The secretory pathway in peptide hormone-producing cells

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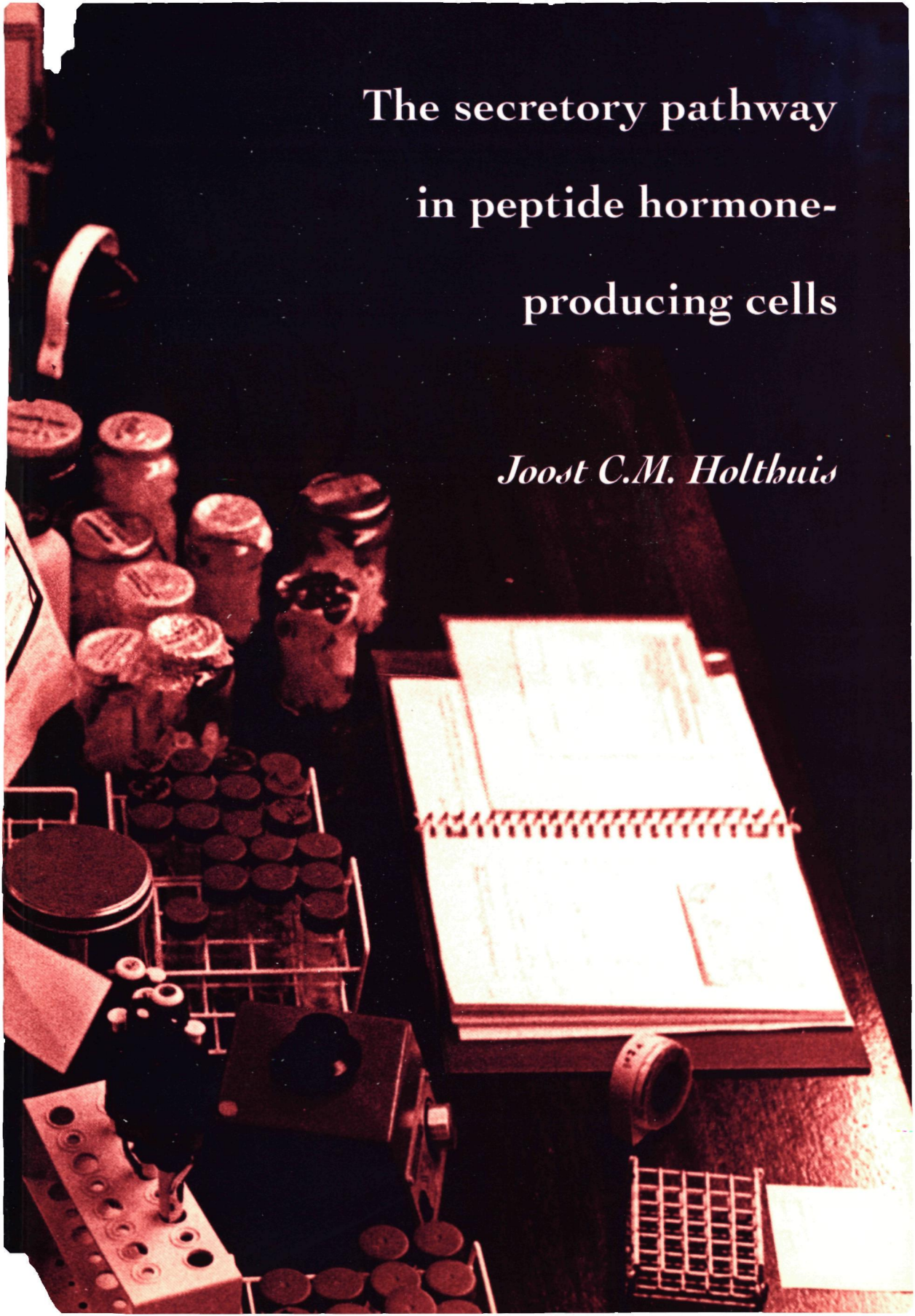

The secretory pathway in peptide hormone-producing cells

een wetenschappelijke proeve op
het gebied van de Natuurwetenschappen

Proefschrift

ter verkrijging van de graad van doctor
aan de Katholieke Universiteit Nijmegen
volgens besluit van het College van Decanen
in het openbaar te verdedigen op

donderdag 21 november 1996
des namiddags te 3.30 uur precies

door

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geboren op 23 februari 1964
te Horst (L)

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Manuscript Commissie:

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Omslag-ontwerp: Sam Gobin

The research presented in this thesis was carried out at the Department of Animal Physiology of the University of Nijmegen, The Netherlands, and financially supported by the Netherlands Organization for Scientific Research (NWO).

CIP-DATA Koninklijke Bibliotheek, Den Haag

Holthuis, Josephus Carolus Maria

The secretory pathway in peptide hormone-
producing cells / Josephus Carolus Maria Holthuis. -[S.1. : s.n.] - Ill.
Thesis Katholieke Universiteit Nijmegen. - With ref. - With summary in Dutch -
With summary in French.
ISBN 90-9009852-6
Subject headings: neuroendocrinology / secretory pathway / hormone biosynthesis.

Moge de waarde van dit proefschrift
evenredig zijn aan de "ellende"
die de schrijver ermee heeft veroorzaakt
in zijn naaste omgeving.

voor Laurence

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Chapter 1

General Introduction

General Introduction

The principle task of endocrine cells and peptidergic neurons (collectively referred to as neuroendocrine cells) is to synthesize, store and release peptide signaling molecules in accordance with the physio-logical demand of an organism To meet this highly specialized function, neuroendocrine cells are equipped with a unique secretory pathway whose major features are still poorly understood To explore this pathway at the molecular level, we developed a strategy for the systematic identification of genes whose products participate in steps of peptide hormone biosynthesis and secretion

This introductory section serves to provide general information on several topics relevant to the research described in this thesis These topics include a general outline of the secretory pathway, the main characteristics of the secretory pathway in neuroendocrine cells, the experimental approaches used to unravel the molecular organization of the secretory pathway and a description of the experimental model system used in this work

THE SECRETORY PATHWAY: A GENERAL OUTLINE

Most, if not all, eukaryotic cells are capable of exporting proteins to the extracellular environment Protein secretion plays a fundamental role in cell growth, in the communication between cells, in creating the extracellular matrix by which cells are organized in tissues, in the destruction of invading microorganisms and in providing the digestive enzymes by which external food particles are converted into simpler nutrients Before reaching the extracellular environment, secretory proteins pass through a series of membrane-bounded compartments that are interconnected by the budding and fusion of transport vesicles (Palade, 1975) (Fig 1) Each compartment along this secretory pathway serves unique functions and contains a distinct set of resident proteins A major advantage of this compartmental organization is that newly synthesized secretory proteins can be exposed to an ordered array of biochemical reactions, thus enabling complex covalent modifications and conformational transitions to occur

Proteins enter the secretory pathway by crossing the membrane of the endoplasmic reticulum (ER) Besides secretory proteins, this organelle imports its own resident proteins and virtually all proteins destined for the Golgi complex, the lysosomes, the endosomes and the plasma membrane These proteins carry, usually at their amino-terminal ends, a hydrophobic signal peptide which is recognized by the signal recognition particle (SRP) as soon as it emerges from the ribosome Binding of SRP ensures that the nascent protein is targeted to the cytosolic face of the ER and translocated into the lumen, most typically as translation proceeds (Blobel and Dobberstein, 1975) Translocation occurs through a hydrophylic protein-conducting channel (Simon and Blobel, 1991), a structure often referred to as the translocon Soluble proteins are fully translocated and released into the ER lumen after their signal peptide is cleaved off by a signal peptidase Transmembrane proteins undergo partial translocation and become anchored in the ER membrane by internal signal peptides or hydrophobic stop-transfer sequences (Rapoport, 1992) *N*-linked glycosylation is one of the key reactions performed in the ER It involves the *en bloc* transfer of a large oligosaccharide to the amino groups of selected asparagine residues by oligosaccharyl transferases (Kornfeld and Kornfeld, 1985, Paulson and Colley, 1989) In the ER lumen, molecular chaperones associate with the freshly translocated proteins in order to control and facilitate their proper folding and assembly (Gething and Sambrook, 1992) Protein disulfide isomerases catalyze the formation of disulfide bonds (Freedman, 1989) A quality control device ensures that incorrectly folded proteins or protein subunits that failed to oligomerize into their final complex are retained in the ER and eventually degraded (Hurtley and Helenius, 1989)

Proteins leaving the ER are carried forward in transport vesicles to the Golgi complex and arrive at the *cis*-Golgi network (CGN) The CGN forms the port of entry to the Golgi stack and in addition functions as a salvage compartment for escaped ER resident proteins (Pelham, 1991) Proteins traversing the *cis*-, *medial*-, and *trans*-cisternae of the Golgi stack are subject to various kinds of post-translational modifications, most remarkably the remodeling of *N*-linked oligosaccharide side chains of glycoproteins and the step-wise biosynthesis of *O*-linked glycans on selected serine or threonine residues (Roth, 1988, Lis and Sharon, 1993) Protein sulfation at tyrosine residues is carried out in the *trans*-Golgi network (TGN) by tyrosine-sulfotransferases (Niehrs and Huttner, 1990)

The main function of the TGN is to distribute proteins for different post-Golgi destinations (Griffiths and Simons, 1986) Lysosomal enzymes are collected in clathrin-coated vesicles by receptors recognizing the mannose 6-phosphate groups linked

to these proteins and transported to an endosomal compartment, en route to lysosomes (Kornfeld and Mellman, 1989) Plasma membrane proteins and secretory proteins such as growth factors, immunoglobulins and matrix proteins are captured in small transport vesicles and rapidly delivered to the cell surface This route of protein secretion is known as the *constitutive pathway* because transport vesicles discharge their contents immediately without internal storage (Gumbiner and Kelly, 1981) Constitutive secretion is a basic function of all animal cells but in certain cell types, such as those of exocrine, endocrine and neuronal origin, there exists in addition a specialized pathway which arises at the TGN This pathway involves the sorting and packaging of a subset of secretory proteins in storage organelles called secretory granules Once sequestered into these organelles, secretory proteins are delivered to the cell surface only in response to a specific extracellular stimulus and, therefore, this route of protein secretion is called the *regulated pathway* (Kelly, 1985, Burgess and Kelly, 1987) Typical examples of regulated secretory proteins are zymogens (in exocrine cells) and the precursors of neuropeptides and peptide hormones (in neuroendocrine cells) Whereas constitutive secretion occurs at an essentially constant rate and is limited mainly by the availability of product, regulated secretory proteins can be stored for considerable periods of time before their release is stimulated by a secretagogue Moreover, regulated secretory proteins are usually highly concentrated (as much as 200-fold) during their passage from the Golgi to secretory granules, and eventually aggregate to form the electron-opaque dense core found in electron micrographs Cells equipped with a regulated secretory pathway are therefore specialized to release, for a brief period, large amounts of products at a rate much higher than the rate of protein biosynthesis

THE REGULATED SECRETORY PATHWAY IN NEUROENDOCRINE CELLS

The regulated secretory pathway in neuroendocrine cells is marked by three major events, namely (1) the sorting and packaging of peptide precursors into secretory granules, (2) the subsequent proteolytic maturation of the precursors, and (3) the stimulus-dependent release of bioactive peptides

Sorting and packaging of peptide precursors into secretory granules

The packaging of peptide precursors and other regulated proteins into secretory granules is associated with a sorting event that separates these molecules from lysosomal proteins, plasmamembrane constituents and constitutively secreted products The mechanisms used by neuroendocrine cells to accomplish this separation are still poorly understood Several interesting models have been proposed, and three of these (schematically depicted in Fig 2) will be briefly discussed below As will become apparent, these models are not mutually exclusive, and in reality the sorting process may well involve aspects of each

Receptor-mediated sorting — This model predicts that a sorting signal is necessary to direct proteins into secretory granules and to separate them from those secreted constitutively, with a membrane-bound receptor to recognize the sorting signal and cause the separation (Kelly, 1985) In fact, the model is analogous to lysosomal sorting where a specific signal (mannose-6-phosphate) targets proteins through a recycling receptor to an endosomal compartment Thus, regulated secretory proteins (and not other proteins) could be selected from the mixed contents of the TGN lumen by receptors that, when occupied, cluster in a specific region of the membrane (as schematically represented in Fig 2A) Subsequently, an immature granule could form by budding of this particular region of the TGN Dissociation of the receptor-ligand complex could then occur as the result of a reduction in pH caused by pumping of protons across the immature secretory granule membrane Finally, recycling of the receptor and further delivery of ligand could cause an increase in protein concentration and eventually lead to aggregation and formation of a dense core Given the large variety of proteins delivered to secretory granules, it is most unlikely that discrete receptors exist for each type of regulated protein It is therefore believed that if sorting receptors indeed exist, they must have an extremely broad specificity Efforts to identify such receptors (Chung et al , 1989) have remained unsuccessful, and thus direct evidence in support of a 'receptor-mediated sorting' model is lacking

Condensation-mediated sorting — According to this model, which has gained increasing support over the last years, sorting could be achieved by the tendency of regulated secretory proteins to form insoluble aggregates in the TGN from which other proteins are excluded (Burgess and Kelly, 1987, Chanat and Huttner, 1991) Selective enwrapment of such aggregates and budding of the TGN membrane could lead to the formation of immature granules, thereby excluding proteins that remain in the fluid phase (Fig 2B) This 'condensation-mediated sorting' model was initially based on mor-

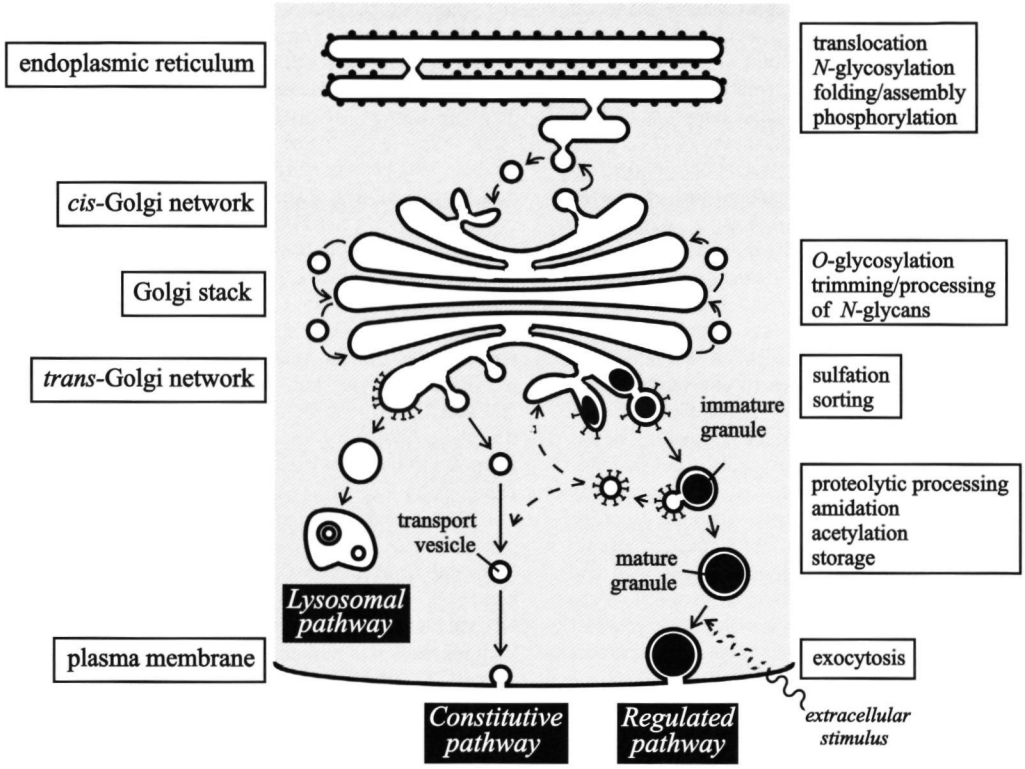

FIG. 1. **The secretory pathway**

phological data showing that regulated secretory proteins form electron-dense cores in the TGN (Orci et al., 1987). Additional support came from studies on the aggregative properties of the granins, a family of acidic secretory proteins found in the granules of many neuroendocrine cell types (Huttner et al., 1991). Permeabilization of rough ER-derived vesicles from neuroendocrine PC12 cells under conditions thought to exist in the TGN (pH 6.4, 10 mM Ca^{2+}) was sufficient to cause an aggregation of the newly synthesized granins (Chanat and Huttner, 1991). Furthermore, these aggregates largely excluded glycosaminoglycan chains which served as constitutively secreted bulk flow markers. A similar selective aggregation of granins and other secretory granule proteins can be reconstituted *in vitro* at mildly acidic pH (Gerdes et al., 1989; Colomer et al., 1996). A major question concerning condensation-mediated sorting which has not been fully addressed is what exactly causes regulated proteins, but not others, to aggregate in the TGN. Furthermore, it is unknown whether the formation of aggregates can induce granule formation *per se*, or whether other membrane proteins are involved, serving the role of a receptor or guide. If receptors would be involved, only a few of the many molecules in an aggregate would need a sorting signal in order to target the entire cast to a secretory granule ('piggy-back targeting', Fig. 2B).

Sorting by retention — Since the bulk of peptide precursors is processed in the immature granule (Halban and Irminger, 1994), a conceptual difficulty with condensation-mediated sorting in the TGN (Fig. 2B) is that it may sterically hinder the cleavages catalyzed by the processing enzymes. Therefore, the 'sorting by retention' model (Kuliawat and Arvan, 1992, 1994) proposes that regulated and non-regulated proteins enter immature secretory

granules largely unsorted and that small regions of maturing granules can pinch off to form transport vesicles, carrying within them a random sampling of any constituent in the fluid phase while the condensation of regulated proteins favours their retention in the granular interior (Fig 2C) Post-granular vesicles may shuttle soluble proteins to the plasma membrane (via a 'constitutive-like' pathway), to lysosomes or back to the TGN Thus, according to the 'sorting by retention' model, the immature secretory granule rather than the TGN represents the major sorting station In addition, the model predicts that sorting is accomplished by passive (self-associating) rather than active (receptor-binding) protein-protein interactions The 'sorting by retention' model was initially based on observations that the formation of insoluble aggregates in immature granules of pancreatic ß-cells lags behind the conversion of proinsulin to insulin (Kuliawat and Arvan, 1992) Subsequently, it was shown that immature granules contain considerable amounts of lysosomal hydrolases that are actively removed from the condensing granule contents (Kuliawat and Arvan, 1994) Furthermore, morphological and biochemical studies have indicated that the immature granule membrane is partially coated with clathrin whereas the surface of mature granules does not have a clathrin coat, suggesting that budding of clathrin-coated vesicles is part of the maturation process (Tooze and Tooze, 1986, Dittié et al , 1996) An important question left unanswered by the above model is what exactly triggers the formation of an immature secretory granule if proteins enter that compartment in a soluble phase

The experimental data collected so far on protein sorting to the regulated secretory pathway have been insufficient to clearify the underlying mechanisms Further insight into these mechanisms requires a precise knowledge of all the molecules involved

Proteolytic maturation of peptide precursors

The majority of peptide hormones and neuropeptides are synthesized as inactive precursor proteins from which the functional molecules need to be liberated by proteolytic processing Peptide precursors, also termed prohormones, may contain an amino-terminal prosequence that is cleaved off to yield the bioactive product In some cases, peptides are excised from prohormones that contain multiple copies of the same amino acid sequence In even more complex cases, a single prohormone encompasses a variety of peptides, discrete subpopulations of which can be selectively generated in a tissue-specific fashion by differential processing A well characterized example of such a multifunctional precursor is proopiomelanocortin (POMC) In the anterior pituitary, POMC is processed to adrenocorticotropic hormone (ACTH) and ß-lipotropic hormone (ß-LPH) as major end products, whereas in the intermediate pituitary ACTH and ß-LPH are further processed to α-melanophore-stimulating hormone (α-MSH), corticotrophin-like intermediate lobe peptide (CLIP) and ß-endorphin (Smith and Funder, 1988) The proteolytic con-version of prohormones is a multistep process that involves (1) cleavage at pairs of basic amino acid residues by prohormone convertases PC1 (also referred to as PC3) and PC2 (Seidah et al , 1990, Smeekens and Steiner, 1990, Thomas et al , 1991), (2) removal of the carboxy-terminal basic residues by carboxypeptidase H/E (Fricker, 1988) and (3) amidation and acetylation of some of the resulting peptides by peptidyl-α-amidating mono-oxygenase (PAM) and acetyl-transferase, respectively (Eipper and Mains, 1988, Bradbury and Smyth, 1991)

Although endoproteolytic cleavage of some prohormones has been reported to start in the TGN (Schnabel et al , 1989, Xu and Shields, 1993), the bulk of processing events is thought to occur in the secretory granules (Halban and Irminger, 1994) In keeping with this notion, prohormone convertases (PC1/PC3 and PC2) as well as post-cleavage processing enzymes (e g carboxy peptidase H/E, PAM) have been localized to secretory granules (Christie et al , 1991, Eipper and Mains, 1988, Fricker, 1988) Moreover, prohormone convertases have been found to exhibit maximum activity at an acidic pH close to that thought to exist in the immature secretory granule (Davidson et al , 1988, Bailyes et al , 1992, Anderson and Orci, 1988) Acidification of the granular milieu is mediated by a vacuolar H^+-ATPase (Mellman et al , 1986) It is believed that this acidification plays a fundamental role in the proper timing of the processing events in the regulated secretory pathway (Rhodes et al, 1987, Anderson and Orci, 1988)

