffin cells. *Nature* **340**: 313-315.

Almers W. (1990) Exocytosis. *Annu. Rev. Physiol.* **52**: 607-649.

Apps D.K., Boisclair M.D., Gavine F.S. & Pettigrew G.W. (1984) Unusual redox behaviour of cytochrome b_{561} from bovine chromaffin granule membranes. *Biochem. Biophys. Acta* **764**: 8-16.

Apps D.K., Percy J.M. & PérezCastiñeira J.R. (1989) Topography of a vacuolar-type ATPase: The chromaffin granule membrane ATPase I. *Biochem. J.* **263**: 81-88.

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Apps D.K. & Schatz G (1979) Adenosine triphosphatase and adenosine diphosphate/ adenosine triphosphate isotope-exchange of the chromaffin-granule membrane. *Biochem. J.* **167**: 297-300.

Amsterdam A. & Jamieson J.D. (1974) Studies on dispersed pancreatic exocrine cells. II. Functional chatacterisation of separated cells. *J. Cell Biol.* **63**: 1057-1073.

Ahnert-Hilger G., Mach W., Fohr K.J. & Gratzl M. (1989) Poration by alpha-toxin and streptolysin O: An approsach to analyse intracellular processes. *Methods Cell Biol.* **31**: 63-90.

Asamer H., Hörtnagl H. & Winkler H. (1971) Immunohistochemical demonstration of a membrane protein of chromaffin granules in adrenal medulla and sympathetic nerves *Naunyn-Schmiedeberg's Arch. Pharmak.* **270**: 87-89.

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Glycosylation and transmembrane topography of bovine chromaffin granule p65

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The bovine homologue of p65, a calmodulin-binding protein located in the membranes of synaptic vesicles and endocrine secretory granules, has been studied by the use of monoclonal antibodies directed against this antigen and against dopamine β-mono-oxygenase. The protein (apparent molecular mass 67 kDa; pI = 5.5–6.2) is partially degraded by treatment with neuraminidase or endoglycosidase F. Trypsin treatment of intact adrenal chromaffin granules or of granule membranes releases a soluble 39 kDa fragment of p65 which corresponds to the whole of its cytoplasmic domain. This domain contains both the epitope for the monoclonal antibody cgm67 and the calmodulin-binding site. The 20 amino acids at the *N*-terminus of this fragment are identical to part of the rat p65 sequence.

INTRODUCTION

Although the process of regulated secretion is reasonably well characterized in pharmacological terms, its molecular details have yet to be elucidated. One approach is to use monoclonal antibodies, raised against secretory granule membranes, in the biochemical characterization of proteins on the granule surface. By this means several granule antigens have been recognized, although their functions have yet to be fully defined; these include p38 (synaptophysin; Wiedenmann & Franke, 1985), SV2 (Buckley & Kelly, 1985) and p65 (Matthew *et al.*, 1981).

This last protein was first identified as a component of rat brain synaptic vesicles, and monoclonal antibodies against it were found to precipitate chromaffin granules from bovine adrenal medulla (Lowe *et al.*, 1988). Subsequently p65 was shown to be identical with a 65 kDa calmodulin-binding protein that had already been identified in the membranes of chromaffin granules (Fournier & Trifaró, 1988; Fournier *et al.*, 1989*b*). The amino acid sequence of rat p65, deduced by sequencing a cDNA clone (Perin *et al.*, 1990), suggested that the protein had a single membrane-spanning sequence; its *N*-terminal domain contained a single putative *N*-glycosylation site and was predicted to lie within the lumen of the granule, whereas the cytoplasmic *C*-terminal domain contained two related sequences with sequence similarity to a regulatory domain of protein kinase C.

We now report some studies on the structure and topography of p65, using a monoclonal antibody (cgm67) raised against bovine chromaffin granules. Our results provide direct biochemical evidence in support of the structural model of Perin *et al.* (1990).

MATERIALS AND METHODS

Triton X-114 was obtained from Fluka AG, Buchs, Switzerland, and purified by the method of Bordier (1981). All other reagents were of AnalaR grade. Monoclonal antibody asv48 was kindly given by Dr. John Bixby (University of Miami, Miami, FL, U.S.A.).

Preparation of chromaffin granule membranes

Chromaffin granules were isolated from fresh bovine adrenal glands (obtained from the local slaughterhouse) by the method of Phillips (1974), and purified granule membranes were prepared from them according to Apps & Schatz (1979). All procedures were carried out at 4 °C in media buffered with 10 mM-Hepes/NaOH, pH 7.2, and containing 2 mM-EGTA, 1 mM-benzamidine and 0.2 mM-phenylmethanesulphonyl fluoride (PMSF). The membranes were finally resuspended in 10 mM-Hepes/NaOH (pH 7.2)/5 mM-dithiothreitol (DTT) to a protein concentration of 10 mg/ml.

Triton X-114 fractionation of chromaffin granule membranes

Chromaffin granule membranes were resuspended to 4 mg of protein/ml in Tris-buffered saline (20 mM-Tris/150 mM-NaCl), pH 7.4, with 2% (w/v, final) Triton X-114, and fractionation was carried out according to Pryde & Phillips (1986). Most experiments were carried out using the 'aqueous' phase, which contains residual Triton X-114. Removal of this detergent precipitated the chromaffin granule membrane glycoproteins, producing the 'glycoprotein-rich' fraction.

pH 11 treatment of membranes

Chromaffin granule membranes (approx. 1 mg/ml) were suspended in 0.1 M-Na_2CO_3, pH 11, containing 10 mM-EDTA. After 30 min at 0 °C the membranes were separated from solubilized proteins by centrifugation.