Regulated release of bioactive peptides

As already mentioned, secretory proteins destined for the constitutive pathway are exported shortly after they have been made, and do not accumulate to a significant extent in the cell Bioactive peptides and other granular proteins, on the other hand, are stored (sometimes for days), rather than undergoing exocytosis within minutes Moreover, their release occurs strictly in response to a secretagogue (which can be ions, hormones, neuropeptides and neuro-

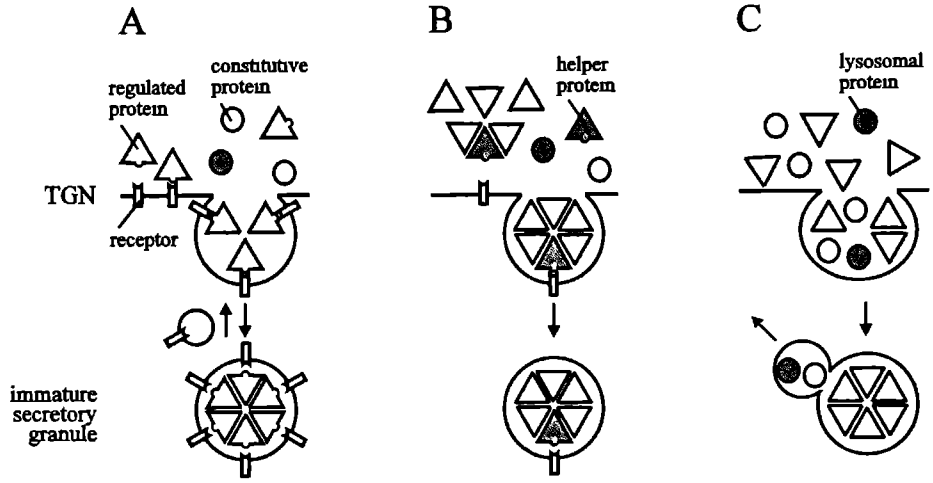

FIG 2. **Possible mechanisms for the sorting of proteins to the regulated secretory pathway.** (A) Receptor-mediated sorting (B) Condensation-mediated sorting (C) Sorting by retention Each model is discussed in detail in the text

transmitters), and usually through a rise in Ca^{2+} at the release sites (Burgoyne and Morgan, 1993) Consequently, regulated exocytosis is a complex event since, in addition to the essential components required in the fusion process, a mechanism should exist that allows a second messenger (e g a Ca^{2+} signal) to be transduced into activation of the dormant fusion machinery This could involve removal of inhibition as well as direct activation Added complexity is due to the variation between cell types in the regulation of exocytosis Whereas the regulatory mechanisms involved in the targeting, docking and fusion of synaptic vesicles in neurons are rapidly being uncovered (Sudhof, 1995), for secretory granules these mechanisms have remained largely obscure

UNRAVELING THE MOLECULAR ORGANIZATION OF THE SECRETORY PATHWAY

Molecular dissection of the constitutive pathway

Both genetics in yeast and biochemistry in mammalian cell-free systems have been used to elucidate the mechanisms of constitutive protein secretion (Pryer et al , 1992, Rothman, 1994) Over the last few years these distinct experimental approaches have converged and provided complementary information that has significantly improved our understanding of the molecular organization of the constitutive secretory pathway

The genetic approach — Although yeast contains only a poorly developed secretory apparatus when compared to that found in higher animal cells, its use as a model system has been of great importance for the systematic identification of gene products with a role in constitutive protein secretion In *Saccharomyces cerevisiae*, or budding yeast, secretion primarily serves to incorporate new cell wall material at the growing bud prior to cell division Novick and Schekman (1979) were the first to describe a screening strategy for the identification of mutant strains that are temperature-sensitive for secretion and cell surface growth When incubated at the non-permissive temperature, such mutants fail to export the cell wall-bound enzymes invertase and acid phosphatase but continue to synthesize proteins and consequently become dense The sedimentation of randomly mutagenized cells on density gradients allowed the isolation of a large number of secretory (*sec*) mutants with abnormally large intracellular pools of invertase at the non-permissive temperature (Novick et al , 1980) Subsequent complementation analysis revealed at least 23 distinct gene products (*sec* 1-23) whose functions are indispensible for the transport of secretory proteins *Sec* mutants were classified by analyzing the glycosylation and processing state of the accumulated secretory proteins and by electron microscopic observations of abbarent membranes and vesicles that accumulated at the restrictive temperature Double mutant analysis was then used to determine the precise

order in which the affected genes function Thus, some mutants could be identified as being blocked in ER to Golgi transport, while others were blocked in transport within the Golgi or from the Golgi to the plasma membrane (Novick et al , 1981) Within these categories, mutants could be further subdivided in those blocked prior to vesicle formation and those blocked in vesicle consumption (Kaiser and Schekman, 1991) Many additional genes with a role in protein secretion were isolated on the basis of their ability to suppress the conditional defect in one of the *sec* mutants (Pryer et al , 1992) This suppressor approach allowed the identification of redundant genes whose phenotypes would not have shown up in the original selection, and provided clues for physical interactions between two gene products Cloning and sequencing of these genes revealed a large collection of molecules required for the transport of secretory proteins from their site of synthesis to the cell surface Insight into the roles of these gene products came with the development of biochemical assays to measure vesicular protein transport between subcellular compartments of the secretory pathway

The biochemical approach — The first insights into the mechanisms of vesicle-mediated protein transport came through the pioneering work of Rothman and colleagues They established a mammalian cell-free system to study vectorial transport of a viral glycoprotein, vesicular stomatitis virus (VSV) G protein, through compartments of the Golgi complex (Fries and Rothman, 1980, Balch et al , 1984) The assay made use of a mutant Chinese hamster ovary (CHO) cell line that lacks functional N-acetylglucosamine (GlcNAc) transferase I, an enzyme normally present in the medial cisternae of the Golgi stack By incubating purified Golgi fractions from VSV-infected mutant CHO cells with that of wild type cells, transport from the *cis* to the medial cisternae could be detected by monitoring the incorporation of [^{3}H]GlcNAc into the viral G protein Transport required the presence of a crude cytosolic fraction, ATP, and proteins on the surface of the Golgi membranes Electron microscopic studies and autoradiographic methods indicated that G protein was transported in sealed 75-nm vesicles (Braell et al , 1984) These vesicles came in two varieties those coated with electron-dense material (distinct from clathrin) on their outer surface and those uncoated Transport-coupled glycosylation of G protein was blocked by non-hydrolysable GTP analogues, such as GTP-γ-S (Melancon et al , 1987), or by pretreatment of the Golgi membranes with the sulphydryl reagent *N*-ethylmaleimide (NEM) (Glick and Rothman, 1987) The GTP-γ-S block caused an accumulation of coated buds and coated vesicles whereas the NEM block caused an accumulation of uncoated vesicles Combination of these treatments resulted in the exclusive accumulation of coated vesicles These and other observations led to the hypothesis that vesicle budding is driven by the assembly of a cytoplasmic coat on the donor membrane whereas vesicle fusion relies on the removal of this coat Purification of the vesicles that accumulated during the GTP-γ-S block allowed the identification of the coat proteins which included a GTPase called ARF (for ADP-ribosylation factor) and seven COPs (for coatomer proteins, Rothman, 1994) The essential role of COPs in vesicle-mediated transport was confirmed by the finding that one of the coat proteins (γ-COP) represented the homolog of a gene product (Sec21p) required for ER-to-Golgi transport in yeast Subsequent studies revealed that GTP-bound ARF triggers coat assembly whereas GDP-bound ARF causes release of the coat The finding that isolated Golgi membranes vesiculated extensively when incubated with ARF and COPs further emphasized the critical roles of these proteins in the budding of transport vesicles

The NEM-sensitive fusion protein (NSF) was identified through its ability to restore vesicular transport between Golgi cisternae after blockade by NEM (Rothman, 1994) NSF is homologous to Sec18p, another gene product required for yeast ER-to-Golgi transport, indicating that an NSF-dependent fusion mechanism operates at diverse locations in the secretory pathway Binding of NSF to Golgi membranes was found to be dependent on the presence of crude cytosol, providing an assay for the purification of the soluble NSF attachment proteins (SNAPs) which are also necessary for vesicle fusion A search for the membrane-localized receptors for the SNAPs led to the discovery of a family of integral membrane proteins, called SNAREs (for SNAP receptors), that play an essential role in the targeting of transport vesicles to their appropriate destinations (Rothman and Warren, 1994) Two categories of SNAREs were found v-SNAREs (which function on the transport vesicles) and t-SNAREs (on the target membranes) The ER and Golgi each express their own species of v-SNAREs that are efficiently packaged into departing transport vesicles The Golgi and plasma membrane also express specific t-SNAREs required for docking of the ER-derived and Golgi-derived transport vesicles, respectively The partnering of a v-SNARE with its cognate t-SNARE allows the binding of NSF and SNAP proteins and renders the docked vesicle competent for fusion

Thus, a considerable portion of the machinery that mediates vesicular transport along the constitutive secretory pathway has been uncovered. This machinery includes coat proteins which act as a mechanical device to bud off a vesicle from a donor membrane; the vesicle- and target-specific identifiers (v-SNAREs and t-SNAREs) which bind each other and thereby dock the vesicle to the membrane of the acceptor compartment; and NSF and SNAP proteins which bind to the SNARE complex and initiate fusion.

Molecular dissection of the regulated pathway

Whereas the genetic and biochemical approaches described above have been very succesfull in alleviating the general mechanisms of protein secretion, the unique features associated with the regulated secretory pathway found in neuroendocrine cells have remained largely unexplored. Unfortunately, neuroendocrine cells are not particularly amenable to the type of genetic analysis that has been so informative for the constitutive pathway. Moreover, the CHO glycosylation mutants which have served as powerful tools for the analysis of protein transport steps occurring early in the secretory pathway are of limited value for a bio-chemical dissection of the regulated secretory pathway. Hence, a systematic identification of gene products associated with the regulated secretory pathway requires other strategies than the ones described above.

Biochemical approaches — Cell-free and permeabilized cell systems derived from neuroendocrine cell lines have been used successfully to reconstitute individual steps of the regulated secretory pathway, such as the budding of immature secretory granules (ISGs) from the TGN (Tooze and Huttner, 1990; Xu and Shields, 1993), their conversion into mature secretory granules (Tooze et al., 1991; Grimes and Kelly, 1991) and their Ca^{2+}-dependent fusion with the plasma membrane (Morgan and Burgoyne, 1992; Hay and Martin, 1992; Carnell and Moore, 1994). Thus far, only a limited number of the proteins involved in these steps have been identified.

The budding of ISGs from the TGN was found to be dependent on ATP and cytosolic factors, and could be blocked by GTP-γ-S (Xu and Shields, 1993). Furthermore, budding was stimulated by pertussis toxin and inhibited by cholera toxin, indicating that heterotrimeric GTP-binding proteins participate in the regulation of the process (Leyte et al., 1992). In a recent study, the putative target of cholera toxin was identified and found to represent a novel, 'extra large' αs subunit (XLαs) of heterotrimeric GTP-binding proteins (Kehlenbach et al., 1995). XLαs was specifically located on the TGN, occurs selectively in neuroendocrine cell types, and likely represents one of the regulatory components involved in the formation of secretory granules.

A requirement for cytosolic proteins in Ca^{2+}-dependent exocytosis of secretory granules became apparent from the run-down of secretory responsiveness of permeabilized neuroendocrine cells as they leak such proteins. This has formed the basis of reconstitution assays with fractionated brain cytosol, allowing the identification of several of the proteins involved in regulated exocytosis. The first to be identified in this way was annexin II, a member of the annexin family of Ca^{2+}- and phospholipid binding proteins (Ali et al., 1989). Another protein capable of stimulating Ca^{2+}-dependent exocytosis belongs to the 14-3-3 protein family (Morgan and Burgoyne, 1992). 14-3-3 proteins act as regulators in a wide range of signal transduction pathways (Aitken et al., 1992). The precise functions of these and other isolated cytosolic proteins in regulated exocytosis remain to be characterized.

The identification of some of the processing enzymes operating in the regulated secretory pathway (e.g. carboxypeptidase H/E, PAM) was accomplished following their purification from secretory granule preparations (Fricker, 1988; Eipper and Mains, 1988). Such an approach, however, did not work for all processing enzymes (e.g. prohormone convertases).

Alternative approaches — The isolation of the prohormone convertases from secretory granules was seriously hampered due to the presence of contaminating lysosomal enzymes. The key to the identification of these processing enzymes was the discovery of the yeast endoprotease Kex2 and its structural resemblance to the human *fur* gene product (furin). A polymerase chain reaction (PCR) strategy with degenerate primers based on sequences conserved between Kex2 and furin led to the discovery of a family of mammalian endoproteases which included the prohormone convertases PC1/PC3 and PC2 (reviewed by Halban and Irminger, 1994).

Another and more systematic approach of identifying components from the regulated secretory pathway took advantage of differential gene expression in neuroendocrine cells exposed to secretagogues. For instance, in the case of pancreatic ß-cells, it has been shown that a number of the secretory granule proteins are regulated by glucose in parallel with proinsulin (Guest et al., 1989; Schuppin and Rhodes, 1996). Similarly, in the melanotrope cells of the rat intermediate pituitary, dopamine receptor

antagonists enhance the expression of POMC and several neuroendocrine processing enzymes, whereas dopamine receptor agonists reduce their expression (Pardy et al , 1990, Bloomquist et al , 1991) Using cDNA libraries prepared from intermediate pituitary mRNA of rats that were chronically treated with dopaminergic agents, Bloomquist et al (1994) succeeded in identifying several dopamine-responsive genes, including one encoding a novel neuroendocrine-specific secretory protein, called RESP18 A major drawback of the mammalian intermediate pituitary, however, is that its function is not well understood and thus physiological manipulations to activate or inactivate the melanotrope cells are difficult In contrast, the melanotropes in the amphibian intermediate pituitary have a well-defined neuroendocrine function and therefore these cells represent an attractive model system to explore the molecular mechanisms underlying neuroendocrine secretion

XENOPUS MELANOTROPE CELLS AS A MODEL SYSTEM TO STUDY MECHANISMS OF NEUROENDOCRINE SECRETION

The melanotrope cells in the intermediate pituitary of the South African clawed toad *Xenopus laevis* play a pivotal role in the process of background adaptation (Jenks et al , 1993) Information concerning the colour of the environment is perceived by the animals visual system and is processed in the central nervous system resulting in a neural signal that reaches the melanotrope cells By placing the animal on a black background, the melanotrope cells are triggered to release α-MSH, which stimulates the dispersion of the black pigment melanin in the melanophores of the skin, causing a darkening of the animal When the animal is placed on a white background, the secretion of α-MSH is inhibited, leading to an aggregation of melanin in the melanophores and a pale-coloured skin As mentioned above, α-MSH is cleaved from the prohormone POMC An elevated release of α-MSH is accompanied by a higher rate of POMC gene transcription (Martens et al , 1987, Ayoubi et al , 1991) Consequently, melantrope cells of black-adapted *Xenopus* contain 30-times more POMC mRNA and display a much higher biosynthetic activity than those of white-adapted animals Hence, the biosynthetic and secretory activity of *Xenopus* melanotrope cells can be manipulated *in vivo* simply by changing the background colour of the animal These features have led us to exploit *Xenopus* melanotropes as an experimental model system in a search for genes whose products are associated with the specialized secretory function of neuroendocrine cells

AIM AND OUTLINE OF THE THESIS

To further elucidate the molecular organization of the secretory pathway in neuroendocrine cells, we developed a strategy for the identification of genes that are coordinately expressed with the POMC gene in *Xenopus* melanotrope cells Differential hybridization of an intermediate pituitary cDNA library of black-background adapted *Xenopus* with cDNA probes derived from intermediate pituitary mRNA of black- and white-adapted animals allowed the identification of twelve distinct genes whose transcript levels parallel that of POMC during background adaptation (Chapter 2) Four of these genes are novel while the others code for a translocon-associated protein, a lumenal cysteine protease of the ER, prohormone processing enzymes, members of the granin family, and an accessory subunit of vacuolar H^+-ATPase from secretory granules Five of the identified genes were selected for further analysis

The isolation and characterization of full-length cDNAs for the translocon-associated protein TRAPδ and a novel protein structurally related to a second component of the translocon are described in Chapter 3 It appears that alternative splicing of the TRAPδ transcript gives rise to two structurally distinct proteins Our findings suggest that the group of ER proteins in proximity of translocating polypeptide chains is more complex than previously expected

Chapter 4 reports on the primary structure and expression of *Xenopus* secretogranin II and secretogranin III A comparative analysis of the *Xenopus* and mammalian proteins uncovered some interesting aspects concerning the evolutionary history of these granins

Chapter 5 describes the biosynthesis of secretogranin III in *Xenopus* melanotrope cells We find that secretogranin III is a sulfated precursor protein and demonstrate that proteolytic processing occurs at two dibasic sites that are recognized by the prohormone convertases PC1/PC3 and PC2

In Chapter 6, we show that Ac45, an accessory subunit of vacuolar H^+-ATPase from secretory granules, is an *N*-glycosylated type I transmembrane protein and demonstrate that the protein is retrieved from the surface of melanotrope cells following exocytosis Transient expression studies in fibroblasts suggest that Ac45 overexpression interferes with a proper functioning of vacuolar H^+-ATPases in the secretory pathway

Chapter 7 provides evidence for the presence of a novel type of sorting motif in the cytoplasmic tail of Ac45 Interestingly, this motif occurs in the cytoplasmic tails of two other membrane proteins found in secretory granules.

The role of vacuolar H^+-ATPases in neuroendocrine secretion is evaluated in Chapter 8 It appears that the acidification mediated by these proton pumps is essential for a proper processing and delivery of regulated secretory proteins

Finally, in Chapter 9, the results obtained with the differential screening approach are discussed in more general terms and directions for future research are given

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Chapter 2

Molecular Probing of the Secretory Pathway in Peptide Hormone-Producing Cells

The Journal of Cell Science (1995) **108**, 3295-3305

Journal of Cell Science 108 3295 3305 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995
JCS9359

Molecular probing of the secretory pathway in peptide hormone-producing cells

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SUMMARY

The biosynthetic machinery in the melanotrope cells of the *Xenopus* intermediate pituitary is primarily dedicated to the generation of proopiomelanocortin (POMC)-derived, melanophore-stimulating peptides. Transfer of the animal to a black background stimulates the production of these peptides and causes a dramatic increase in POMC mRNA levels. To identify genes involved in the biosynthesis and regulated release of peptide hormones, we differentially screened an intermediate pituitary cDNA library of toads adapted to a black background with cDNA probes derived from intermediate pituitary mRNA of black- and white-adapted animals. Here we report the identification of twelve distinct genes whose expression levels in the melanotropes are regulated in coordination with that of POMC. Four of these genes are novel while the others code for translocon-associated proteins, a lumenal cysteine protease of the endoplasmic reticulum, prohormone-processing enzymes, members of the granin family and a transmembrane protein presumably involved in the assembly and/or specific functioning of vacuolar H^+-ATPase from secretory granules. Our results indicate that a wide variety of both soluble and membrane-associated components of the secretory pathway is recruited in physiologically activated, peptide hormone-producing cells.

Key words pituitary, secretory pathway differential screening, *Xenopus laevis*

INTRODUCTION

During the last decade, considerable contributions have been made towards an understanding of the mechanisms by which proteins are secreted from eukaryotic cells A number of the key molecules involved have been identified by genetic analysis of secretion-defective mutants in yeast (Pryer et al , 1992), whereas the utilization of mammalian cell-free systems led to a biochemical definition of vesicle mediated protein transport (Rothman and Orci, 1992) Both lines of investigation primarily dealt with the constitutive route of protein secretion, which is common to all cell types Specialized secretory cells, however, often contain additional pathways by which proteins are delivered to the cell surface (Burgess and Kelly, 1987) For instance, the production and release of peptide hormones by endocrine cells or peptidergic neurons is mediated through a regulated secretory pathway that, in various aspects, is clearly distinct from the constitutive route Firstly, peptide hormones arise from inactive precursor molecules or prohormones, which acquire full biological activity by undergoing multiple post-translational modifications during their intracellular transport Such modifications may include glycosylation (Loh and Gainer, 1982), sulfation (Huttner, 1988), endoproteolytic cleavage (Halban and Irminger, 1994), exoproteolytic cleavage (Fricker, 1988), amidation (Eipper and Mains, 1988) and acetylation (Glembotski, 1982) Several of the responsible processing enzymes have been identified and it appears that a number of these reside exclusively in the secretory pathway of peptidergic cells (Eipper and Mains, 1988, Halban and Irminger, 1994) Secondly, prohormones arriving in the *trans*-Golgi network (TGN) are segregated from proteins travelling via the constitutive route and packaged separately into secretory granules (Orci et al , 1987) The molecular systems controlling prohormone sorting and packaging are poorly understood but may involve specific targeting signals that interact with sorting receptors in the TGN lumen (Chidgey, 1993) Alternatively, these events may be the consequence of a selective aggregation of prohormones triggered by the low pH and calcium-rich milieu of the TGN lumen (Reaves and Dannies, 1991, Chanat and Huttner, 1991) Thirdly, whereas the contents of transport vesicles derived from the constitutive route of secretion is released continuously into the extracellular environment, the secretory granules containing mature peptides are stored in the cytosol and will be delivered to the cell surface only in response to a specific extracellular stimulus This regulated form of protein export has been studied in various experimental settings and, thus far, only a limited number of the molecular components involved have been identified (Wollheim and Lang, 1994) Further insight into the mechanisms by which peptide hormones are produced and released is desirable, as their malfunctioning may constitute the basis of many neuroendocrine disorders

To explore the pathway of peptide hormone secretion at the

molecular level we use as a model system, the neurointermediate lobe (NIL) from the pituitary gland of *Xenopus laevis*. This tissue consists of a nearly homogeneous population of melanotrope cells with a well defined neuroendocrine function namely the production and release of α-melanophore stimulating hormone (α-MSH) during adaptation of the animal to a dark background (Jenks et al. 1977). α-MSH is cleaved from the prohormone proopiomelanocortin (POMC) and stimulates the dispersion of black pigment in dermal melanophores, thus imparting a dark colour to the toad. Consequently, in the NIL of black background adapted animals the POMC gene is highly expressed and the level of POMC mRNA is up to 30-fold higher than in that of white adapted animals (Martens et al., 1987). Moreover the melanotropes of black adapted animals display the ultrastructural features of cells with high biosynthetic and secretory activities including an enlarged cell nucleus, extensively elaborated endoplasmic reticulum and Golgi complex and a low amount of storage granules (Hopkins, 1970; de Rijk et al. 1990). Conversely the melanotropes of white adapted animals resemble inactive storage cells with a poorly developed synthetic apparatus and a high content of storage granules. Hence the biosynthetic and secretory activity of *Xenopus* melanotropes can be manipulated at the physiological level simply by changing the background colour of the animal. As an approach to identify genes associated with the specialized secretory function of peptide hormone producing cells, we differentially screened a NIL cDNA library of black-adapted *Xenopus* with cDNA probes derived from NIL mRNA of black and white adapted animals. Here we report the identification of twelve distinct genes whose transcript levels in the melanotropes are regulated in coordination with that of POMC during background adaptation.