Preparation of antibodies

cgm67 and dbh1 monoclonal antibodies were prepared essentially as described by Bastin *et al.* (1982). Mice were immunized with the glycoprotein-rich Triton X-114 fraction of chromaffin granule membranes. Hybridomas were screened against the immunogen.

Calmodulin-affinity chromatography of chromaffin granule membrane glycoproteins

The aqueous fraction from Triton X-114 fractionation of chromaffin granule membranes was incubated for 20 min with calmodulin–agarose gel (Sigma) in 10 mM-Hepes/NaOH (pH 7.2)/0.1 mM-$CaCl_2$. After extensive washing in the same buffer, bound proteins were eluted by replacing $CaCl_2$ by 5 mM-EGTA. Controls were performed with the addition of 2 mM-EGTA during the binding incubation.

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; Tos-Lys-CH_2Cl, *N*-α-*p*-tosyl-2-lysine chloromethyl ketone ('TLCK'); DTT, dithiothreitol.

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Enzymic deglycosylation of gp67

N-Deglycosylation was carried out using endoglycosidase F (EC 3.2.1.96; Boehringer) and desialylation was carried out with neuraminidase (sialidase, EC 3.2.1.18; Sigma, type VI). For deglycosylation, 100 μg of the aqueous fraction from Triton X-114 fractionation of chromaffin granule membranes was lyophilized and resuspended with the following reagents to give a final protein concentration of 1.6 mg/ml: 50 mM-Mes/NaOH, pH 6.5, 0.5% deoxycholate, 1% Triton X-100, 5 mM-DTT, 15 mM-EDTA, pH 6.5, and 33 units of endoglycosidase F/ml. The digestion was carried out at 30 °C for 6 h in the presence of 5 mM-benzamidine, 200 μM-PMSF, 5 μg of leupeptin/ml, 5 μg of pepstatin/ml and 50 μg of Tos-Lys-CH_2Cl (*N*-α-*p*-tosyl-2-lysine chloromethyl ketone; TLCK)/ml. For digestion with neuraminidase, 100 μg of the Triton X-114 aqueous fraction was lyophilized and resuspended in 50 mM-Mes/NaOH, pH 6.0, to a final concentration of 1.6 mg/ml in the presence of 0.2% Triton X-100, 5 mM-DTT and the above proteinase inhibitors. Neuraminidase was added to a final concentration of 1 unit/ml and the reaction mixture was incubated at 30 °C for 6 h. The samples were lyophilized and dissolved in SDS sample buffer with 5 mM-DTT for analysis by gel electrophoresis and immunoblotting.

Proteinase digestion of chromaffin granule membranes

Chromaffin granule membranes were resuspended to 1 mg/ml and incubated with various concentrations of trypsin for 60 min at 20 °C in the presence of 2 mM-DTT. The digestions were stopped by the addition of 50 μg of Tos-Lys-CH_2Cl and 200 μM-PMSF. The soluble peptides were separated from the membranes by centrifugation (412000 $g_{av.}$, 15 min, 2 °C). The resulting pellet was washed twice by resuspension and centrifugation in 10 mM-Hepes/NaOH, pH 7.2, containing the same inhibitors. The supernatant fraction was recentrifuged as above to remove any residual membrane fragments. The membrane fractions were resuspended and then denatured by the addition of 0.25 vol. of hot (100 °C) pH 4.6 SDS sample buffer [0.2 M-potassium biphthalate, pH 4.6, 0.2 mM-EDTA, 20% (w/v) SDS, 40% (v/v) glycerol, 0.015% (w/v) Bromophenol Blue] in the presence of 5 mM-DTT. The soluble fractions were lyophilized and taken up in one-tenth of the original volume of 4-fold diluted SDS sample buffer (pH 4.6, as above). The samples were then analysed by electrophoresis and immunoblotting.

Electrophoresis and immunoblotting

One- and two-dimensional gel electrophoresis was performed as described by O'Farrell (1975) and by Gavine *et al.* (1984), with the exception that samples were lyophilized before use. Electrophoretic transfer to nitrocellulose was done according to Towbin *et al.* (1979). Incubation with primary antibody was carried out overnight, with bound antibody being detected either with ^{125}I-labelled second antibody or with biotinylated second antibody followed by ^{125}I-streptavidin.

Purification and partial amino acid sequencing of the cytosolic domain of p65

Chromaffin granule membranes (10 mg of protein in 10 ml of 10 mM-Hepes/NaOH, pH 7.2, 5 mM-DTT) were incubated with 10 μg of trypsin for 60 min at 20 °C, and the reaction was stopped by the addition of 50 μg of Tos-Lys-CH_2Cl/ml and 200 μM-PMSF. The membranes were pelleted and the supernatant was removed and made 1.25 M in ammonium sulphate and 50 mM in sodium phosphate, pH 7.0. This sample was applied to a hydrophobic interaction column (Phenyl-5PW; TSK) equilibrated in the same medium, washed with this medium and eluted with a 30 ml linear gradient of 1.25–0 M-ammonium sulphate in 50 mM-phosphate buffer, pH 7.0. Fractions were assayed for the cgm67 epitope by immunodotting, and active fractions containing an immunoreactive 39 kDa peptide were pooled, dialysed against distilled water and concentrated by rotary evaporation. Samples from six such runs were combined. The dialysed sample was made 10% in acetonitrile and 0.1% in trifluoroacetic acid, applied to a C_{18} reverse-phase h.p.l.c. column and eluted with a linear gradient of 10–70% acetonitrile in water containing 0.1% trifluoroacetic acid. The peak containing the cgm67-immunoreactive 39 kDa peptide was checked for purity by gel electrophoresis and Coomassie Blue staining, and was sequenced on an Applied Biosystems type 477 microsequencer by automated Edman degradation.