MATERIALS AND METHODS

Animals

South African clawed toads *Xenopus laevis* (40-60 g) were adapted to black or white backgrounds under constant illumination for three weeks at 22°C.

Metabolic cell labeling studies

Neurointermediate lobes (NILs) and anterior lobes (ALs) from black and white *Xenopus* were dissected and preincubated in incubation medium (IM: 112 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 15 mM Hepes pH 7.4, 0.3 mg/ml BSA, 2 mg/ml glucose) at 22°C for 15 minutes. Pulse labeling of newly synthesized proteins was performed by incubating lobes in IM containing 1.7 mCi/ml [^{35}S]methionine (Tran^{35}S-label, ICN Radiochemicals) for 10 minutes at 22°C. Lobes were homogenized on ice in lysis buffer containing 50 mM Hepes pH 7.2, 140 mM NaCl, 1% Tween 20, 0.1% Triton X-100, 0.1% deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mg/ml soybean trypsin inhibitor. Homogenates were cleared by centrifugation before addition of 0.1 volume of 10% SDS. Newly synthesized proteins were resolved by SDS-PAGE and visualized by fluorography.

cDNA library construction and prescreening

Cytoplasmic RNA was isolated from NILs of 100 black adapted *Xenopus* toads using the NP40 method and subjected to oligo(dT) chromatography according to Sambrook et al. (1989). cDNA appropriate for directional cloning was synthesized using a commercial cDNA synthesis kit (Stratagene), size fractionated on CL4B Sepharose and ligated into lambda uni-ZAP XR (Stratagene). About 50 000 primary plaques were hybridized on duplicate filters at a density of 10 plaques per cm^2 using standard procedures (Sambrook et al. 1989). Hybridization was with a random prime labeled POMC cDNA (poly(A) tail removed) and with a single stranded (ss) cDNA probe synthesized from oligo(dT) primed *Xenopus* liver RNA using Superscript reverse transcriptase (Gibco BRL). Filters were washed at 63°C to a final stringency of 0.2× SSC and exposed to X-ray film at −70°C with two intensifying screens.

Preparation of cellulose-coupled cDNAs

As the first step towards the generation of the probes used in the differential hybridization, cellulose coupled cDNAs were prepared from RNA from various *Xenopus* tissues using a modified version of the method from Rodriguez and Chader (1993). A 100 μg sample of total RNA from brain, liver or heart was annealed to 5 mg oligo(dT) cellulose (Pharmacia) in 100 μl of 10 mM Tris HCl pH 7.5, 1 mM EDTA pH 8.0, 1 M NaCl and 0.25% SDS by preheating the mixture to 70°C and rotation for 20 minutes at room temperature. Excess RNA was removed by washing the cellulose three times in 10 mM Tris HCl pH 7.5, 1 mM EDTA pH 8.0, 100 mM NaCl and two times in RT buffer (50 mM Tris HCl pH 8.3, 75 mM KCl and 3 mM $MgCl_2$). For cDNA synthesis the cellulose was suspended in 50 μl RT buffer supplemented with 10 mM DTT, 500 μM dNTPs, 5 μCi [α ^{32}P]dATP (3000 Ci/mmol, Amersham), 35 units RNasin and 400 units Superscript reverse transcriptase. After 45 minutes at 42°C the cellulose was washed three times at room temperature and two times at 94°C with TE pH 7.5. cDNA synthesized was quantified by measuring the amount of incorporated ^{32}P. In a similar way cDNA from total NIL RNA of six black and six white animals was synthesized on 2 mg oligo(dT) cellulose.

Differential hybridization

Recombinant pBluescript SK⁻ phagemids were excised in vivo from selected lambda ZAP clones and rescued as ss antisense DNA according to the instructions of the manufacturer. A 5 ng sample of ss antisense DNA derived from 204 isolated cDNA clones was spotted on four separate nitrocellulose filters. Each of these was then hybridized with different ss sense cDNA probes prepared by random prime labeling of cellulose coupled antisense cDNAs. For this the cellulose coupled cDNAs were suspended in 15 μl water, heated for 2 minutes at 95°C and cooled on ice before addition of 35 μl buffer containing random primers (Sambrook et al. 1989), 1 mg/ml BSA, 60 μCi [α ^{32}P]dATP and 8 units Klenow fragment (Pharmacia). Following 30 minutes incubation at 37°C and 60 minutes rotation at room temperature, unincorporated label was removed by washing the cellulose three times in 10 mM Tris HCl pH 7.5, 1 mM EDTA and 100 mM NaCl. Labeled ss cDNA fragments were recovered by incubating the cellulose twice at 94°C for 2 minutes in 75 μl water containing 2 μg yeast tRNA and collected by centrifugation. Probes were generated from cellulose coupled NIL cDNA of white and black animals (white probe: total activity 0.5×10^6 cpm, black probe: total activity 4.0×10^6 cpm). For a probe enriched in brain specific sequences, labeled ss cDNA fragments (total activity 6×10^6 cpm) were generated from 70 ng of cellulose coupled brain cDNA mixed with 140 ng cellulose coupled liver cDNA in 200 μl 5× SSPE, denatured at 94°C for 2 minutes and allowed to anneal at 55°C for 1 hour. Non hybridizing ss cDNA fragments were annealed to 220 ng cellulose coupled heart cDNA. Consequently two fractions of the original probe were isolated: one that fails to anneal to liver and heart cDNA, thus enriched in brain specific sequences (brain probe: total activity 3.5×10^6 cpm) and another fraction eluted from liver and heart cDNA containing mainly common sequences (common probe: total activity 2.5×10^6 cpm). Prehybridized filters were screened separately with black, white, brain and common probes overnight at 63°C.

and washed to a final stringency of 0.2× SSC at 63°C. Exposure to X-ray film was for three days at −70°C with two intensifying screens.

DNA sequence analysis and database matching

Sequencing of selected cDNA clones on both strands and with pBluescript subclones or specific primers was performed with single- and double-stranded DNA using T7 DNA polymerase (Pharmacia) and the dideoxy chain termination method (Sanger et al., 1977). Nucleotide sequences and deduced protein sequences were compared with those present in the EMBL/GenBank and Swissprot/PIR databases using computer facilities of the CAOS/CAMM center at the University of Nijmegen.

RNA isolation

For expression studies, total RNA was prepared according to the method of Chomczynski and Sacchi (1987), using acid-guanidine isothiocyanate-phenol-chloroform extraction. After recovery by ethanol precipitation, the RNA was quantified by spectrophotometry and its integrity checked by running samples on denaturing agarose gels followed by ethidium bromide staining. Poly(A)$^+$ RNA was selected by oligo(dT) chromatography and quantified by spectrophotometry. The recovery of RNA from NILs and ALs was aided by using yeast tRNA as a carrier.

RNase protection assay

Constructs to be used in the RNase protection assay were generated by subcloning appropriate restriction fragments from selected cDNA clones into pBluescript SK$^-$ vector. After verification by sequencing, constructs were linearized by restriction digestion and antisense run-off transcripts were generated from the T3 or T7 RNA polymerase promotor. Transcripts labeled with [α-^{32}P]UTP (800 Ci/mmol, Amersham) were purified on 5% polyacrylamide/8 M urea gels. Transcript sizes generated from the constructs (with size of protected bands in parentheses) were: X0286, 402 nt (346); X1035, 359 nt (277); X1262, 216 nt (156); X1267, 347 nt (277); X1311, 362 nt (279); X6227, 319 nt (244); X8211, 429 nt (355); X8556, 385 nt (343); X8591, 301 nt (254); X8596, 297 nt (276); X8675, 410 nt (311); fascin, 406 nt (311); actin, 233 nt (210). About 1×10^5 cpm of each transcript was combined with total RNA samples in 25 μl hybridization mix (80% formamide, 400 mM NaCl, 40 mM Pipes, pH 6.4, and 1 mM EDTA). Samples were incubated at 80°C for 5 minutes before hybridization overnight at 50°C. Non-hybridized RNA was digested with RNase A and T_1 for 30 minutes at 37°C. Samples were treated with proteinase K, phenol/chloroform/isoamyl-alcohol extracted, supplemented with 10 μg yeast tRNA, ethanol precipitated and run on 5% polyacrylamide/8 M urea gels. Following autoradiography, quantification of protection signals was performed with a Ultrascan XL laser densitometer (LKB/Pharmacia).

Northern blot analysis

RNA was separated by electrophoresis on 2.2 M formaldehyde-containing 1.2% agarose gels in MOPS buffer and blotted onto nitrocellulose filters as described by Ausubel et al. (1989). Hybridization was overnight at 45°C in 5× SSPE, 50% formamide, 5× Denhardt's, 0.5% SDS and 0.1 mg/ml denatured salmon sperm DNA, using 1×10^6 cpm/ml of probe. A cDNA probe prepared from oligo(dT)-primed NIL RNA of black animals was used to hybridize a blot containing RNA of NILs and ALs from black and white animals. Other tissue RNA blots were hybridized with random prime-labeled inserts from selected cDNA clones. Blots were washed at 63°C to a final stringency of 0.1× SSPE and autoradiographed at −70°C using two intensifying screens.

In situ hybridization

Black and white animals were anesthetized in water containing 1 mg/ml 3-aminobenzoic acid ethyl ester (Sigma). Brains with pituitaries still attached were perfused with XPBS (101 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 and 1.4 mM KH_2PO_4) and subsequently with 4% paraformaldehyde in XPBS (PFA/XPBS), dissected and incubated in 4% PFA/XPBS for 4 hours at 4°C. Following overnight incubation in 30% sucrose/XPBS at 4°C, tissues were frozen and cut into 15 μm sections that were collected on binding silane-treated glass slides. Each slide contained pituitary sections of both black and white animals to be processed under identical conditions. Sections were incubated with 0.2% pepsin in 0.1 M HCl for 10 minutes, fixed in 2% PFA/XPBS for 10 minutes, acetylated with acetic anhydride and dehydrated in a graded series of ethanol at room temperature. Hybridization was overnight at 55°C in 5× SSC, 50% formamide, 50 mM sodium phosphate, pH 7.4, 2.5× Denhardt's, 10% dextran sulfate, 0.1% SDS, 0.2 mg/ml denatured proteinase K-treated salmon sperm DNA and 0.2 mg/ml yeast tRNA. Digoxigenin-labeled antisense RNA probes were prepared according to the instructions of the manufacturer (Boehringer Mannheim). Linearized plasmid constructs used in the RNase protection assay served as templates. After hybridization with 0.2 μg/ml of probe, sections were washed at 63°C to a final stringency of 0.1× SSC and hybrids were detected by an enzyme-linked immunoassay (Boehringer Mannheim). Specificity of the hybridization signals obtained was assessed by using sense RNA probes as a control.

RESULTS AND DISCUSSION

Xenopus melanotropes as a model system for identifying genes involved in peptide hormone production and release

The biosynthetic activity of the melanotrope cells in the NIL of *Xenopus* can be manipulated in vivo by changing the background colour of the animal (Fig. 1). Metabolic cell labeling experiments revealed that in the melanotropes of black-adapted toads the production levels of a subset of the newly synthesized proteins are dramatically increased compared to those in melanotropes of white-adapted animals (Fig. 1B). Within this group, POMC clearly represents the major newly synthesized protein as it constitutes over 80% of all radiolabeled products. These findings are consistent with northern blot experiments in which NIL RNA from black and white animals was hybridized with a cDNA probe prepared from NIL RNA of black animals. As shown in Fig. 1C, POMC transcripts represent by far the most abundant mRNA species in the NIL. Moreover, as reported previously (Martens et al., 1987), the levels of POMC mRNA are 20- to 30-fold higher in the NILs of black animals compared to that in white animals. These physiologically induced changes in protein biosynthesis and RNA expression are confined to the melanotrope cells of the NIL and do not occur in cells of the anterior lobe (AL) of the pituitary (Fig. 1B,C).

The above results led us to use *Xenopus* melanotropes in a strategy aimed at the identification of genes involved in peptide hormone production and release. Our strategy is based on the assumption that, following physiological manipulation of these cells, at least part of the genes implicated in the translocation, sorting, processing and exocytotic release of peptide hormones will be coordinately expressed with the POMC gene.

Differential screening strategy

As outlined in Fig. 2, the first step in our strategy concerned the construction of a cDNA library from NIL mRNA of fully black-adapted *Xenopus*. Screening of the library with a POMC

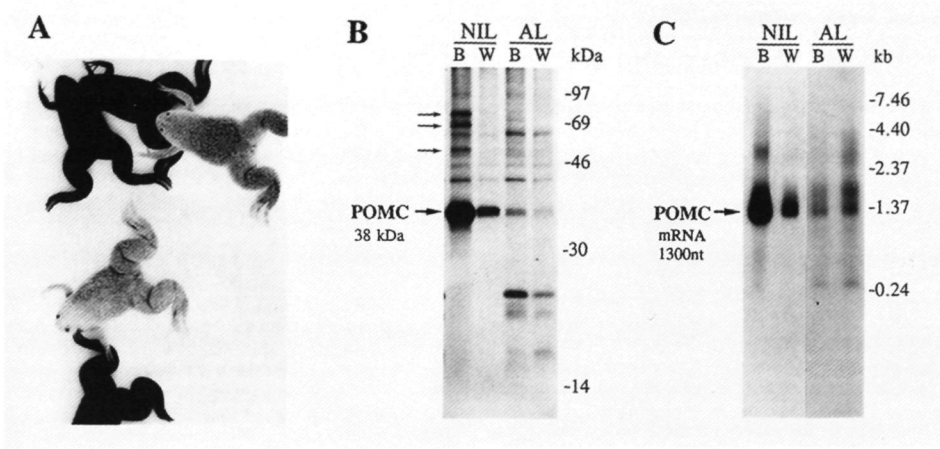

Fig. 1. Effect of background adaptation on levels of newly synthesized proteins and RNA expression in the pituitary gland of *Xenopus laevis*. (A) Animals adapted to black or white backgrounds for three weeks. (B) Radiolabeled proteins produced by pulse-incubated neurointermediate lobes (NIL) and anterior lobes (AL) of black (B) and white (W) animals. Arrows indicate POMC and three additional proteins whose production levels in the NIL are clearly enhanced in black animals with respect to white animals. Each lane contains protein extract equivalent to 1/25 part of a lobe. (C) Northern blot analysis of total RNA extracted from NILs and ALs of black (B) and white (W) animals using a cDNA probe derived from oligo(dT)-primed NIL RNA of black animals. The migration of POMC transcripts is indicated. Each lane contains RNA extracted from five pooled lobes.

cDNA probe revealed that 75% of the 50,000 primary clones harbour POMC-encoding sequences. This proportion of POMC cDNAs is in line with the high rate of POMC biosynthesis and the abundant presence of POMC transcripts in NILs of black animals (Fig. 1). The library was subsequently hybridized with a liver mRNA-derived cDNA probe to eliminate clones encoding ribosomal proteins, mitochondrial enzymes and other highly expressed 'housekeeping' proteins. Following screening with the POMC and liver probes, about 7,500 non-hybridizing clones remained for further analysis. From this group, clones representing genes that are coordinately expressed with the POMC gene were selected by differential hybridization with cDNA probes derived from NIL RNA of black and white animals (black and white probes, respectively). To determine which of the differentially hybridizing clones represent neuroendocrine-specific genes, an additional screening was performed with cDNA probes enriched in brain-specific or commonly expressed sequences (brain and common probes, respectively).

For testing the specificity of our differential hybridization procedure, we first screened a number of NIL cDNAs that had been isolated and identified following a random pick approach. Differential hybridization signals in the black/white screening were obtained for cDNAs encoding POMC, prohormone convertase PC2, carboxypeptidase H (CPH), the neuroendocrine polypeptide 7B2, calpactin light chain, calreticulin and binding protein BiP (Fig. 3A and data not shown). Non-differentially hybridizing cDNAs or cDNAs showing only a minor (below threefold) difference in signals included those for ribosomal proteins, translation initiation factors, ferritin-H, mitochondrial NADH-oxidoreductase chain 4, actin and the actin-bundling protein fascin (Fig. 3A and data not shown). Only cDNAs encoding neuroendocrine-specific proteins (POMC, PC2, CPH and 7B2) were positive with the brain probe, not with the common probe (Fig. 3A).

Selection of differentially expressed genes

Having established the feasability of the various probes for differential screening, we then randomly picked 204 clones from the group of 7,500 non-POMC and non-liver clones, and single-stranded DNA corresponding to these was spotted onto nitrocellulose filters. Screening of the filters with the black and white probes yielded 58 differentially hybridizing cDNAs (Fig. 3B). Seven of these were removed, since they displayed strong hybridization signals with the common probe. Of the remaining 51 cDNAs, 36 were brain-specific, whereas no hybridization signals above background were observed for the other 15 cDNAs. Partial sequence analysis and cross-hybridization experiments with the cDNA inserts as probes revealed that the 51 cDNAs were derived from 27 distinct genes (data not shown). Fifteen genes were excluded from subsequent studies, since their presumed differential expression, based on the dot blot hybridization procedure, could not be confirmed in RNase protection assays (data not shown). The RNA levels of the remaining twelve genes were at least sixfold higher in the NILs of black animals than in those of white animals (Fig. 4, Table 1). These physiologically induced changes in gene expression are tissue specific because they were not observed in the AL (Fig. 4). Eight of the differentially regulated genes are selectively or preferentially expressed in *Xenopus* brain and pituitary (clones X1035, X1311, X8211, X8290, X8556, X8591, X8596 and X8675) whereas four represent ubiquitously expressed genes (clones X0286, X1262, X1267 and X6227) (Fig. 4). The above results were highly reproducible

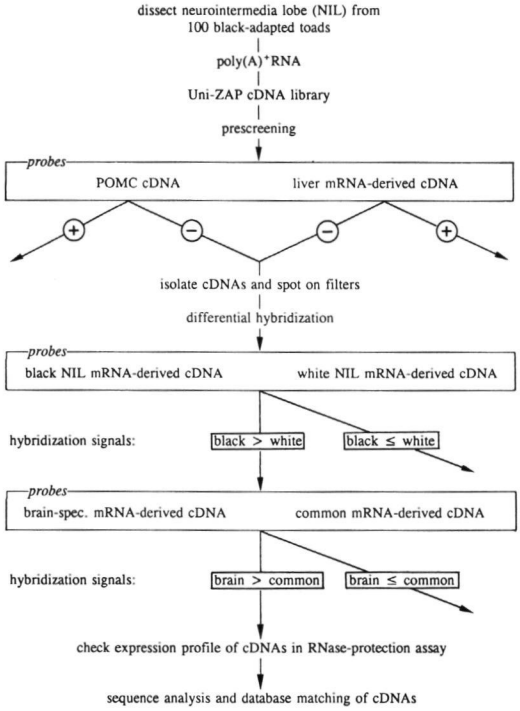

Fig. 2. Strategy for the identification of differentially expressed genes. See text for details.

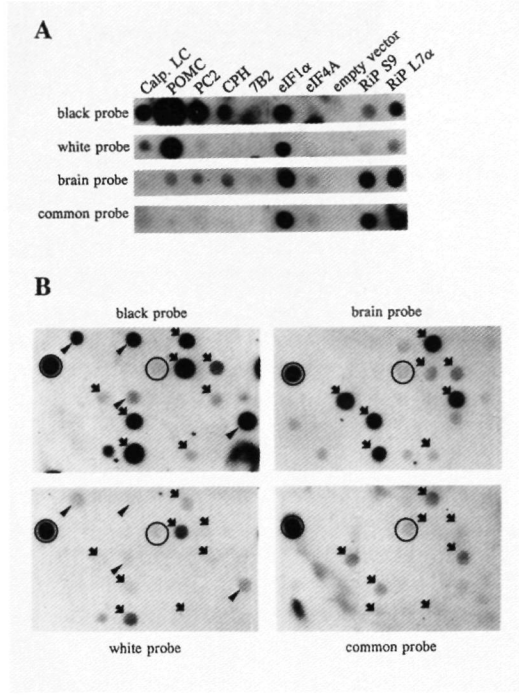

Fig. 3. Differential hybridization of NIL cDNA clones. Filters containing spotted DNA from isolated NIL cDNA clones were hybridized with cDNA probes derived from NIL mRNA of black and white animals (black and white probes), or cDNA probes enriched in brain-specific (brain probe) or commonly expressed sequences (common probe). (A) Previously identified NIL cDNA clones serving as a reference for the differential screening procedure. Calp. LC, calpactin light chain; POMC, proopiomelanocortin; PC2, prohormone convertase 2; CPH, carboxypeptidase H; 7B2, neuroendocrine protein 7B2; eIF1α, translation initiation factor 1α; eIF4A, translation initiation factor 4A; empty vector, pBluescript SK$^-$; RiP S9, ribosomal protein S9; RiP L7α, ribosomal protein L7α. (B) Differential hybridization of newly isolated NIL cDNA clones. Arrows indicate clones displaying differential hybridization signals in both the black/white and brain/common screenings. Clones giving differential hybridization signals in the black/white screening only are marked with an arrowhead. Non-differentially hybridizing clones are encircled.