RESULTS AND DISCUSSION

The cgm67 epitope is on p65

The non-ionic detergent Triton X-114 may be used to fractionate membrane proteins according to their hydrophobicities (Pryde & Phillips, 1986). After fractionation of bovine chromaffin granule membranes by this method, the glycoprotein-rich fraction was used as the immunogen in the preparation of a class IgM mouse monoclonal antibody, which was named cgm67. This antibody recognizes an epitope on a bovine chromaffin

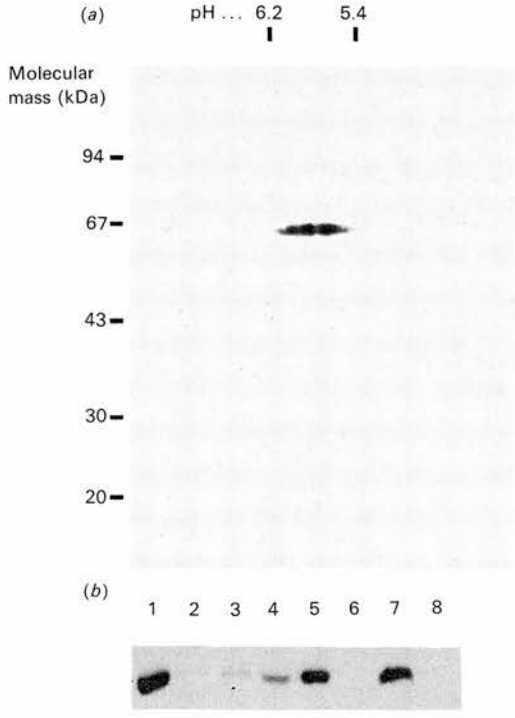

Fig. 1. Detection of cgm67-reactive epitopes in chromaffin granule membranes

(*a*) Immunoblot of a two-dimensional electrophoretogram of chromaffin granule membranes using the monoclonal antibody cgm67. (*b*) Immunoblot showing the distribution of cgm67 immunoreactivity on fractionation of chromaffin granule membranes with Triton X-114 (tracks 1–6) or washing at pH 11 (tracks 7 and 8). Track 1, chromaffin granule membranes; track 2, phospholipid-rich fraction; track 3, detergent-rich fraction; track 4, aqueous fraction; track 5, glycoprotein-rich fraction; track 6, supernatant remaining after removal of glycoproteins from aqueous fraction; tracks 7 and 8, pellet and supernatant obtained on washing chromaffin granule membranes at pH 11. In each track 15 μg of protein was loaded.

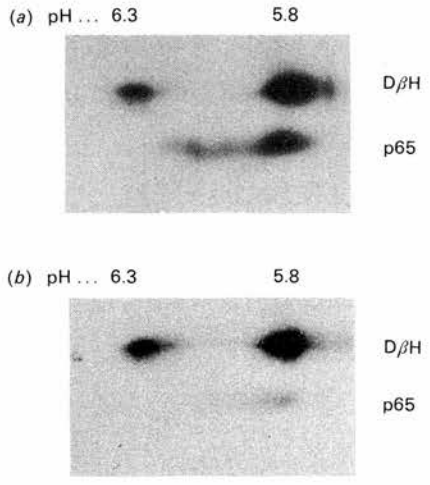

Fig. 2. Two-dimensional immunoblots

(*a*) and (*b*) show the position of protein p65 relative to that of dopamine β-mono-oxygenase (DβH, 75 kDa), and were developed with monoclonal antibodies cgm67 (*a*) and asv48 (*b*), together with dbh1, a monoclonal antibody directed against dopamine β-mono-oxygenase.

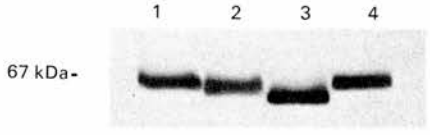

Fig. 3. Enzymic degradation of the oligosaccharide chains of p65

The aqueous fraction from Triton X-114 treatment of chromaffin granule membranes was treated with neuraminidase (track 2) or endoglycosidase F (track 3); the product was analysed by immune blotting with cgm67. Tracks 1 and 4 are controls, incubated without enzyme.

granule membrane protein of apparent molecular mass 67 kDa and pI 5.5–6.2 (Fig. 1*a*). The antigen was shown to be located in the granule fraction by subcellular fractionation, and its distribution paralleled that of dopamine β-mono-oxygenase (results not shown).

When chromaffin granule membranes were fractionated with Triton X-114, immunoreactivity with cgm67 appeared predominantly in the aqueous phase (Fig. 1*b*, track 4). Removal of residual Triton X-114 from this fraction concentrated the immunoreactivity in the insoluble glycoprotein-rich fraction (Fig. 1*b*, track 5), leaving the supernatant, which contained secretory proteins, devoid of immunoreactivity (Fig. 1*b*, track 6). The immunoreactive protein also remained membrane-bound on treatment with 100 mM-Na_2CO_3, consistent with it being an integral membrane protein (Fig. 1*b*, track 7).