(Fig. 5) and consistent with northern blot analysis (Fig. 6A,B) and in situ hybridization experiments (Fig. 7 and data not shown). The cDNAs corresponding to the twelve differentially expressed genes were therefore sequenced, and their nucleotide and deduced protein sequences compared with those present in the EMBL/GenBank and NBRF/SWISS databases.

Characterization of differentially expressed genes

As summarized in Table 1, the database search revealed that eight of the twelve differentially expressed genes correspond to previously identified genes. These genes can be classified according to the functions and/or subcellular distributions of the proteins they encode.

Prohormone processing enzymes

Clone X1035 encodes the prohormone convertase PC2, a neuroendocrine-specific endoprotease that cleaves its substrate at pairs of basic amino acids (Benjannet et al., 1991; Thomas et al., 1991). Identification of this gene is reassuring as its transcript levels in *Xenopus* melanotropes were previously found to be regulated in parallel with POMC mRNA (Braks et al., 1992). Clone X8675 codes for the *Xenopus* homolog of rat carboxypeptidase H (CPH), an enzyme removing the basic amino acid residues from C-terminal ends of prohormone-derived peptides (Fricker, 1988). An amino acid sequence identity of 84% over 197 matched residues reflects a high degree of conservation for this enzyme between mammals and amphibians. Both the PC2 gene and CPH gene produce two differentially regulated transcripts (Fig. 6A, Table 1) that differ in size due to the use of alternative polyadenylation sites (data not shown). The frequency by which cDNAs for these transcripts were selected in our study indicates that they represent the most abundant of all regulated non-POMC mRNAs in the NIL (Table 1). RNase protection assays revealed 35-fold higher levels of CPH mRNA in the NILs of black animals than in those of white animals (Fig. 5). This black/white ratio in transcript levels is 25 for PC2. In situ hybridization experiments

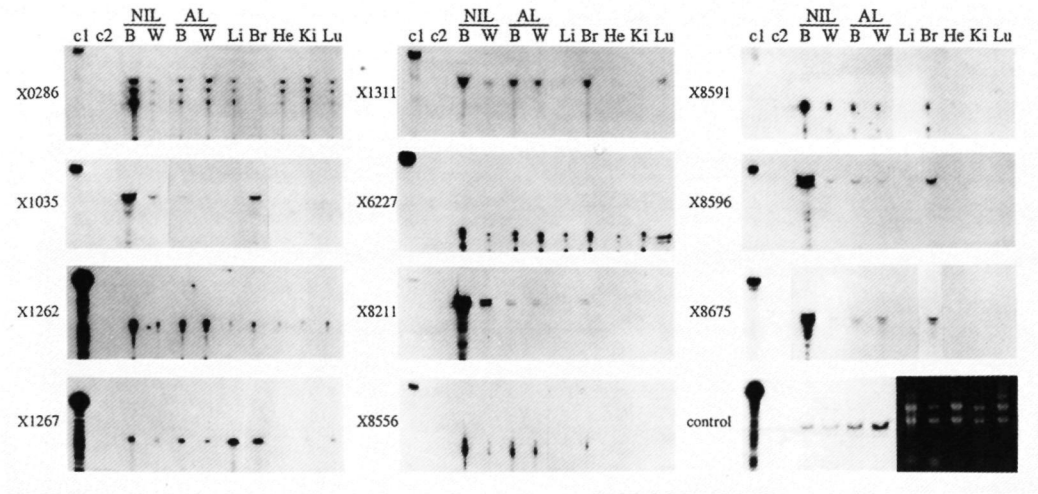

Fig. 4. RNase-protection analysis of differentially expressed genes. Radiolabeled anti-sense RNA was transcribed from selected NIL cDNA clones and hybridized to total RNA extracted from the NIL and AL of black (B) and white (W) animals, or to 5 µg total RNA from liver (Li), brain (Br), heart (He), kidney (Ki) and lung (Lu). In each assay, total RNA from 2 NILs or ALs was used (except for clone X8211, 1/5 part of a NIL, one AL; clones X1035 and X8675, 1/25 part of a NIL and one AL each). RNase-treated samples were loaded onto denaturing polyacrylamide gels and autoradiographed. Undigested RNA probes (c1) or probes hybridized to 20 µg yeast tRNA prior to digestion (c2) served as controls for specificity of protection signals. Quality of RNA was checked by hybridizing samples to antisense RNA probe against the non-regulated transcript for fascin (Holthuis et al., 1994) or by running samples on ethidium bromide-stained gels (see control).

demonstrate that these dynamics in gene expression occur in the melanotrope cells of the intermediate pituitary (Fig. 7; Braks et al., 1992). Since both enzymes are indispensible for the conversion of prohormones into bioactive peptides, the vigorous regulation of their mRNAs in *Xenopus* melanotropes is not surprising. Our findings concerning PC2 and CPH are in line with those of other investigators who previously demonstrated that in rat intermediate pituitary these genes are coregulated with POMC by dopaminergic agents (Bloomquist et al., 1991, 1994).

Granins

Clone X8211 corresponds to a previously isolated *Xenopus* gene encoding the neuroendocrine protein 7B2 (Martens et al.,

Table 1. Identification of differentially expressed genes

Clone	Freq*	Black/white Ratio†	mRNA (kb)‡	Protein (kDa)§	Database match	Entry	Species¶	Matched length (aa)	% Identity
X1035	10	25	2.2	71	Prohormone convertase PC2	S23118	X	614	100
X6243	4	25	4.4	71	Prohormone convertase PC2‖	S23118	X	183	100
X8211	4	20	1.2	23	Neuroendocr. polypeptide 7B2	S03938	X	209	100
X8675	4	35	4.4	–	Carboxypeptidase H‖	A40469	R	65	79
X1151	3	35	2.0	–	Carboxypeptidase H	A40469	R	132	89
X8556	2	15	n.d.	68	Secretogranin II	S02180	R	574	49
X8596	2	30	2.1	57	Secretogranin III	A37180	R	435	61
X0286	1	20	0.8	18	Translocon-associated protein δ	S33295	R	150	81
X1262	1	20	1.2	20	Glycoprotein 25L precursor	G25lcanfa	D	183	32
X1267	1	6	2.5	–	ERV1 protein	ERV1yeast	Y	72	27
X1311	1	10	2.3	48	Vacuolar H^+-ATPase subunit	BT10039	B	430	60
X6227	1	10	n.d.	–	ER60 protease precursor	ER60mouse	M	165	72
X8290	1	8	8.0	–	no match	–	–	–	–
X8591	1	10	n.d.	–	no match	–	–	–	–

*Frequency by which independent cDNAs representing one transcript were isolated.
†Ratio of transcript levels in the intermediate pituitary between black- and white-adapted animals.
‡Transcript size as determined by northern blot analysis.
§Calculated size of protein without signal peptide as deduced from cDNA sequence.
¶B, bovine; D, dog; M, mouse; R, rat; X, *Xenopus*; Y, yeast.
‖cDNAs corresponding to larger transcripts generated by the use of alternative polyadenylation sites.
n.d., not determined.

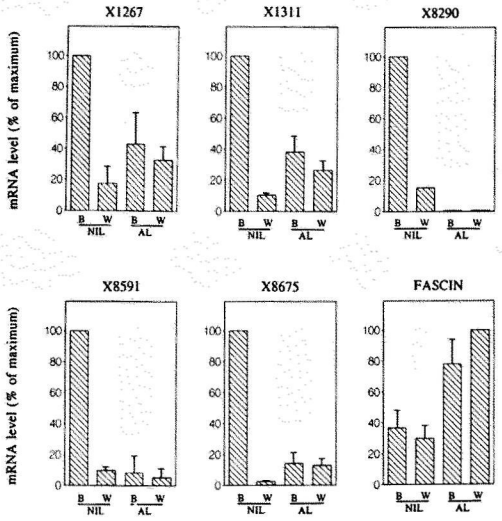

Fig. 5. mRNA levels of differentially expressed genes in the NIL and AL of black (B) and white (W) animals. mRNA levels were determined by RNase-protection analysis using radiolabeled antisense RNA transcribed from selected NIL cDNA clones (X1267, X1311, X8290, X8591 and X8675) and aliquots of total RNA extracted from pooled lobes. Aliquots of RNA from each pool were used to determine the level of the non-regulated transcript for fascin. mRNA levels were quantified by densitometric scanning of autoradiographs. Data shown are the mean ± s.e.m. of three independent experiments.

1989). The protein sequences deduced from clones X8556 and X8596 display 49% and 61% identity with rat secretogranin (SG)II (Gerdes et al., 1989) and SGIII (Ottiger et al., 1990), respectively. These clones therefore likely represent the *Xenopus* homologs of SGII and SGIII. Transcripts for 7B2, SGII and SGIII are selectively present in neuroendocrine tissues (brain and pituitary; Fig. 4) and are fairly abundant among the regulated messengers in the NIL (Table 1). The black/white ratio in transcript levels is 20 for 7B2, 15 for SGII and 30 for SGIII. All three proteins belong to a class of acidic secretory granule-associated proteins, also known as the granin family (Huttner et al., 1991). Several functions for these proteins have been proposed. They may represent precursors of biologically active peptides (Saria et al., 1993), assist in sorting and secretory granule biogenesis (Chanat and Huttner, 1991; Huttner and Natori, 1995) or serve as molecular chaperones for prohormone-processing enzymes (Braks and Martens, 1994). The differential regulation of three distinct granins in *Xenopus* melanotropes during background adaptation reinforces the physiological relevance of this class of proteins in neuroendocrine cells.

ER resident proteins

Two differentially regulated genes represent *Xenopus* homologs of known mammalian genes for proteins residing in the endoplasmic reticulum (ER): namely, the cysteine protease precursor ER60 (clone X6227) and the translocon-associated protein TRAPδ (clone X0286). Both ER60 and TRAPδ are encoded by ubiquitously expressed genes (Fig. 4) and exhibit a high degree of conservation during vertebrate evolution (Table 1). ER60 transcript levels in the NIL show a black/white ratio of 10 whereas this ratio is 20 for TRAPδ mRNA. ER60 belongs to the class of reticuloplasmins, a group of soluble and abundant proteins that is retained in the ER lumen by the C-terminal retention signal KDEL. ER60 protease shows cysteine proteolytic activity and catalyzes proteolytic cleavage of itself as well as of other reticuloplasmins (Urade et al., 1992; Urade and Kito, 1992). Therefore, ER60 protease could well be part of the proteolytic system that clears the ER of abnormal or incorrectly folded secretory proteins. TRAPδ is a subunit of a tetrameric complex of transmembrane proteins that is located in proximity to the site where secretory proteins are translocated across the ER membrane (Hartmann et al., 1992). The TRAP complex is not essential for the translocation process (Migliaccio et al., 1992) and its function is unknown. It may have a role in the proper release of newly synthesized proteins from the translocon or participate in the folding or quality control systems of the ER. The enhanced transcript levels observed for ER60 protease and TRAPδ in the melanotropes of black-adapted *Xenopus* probably relate to an expanding capacity of the ER to accommodate the high quantity of newly synthesized prohormone.

An accesory subunit for vacuolar H^+-ATPase

Clone X1311 corresponds to a single mRNA of 2.3 kb, which is expressed predominantly in pituitary and brain (Figs 4, 6B). NILs of black animals contain ten times more of this transcript than those of white animals (Fig. 5). In situ hybridization experiments indicate that these changes in gene expression originate from the melanotrope cells (Fig. 7). The 48 kDa protein encoded by X1311 carries an N-terminal signal sequence and contains seven potential N-linked glycosylation sites as well as a membrane-spanning segment close to its C-terminal end. A database search revealed 60% amino acid sequence identity with Ac45 (Table 1), a novel accessory subunit of the vacuolar H^+-ATPase (V-ATPase) from bovine adrenal medulla chromaffin granules (Supek et al., 1994). X1311 therefore probably represents the *Xenopus* homolog of Ac45. The function of Ac45 is not known. V-ATPases are ancient multimeric enzymes responsible for the generation of the acidic gradients within the central vacuolar system of eukaryotic cells (Forgac, 1989). In neuroendocrine cells, the perturbation of these gradients by weak bases or with specific V-ATPase inhibitors leads to missorting and impaired proteolytic processing of regulated secretory proteins (Moore et al., 1983; Henomatsu et al., 1994). How V-ATPases are directed to, and achieve their specialized functions in such diverse organelles as endosomes, lysosomes and secretory granules is unclear. Studies with yeast mutants lacking genes for specific V-ATPase subunits have indicated that the assembly of the enzyme starts with its membrane sector (Manolson et al., 1992; Kane et al., 1992). Consequently, specific accessory membrane proteins are likely to be required for correct assembly and specific functioning of V-ATPases in the various organelles. Hence, an attractive possibility is that Ac45 is a membrane-associated modulator of V-ATPase activity in secretory granules. Such a role would be consistent with our finding that transcripts for this protein are primarily expressed in neuroen-

docrine tissues and regulated in coordination with the biosynthetic activity of peptide hormone-producing cells.

Novel gene products

Four of the regulated transcripts isolated in this study are derived from previously unknown genes. Two of these, clones X8290 and X8591, gave no match with sequences in the database. Northern blot experiments revealed a single brain- and pituitary-specific transcript of 8 kb for X8290 (Fig. 6A,B), yet failed to produce a specific hybridization signal for X8591. RNase protecion analysis showed that the X8591 transcript is predominantly, but not exclusively, expressed in brain and pituitary (Fig. 4). Levels of X8290 and X8591 transcripts in NILs of black animals are 8- and 10-fold higher, respectively, than in those of white animals (Fig. 5). The X8290 and X8591 cDNAs lack open-reading frames of significant length. Rescreening of a *Xenopus* hypothalamus cDNA library yielded clones with insert sizes up to 3.5 kb. None of these, however, contained open-reading frames and they all seem to represent 3′-untranslated regions of long transcripts. Work is in progress to obtain 5′-extended cDNAs.

Clone X1267 represents a transcript of 2.5 kb, which was detected by northern blot analysis in *Xenopus* liver (Fig. 6B). RNase protection assays revealed lower amounts of the transcript in a variety of other tissues (Fig. 4). NILs of black animals contain sixfold higher levels of this mRNA than those of white animals (Fig. 5). X1267 cDNA contains an open-reading frame that shares 27% identity over 72 matched amino acids with the yeast *ERV1* gene product (Lisowsky, 1992). A zinc finger-like motif (HXF/YXCXXCAXXFXR/K, corresponding to Erv1p residues 54-67) was found within a well-conserved region. The *ERV1* gene is essential for yeast viability and experimental data suggest that it encodes an important component for the expression or stability of nuclear and mitochondrial transcripts (Lisowsky, 1992). The structural resemblance between the X1267 protein and the *ERV1* gene

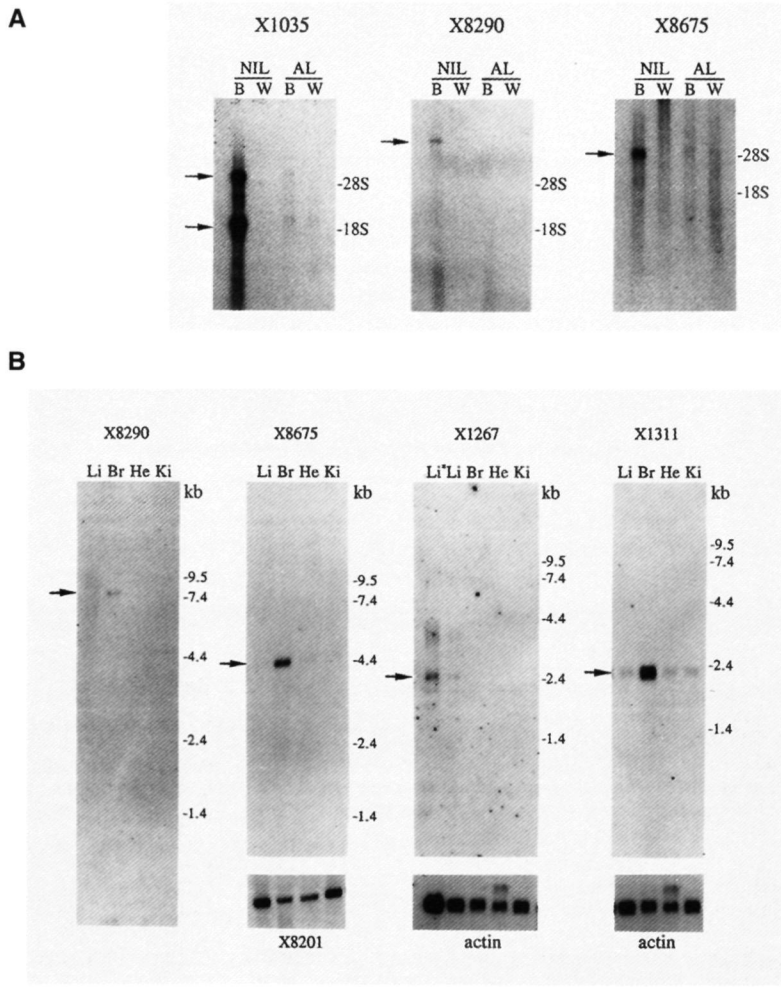

Fig. 6. Northern blot analysis of differentially expressed genes. (A) Random prime-labeled inserts from NIL cDNA clones X1035, X8290 and X8675 were hybridized to total RNA extracted from NILs and ALs of black (B) and white (W) animals. Each lane contains RNA extracted from five pooled lobes, except those hybridized with the X8290 probe (ten pooled lobes). The mobility of 18 S and 28 S ribosomal RNA is indicated. (B) Hybridization of random prime-labeled inserts from NIL cDNA clones X8290, X8675, X1267 and X1311 to 6 μg poly(A)$^+$ RNA from liver (Li), brain (Br), heart (He) and kidney (Ki) or 20 μg liver poly(A)$^+$ RNA (Li*). As a control for loading, blots were stripped and rehybridized with radiolabeled inserts from cDNA clones representing ubiquitously expressed transcripts (X8211 or actin). The mobility of size markers is indicated.

product together with the finding that its transcript levels in *Xenopus* melanotropes are regulated in parallel with those of POMC make X1267 an attractive candidate for a regulator of gene expression in these cells.

The 1.3 kb transcript corresponding to clone X1262 was detected in all tissues examined, both by RNase protection assay (Fig. 4) and northern blot analysis (data not shown). About 20-fold more X1262 mRNA was measured in NILs of black animals compared to those of white animals. The protein encoded by X1262 has a low but significant degree of identity (32% over 183 matched residues) with ER-associated glycoprotein gp25L (Table 1). The proteins are similar in size and both carry an N-terminal signal sequence, a C-terminal transmembrane domain and a double lysine motif that may function as a retention signal for ER transmembrane proteins. gp25L has been isolated from dog pancreatic microsomes as a complex together with two members of the TRAP tetramer (TRAPα and TRAPβ) and the Ca^{2+} binding protein calnexin (Wada et al., 1991). The role of gp25L and the significance of its association with calnexin and members of the TRAP complex remain to be established. We noticed that gp25L and the X1262 protein share an overall sequence identity of 27% with protein P24B from yeast. P24B is a component of ER-derived transport vesicles with an essential role in the delivery of a subset of secretory proteins to the Golgi complex (Schimmöller et al., 1995). X1262 is clearly not the *Xenopus* homolog of gp25L or P24B. An amino acid sequence deduced from two overlapping human expressed sequence tags (EMBLnew accession numbers hs23814 and t10797) displays 85% sequence identity over 100 matched residues with the X1262 protein. Hence, the X1262 protein is highly conserved during evolution and belongs to a novel family of ER transmembrane proteins whose members occur throughout the animal kingdom.

The nature of the genes described above demonstrates that our differential screening method can be successfully applied as a strategy for the isolation of components from the secretory pathway in peptide hormone-producing cells. We identified a number of regulated secretory proteins (PC2, CPH, 7B2, SGII and SGIII) whose recruitment in the melanotropes of black-adapted *Xenopus* is not an unexpected finding, given that such proteins are generally stored and released together with peptide hormones. We also tracked down one soluble and several transmembrane proteins representing permanent residents of the secretory pathway. Three of these (ER60, TRAPδ and the gp25L-like protein) are retained in the ER, whereas a fourth protein (Ac45) belongs to the membrane moiety of V-ATPase from secretory granules. The impressive (10- to 20-fold) rise in transcript levels for these non-secreted proteins in stimulated melanotropes most likely reflects, at the molecular level, the

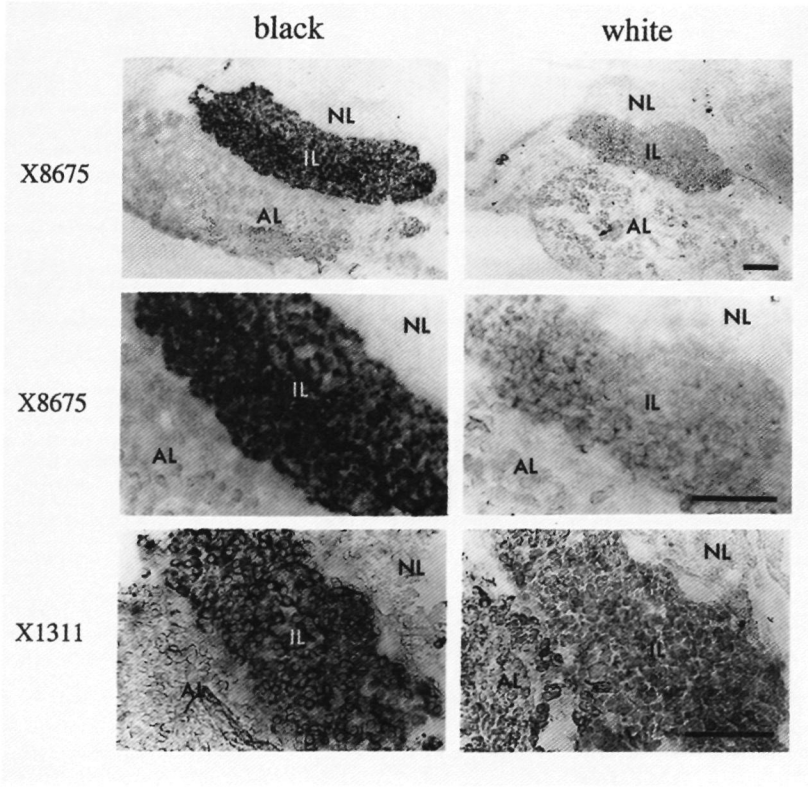

Fig. 7. In situ hybridization analysis of X8675 and X1311 transcripts in pituitary glands of black- and white-adapted *Xenopus*. Cryosections of pituitaries from black and white animals were hybridized under identical conditions with anti-sense digoxigenin-labeled RNA probes. Both probes give strong hybridization signals in the melanotropes of the intermediate lobe (IL) of black animals, whereas only weak signals are obtained in melanotropes of white animals. Note that cells of the anterior lobe (AL) are moderately stained without any difference in intensity between black and white animals. No staining is present in the neural lobe (NL). Control experiments using sense digoxigenin-labeled RNA probes failed to produce any signal above background (not shown). Bar, 250 μm.

extensive elaboration of the synthetic apparatus observed within these cells (Hopkins, 1970, de Rijk et al., 1990). Thus far, our collection of differentially expressed genes does not include genes for cytosolic components involved in vesicular traffic. Such genes may have simply not been encountered in this study due to their relatively low levels of expression.

Clearly the most abundant species among the regulated transcripts in *Xenopus* melanotropes is that for POMC, which constitutes approximately 75% of all mRNAs. Our data indicate that transcripts for PC2 represent the second most abundant type (~1% of total mRNA), followed by those for CPH, 7B2, SGII and SGIII (~0.5%, ~0.3%, ~0.2% and ~0.2% of total mRNA, respectively). In agreement with our metabolic cell labeling studies, these data indicate that the biosynthetic machinery in *Xenopus* melanotropes is primarily dedicated to the production of POMC and does not produce any other protein in stoichiometric amounts with this prohormone. Interestingly, we recently showed that 7B2 is a molecular chaperone in the secretory pathway of *Xenopus* melanotropes, which is produced in comparable amounts with its physiological target PC2 (Braks and Martens, 1994). Our screening results suggest that a similar neuroendocrine secretory protein with chaperone-like activity towards prohormones does not exist.

Undoubtedly, many more components from the biosynthetic and secretory machinery in neuroendocrine cells remain to be identified. The in vivo model system of *Xenopus* melanotropes can be put forward as a valuable means to trace some of these and to gain further insight into the molecular mechanisms responsible for the proper generation and release of bioactive peptides.

We are grateful to Tony Coenen, Peter Cruijsen, Karel Janssen, Judith Jeuken and Helma Van den Hurk for technical assistance, and to Ron Engels for animal care. We thank Doug Eib and Bruce Jenks for critically reading the manuscript. This work was supported by a PIONIER grant from the Netherlands Organization for Scientific Research (NWO) and European Community grant BIOT-CT91-0302 to G.J.M.M.

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(Received 24 March 1995 - Accepted 10 July 1995)

Chapter 3

Translocon-Associated Protein TRAPδ and a Novel TRAP-Like Protein are Coordinately Expressed with Proopiomelanocortin in *Xenopus* Intermediate Pituitary

The Biochemical Journal (1995) **312**, 205-213

Biochem J (1995) 312 205–213 (Printed in Great Britain)

Translocon-associated protein TRAPδ and a novel TRAP-like protein are coordinately expressed with pro-opiomelanocortin in *Xenopus* intermediate pituitary

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In the intermediate pituitary gland of *Xenopus laevis*, the expression levels of the prohormone pro-opiomelanocortin (POMC) can be readily manipulated When the animal is placed on a black background, the gene for POMC is actively transcribed, whereas on a white background the gene is virtually inactive In this study, we characterized two genes whose transcript levels in the intermediate pituitary are regulated in coordination with that for POMC One of these codes for a protein homologous to translocon-associated protein TRAPδ, a subunit of a transmembrane protein complex located at the site where nascent secretory proteins enter the endoplasmic reticulum (ER) Both *Xenopus* and mice were found to express an alternatively spliced transcript that gives rise to a previously unknown version of the TRAPδ protein The product of the second gene is a novel and highly conserved protein with structural similarity to glycoprotein gp25L, a constituent of another translocon-associated protein complex A database search revealed the existence of a novel family of gp25L-related proteins whose members occur throughout the animal kingdom Together, our data imply that (i) the group of ER proteins surrounding translocating polypeptide chains may be far more complex than previously expected, and (ii) a number of the accessory components of the translocon participate in early steps of prohormone biosynthesis

INTRODUCTION

The endoplasmic reticulum (ER) forms the entrance of a biosynthetic transport route by which a complex mixture of both secretory and transmembrane proteins reach their final destinations Virtually all proteins that require transport to the cell surface lysosomes or storage vesicles, as well as those residing in the ER or Golgi complex, first undergo translocation across or insertion into the ER membrane These events take place at specific sites (translocons) and involve hydrophilic protein-conducting channels whose major components were initially identified in yeast by genetic screening for translocation defects [1,2] Experiments in which the mammalian translocation process was reconstituted from purified components have indicated that transport of polypeptides essentially requires only two integral membrane–protein complexes, namely the signal recognition particle receptor, which is responsible for targeting the nascent polypeptide chain to the ER, and the Sec61p complex, which is believed to form the actual protein-conducting channel [3] The translocation of a subset of polypeptides also depends on the presence of a third membrane component, the translocating chain-associating membrane (TRAM) protein [3,4] By means of (photo)chemical cross-linking techniques, several additional membrane proteins have been identified that are in close molecular proximity to translocating nascent chains Among these is the translocon-associated protein TRAPα (previously the signal sequence receptor SSRα), a type I transmembrane glycoprotein that interacts with both nascent secretory as well as membrane protein precursors during translocation [5,6] TRAPα can be cross linked to membrane-bound ribosomes [7], and Fab fragments of antibodies raised against the protein were found to inhibit the translocation of several secretory proteins *in vitro* [8] However, immunodepletion of TRAPα from detergent extracts of rough microsomes had no effect on translocation activity [9] It therefore appears that TRAPα, although representing another constituent of the translocon is not required in the translocation process itself At present, the function of TRAPα is unknown Interestingly, the protein was found to be tightly and stoicheiometrically associated with a second single-spanning membrane glycoprotein, named TRAPβ (previously SSRβ) [10] Wada et al [11] have isolated a hetero-tetrameric protein complex in which TRAPα and TRAPβ are associated with calnexin and a small transmembrane glycoprotein, gp25L In contrast, Migliaccio et al [9] and Hartmann et al [12] purified another complex in which the TRAPα–β dimer is associated with a type IV transmembrane protein TRAPγ, and a single membrane-spanning protein, TRAPδ It is possible that differences in the purification procedures employed by these research groups have led to the isolation of distinct protein complexes Alternatively, the variability observed in these protein complexes may reflect different substrate specificities of the translocon apparatus or be related to discrete steps in the translocation event

Our research interest is focused on the molecular mechanisms involved in the biosynthesis of peptide hormones and their stimulus-dependent release from neuroendocrine cells We recently developed a strategy for the selective cloning of components from the secretory pathway in *Xenopus* melanotropes, taking advantage of the fact that the biosynthetic and secretory activity of these intermediate pituitary cells can be readily modulated *in vivo* (J Holthuis, E Jansen, M van Riel and G Martens, unpublished work) When the animal is placed on a black background, the melanotropes produce and release high quantities of pro-opiomelanocortin (POMC) derived, melanophore-stimulating peptides which cause a darkening of the skin [13] In contrast, melanotrope cells of animals on a white background are relatively inactive, resulting in a light skin Consequently, the melanotropes of black adapted animals contain up to 30-fold more POMC mRNA than those of white-

Abbreviations used ER endoplasmic reticulum EST expressed sequence tag NIL neurointermediate lobe ORF open reading frame POMC pro opiomelanocortin RT-PCR reverse transcription PCR ss single stranded TRAP translocon associated protein

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adapted animals [14] We differentially screened an intermediate pituitary cDNA library with cDNA probes derived from intermediate pituitary mRNA of black- and white-adapted animals We here describe the characterization of two genes whose transcript levels are regulated in coordination with that of POMC One of these codes for the *Xenopus* homologue of TRAPδ whereas the other gives rise to a member of a novel family of gp25L-related proteins

MATERIALS AND METHODS

Animals

South African clawed toads *Xenopus laevis* were bred and reared in the aquarium facility of the Department of Animal Physiology at the University of Nijmegen Animals (40–60 g) were adapted to black or white backgrounds under constant illumination for 3 weeks at 22 °C

cDNA library screenings

Cytoplasmic RNA was isolated from neurointermediate lobes (NILs) of 100 black-adapted *Xenopus* toads by using the Nonidet P40 method and subjected to oligo(dT) chromatography according to Sambrook et al [15] cDNA appropriate for directional cloning was synthesized with a commercial cDNA synthesis kit (Stratagene), size-fractionated on CL4B Sepharose and ligated into λ uni-ZAP XR (Stratagene) About 50000 primary plaques were hybridized with a random-prime-labelled POMC cDNA fragment and a single-stranded (ss) cDNA probe synthesized with Superscript reverse transcriptase (Gibco-BRL) from oligo(dT)-primed *Xenopus* liver RNA, by using standard procedures [15] Recombinant pBluescript SK phagemids were excised *in vivo* from 204 non-hybridizing λ ZAP clones, rescued as ss antisense DNA and spotted on to a duplicate set of nitrocellulose filters As a prerequisite for the generation of probes for the differential screening, total NIL RNA of six black-adapted and six white-adapted animals was used for the synthesis of oligo(dT) cellulose-coupled cDNA according to the method of Rodriguez and Chader [16] Ss-sense cDNA probes (the black and white probes) were then prepared by random prime-labelling of each cellulose-coupled cDNA Filters were hybridized separately overnight at 63 °C with the black probe (total radioactivity 4×10^6 c p m) and white probe (total radioactivity $0\,5 \times 10^6$ c p m), washed to a final stringency of $0\,2 \times$ SSC (where SSC is 0 15 M NaCl/0 015 M sodium citrate) at 63 °C and exposed to X-ray film for 3 days at −70 °C with two intensifying screens Next, cDNA inserts from two selected clones, X0286 and X1262, were random-prime-labelled and used to rehybridize the primary *Xenopus* NIL cDNA library Since this procedure yielded a full-length cDNA for X0286 only, $1\,2 \times 10^6$ plaques from an amplified λ ZAP-II *Xenopus* hypothalamus cDNA library [17] were hybridized to obtain the full-length X1262 cDNA clone About 6×10^5 plaques from an amplified human fetal brain λ ZAP II cDNA library (Stratagene) and 8×10^5 plaques from an amplified mouse λ uni-ZAP XR brain cDNA library (Stratagene) were screened for X0286 cDNA homologues Hybridization of the human and mouse libraries was performed overnight in hybridization buffer ($6 \times$ SSC, 0 5 °$_0$ SDS, 0 1 °$_0$ pyrophosphate, 40 mM sodium phosphate, pH 7 0, 1 mM EDTA, $3 \times$ Denhardt's and 0 1 mg/ml denatured salmon sperm DNA) at 42 °C and filters were washed to a final stringency of $0\,4 \times$ SSC at 60 °C

DNA sequence analysis and database matching

Sequencing of selected cDNA clones on both strands and with pBluescript subclones or specific primers was performed with single- and double-stranded DNA by using T7 DNA polymerase (Pharmacia) and the dideoxy chain-termination method [18] Nucleotide sequences and deduced protein sequences were compared with those present in the EMBL/Genbank and SwissProt/PIR databases using the computer facilities of the CAOS/CAMM centre at the University of Nijmegen

RNA isolation

For expression studies, total RNA was prepared according to the method of Chomczynski and Sacchi [19], using acid/guanidine isothiocyanate/phenol/chloroform extraction After recovery by ethanol precipitation, the RNA was quantified by spectrophotometry and its integrity checked by running samples on denaturing agarose gels followed by ethidium bromide staining Poly(A)$^+$ RNA was selected by oligo(dT) chromatography and quantified by spectrophotometry For the recovery of RNA from *Xenopus* neurointermediate and anterior lobes, yeast tRNA was used as a carrier

RNase protection assay

Constructs to be used in the RNase protection assay were generated by subcloning appropriate restriction fragments from selected cDNA clones into pBluescript SK vector After verification by sequencing, ~100 ng of linearized construct was used for the generation of antisense run-off transcripts from the T3 or T7 RNA polymerase promotor Transcripts labelled with [α-^{32}P]UTP (800 Ci/mmol, Amersham) were purified on 5 °$_0$ (w/v) polyacrylamide/8 M urea gels Sizes of transcripts generated from the constructs (with sizes of protected bands in parentheses) were X0286, 402 nt (346), X1262, 216 nt (156), POMC, 301 nt (271), fascin, 406 nt (311) About 10^5 c p m of each transcript was combined with total RNA samples in 25 μl of hybridization mix [80 °$_0$ (v/v) formamide, 400 mM NaCl, 40 mM Pipes, pH 6 4, containing 1 mM EDTA] Samples were incubated at 80 °C for 5 min before hybridization overnight at 50 °C Non-hybridized RNA was digested with RNase A and RNase T1 for 30 min at 37 °C Samples were treated with proteinase K, extracted with phenol/chloroform/isoamyl alcohol, supplemented with 10 μg of yeast tRNA, precipitated with ethanol and run on a 5 °$_0$ (w/v) polyacrylamide/8 M urea gel After autoradiography, quantification of protection signals was performed with an Ultrascan XL laser densitometer (LKB/Pharmacia)

Northern blot analysis

Poly(A)$^+$ RNA from *Xenopus* liver, brain, heart and kidney was separated by electrophoresis on 2 2 M formaldehyde-containing 1 2 °$_0$ (w/v) agarose gels in Mops buffer and blotted onto nitrocellulose filters as described by Ausubel et al [20] Hybridization was overnight at 45 °C in $5 \times$ SSPE [where SSPE is 0 15 M NaCl/10 mM sodium phosphate (pH 7 4)/1 mM EDTA], 50 °$_0$ (v/v) formamide, $5 \times$ Denhardt's, 0 5 °$_0$ SDS and 0 1 mg/ml denatured salmon-sperm DNA Probes (10^6 c p m per ml of hybridization buffer) were prepared by random prime-labelling of cDNA inserts from clones X0286 and X1262 Blots were washed at 63 °C to a final stringency of $0\,1 \times$ SSPE and autoradiographed for 3 days at −70 °C with two intensifying screens

Reverse transcription–PCR analysis

Total RNA extracted from various tissues was annealed to oligo(dT) primer, and ss cDNA was synthesized with Superscript

reverse transcriptase (RT). The cDNA was amplified with 50 pmol each of primer 1 (5′-CAGAAGTGGTTTTCATCGTGGAGAT-3′) and primer 2 (5′-TTAAGCCTGGATGTTGCTCTTTGC-3′) for 30 cycles (93 °C, 1 min; 60 °C, 1.5 min; 70 °C, 1 min; Perkin Elmer-Cetus Thermal Cycler) with Super-*Taq* DNA polymerase (Perkin Elmer-Cetus). Primer 1 corresponds to nucleotides 179–203 and primer 2 is the reverse complement of nucleotides 544–567 in cDNA clone X0286 (see Figure 3). PCR products were run on a 1.5% agarose gel, blotted onto nitrocellulose and hybridized under standard conditions [15] with random-prime-labelled X0286 cDNA insert as a probe. Autoradiography was for 3 h at room temperature.

RESULTS

Isolation and characterization of mRNAs co-ordinately expressed with POMC in *Xenopus* intermediate pituitary during background adaptation

As an approach towards the identification of genes involved in peptide hormone biosynthesis and release, we have screened an intermediate pituitary cDNA library of black-background-adapted *Xenopus* with cDNA probes derived from intermediate pituitary mRNA of black- and white-adapted animals. This screening yielded 37 differentially hybridizing clones corresponding to a total of 14 distinct transcripts whose levels in the melanotropes are regulated in coordination with that of POMC (J. Holthuis, E. Jansen, M. van Riel and G. Martens, unpublished work). Multiple independent cDNAs were isolated for transcripts encoding prohormone processing enzymes (carboxypeptidase H and prohormone convertase PC2) and members of the granin family of proteins (secretogranins SGII, SGIII and the neuroendocrine polypeptide 7B2). Because these proteins are all packaged and released together with peptide hormones, their co-expression with POMC in the melanotropes was not unexpected. Of the remaining transcripts, those represented by clones X0286 and X1262 displayed the strongest alterations in expression levels. RNase protection experiments indicated that NILs of black-adapted animals contain 20-fold more X0286 transcripts and 15-fold more X1262 transcripts than those of white-adapted animals (Figure 1). These magnitudes of regulation are similar to that observed for POMC mRNA, whose level is altered 20–30-fold during background adaptation [14]. As previously demonstrated for POMC, the physiologically induced changes in X0286 and X1262 transcript levels are confined to the melanotropes of the NIL and do not occur in cells from the anterior lobe of the pituitary (Figure 1). Such an expression pattern is restricted to a limited set of genes, excluding for instance those encoding ferritin, actin or the actin-bundling protein fascin [21] (Figure 1, and results not shown). Northern blot analysis showed that clone X0286 represents an mRNA of 0.8 kb that is expressed in *Xenopus* liver, brain, heart, kidney and lung (Figure 2). Clone X1262 corresponds to a 1.2 kb transcript with an expression

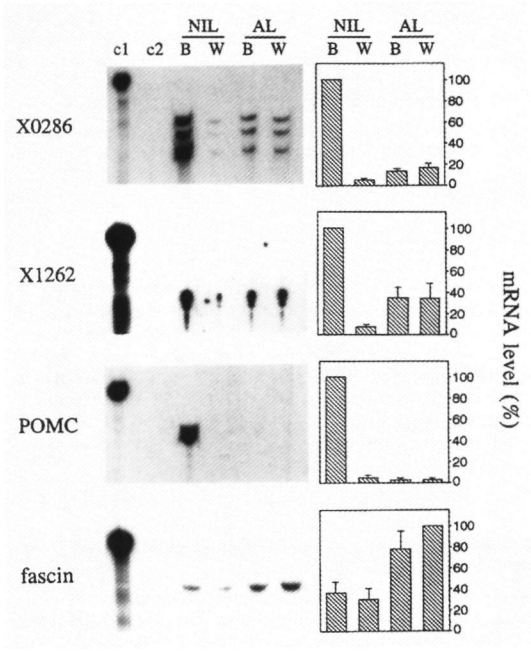

Figure 1 RNase protection analysis of X0286, X1262 and POMC transcripts in the *Xenopus* pituitary

Radiolabelled anti-sense RNA was hybridized to total RNA extracted from NILs and anterior lobes (AL) of black- (B) or white-adapted (W) *Xenopus*. In each experiment, total RNA from two lobes was used, except for POMC (1/25 part of each lobe). Samples were digested with RNase A and RNase T1, loaded onto denaturing polyacrylamide gels and autoradiographed. An anti-sense probe against the non-regulated fascin mRNA served as a control. The multiple protection signals observed for X0286 probably relate to the divergence observed in the nucleotide sequences of two related transcripts (see Figure 2 and text). Transcript levels were quantified by densitometric scanning of autoradiographs. Data shown are the means ± S.D. of three independent experiments. c1, undigested RNA probe; c2, RNA probe hybridized to 20 μg of yeast tRNA before digestion.

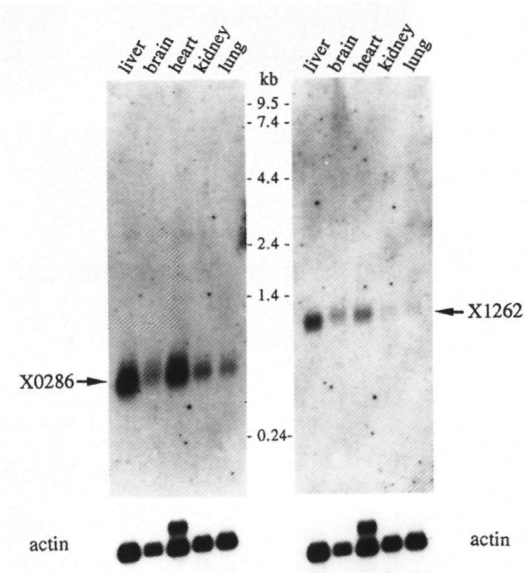

Figure 2 Northern blot analysis of X0286 and X1262 transcripts

Random-prime-labelled inserts from cDNA clones X0286 and X1262 were hybridized to 7.5 μg of poly(A)$^+$ RNA from *Xenopus* liver, brain, heart, kidney and lung. As a control for loading, blots were stripped and rehybridized with an actin cDNA probe. The mobilities of RNA size markers are indicated.