The apparent molecular mass of the cgm67-reactive protein is similar to that of p65, a 65 kDa protein of rat synaptic vesicle membranes (Matthew *et al.*, 1981) also shown to be one of the calmodulin-binding proteins of bovine chromaffin granule membranes (Fournier & Trifaró, 1988). To determine whether the protein recognized by cgm67 was indeed p65, we obtained a calmodulin-binding fraction from solubilized chromaffin granule membranes by calmodulin–agarose affinity chromatography (see the Materials and methods section), separated the proteins by two-dimensional electrophoresis and blotted them both with cgm67 and with asv48, the monoclonal antibody originally used in characterizing p65 (Matthew *et al.*, 1981). A trace of dopamine β-mono-oxygenase was added to the material loaded on to the gel, and was used as an internal pI and molecular mass marker by detection with dbh1, a class IgG anti-(dopamine β-mono-oxygenase) monoclonal antibody. The positions of the asv48- and cgm67-immunoreactive spots were coincident, although that due to cgm67 was much stronger than that due to asv48 (Figs. 2*a* and 2*b*). As p65 has been shown to bind both asv48 and calmodulin and to be present in bovine chromaffin granule membranes (Fournier & Trifaro, 1988), we conclude that cgm67 binds to an epitope on this protein, although we found an apparent molecular mass of 67 kDa rather than the 65 kDa reported in the rat (Matthew *et al.*, 1981). Both of these values are much larger than the peptide molecular mass calculated from the rat sequence, which is 47576 Da.

Bovine chromaffin granule p65 is *N*-glycosylated

Incubation of the aqueous fraction of chromaffin granule membranes with endoglycosidase F or neuraminidase produced partial degradation of p65, as shown by SDS/PAGE. Neuraminidase, which removes terminal sialic acid residues from oligosaccharides, decreased the molecular mass of p65 by about 1 kDa (Fig. 3, tracks 1 and 2), whereas endoglycosidase F, which completely removes *N*-linked high-mannose and complex oligosaccharides, produced a greater decrease, of about 5 kDa (Fig. 3, tracks 3 and 4). We also found that p65 did not bind the lectins concanavalin A or wheatgerm agglutinin, but that it did bind peanut agglutinin after neuraminidase treatment (results not shown). Together, these results indicate that bovine p65 contains one or more *N*-linked oligosaccharide chains, terminating in sialic acid and galactose. The decrease in molecular mass on deglycosylation is not large enough to account for the discrepancy between the calculated molecular mass and that determined by SDS/PAGE (see above).

Isolation and partial amino acid sequencing of the cytosolic domain of bovine p65

Treatment of intact chromaffin granules or of their broken membranes with low concentrations of trypsin released soluble cgm67-reactive cleavage products, the major one being a fragment of about 39 kDa. As the trypsin concentration was increased from 0.1 to 20 μg/ml, more of the 39 kDa fragment was released (Figs. 4*c* and 4*d*, tracks 4–10), but at higher concentrations (> 20 μg/ml) this began to be degraded to a 27 kDa fragment (tracks 9–11). As the detection of the fragments depends upon their immunoreactivities, p65-derived polypeptides that do not contain the cgm67 epitope are not seen in these blots.

On trypsin treatment of broken membranes the amount of residual intact p65 decreased as the trypsin concentration was increased from 0.01 to 2.5 μg/ml (Fig. 4*b*, tracks 1–6), and a new membrane-bound form of molecular mass 45 kDa appeared, with a smaller amount of a 28 kDa form being seen at trypsin concentrations around 200 μg/ml (Fig. 4*b*, tracks 5–11). This 45 kDa species was not seen when intact granules were incubated with trypsin (Fig. 4*a*), nor was any other membrane-bound cgm67-immunoreactive product.

These results are consistent with the release of a large (39 kDa) domain of p65 into the soluble fraction from both broken membranes and intact granules and with the release of a smaller, luminal domain (22 kDa; non-immunoreactive) from broken membranes only. The cgm67 epitope is present on the larger domain, and this must be cytosolic, as it is released from intact chromaffin granules by trypsin, and no membrane-bound degradation product can then be detected (Fig. 4*a*). According to the structure prediction by Perin *et al.* (1990), this cytosolic domain is *C*-terminal to the membrane-spanning sequence (see Fig. 6).

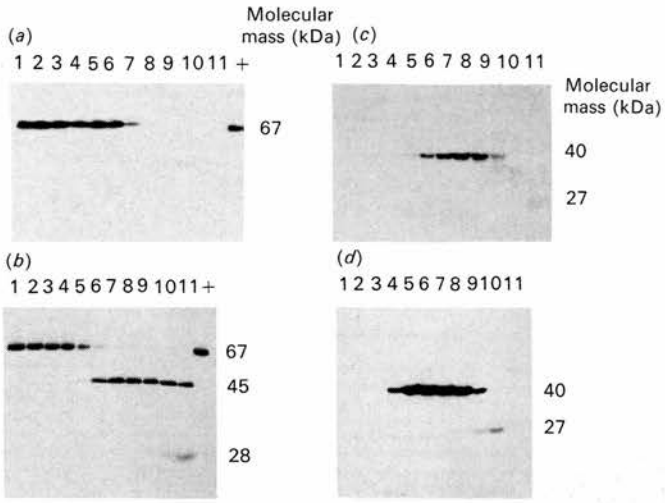

Fig. 4. Trypsin degradation of p65

Intact chromaffin granules or chromaffin granule membranes were incubated with a range of trypsin concentrations (μg/ml: track 1, 0.01; track 2, 0.03; track 3, 0.09; track 4, 0.27; track 5, 0.81; track 6, 2.5; track 7, 7.4; track 8, 22; track 9, 67; track 10, 200; track 11, 600) and the insoluble and soluble fractions were examined separately by immune blotting with cgm67. Tracks marked + are controls without trypsin. (*a*) and (*b*) show insoluble fractions from intact granules and granules membrane respectively; (*c*) and (*d*) show soluble fractions from intact granules and granule membranes respectively.