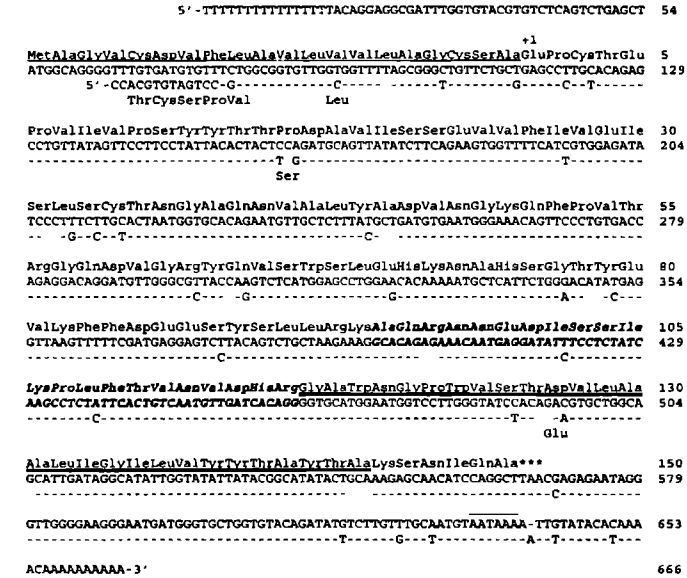

Figure 3 Nucleotide sequence and deduced amino acid sequence of *Xenopus* intermediate pituitary cDNA clone X0286

Substitutions in the sequence of a closely related cDNA are indicated The signal peptide sequence is underlined Positive numbering of amino acids starts in the mature protein Doubly underlined amino acids are predicted to span the membrane The nucleotide sequence and deduced amino acid sequence shown in bold italics are deleted in an alternatively spliced form of the X0286 transcript Overlined is the consensus for polyadenylylation

profile similar to that of the X0286 mRNA, yet with lower levels of expression (Figure 2). RNase protection experiments demonstrated that both transcripts are also present in muscle, gut, pancreas, spleen, adipose tissue, testis, ovaria and oocytes (results not shown). Together, these results indicate that the expression of X0286 and X1262 is not limited to specific organs or cell types but that both transcripts are derived from ubiquitously expressed genes.

Transcript X0286 encodes the *Xenopus* homologue of TRAPδ

Clone X0286 contained a cDNA insert of 0 6 kb Nucleotide sequence analysis revealed an open reading frame (ORF) of 167 amino acids lacking a translation initiation codon. Rescreening of the intermediate pituitary cDNA library gave five positive clones with insert sizes ranging from 0.5 to nearly 0.7 kb Sequence analysis of the largest insert revealed a 54 bp 5′-untranslated region, a 510 bp ORF and a 102 bp 3′-untranslated region with a putative polyadenylation signal 17 bp upstream of the poly(A) tract (Figure 3) A potential translation initiation site (5′-TGAGCT*ATG*G-3′, where the start codon is italic) [22] is present at nucleotide positions 49–58 Translation from this site would generate a polypeptide of 170 amino acids with a predicted molecular mass of 18574 Da. The initiator methionine precedes a predominantly hydrophobic region of 20 amino acids, strongly resembling a signal sequence with a cleavage site conforming to the -1, -3 rule [23]. Removal of this region would leave a polypeptide of 150 amino acids with a molecular mass of 16654 Da A potential membrane-spanning segment is found at residue positions 117–144 A database search indicated that the X0286 sequence is closely related to that of TRAPδ from the rat (Figure 4) When excluding the signal peptide region, X0286 shares 80°$_0$ identity and 90°$_0$ similarity with TRAPδ over 150 matched amino acid residues. From this high degree of structural identity we conclude that X0286 encodes the *Xenopus* homologue of TRAPδ No other entries with significant sequence similarity to X0286 were present in the database

Generation of multiple TRAPδ transcripts by gene duplication and alternative mRNA splicing

Nucleotide sequence analysis and subsequent alignment of all six isolated X0286 cDNA clones demonstrated the existence of at least three distinct transcripts for TRAPδ in *Xenopus*. First, we noticed 43 substitutions over 602 matched nucleotides (7°$_0$) between the original X0286 clone and the five clones from the rescreening (Figure 3) These substitutions caused considerable alterations in the signal peptide sequences, whereas structural changes in the mature protein are limited to two conservative substitutions $Pro^{16} \rightarrow Ser$ and $Asp^{127} \rightarrow Glu$ It therefore seems that two structurally distinct TRAPδ proteins are expressed from a closely related pair of genes. In *Xenopus*, several other gene pairs have been described whose exon sequences diverge by between 4°$_0$ and 9°$_0$ Included are the genes for vitellogenins [24], albumins [25], proenkephalins [26], L1 ribosomal proteins [27] and POMCs [28] These gene pairs are believed to originate

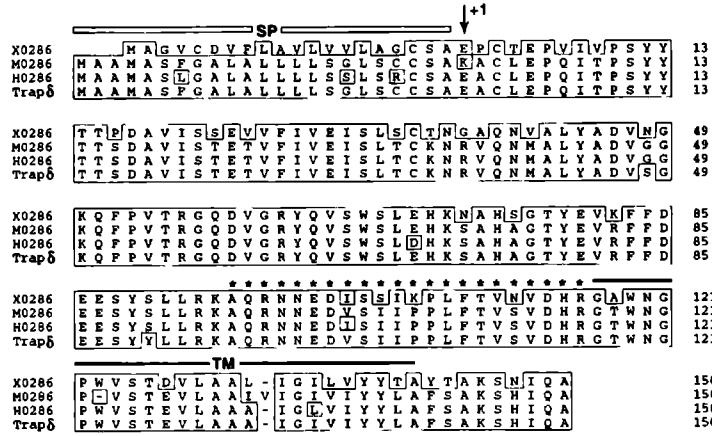

Figure 4 Alignment of amino acid sequences deduced from *Xenopus* cDNA X0286, mouse cDNA M0286, human cDNA H0286 and rat TRAPδ

The single letter amino acid code is used Gaps are introduced for optimal alignment Signal peptide (SP) and transmembrane (TM) regions are overlined and the putative signal peptide cleavage site is indicated by an arrow Residues deleted in alternatively spliced forms of the *Xenopus* and mouse mRNAs are starred The rat TRAPδ sequence was taken from Hartmann et al [12] The cDNA sequences of X0286 M0286 and H0286 are available from the EMBL database under accession numbers X90584 X90582 and X90583 respectively

from a duplication of the entire *Xenopus* genome that took place about 30 million years ago [29,30]

A second remarkable finding was that three of the six isolated X0286 cDNA clones showed a deletion of 66 nucleotides from position 397 to 462 Consequently, the proteins encoded by these cDNAs lack a stretch of 22 amino acid residues (from Ala^{95} to Arg^{116}) immediately N-terminal of the membrane-spanning segment (Figure 3) This finding suggests that the transcript of at least one of the two TRAPδ genes undergoes alternative splicing, thereby giving rise to two structurally distinct TRAPδ proteins of ~14 and ~17 kDa RT–PCR was used to study the expression of alternatively spliced TRAPδ transcripts in a variety of *Xenopus* tissues (for details see the Materials and methods section) After Southern blot analysis, PCR products of approximately 325 bp and 400 bp, each corresponding to one of the alternatively spliced transcripts, were detected in pituitary (NIL and anterior lobe) pancreas, kidney, liver, heart, gut, spleen, muscle and skin (results not shown) Alternative splicing of the TRAPδ transcript therefore occurs in a wide variety of tissues and probably does not represent a cell type-specific event

To analyse further the conservation of TRAPδ during evolution and to find out whether alternative splicing of its transcript also occurs in mammals, we screened brain cDNA libraries from mouse and human, with radiolabelled X0286 cDNA as a probe Six positive clones from the mouse library and two from the human library were isolated with cDNA inserts varying from 0 7 kb to nearly 1 kb in size These clones each contained an ORF for a protein highly homologous to TRAPδ (Figure 4) The mouse and human proteins share an overall sequence identity of 80 °$_0$ with *Xenopus* TRAPδ, 97 °$_0$ identity with rat TRAPδ and 97 °$_0$ identity with each other One of the six isolated mouse cDNA clones showed a deletion of 66 nucleotides within the ORF and thus the protein encoded by this cDNA lacks TRAPδ residues Ala^{95} to Arg^{116} (see Figure 4) This in-frame deletion is located at exactly the same position as that observed in some of the X0286 cDNAs Hence alternative splicing of the TRAPδ transcript is a conserved phenomenon that takes place in both amphibians and mammals

Transcript X1262 encodes a protein structurally related to mammalian glycoprotein gp25L and two yeast transmembrane proteins

Clone X1262 contained a cDNA insert of 1 kb with an ORF of 186 amino acids from which the start methionine is lacking Rescreening of the melanotrope cDNA library did not yield clones with 5 -extended sequences We therefore hybridized a *Xenopus* hypothalamus cDNA library using the X1262 insert as a probe Six of the 21 positive clones were selected for sequence analysis The largest of these contained a 1 1 kb cDNA comprising a 29-bp 5 -untranslated region, a 615-bp ORF and a 484-bp 3 -untranslated region with three polyadenylation signals upstream of the poly(A) tail (Figure 5) Translation from the start codon at nucleotide position 30 would produce a polypeptide of 23786 Da with an N-terminal 18-residue hydrophobic sequence reminiscent of a signal peptide Use of the predicted peptide cleavage site would leave a 187-residue polypeptide with a calculated molecular mass of 21795 Da A potential N-linked glycosylation site was found at residue position 147 and a stretch of hydrophobic residues, long enough to span a lipid bilayer, is located close to the C-terminus at residue positions 154–176 Alignment of the cDNA sequences from all six X1262 clones revealed that one of these contains nine substitutions over 1042 matched nucleotides (1 °$_0$) Two amino acid substitutions were found in the ORF, namely $Pro^{48} \rightarrow$ His and $Tyr^{49} \rightarrow$ Ile (Figure 5) Hence, like TRAPδ, X1262 in *Xenopus* is generated by a closely related pair of transcriptionally active genes

A database search indicated that the X1262 nucleotide sequence is 70 °$_0$ identical with two human expressed sequence tags (HS23814 and HBC1303) over a total length of 321 nucleotides At the protein level, X1262 shares 85 °$_0$ sequence identity (91 °$_0$ similarity) over 100 matched amino acid residues with the

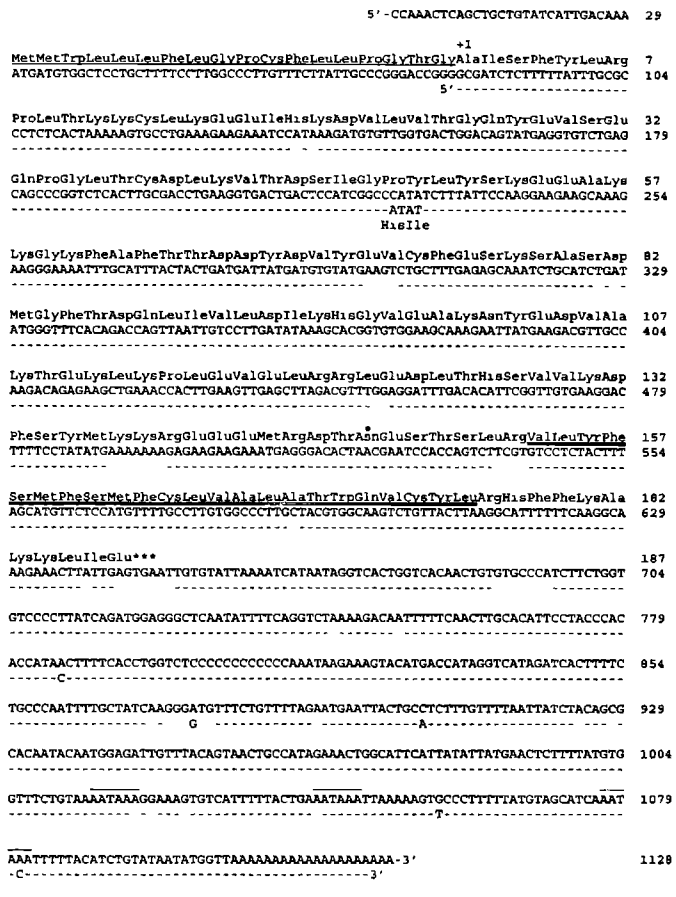

Figure 5 Nucleotide sequence and deduced amino acid sequence of *Xenopus* intermediate pituitary cDNA clone X1262

Substitutions in the sequence of a closely related cDNA are indicated. A potential Asn-linked glycosylation site is marked by a black dot. Other designations are as in Figure 3.

expressed sequence tag (EST)-derived polypeptide sequences (Figure 6, Table 1). The EST-encoded protein thus probably represents the human homologue of X1262. The database search revealed three additional entries showing low but significant degrees of similarity between the X1262 protein and dog glycoprotein gp25L, yeast hypothetical protein Yhr110w, and yeast protein p24B (Figure 6). In its mature form, the X1262 protein shares an overall sequence identity of 31% (52% similarity) with gp25L, 25% identity (51% similarity) with Yhr110w and 23% identity (46% similarity) with p24B. The structural relationship between these proteins, as summarized in Table 1, suggests that each represents a seperate member of a novel protein family. Common (structural) features found within this protein family include an N-terminal signal sequence, a conserved pair of cysteines (residues 13 and 74 in X1262), a transmembrane domain close to the C-terminus and a calculated molecular mass of ~22 kDa (excluding signal peptide). Single Asn-linked glycosylation sites are present in all proteins, except in p24B. Both the X1262 protein and its human homologue contain a strikingly high number of positively charged residues at their C-terminal ends. Included is a double lysine motif (residues 183 and 184 in X1262) that may function as a signal for the retrieval of transmembrane proteins to the ER [31,32]. The same motif is present in the gp25L protein, whereas two lysine residues at positions −3 and −5 from the Yhr110w C-terminus also match the consensus for this ER retrieval signal.

DISCUSSION

We have identified transcripts for TRAPδ and a novel protein similar to gp25L among a distinct set of messengers whose expression levels parallel that of POMC in physiologically activated *Xenopus* melanotropes. TRAPδ and gp25L are constituents of distinct tetrameric protein complexes that reside in the ER membrane within a short distance from translocating polypeptides.

TRAPδ was initially isolated by Migliaccio et al. [9] as part of a complex whose other components are TRAPα, -β and -γ

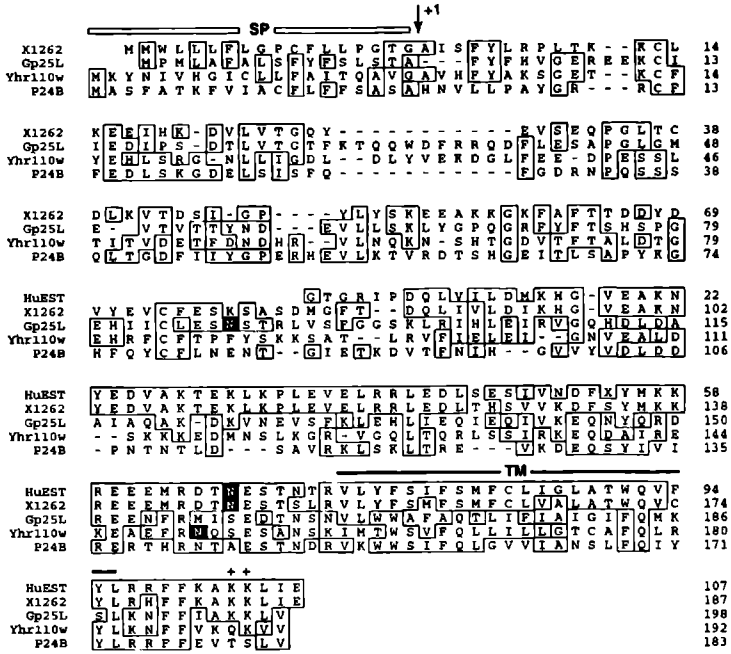

Figure 6 Alignment of amino acid sequences deduced from *Xenopus* cDNA X1262, human ESTs HuEST, dog glycoprotein gp25L and yeast proteins Yhr110w and p24B

The single-letter code is used Gaps are introduced for optimal alignment Potential Asn linked glycosylation sites are indicated by white letters Other designations are as in Figure 4 The HuEST sequence is a compilation of EMBL database entries HS23814 and HBC1303 (accession numbers T32283 and T10797) The dog gp25L protein sequence was taken from Wada et al [11] The yeast protein Yhr110w and p24B sequences were taken from the SwissProt database (accession numbers P38819 and P32803) The X1262 cDNA sequence is available from the EMBL database under accession number X90517

Table 1 Sequence identity/similarity between gp25L-related proteins

	X1262	HuEST	gp25L	Yhr110w	P24B	
X1262	—	83	31	25	23	
HuEST	91	—	27	26	29	
gp25L	52	49	—	32	27	Identity (%)
Yhr110w	51	55	52	—	30	
P24B	46	51	42	49	—	
	Similarity (%)					

Cross-linking experiments have indicated that the four proteins are genuine neighbours in intact ER membranes [12] TRAPδ is capable of forming homodimers and may connect two or more copies of the TRAP complex in the membrane At present, the function of the TRAP complex is unknown Sequence comparison between cDNAs from distantly related species (mammals, fish and nematodes) has revealed that TRAPα and TRAPβ are highly conserved proteins [12] Our present findings demonstrate that the same applies for TRAPδ, the mature *Xenopus* protein and its mammalian counterparts share an overall sequence identity of ~ 80 %. Hence the TRAP complex probably serves a role that has been highly conserved during evolution. TRAPδ is encoded by a ubiquitously expressed gene, as in *Xenopus* the transcript is present in all 14 different tissues examined In addition, we noticed that the TRAPδ transcript undergoes alternative splicing and as a result generates two structurally distinct proteins of ~14 kDa and ~17 kDa Alternative splicing of the transcript was detected in a wide range of tissues and represents an evolutionarily conserved event that takes place in both amphibians and mammals Migliaccio et al [9] and Hartmann et al [12] previously described only the 17 kDa form of TRAPδ, their studies did not contain any indication of the existence of a smaller form of this protein A likely explanation for this would be that 17 kDa TRAPδ, but not the 14 kDa protein, is part of the TRAP complex In that case, the region 22 amino acids long upstream of the transmembrane segment that is deleted in the 14 kDa protein could be essential for the interaction of TRAPδ with the other subunits of the complex. Apart from the high structural conservation of TRAPδ and alternative splicing of its transcript, the functional importance of this protein is underscored by a dynamic regulation of its gene in stimulated neuroendocrine cells (as discussed below)

In addition to TRAPδ mRNA, we identified a ubiquitously expressed *Xenopus* transcript for a novel and highly conserved protein with structural similarity to dog glycoprotein gp25L Like gp25L, the *Xenopus* protein has a single transmembrane

segment positioned close to its C-terminus and a double lysine motif that may function as a retrieval signal for ER transmembrane proteins Moreover, gp25L and the *Xenopus* gp25L-like protein are structurally related to yeast proteins Yhr110w and p24B Yhr110w is a hypothetical type I membrane glycoprotein and also contains the ER retrieval signal Its gene resides in the intergenic region CDC12–MSH1 of yeast chromosome VIII [33] P24B is a type I membrane protein isolated from a crude preparation of yeast endosomes [34] As the endosome-enriched fraction was found to contain several ER resident proteins, it remains to be established whether P24B is a true marker of the endosome Altogether, our findings provide evidence that the gp25L-like protein identified in this study belongs to a novel family of ER-resident transmembrane proteins whose members are spread throughout the animal kingdom At present, the functions of these proteins are unknown Wada et al [11] reported that gp25L is associated with calnexin and two subunits of the TRAP complex, namely TRAPα and TRAPβ The authenticity of this protein complex was confirmed with different purification procedures and by co-immunoprecipitation However, gp25L and calnexin were not found in the TRAP complex identified by Migliaccio et al [9] and Hartmann et al [12] The isolation of the two different protein complexes may be due to differences in the purification protocols employed by these research groups In particular, variations in the methods by which the membrane proteins were extracted from the microsomes could be important, such variations have previously caused conflicting results about the composition of the signal peptidase protein complex ([35] and references therein) Interestingly, Hartmann et al [12] noted that the TRAP complex is stable in mild detergents such as cholate, and that upon application of stronger detergents (e g Nonidet P-40) only the interaction of TRAPα with TRAPβ remained intact Hence it can be proposed that a tightly associated TRAPα–β dimer in proximity to the translocon may interact with different accessory subunits to form TRAP complexes of varying composition Deshaies et al [36] have presented results suggesting that also the integral components of the translocon in yeast exist as a dynamic assembly in the FR Our identification of a gp25L-like protein suggests that TRAP complexes containing other components than those reported to date may indeed exist The various assembly states of the TRAP proteins could meet differential requirements by subsets of precursor proteins or, alternatively, reflect cycles of subunit association and dissociation that mark discrete steps in the translocation event

The data presented in this study suggest that the protein environment surrounding a translocating polypeptide in the ER is even more complex than previously expected, and possibly includes protein variants generated by alternative RNA splicing (e g 14 kDa and 17 kDa TRAPδ) as well as distinct members of protein families (e g gp25L and gp25L-like proteins) In addition, the genes for some of these proteins seem to be vigorously regulated in specialized secretory cells In the *Xenopus* pituitary gland we observed marked cell-type-specific changes in the transcript levels for TRAPδ and the gp25L-like protein during the physiological process of background adaptation Similar dynamics in gene expression have been observed for the prohormone POMC [14], prohormone-processing enzymes (PC2 and CPH) [37] and several additional constituents of secretory granules (SGII, SGIII and 7B2) ([38] and J Holthuis, E Jansen, M van Riel and G Martens, unpublished work) The biosynthetic machinery in activated *Xenopus* melanotropes is primarily dedicated to the production of POMC derived peptide hormones POMC or POMC-derived cleavage products constitute up to 80% of all newly synthesized proteins in these cells (J Holthuis, E Jansen, M van Riel and G Martens, unpublished work) Taken together, these data suggest that in *Xenopus* melanotropes the TRAP complex, or subunits thereof, are important for the biosynthesis of POMC either during or shortly after the emergence of the prohormone in the ER lumen One could think of a general role in the unidirectional transfer of nascent polypeptides across the ER membrane, in the proper release of newly synthesized polypeptides from the translocation site or in a hithertho unrecognized protein modification step It is also conceivable that these proteins participate in the folding or quality control systems of the ER It has been well established that on hyperstimulation of exocrine and endocrine cells, regulated secretory proteins entering the ER tend to condensate into intracisternal granules from which other soluble ER proteins are excluded [39,40] This premature condensation, which has also been observed in stimulated *Xenopus* melanotropes [41], is thought to develop when export of regulated secretory proteins from the ER fails to keep pace with the rate of their synthesis Under these circumstances, the folding and quality control systems of the ER are likely to enter a highly alarmed state in which the production rates of the components involved will be elevated

Note added in proof (received 9 October 1995)

A recent report has shown that the yeast P24B protein is a component of ER-derived COPII-coated vesicles and has a role in the correct delivery of a subset of secretory proteins to the Golgi complex [42]

We gratefully acknowledge Elma Loomans and Yvonne Croes for technical assistance Ron Engels for animal care and Dr B Wieringa (University of Nijmegen) for providing the mouse and human brain cDNA libraries This work was supported by a PIONIER grant from the Netherlands Organization for Scientific Research (NWO) and European Community grant BIOT CT91 0302 to G J M M

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Received 12 May 1995/26 July 1995, accepted 27 July 1995

Chapter 4

The Neuroendocrine Proteins Secretogranin II and III are Regionally Conserved and Coordinately Expressed with Proopiomelanocortin in *Xenopus* Intermediate Pituitary

The Journal of Neurochemistry (1996) **66**, 2248-2256

Journal of Neurochemistry
Lippincott–Raven Publishers Philadelphia

The Neuroendocrine Proteins Secretogranin II and III Are Regionally Conserved and Coordinately Expressed with Proopiomelanocortin in *Xenopus* Intermediate Pituitary

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Abstract: Chromogranins and secretogranins are acidic secretory proteins of unknown function that represent major constituents of neuroendocrine secretory granules Using a differential screening strategy designed to identify genes involved in peptide hormone biosynthesis and secretion, we have isolated cDNA clones encoding the first nonmammalian homologues of secretogranin II (SgII) and secretogranin III (SgIII) from a *Xenopus* intermediate pituitary cDNA library A comparative analysis of the *Xenopus* and mammalian proteins revealed a striking regional conservation with an overall sequence identity of 48% for SgII and 61% for SgIII One of the highly conserved and thus potentially functional domains in SgII corresponds to the bioactive peptide secretoneurin However, in SgII and especially in SgIII, a substantial portion of the potential dibasic cleavage sites is not conserved, arguing against the idea that these granins serve solely as peptide precursors Moreover, SgIII contains a conserved and repeated motif (DSTK) that is reminiscent of a repeat present in the *trans*-Golgi network integral membrane proteins TGN38 and TGN41, a finding more consistent with an intracellular function for this protein When *Xenopus* intermediate pituitary cells were stimulated in vivo, the mRNA levels of SgII and SgIII increased dramatically (15- and 35-fold, respectively) and in parallel with that of the prohormone proopiomelanocortin (30-fold increase) Our results indicate that the process of peptide hormone production and release in a neuroendocrine cell involves multiple members of the granin family **Key Words:** Secretogranin—Proopiomelanocortin—Intermediate pituitary—*Xenopus laevis*—Differential screening
J Neurochem **66,** 2248–2256 (1996)

Neuroendocrine cells store their newly synthesized peptide hormones in specialized vesicular organelles, the secretory granules Besides peptides, secretory granules contain various other gene products including enzymes involved in the posttranslational modification of peptide precursors and a group of acidic secretory proteins collectively known as the granin (chromogranin/secretogranin) family (Huttner et al 1991) In addition to the three classical granins chromogranin A (CgA), chromogranin B (CgB, previously called secretogranin I) and secretogranin II (SgII, previously called chromogranin C), other neuroendocrine proteins like the 1B1075 protein [secretogranin III (SgIII)], the HISL-19 antigen (secretogranin IV), and the 7B2 protein (secretogranin V) have been proposed to belong to this family The granins are structurally unrelated (except for some sequence homology between CgA and CgB) yet share several physicochemical features, including the presence of an N-terminal signal sequence, a hydrophilic and acidic amino acid composition, and a large number of paired basic amino acids that may serve as recognition sites for endoproteolytic enzymes Despite many years of research, the physiological roles of these proteins have remained unclear Several granins have been reported to undergo proteolytic processing in secretory granules, leading to the hypothesis that they represent precursors of bioactive peptides Consistent with this notion was the finding that the CgA-derived peptides pancreastatin (Konecki et al, 1987) and chromostatin (Galindo et al, 1991) and a cleavage product of SgII, named secretoneurin (Vaudry and Conlon, 1991, Kirchmair et al, 1993), exhibit some activity as autocrine or paracrine regulators of neuroendocrine secretion (Tatemoto et al, 1986, Galindo et al, 1991, Saria et al, 1993) However, there is growing evidence that granins have important intracellular functions as helper proteins in the proteolytic processing and sorting of prohormones (Huttner and Naton, 1995) In vitro data suggest that CgA is able to modulate the processing of peptide precursors by acting as a competitive substrate for endoproteolytic enzymes (Seidah et al, 1987) Recent

Received November 16 1995 revised manuscript received January 22 1996 accepted January 22 1996

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Abbreviations used AL anterior lobe CgA chromogranin A CgB chromogranin B NIL neurointermediate lobe POMC proopiomelanocortin SgII secretogranin II SgIII secretogranin III PSPE saline sodium phosphate EDTA TGN *trans* Golgi network

studies have disclosed a role for 7B2 as a molecular chaperone involved in the intracellular transport and activation of the prohormone convertase PC2 (Braks and Martens, 1994, 1995; Martens et al., 1994; Benjannet et al., 1995; Zhu and Lindberg, 1995). Finally, CgB has been found to promote the aggregation-dependent sorting of proopiomelanocortin (POMC)-derived cleavage products into secretory granules of anterior pituitary-derived AtT20 cells (Huttner and Natori, 1995).

The melanotrope cells in the intermediate pituitary gland of *Xenopus laevis* form an attractive in vivo model system to study the functioning of neuroendocrine cells at the molecular level. The biosynthetic activity of the melanotropes is primarily focused on the production of melanophore-stimulating peptides that are generated by specific endoproteolytic processing of the prohormone POMC. In these cells, the expression levels of POMC can be readily manipulated in vivo: When *Xenopus* is placed on a black background, the POMC gene is actively transcribed, whereas on a white background, the gene is virtually inactive. As a consequence of this physiological process of background adaptation, the melanotropes of black-adapted toads contain up to 30-fold more POMC transcripts and produce much higher quantities of melanophore-stimulating peptides than those of white-adapted animals (Martens et al., 1987). We are interested in genes coexpressed with POMC and whose expression is associated with the specialized secretory function of a neuroendocrine cell. Our search for such genes previously led to the identification of a *Xenopus* intermediate pituitary mRNA encoding the 7B2 (secretogranin V) protein (Martens et al., 1989). Here we report the identification and characterization of two other regulated intermediate pituitary transcripts encoding *Xenopus* homologues of the granin family members SgII and SgIII.

MATERIALS AND METHODS

Animals

South-African clawed toads, *Xenopus laevis*, were bred and reared in the aquarium facility of the Department of Animal Physiology at the University of Nijmegen. Animals (weighing 40–60 g) were adapted to black or white backgrounds under constant illumination for 3 weeks at 22°C.

cDNA library screenings

A *Xenopus* neurointermediate lobe (NIL) cDNA library from black background-adapted toads was differentially screened with cDNA probes derived from NIL mRNA of black- and white-adapted animals as described previously (Holthuis et al., 1995). This screening included the isolation of two partial cDNAs coding for SgII (clones X8556 and X8564) and two partial cDNAs encoding SgIII (clones X8596 and X8752). cDNA inserts from clone X8556 and X8596 were random prime-labeled with [α-^{32}P]dATP (3.000 Ci/mmol; ICN Radiochemicals) and used to screen an amplified *Xenopus* hypothalamus cDNA library in λ uniZAP XR (Van Riel et al., 1993) according to standard procedures (Sambrook et al., 1989). Of the positively hybridizing plaques, those representing clones with the largest cDNA inserts were selected by PCR analysis using specific SgII and SgIII primers in combination with a pBluescript primer. This procedure yielded a cDNA covering nearly the entire open reading frame of SgII (clone X8556-31) as well as a full-length cDNA encoding SgIII (clone X8596-16).

DNA sequence analysis and database matching

Sequencing of cDNA clones X8556-31 and X8596-16 on both strands and with pBluescript subclones or specific primers was performed with single- and double-stranded DNA using T7 DNA polymerase (Pharmacia) and the dideoxy chain termination method (Sanger et al., 1977). Nucleotide sequences and deduced protein sequences were compared with those present in the EMBL/Genbank and Swissprot/PIR databases using computer facilities of the CAOS/CAMM Center at the University of Nijmegen.

RNA isolation

For expression studies, total RNA was prepared according to the method of Chomczynski and Sacchi (1987), using acid-guanidine isothiocyanate-phenol-chloroform extraction. After recovery by ethanol precipitation, the RNA was quantified by spectrophotometry, and its integrity was checked by assaying samples on denaturing agarose gels followed by ethidium bromide staining. Poly(A)$^+$ RNA was selected by oligo(dT) chromatography and quantified by spectrophotometry. For the recovery of RNA from *Xenopus* NILs and anterior lobes (ALs), yeast tRNA was used as a carrier.

RNase protection assay

Constructs to be used in the RNase protection assay were generated by subcloning appropriate restriction fragments from selected cDNA clones into pBluescript SK vector. After verification by sequencing, ~100 ng of linearized construct was used for the generation of antisense run-off transcripts from the T3 or T7 RNA polymerase promoter. Transcripts labeled with [α-^{32}P]UTP (800 Ci/mmol; Amersham) were purified on 5% polyacrylamide/8 *M* urea gels. Sizes of transcripts generated from the constructs (with sizes of protected bands in parentheses) were as follows: SgII, 385 nucleotides (343); SgIII, 297 nucleotides (276); POMC, 301 nucleotides (271); and fascin, 406 nucleotides (311). About 1×10^5 cpm of each transcript was combined with total RNA samples in 25 μl of hybridization mix [80% formamide, 400 m*M* NaCl, 40 m*M* PIPES (pH 6.4), and 1 m*M* EDTA]. Samples were incubated at 80°C for 5 min before hybridization overnight at 50°C. Nonhybridized RNA was digested with RNase A and T1 for 30 min at 37°C. Samples were treated with proteinase K, phenol/chloroform/isoamyl alcohol-extracted, supplemented with 10 μg of yeast tRNA, ethanol-precipitated, and electrophoresed on a 5% polyacrylamide/8 *M* urea gel. Following autoradiography, quantification of protection signals was performed with an Ultrascan XL laser densitometer (LKB/Pharmacia).

Northern blot analysis

Total RNA from *Xenopus* NILs and ALs or poly(A)$^+$ RNA from *Xenopus* liver, brain, heart, kidney, and lung was separated by electrophoresis on 2.2 *M* formaldehyde-containing 1.2% agarose gels in MOPS buffer and blotted onto nitrocellulose filters as described by Ausubel et al. (1989). Hybridization was overnight at 45°C in 5× saline-sodium phosphate-EDTA (SSPE), 50% formamide, 5× Denhardt's solution, 0.5% sodium dodecyl sulfate, and 0.1

mg/ml of denatured salmon sperm DNA Probes (1×10^6 cpm/ml of hybridization buffer) were prepared by random prime-labeling of cDNA inserts from clones X8556 31 and X8596 16 Blots were washed at 63°C to a final stringency of 0 2× SSPE and autoradiographed for 3 (total RNA blots) or 10 days [poly(A)$^+$ RNA blot] at −70°C using two intensifying screens

RESULTS

Isolation of cDNAs encoding *Xenopus* SgII and SgIII

To identify genes coexpressed with POMC in *Xenopus* melanotrope cells we have differentially screened an intermediate pituitary cDNA library of black background-adapted toads with cDNA probes prepared from intermediate pituitary mRNA of black- and white-adapted animals This screening yielded 37 differentially hybridizing cDNAs corresponding to a total of 12 distinct genes whose expression levels in the melanotropes parallel that of POMC during background adaptation (Holthuis et al , 1995) Three genes turned out to encode members of the granin family One of these gives rise to the 7B2 protein and has been characterized previously (Martens et al , 1989) As described below, the other two genes code for *Xenopus* homologues of SgII and SgIII

Two of the differentially hybridizing clones selected from the *Xenopus* intermediate pituitary library, clones X8556 and X8564, contained overlapping cDNAs of 1 6 and 1 4 kb, respectively, representing the 3′ noncoding region of an unknown transcript To isolate 5′-extended sequences, we screened a *Xenopus* hypothalamus cDNA library using the X8556 insert as a probe This led to the isolation of a 3 7-kb cDNA, clone X8556-31, whose nucleotide sequence is deposited in the EMBL database (accession no X92873) Alignment of the nucleotide sequences of X8556, X8556-31, and X8564 revealed that all three originated from the same gene The X8556-31 sequence contains an open reading frame of 1,785 bp and a 3′ noncoding region of 1,880 bp with a polyadenylation signal (AATAAA) 15 bp upstream of the poly(A) tail (data not shown) The first 11 of the 595 amino acids deduced from the open reading frame were found to be part of a signal peptide sequence When this hydrophobic region is cleaved off, an acidic protein remains with a calculated molecular weight of 67,852 and an estimated pI of 4 61 The protein sequence contains a single consensus site for Asn-linked glycosylation (Asn^{284}) and 11 pairs of basic amino acids that represent potential recognition sites for endoproteolytic enzymes A database search showed that the X8556-31 protein bears a high degree of sequence identity with rat, human, and bovine SgII (Fig 1 and data not shown) No similarities were found with other protein sequences in the database We therefore conclude that the X8556-31 cDNA encodes the *Xenopus* homologue of SgII

Two other *Xenopus* intermediate pituitary cDNAs selected by the differential screening (clones X8596 and X8752 with inserts of 1 6 and 1 3 kb, respectively) showed significant sequence homology with the rat brain transcript 1B1075 encoding SgIII (Ottiger et al , 1990) Both cDNAs lacked a complete open reading frame Screening of the *Xenopus* hypothalamus cDNA library with the X8596 insert as a probe resulted in the isolation of a full-length 2-kb cDNA (clone X8596-16) The overlapping sequences of X8596-16, X8596, and X8752 were identical However, we noticed that the sequence of another X8596-positive clone selected from the hypothalamus cDNA library (X8596 4) displayed several nucleotide substitutions Given the tetraploid nature of the *Xenopus* genome, these findings indicate the expression of a pair of closely related genes The X8596-16 sequence comprises 307 bp of the 5′-untranslated region, an open reading frame of 1,371 bp, and a 3′-untranslated region of 360 bp that ends with a poly(A) tail (data not shown) This nucleotide sequence is available from the EMBL database under accession no X92872 The open reading frame encodes a protein of 457 amino acids The first 20 residues at the amino-terminus resemble a signal sequence with a hydrophobic core and a cleavage site conforming to the −1, 3 rule (Von Heijne, 1986) Removal of this region leaves a polypeptide with a predominantly acidic amino acid composition (pI, 4 50) and a calculated molecular weight of 49,744 One consensus site for Asn-linked glycosylation (Asn^{47}) and seven pairs of basic amino acids are present The high degree of identity between the amino acid sequence deduced from X8596-16 and SgIII protein sequences from the rat, mouse, and bovine (Fig 2) indicates that this cDNA clone encodes *Xenopus* SgIII

Conserved structural features of SgII

The overall amino acid sequence identity between *Xenopus* SgII and its rat and human counterparts (excluding signal peptide regions) is 47 and 48%, respectively (Fig 1) In both cases, a sequence similarity of 72% was found The putative acceptor site for tyrosine sulfation [Tyr^{124} in human SgII according to the sulfation consensus motif (Huttner, 1987)] is conserved in the three species, whereas the Asn-linked glycosylation site is not Seven of the 13 pairs of basic amino acids present in *Xenopus*, rat, and mouse SgII are conserved These include all pairs involved in the generation of secretoneurin and LA 42, two SgII-derived peptides previously isolated from neuroendocrine tissues in the frog (Vaudry and Conlon, 1991) and mammals (Kirchmair et al , 1993, Tilemans et al , 1994) The amino acid sequence identity between secretoneurin from *Xenopus* and frog is 100%, and that between *Xenopus* and rat or human secretoneurin is 76% The degree by which the primary structure of secretoneurin is conserved during evolution therefore clearly exceeds that of intact SgII In contrast, the LA-42 peptide displays a much lower level of sequence identity, namely, 44% between *Xenopus* and rat and 56% between *Xeno-*

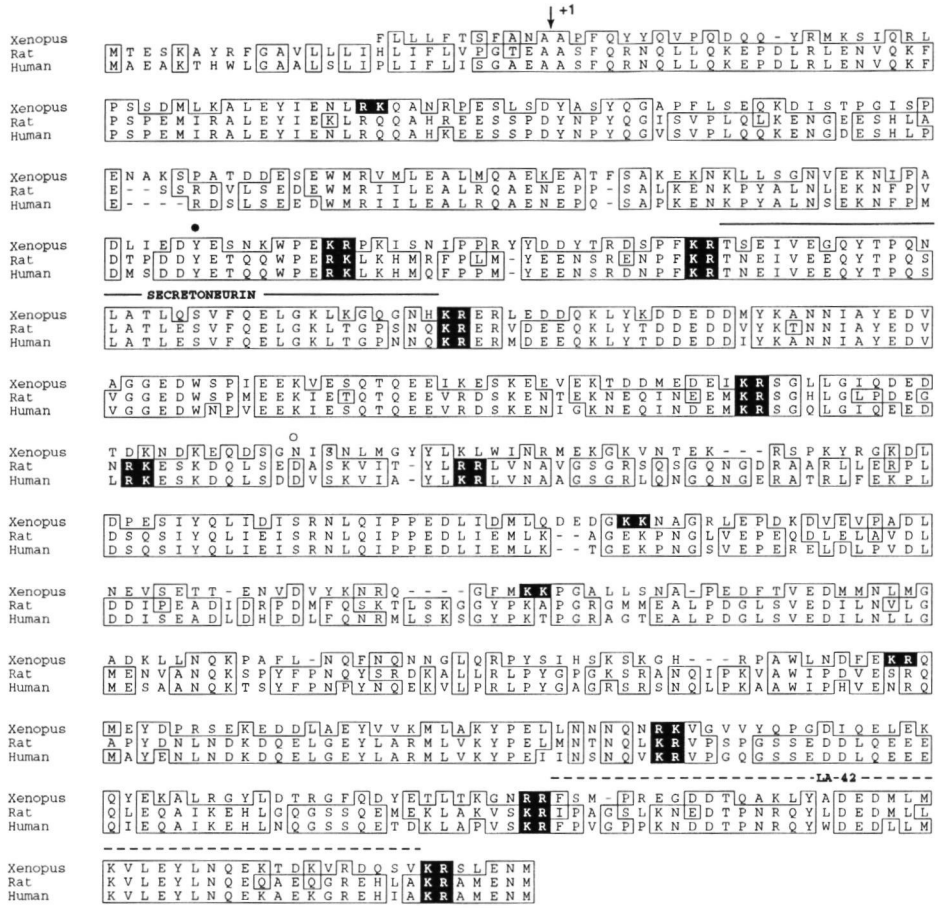

FIG. 1. Alignment of the amino acid sequences of *Xenopus*, rat, and human SgII. The single-letter amino acid code is used. Identical amino acids are boxed. Gaps are introduced for optimal alignment. The putative signal peptide cleavage site is indicated by an arrow, and positive numbering of amino acids starts in the mature protein. Pairs of basic amino acids are shown in white letters. A putative tyrosine sulfation site is marked by a solid circle; a potential Asn-linked glycosylation site is marked by an open circle. Amino acid sequences corresponding to the SgII-derived peptides secretoneurin and LA-42 are overlined. The rat and human SgII sequences were taken from Gerdes et al. (1988, 1989). The *Xenopus* SgII cDNA sequence is available from the EMBL database under accession no. X92873.

pus and human. Apart from secretoneurin and LA-42, the primary structure of SgII harbors four additional peptides flanked by conserved pairs of basic amino acids. Only one of these peptides, corresponding to *Xenopus* SgII residues 195–260, shows a considerable degree of conservation as indicated by sequence identities of 65% between *Xenopus* and rat, 67% between *Xenopus* and human, and a sequence similarity of 94% in both cases. Finally, the chromogranin/secretogranin motif (consensus sequence E[N/S]LX[A/D]X[D/E]XEL), which is present in the carboxy-terminal regions of mammalian CgA, CgB, and SgII (Huttner et al., 1991), is only poorly conserved in *Xenopus* SgII, where it corresponds to residues 463–472. Hence, the functional significance of this motif is questionable.