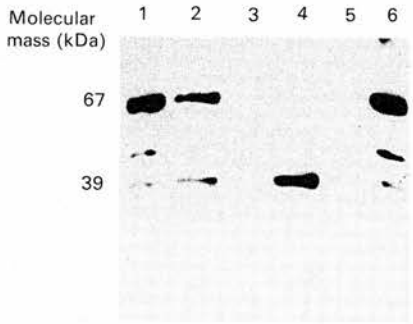

Fig. 5. Binding of p65 and its cytoplasmic domain to calmodulin

The aqueous fraction from Triton X-114 treatment of chromaffin granule membranes (tracks 1 and 6) was incubated with immobilized calmodulin in the presence of 100 μM-Ca^{2+} (total) and eluted with 5 mM-EGTA. Track 2, eluate; track 3, eluate when 2 mM-EGTA was included during the incubation. Tracks 4 and 5 show a similar experiment in which the cytoplasmic domain of p65, liberated by treatment of chromaffin granule membranes with trypsin (see Fig. 4*d*), was incubated with immobilized calmodulin. Proteins were visualized by immunoblotting with cgm67.

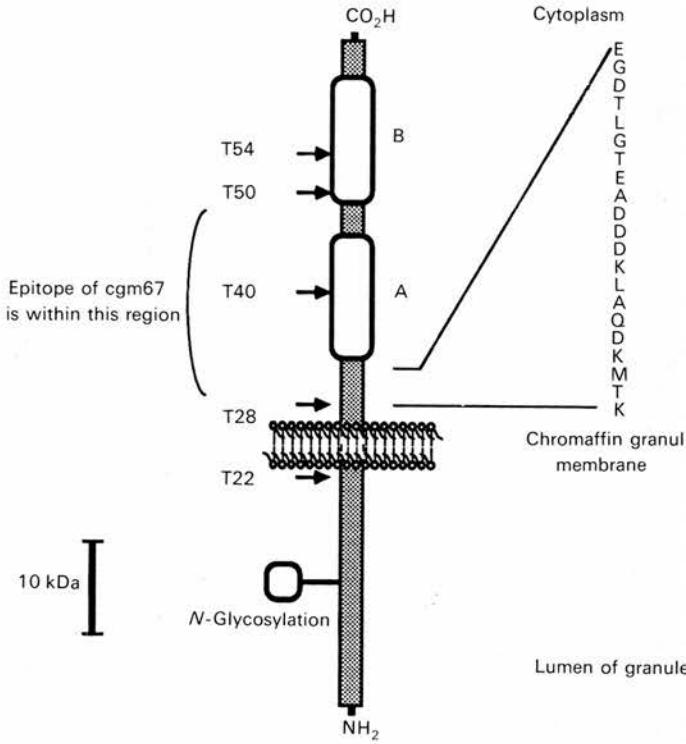

Fig. 6. Diagram of the structure of bovine p65

T22, T28, T40, T50 and T55 are trypsin cleavage sites; boxes A and B indicate regions which in rat p65 contain sequences similar to protein kinase C. For the amino acid sequence, see Table 1.

The 39 kDa tryptic fragment showed Ca^{2+}-dependent binding to immobilized calmodulin, from which it was eluted by EGTA (Fig. 5, track 4). An endogenous 39 kDa degradation product which was present in the aqueous phase from Triton X-114 fractionation (Fig. 5, track 1) behaved similarly (Fig. 5, track 2). A small amount of another (approx. 50 kDa) endogenous degradation product that was detectable in this fraction failed to bind to calmodulin (Fig. 5, tracks 1 and 2).

The 39 kDa tryptic fragment of p65 bound to a phenyl-5PW hydrophobic interaction chromatography gel in the presence of 1.25 M-ammonium sulphate (at which concentration most other peptides were not bound), and was eluted at about 700 mM-ammonium sulphate. Further purification of this fraction by reverse-phase h.p.l.c. on a C_{18} column gave a fraction containing only immunoreactive peptides, a major one having a molecular mass of 39 kDa and a minor one of slightly lower molecular mass. These peptides were sequenced together by automated Edman degradation (Table 1). The sequence of the first 20 amino acids of the major peptide is identical with residues 112–131 of the rat p65 sequence, which follow the potential trypsin-cleavage

Table 1. *N*-Terminal amino acid sequence of the 39 kDa soluble fragment derived from bovine p65 by proteolytic cleavage

About 32 pmol of peptide was loaded on to the sequencer, and the amino acid recoveries are corrected for background. In each case the amino acid quoted is that with the highest recovery in that cycle. The sequence of rat p65 (residues 112–131) is shown for comparison.

Cycle	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Amino acid	T	M	K	D	Q	A	L	K	D	D	D	A	E	T	G	L	T	D?	G	E
Yield (pmol)	28	19	17	14	28	26	28	11	8	10	6	17	9	9	15	7	5	2	7	8
Rat p65	T	M	K	D	Q	A	L	K	D	D	D	A	E	T	G	L	T	D	G	E

site at lysine-111. This sequence lies between the proposed transmembrane span and the first region, with sequence similarity with the C1 regulatory domain of protein kinase C (Fig. 6). The minor peptide was probably generated by cleavage of p65 at the next lysine (i.e. three residues closer to the *C*-terminus), as suggested by partial *N*-terminal sequencing.

The cleavage by trypsin of chromaffin granule p65 is consistent with results reported elsewhere (Fournier *et al.*, 1989*a*; Trifaró *et al.*, 1989) and with the sequence data for rat p65 (Perin *et al.*, 1990), and puts some restrictions on the location of the cgm67 epitope. The trypsin-cleavage sites shown in Fig. 6 are numbered according to their apparent distance (in kDa) from the *N*-terminus. The membrane-bound 45 kDa fragment (Fig. 4*b*, tracks 6–11) is produced by cleavage of p65 at site T22, and that of 28 kDa (Fig. 4*b*, tracks 10 and 11) by a further cleavage at T50. The soluble 39 kDa fragment (Figs. 4*c* and 4*d*, tracks 4–9) is produced by cleavage at T28, and the soluble 27 kDa fragment (Fig. 4*d*, tracks 9 and 10) is produced from this by removal of a 12 kDa fragment, from either its *N*-terminus (cleavage at T40) or its *C*-terminus (cleavage at T55), assuming that it is generated by a single cut. The cgm67 epitope must lie between T28 and T50; more precise location is not possible without further fragmentation and sequencing. In view of the large discrepancy between the molecular mass values determined by electrophoresis and by sequencing, the values quoted only give a rough idea of the relative sizes of these fragments. The extreme sensitivity of sites T22 and T28 to trypsin means that no membrane-bound tryptic fragment of molecular mass greater than 45 kDa is detected by cgm67.

The amino acid sequence data provide direct confirmation of the membrane orientation of p65. The sequence obtained is close to the start of the two regions similar in sequence to protein kinase C, which encompass most of the 39 kDa fragment. The calmodulin-binding domain lies within this fragment (Fig. 6), although it was not detected using the calmodulin overlay technique (Fournier *et al.*, 1989*b*; Trifaró *et al.*, 1989). The complete identity of the rat and bovine sequences for 20 amino acids indicates a strong pressure for conservation in this region, which suggests some role in the function of p65.

Note added in proof (received 27 August 1991)

The results reported here are in agreement with those of a recently published study on the domain structure of p65 (Perin *et al.*, 1991).

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The vacuolar H^+-translocating ATPase of renal tubules contains a 115-kDa glycosylated subunit

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Kidney microsomes were fractionated with Triton X-114, to give a fraction enriched in the renal tubule H^+-translocating ATPase, as judged by the sensitivity of its ATPase activity to bafilomycin A_1, and its content of two polypeptides recognized by antibodies directed against subunits of plant tonoplast ATPases. This fraction contained a polypeptide of apparent molecular mass of 115 kDa, that was recognized by an antibody to the largest (120 kDa) subunit of chromaffin-granule membrane H^+-ATPase, and, like this subunit, was reduced in molecular weight on treatment with glycopeptidase F. We conclude that, like other mammalian vacuolar H^+-ATPases, the kidney H^+-ATPase contains a large, glycosylated subunit.

Kidney; Proton translocation; ATPase; Glycoprotein

1. INTRODUCTION

Three classes of H^+-translocating ATPase have been recognized [1]: F-type (the ATP-synthases of energy-transducing membranes), P-type (proton pumps, located in the plasma membranes of plant, fungal and some specialized animal cells), and V-type (endomembrane proton pumps). The V-type ATPases have a wide distribution, occurring in the membranes of most (probably all) types of acidic intracellular compartments in eukaryotic cells [2]. In renal tubular cells, ATP-driven proton transport is carried out by V-type ATPases that are inserted into the apical plasma membrane by fusion of exocytotic vesicles, in response to changes in acid/base status [3,4].

V-type ATPases are of high molecular weight (400–600 kDa) and complex subunit composition. There is now a consensus that they have a minimum of 3 subunit types: (i) 66–73 kDa; (ii) 55–62 kDa; and (iii) 13–17 kDa, as judged by SDS-polyacrylamide gel electrophoresis. These subunits have been implicated in ATP hydrolysis, regulatory nucleotide-binding and H^+-translocation, respectively [5–8]. Subunits of intermediate size (20–40 kDa) are also present in most preparations. In some cases up to 5 polypeptides in this range have been reported, but none has yet been characterized functionally.

An interesting controversy surrounds the largest (100–120 kDa) subunit. This was originally found in just two types of mammalian V-ATPase [9–11], but a polypeptide of comparable size has now been reported to occur in V-ATPases from beet and yeast vacuoles [12,13], although most groups working with plant or fungal V-type ATPases do not find it in their preparations. A subunit of this size was not found in immunoaffinity-purified ATPase of bovine renal cortex and medulla [14], and it was not detected by immune blotting of liver lysosomal membranes and kidney microsomes [15]. We now report the immunochemical detection of a 115 kDa *N*-glycosylated component in partially-purified kidney microsomal V-ATPase and in the ATPase of chromaffin granules isolated from human phaeochromocytoma.

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2. MATERIALS AND METHODS

Kidney microsomes were prepared by an adaptation of a published procedure [16]. All solutions contained 10 mM Tris-HCl, 1 mM $NaHCO_3$, 1 mM EDTA, 1 mM DTT (pH 8.0, 0°C), plus sucrose at the required concentration. Bovine renal medullary tissue was minced and homogenized in 0.25 M sucrose, and centrifuged for 10 min at g_{av} = 1600. After centrifugation of the supernatant for 60 min at g_{av} = 41 000, crude microsomes were removed from the surface of the pellet by swirling, collected by centrifugation, resuspended in 0.25 M sucrose, overlaid onto step gradients of 0.7, 1.0 and 1.5 M sucrose, and centrifuged for 5 h at 35 000 rpm in a swingout rotor (Beckman SW41). The microsomal fraction was collected from the 0.7 M/1.0 M sucrose interface, diluted, centrifuged and finally resuspended in 0.25 M buffered sucrose containing benzamidine (2 mM), pepstatin (5 μg/ml), leupeptin (5 μg/ml).

Triton X-114 (Fluka) was precondensed as described [17]. It was used to fractionate kidney microsomes essentially as described for chromaffin-granule membranes [18,19], except that the final concentration of detergent was 17.5 mg/ml. The Triton-insoluble fraction was used for immune blotting as described below. Chromaffin granule membranes were prepared from bovine adrenal medulla and human phaeochromocytoma as described [20] and fractionated with Triton X-114 [19]. Antiserum to chromaffin granule H^+-ATPase was

raised by injecting reconstituted holo-ATPase [18] into rabbits. Rabbit antisera to individual subunits of the tonoplast H^+-ATPase of *Kalanchoe daigremontiana* were raised by injection of proteins electroeluted from gels. Immune blots were developed using the ECL system (Amersham). Deglycosylation of proteins in the Triton-insoluble fraction was carried out by incubation (8 h, 20°C) of 100 μg protein with 1 unit endoglycosidase F (Boehringer) in 50 mM HEPES-NaOH, pH 7.0, containing 2 mM benzamidine, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 0.2 mM PMSF, 0.5% deoxycholate, 1.0% Triton X-100, 20 mM EDTA and 10 mM dithiothreitol (total volume 105 μl).

3. RESULTS

The purified kidney microsomes had a specific ATPase activity of 0.37 μmol/min/mg protein, and this could be inhibited 15% by 2 μM bafilomycin A_1, a specific inhibitor of V-type ATPases [21]. We established that the optimal concentration of Triton X-114 for fractionating kidney microsomes was 1.75% (w/v); the Triton-insoluble fraction had only a slightly higher specific ATPase activity than did the membranes (0.48 μmol/min/mg protein), but it was inhibited about 80% by bafilomycin, indicating an enrichment in vacuolar ATPase.

This enrichment is confirmed by the immune blots shown in Fig. 1. Rabbit antisera directed against the 57 kDa and 67 kDa subunits of the tonoplast H^+-ATPase of *Kalanchoe daigremontiana* recognize polypeptides of similar molecular weight in bovine chromaffin granule membranes and kidney microsomes, and in each case the immunoreactive polypeptides appear in the Triton-insoluble fraction. The anti-57 kDa serum recognizes a doublet in the kidney fraction (Fig. 1b, track 4); a similar result was obtained with antiserum directed against the 57 kDa subunit of beet tonoplast H^+-ATPase (not shown).

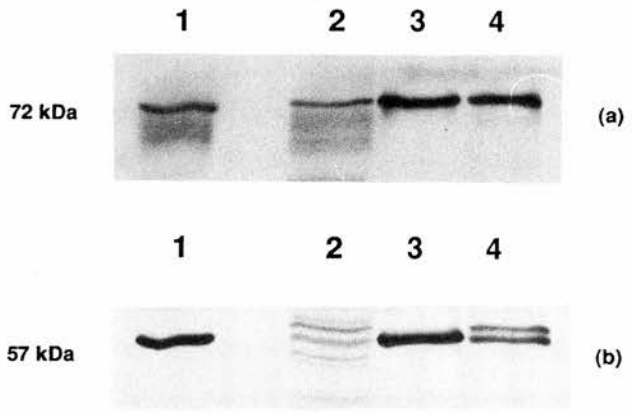

Fig. 1. Immunoblots showing the enrichment in vacuolar ATPase obtained by fractionating membranes with Triton X-114 (Track 1) Chromaffin granule membranes (60 μg protein). (Track 2) Kidney microsomes (60 μg). (Track 3) Triton-insoluble fraction from chromaffin granule membranes (10 μg). (Track 4) Triton-insoluble fraction from kidney microsomes (30 μg). Antibodies were against the 67 kDa (a) and 57 kDa (b) subunits of a plant tonoplast ATPase; note that in animals these recognize proteins of 72 and 57 kDa, respectively. In each case only the relevant part of the blot is shown, but there is no significant immunoreactivity elsewhere.

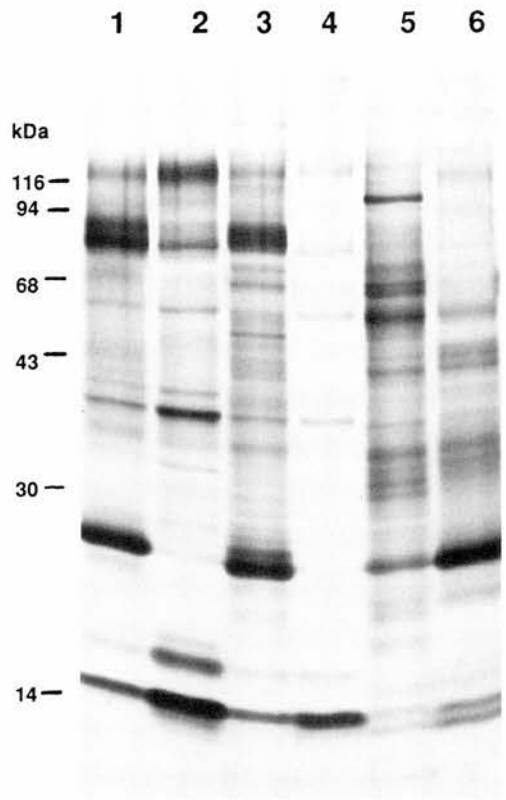

Fig. 2. Silver-stained gel showing unfractionated membranes (tracks 1, 3 and 5) and Triton-insoluble fractions (tracks 2, 4 and 6). (Tracks 1 and 2) Bovine chromaffin granules; (tracks 3 and 4) human phaeochromocytoma granules; (tracks 5 and 6) bovine kidney microsomes. Each track contained 3 μg protein. The positions of marker proteins are shown at the side; major bands in tracks 2 and 4 are at 120, 72, 57, 40, 19 and 16 kDa.

Although fractionation of chromaffin granule membranes with Triton X-114 purifies the H^+-ATPase to near-homogeneity [18], the Triton X-114-insoluble fraction from kidney microsomes contains many polypeptides besides those identified as H^+-ATPase subunits (Fig. 2).

The antiserum produced by immunization of rabbits with reconstituted chromaffin-granule H^+-ATPase vesicles [18] reacted with the 120 kDa subunit of this ATPase, and (more weakly) with dopamine β-monooxygenase (Fig. 3a, track 1). In unfractionated kidney microsomes, immunoreactivity was barely detectable (track 2), but the Triton X-114-insoluble fraction contained a protein of about 115 kDa that was clearly recognized by this serum (track 4).

Digestion of the Triton X-114-insoluble fraction with endoglycosidase F converted the diffuse immunoreactive band at 115 kDa into a sharper one at about 100 kDa (Fig. 3b, tracks 5 and 6). This is similar to the effect of deglycosylation on the 120 kDa H^+-ATPase subunit in both bovine and human chromaffin granules (Fig. 3b, and [10]).

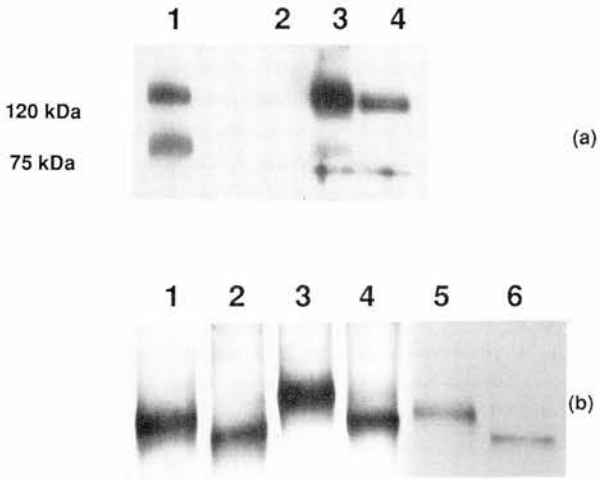

Fig. 3. Immunoblots showing that an antibody to the 120 kDa subunit of chromaffin granule membrane H^+-ATPase recognizes a similar component in kidney membranes. (a) Fractionation with Triton X-114. (Track 1) Chromaffin granule membranes (20 μg protein); (track 2) kidney microsomes (20 μg); (track 3) chromaffin granule membrane Triton-insoluble fraction (5 μg); (track 4) kidney microsome Triton-insoluble fraction (28 μg). The immunoreactive band at 75 kDa is dopamine β-monooxygenase. (b) Digestion with endoglycosidase F; Triton-insoluble fractions from: (tracks 1 and 2) human phaeochromocytoma granule (5 μg protein); (tracks 3 and 4) bovine chromaffin granule (5 μg); (tracks 5 and 6) bovine kidney microsomes (25 μg). Tracks 1, 3 and 5 are controls; tracks 2, 4 and 6 are endoglycosidase F-treated.

4. DISCUSSION

Fractionation with Triton X-114 [16,18] has proved useful in purifying V-type H^+-ATPases from chromaffin granule membranes [18] and plant tonoplasts [22], essentially in one step. When applied to kidney microsomes, it produces a Triton-insoluble fraction that is enriched about 5-fold in V-ATPase, as judged by its sensitivity to bafilomycin, but which contains many other polypeptides (Fig. 2). The activity of this preparation can be increased by the addition of phospholipids (cf. [18]) but the conditions have not yet been optimized.

Our attempts to raise antibodies to chromaffin granule membrane H^+-ATPase by immunization with individual subunits have proved unsuccessful; however, antibodies to the 57 kDa and 67 kDa subunits of plant tonoplast H^+-ATPases crossreact with the mammalian counterparts [23], and antisera raised against individual subunits of the tonoplast H^+-ATPase from the crassulacean plant *Kalanchoe daigremontiana* have been used to follow the kidney H^+-ATPase during Triton X-114 fractionation. Immunoblotting using these antisera confirms the results obtained, using bafilomycin-sensitive ATPase activity as an assay.

Immunization of rabbits with reconstituted chromaffin-granule H^+-ATPase produced an antiserum that reacted with the largest (120 kDa) subunit. Its slight reactivity with dopamine β-monooxygenase, a contaminant of the purified H^+-ATPase, was not a nuisance in these experiments, since this enzyme is absent from kidney; it could in any case be overcome by absorption of the serum with purified dopamine β-monooxygenase, without affecting the reactivity of the serum with the larger component. The antiserum recognizes a polypeptide in kidney microsomes that has an electrophoretic mobility close to that of the 120 kDa subunit of chromaffin-granule H^+-ATPase, and is glycosylated to a similar extent. In fact, the bovine kidney polypeptide is slightly smaller than that in bovine chromaffin granules, with an apparent molecular weight of about 115 kDa; this is reduced by about 15 kDa on treatment with endoglycosidase F (specific for N-linked oligosaccharide chains). It is established that the 120 kDa subunit is a component of the H^+-ATPase of chromaffin granules and clathrin-coated vesicles [9–11] and this result suggests that the kidney H^+-ATPase is similar, this polypeptide having previously been undetected by protein staining and immune blotting. Several of the properties of this subunit make it difficult to detect: it is glycosylated, so appears as a diffuse band in electrophoretograms; it stains poorly with Coomassie blue; it is extremely sensitive to proteolysis; and it aggregates when heated in SDS.

Interestingly, the antibody raised against bovine H^+-ATPase fails to recognize any antigen in plant tonoplasts [22], although the presence of a 100 kDa subunit in beet H^+-ATPase has been reported [13]. The question of whether a polypeptide of 100–120 kDa is a necessary component of all V-type ATPases, or is a species- or tissue-specific subunit, remains open.

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