Conserved structural features of SgIII

Figure 2 shows an alignment of all SgIII amino acid sequences presently available. *Xenopus* SgIII shares 61% sequence identity and 73% sequence similarity with its rat and murine counterparts (excluding signal peptides). In addition, 47% sequence identity (67% similarity) was found with an SgIII-derived peptide purified from chromaffin granules of bovine adrenal

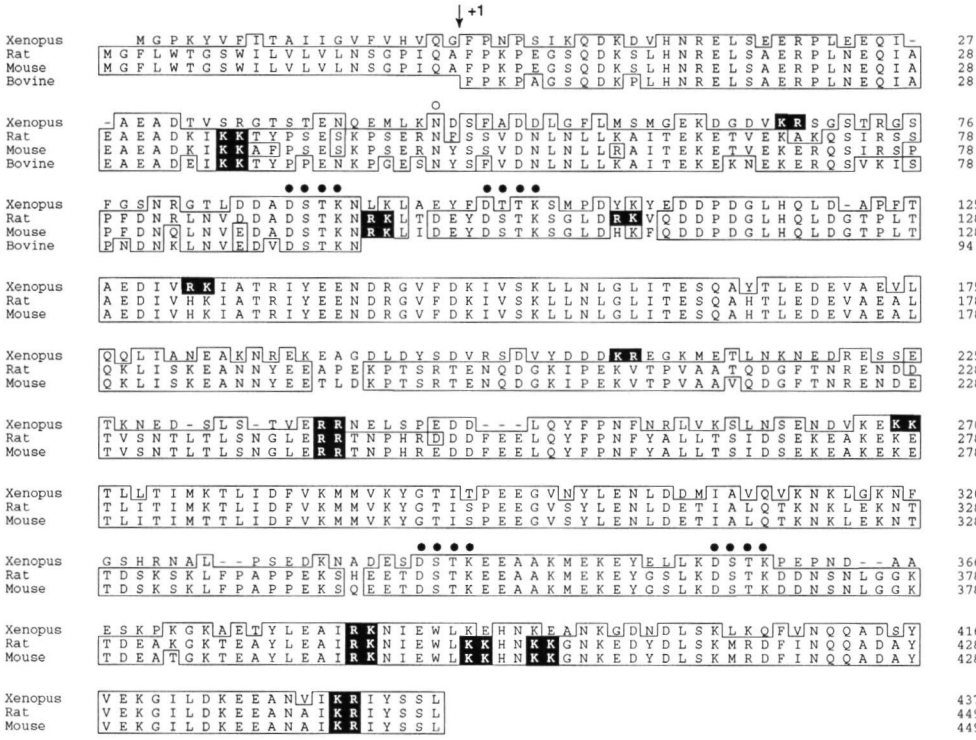

FIG. 2. Alignment of the amino acid sequences of *Xenopus*, rat, mouse, and bovine SgIII. The single-letter amino acid code is used. Identical amino acids are boxed. Gaps are introduced for optimal alignment. The putative signal peptide cleavage site is indicated by an arrow, and positive numbering of amino acids starts in the mature protein. Pairs of basic amino acids are shown in white letters. A repetitive sequence motif (DSTK) is indicated by solid circles. The open circle marks a potential Asn-linked glycosylation site (Asn^{47}). Note that Asn^{3} is part of an Asn-Xaa-Ser/Thr motif but will not likely be glycosylated owing to the presence of Pro at the Xaa position (Bause, 1983). The rat, mouse, and bovine SgIII sequences were taken from Ottiger et al. (1990), Dopazo et al. (1993), and Sigafoos et al. (1993), respectively. The *Xenopus* SgIII cDNA sequence is available from the EMBL database under accession no. X92872.

medulla (Sigafoos et al., 1993). SgIII displays a striking, regional conservation. Highly conserved regions with a sequence identity of >90%, e.g., *Xenopus* residues 112–179, 267–305, 338–359, 377–393, and 408–437, are interrupted by regions in which the degree of sequence identity drops below 30%, e.g., *Xenopus* SgIII residues 32–71, 180–266, and 360–376. The putative Asn-linked glycosylation site (Asn^{47}) and only three of the 12 paired basic amino acids are conserved. A short sequence motif (DSTK) that appears four times in the protein is, apart from one conservative substitution in *Xenopus* SgIII (Ser^{101} to Thr), perfectly conserved during evolution. These DSTK repeats seem to be unique for SgIII as they do not occur in any other protein sequence from the database. However, the DSTK motif displays similarity to the carboxy-terminal half of an octapeptide repeat ([P/T]TGGDS[D/N]K) that occurs six times in the luminal domains of the type I *trans*-Golgi network (TGN) membrane glycoproteins TGN38 and TGN41 (Luzio et al., 1990; Reaves et al., 1992). The significance of the similarity found between these elements is unclear.

Expression of SgII and SgIII mRNAs in *Xenopus* pituitary during background adaptation

In agreement with the outcome of our differential screening approach, RNase protection analysis showed that NILs of toads adapted to a black background contain 15-fold higher levels of SgII mRNA and 35-fold higher levels of SgIII mRNA than those of white-adapted animals (Fig. 3). These magnitudes of regulation are similar to that observed for POMC, whose transcript levels are altered up to 30-fold during background adaptation (Martens et al., 1987). As previously demonstrated for POMC, the physiologically induced changes in the expression levels of SgII and SgIII are confined to the NIL and do not occur in the AL of the pituitary (Fig. 3).

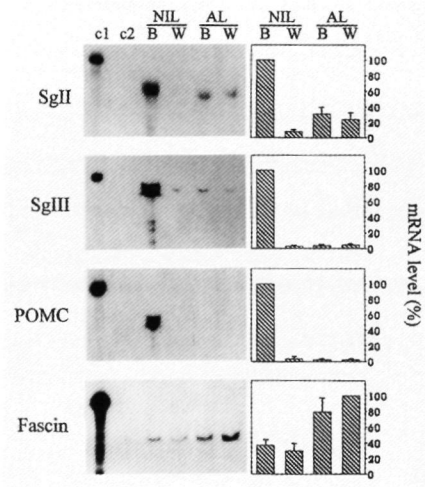

FIG. 3. RNase protection analysis of SgII, SgIII, POMC, and fascin transcripts in *Xenopus* pituitary. Radiolabeled antisense RNA was hybridized to total RNA extracted from NILs and ALs of black- (B) or white (W)-adapted *Xenopus*. In each experiment, total RNA from two lobes was used, except for POMC (1/25th of each lobe). Samples were digested with RNase A and T1, loaded onto denaturing polyacrylamide gels, and autoradiographed. An antisense probe against the nonregulated transcript for the actin-bundling protein fascin (Holthuis et al., 1994) served as a control. Transcript levels were quantified by densitometric scanning of autoradiographs. Data are mean ± SD (bars) values of three independent experiments. c1, undigested RNA probe; c2, RNA probe hybridized to 20 μg of yeast tRNA before digestion.

Northern blot analysis revealed that the *Xenopus* pituitary contains a single SgII transcript of 4.5 kb whose expression profile during background adaptation matches the one found in the RNase protection assay (Fig. 4A). Two SgIII transcripts, 2.2 and 5 kb in size, were detected in pituitary and brain (Fig. 4B and C). Both are strongly regulated in the intermediate pituitary during background adaptation (Fig. 4B). In addition, RNase protection experiments revealed low levels of expression in *Xenopus* lung and kidney (data not shown), indicating a wider tissue distribution of SgIII than reported previously (Ottiger et al., 1990). Because all *Xenopus* SgIII-encoding cDNAs isolated so far are derived from the 2.2-kb transcript, we have no clue with respect to the origin of the larger transcript. It may arise from alternative splicing of the nuclear RNA or from the utilization of an alternative polyadenylation site. The preferential expression of the 2.2-kb mRNA in the pituitary and that of the 5-kb mRNA in brain suggests that these transcripts are subject to tissue-specific regulation.

DISCUSSION

The present study shows that the neuroendocrine proteins SgII and SgIII are expressed in coordination with the prohormone POMC in physiologically manipulated *Xenopus* melanotropes. We previously observed a similar correlation of expression between POMC and another granin family member, namely, the 7B2 protein (Martens et al., 1989). An important clue that the coregulation between POMC and these granins is of physiological significance came with the finding that 7B2 physically interacts with and modulates the endoproteolytic activity of the prohormone convertase PC2 (Braks and Martens, 1994, 1995; Martens et al., 1994; Benjannet et al., 1995; Zhu and Lindberg, 1995). This endoproteolytic enzyme is held responsible for a crucial step in the generation of the POMC-derived, melanophore-stimulating peptides by the melanotrope cells (Benjannet et al., 1991; Thomas et al., 1991; Braks et al., 1992). Hence, our results indicate that in *Xenopus* melanotropes multiple members of the granin family participate in the mechanisms underlying the production and regulated release of these hormones.

The physiological roles of SgII and SgIII are presently unclear. SgII has been reported to undergo extensive proteolytic processing in secretory granules at pairs of basic amino acids (Fischer-Colbrie et al., 1995). At least two SgII-derived peptides occur in vivo in various neuroendocrine tissues: the neuropeptide secretoneurin (Vaudry and Conlon, 1991; Kirchmair et al., 1993) and a peptide designated LA-42, which originates from the C-terminal portion of the protein (Tilemans et al., 1994). Alignment of the SgII sequences from mammals and *Xenopus* shows that the dibasic cleavage sites flanking these peptides are all conserved. Unlike the LA-42 peptide and the SgII protein as a whole, secretoneurin is remarkably well conserved during evolution. Secretoneurin has been found to possess some chemotactic activity toward monocytes, suggesting its contribution to neurogenic in-

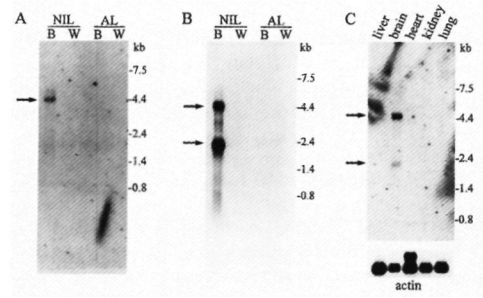

FIG. 4. Northern blot analysis of SgII and SgIII transcripts in several *Xenopus* tissues. Random prime-labeled inserts from (**A**) SgII clone X8556-31 or (**B** and **C**) SgIII clone X8596-16 were hybridized to total RNA extracted from NILs and ALs of five black- (B) or white (W)-adapted *Xenopus* (A and B) or 7.5 μg of poly(A)$^+$ RNA from various *Xenopus* tissues (C). As a control for loading, the poly(A)$^+$ RNA blot was stripped and rehybridized with a *Xenopus* actin cDNA probe. The mobilities of RNA size markers are indicated.

flammatory events (Reinisch et al , 1993) In addition, superfusion and microdialysis studies revealed that secretoneurin can induce the release of dopamine in the striatum of the rat (Saria et al , 1993, Agneter et al , 1995) It is of interest to note that dopamine is one of the major hypothalamic substances controlling secretory activity in the intermediate pituitary In *Xenopus*, dopamine strongly inhibits the secretion of melanophore-stimulating peptides from the melanotropes and is believed to act directly on these cells when discharged from the hypothalamic neurons innervating the intermediate pituitary (Verburg-Van Kemenade et al , 1986) Consequently, secretoneurin may represent a paracrine factor in a regulatory feedback mechanism by which the secretory activity of *Xenopus* melanotropes can be modulated Thus, following stimulation of the melanotropes in the intermediate pituitary, the enhanced production rate of SgII and subsequent secretion of SgII-derived secretoneurin could promote the release of dopamine from local nerve terminals, leading to a down-regulation of the secretory activity of these neuroendocrine cells Whether SgII in *Xenopus* melanotropes is processed to secretoneurin and serves such a role remains to be investigated From the alignment of SgII sequences from *Xenopus* and mammals it appears that SgII may give rise to more than one functionally important peptide For instance, like secretoneurin, the region corresponding to amino acid residues 195–260 in *Xenopus* SgII is flanked by conserved Lys-Arg pairs and shares a considerable degree of sequence identity with the corresponding rat and human peptides However, one should realize that the conservation of dibasic residues does not necessarily imply that these sites are recognized by the cleavage enzyme It appears that both residues surrounding the dibasic residues and higher-order structures are critical for substrate recognition (Rholam et al , 1995)

Our finding that a substantial number of the potential dibasic cleavage sites in *Xenopus* and mammalian SgII is not conserved argues against the idea that this protein serves solely as a peptide precursor Several studies suggest that SgII exerts an intracellular function in the secretory pathway Thompson et al (1992) found that prolactin granulogenesis in GH4C1 pituitary cells is associated with increased SgII expression and aggregation in the Golgi apparatus Chanat and Huttner (1991) provided evidence that CgB and SgII undergo a selective, pH- and Ca^{2+}-dependent aggregation in the TGN, which leads to their segregation from constitutive secretory proteins This milieu-induced aggregation of CgB and SgII in the TGN has been postulated (a) to support the packaging and sorting of prohormones and other regulated secretory proteins into secretory granules and (b) to play an essential role in the early phase of secretory granule biogenesis In agreement with these postulations is the finding that overexpression of CgB in AtT20 pituitary cells significantly improves the efficiency with which the 23-kDa POMC cleavage product formed in the TGN of these cells is sorted to immature secretory granules and subsequently processed to adrenocorticotropin (Huttner and Natori, 1995) Although the data presented in our study would be consistent with a similar role for SgII in *Xenopus* melanotropes, the possible involvement of this protein in prohormone packaging or granule formation requires further investigations

SgIII was initially identified from a rat brain cDNA library following a selective screen for clones whose cognate mRNAs are expressed exclusively or predominantly in the CNS (Otliger et al , 1990) Subsequently, it was shown that in mice the gene for SgIII can be deleted from the genome without causing any detectable impairment in viability, fertility, or locomotor behavior (Kingsley et al , 1990) This would either suggest that many of the cell types that normally express SgIII simply do not require this protein for their own survival and function or that a deficiency of SgIII can be functionally compensated for by other gene products Nevertheless, the fact that SgIII mRNA levels in *Xenopus* melanotropes are regulated by a similar magnitude as that of POMC during background adaptation strongly indicates that the protein is important for the proper functioning of these neuroendocrine cells Like other members of the granin family, SgIII contains a large number of paired basic amino acid residues The isolation of an SgIII derived peptide from bovine chromaffin granules (Sigafoos et al , 1993) suggests that some of these are used by endoproteolytic enzymes, raising the possibility that SgIII, like CgA and SgII, represents a precursor of biologically active peptides However, in view of the comparative analysis of SgIII protein sequences from mammals and *Xenopus*, it is unlikely that this would be its only function First, most of the potential dibasic cleavage sites that could serve to generate bioactive peptides are not conserved during evolution, in contrast to such sites in known peptide hormone and neuropeptide precursors Second, the regions in SgIII with the highest degree of conservation are not flanked by dibasic cleavage sites Third, SgIII contains a short sequence motif (DSTK) that is repeated four times in the protein These DSTK repeats are nearly perfectly conserved during 350 million years of vertebrate evolution and do not occur in any known peptide precursor or in any other protein sequence present in the database It therefore appears that these elements represent a unique and functionally important feature of SgIII Interestingly, we noticed that the DSTK motif resembles the carboxy-terminal half of an octapeptide motif that is repeated six times in the luminal domains of the type I transmembrane glycoproteins TGN38 and TGN41 (Luzio et al , 1990, Reaves et al , 1992) These proteins form dimers that cycle between the TGN and the cell surface and have many of the properties predicted for a "cargo" receptor, which couples the segregation of secretory proteins to the budding of exocytotic vesicles at the TGN (Stanley and Howell, 1993) It has been suggested that the octapeptide repeat in

TGN38 and TGN41 forms a binding site for luminal proteins, although no experimental evidence is yet available to prove this point The presence of the DSTK repeat in SgIII raises the intriguing possibility that this protein specifically interacts with one or a subset of other luminal proteins, for instance, to facilitate their aggregation and subsequent packaging into secretory granules

In conclusion, our study has demonstrated a dynamic regulation of the SgII and SgIII genes during physiological manipulation of peptide hormone production levels in *Xenopus* melanotropes Furthermore, our results uncovered the evolutionary history of SgII and SgIII from mammals down to amphibians, allowing the identification of conserved and thus potentially functional regions in these proteins. These findings will help to explore the functions of SgII and SgIII in the neuroendocrine system

Acknowledgment: We are grateful to Frank Detmers for technical assistance, Ron Engels for animal care, and Martin van Horssen for comments on the manuscript This work was supported by a PIONIER grant from the Netherlands Organization for Scientific Research (NWO) and by European Community grant BIOT-CT91-0302 to G J M M

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Chapter 5

Secretogranin III is a Sulfated Protein Undergoing Proteolytic Processing in the Regulated Secretory Pathway

The Journal of Biological Chemistry (1996) **271**, 17755-17760

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol 271, No 30 Issue of July 26 pp 17755-17760 1996
Printed in U S A

Secretogranin III Is a Sulfated Protein Undergoing Proteolytic Processing in the Regulated Secretory Pathway*

(Received for publication, January 29, 1996, and in revised form, April 9, 1996)

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Secretogranin III (SgIII) is an acidic protein of unknown function that is present in the storage vesicles of many neuroendocrine cells. It is coexpressed with the prohormone proopiomelanocortin in the intermediate pituitary of *Xenopus laevis*. We developed an antiserum to investigate the biosynthesis of SgIII in pulse-chase incubated *Xenopus* neurointermediate lobes. SgIII was synthesized as a 61- or 63-kDa (*N*-glycosylated) protein and processed to a 48-kDa form which, in turn, was partially cleaved to fragments of 28 and 20 kDa. The 48-, 28-, and 20-kDa cleavage products, but not their precursors, were secreted. This secretion is regulated and can be blocked in parallel with that of proopiomelanocortin-derived peptides by the hypothalamic factors dopamine, γ-aminobutyric acid, and neuropeptide Y. Coexpression of *Xenopus* SgIII with prohormone convertase (PC)1 or PC2 in transfected fibroblasts was sufficient to reconstitute the processing events observed in the neurointermediate lobes. Site-directed mutagenesis revealed that *Xenopus* SgIII is cleaved at two dibasic sites, namely Lys^{68}-Arg^{69} and Arg^{237}-Arg^{238}. Pulse-chase incubations of lobes with $Na_2[^{35}S]SO_4$ showed that SgIII is sulfated in the *trans*-Golgi network before it is processed. Finally, SgIII processing was found in several neuroendocrine cell types from various species. We conclude that SgIII is a precursor protein and that the intact molecule can only have an intracellular function, whereas an extracellular role can only be attributed to its cleavage products.

A hallmark of neuroendocrine cells is their ability to synthesize, store, and release biologically active peptides in a regulated fashion Most neuropeptides and peptide hormones are generated from inactive precursor proteins that are proteolytically processed at pairs of basic amino acids and often further modified to yield a functional product (1) The bulk of these modifications occurs subsequent to the sorting of peptide precursors, along with their processing enzymes, into secretory granules These specialized storage vesicles deliver their contents to the cell surface only in response to an external signal Besides peptides and processing enzymes, secretory granules contain a group of acidic secretory proteins, collectively known as the granin (chromogranin/secretogranin) family (2) Within this family, only chromogranin A (CgA)[1] and CgB (secretogranin I, SgI) show a structural relationship Granins are characterized by acidic isoelectric points and by the presence of numerous pairs of basic amino acids, some of which are used by endoproteolytic enzymes Unlike peptide hormones and processing enzymes, which have well defined functions in the neuroendocrine system, the physiological role of the granins is unclear One hypothesis is that granins themselves are precursors for biologically active peptides This notion is supported by the observation that peptides derived from proteolytic processing of CgA and SgII are capable of modulating secretion in an autocrine or paracrine manner (3–5) However, recent experimental evidence suggests that at least some granins function intracellularly as helper proteins in the sorting and proteolytic processing of prohormones For instance, overexpression of CgB in anterior pituitary-derived AtT20 cells was found to promote the aggregation-dependent sorting of proopiomelanocortin (POMC)-derived cleavage products into secretory granules (6) Furthermore, the neuroendocrine protein 7B2 (SgV) physically interacts with the proform of prohormone convertase PC2 and seems to regulate both transport and activation of this processing enzyme in the secretory pathway (7–10)

One of the granins whose function has remained elusive is SgIII Its transcript was originally identified from rat brain during a search for mRNAs that are exclusively expressed in the central nervous system (11) The protein was detected in many brain areas, especially in neurons participating in auditory, olfactory, and extrapyramidal motor functions, as well as in those related to the hypothalamic-pituitary axis Moreover, SgIII was found in cells of the intermediate and anterior pituitary, whereas ultrastructural studies demonstrated its presence in intracellular vesicles (11) Genetic ablation of the gene in mice revealed that animals lacking SgIII survive without any obvious impairment in viability, fertility, or locomotor behavior (12) Consequently, many of the cell types that normally express SgIII can function in the absence of this protein, perhaps because its physiological role can be replaced by that of another gene product Recently, we cloned the first nonmammalian homolog of SgIII from the amphibian *Xenopus laevis* This was achieved by a differential screening strategy designed to identify genes coexpressed with POMC in the melanotrope cells of the intermediate pituitary (13) In these cells, the production levels of POMC-derived melanophore-stimulating peptides can be manipulated *in vivo* by changing the background color of the toad When the animal is placed on a black background, the mRNA levels of SgIII in the intermediate pituitary increase dramatically (up to 35-fold) and in parallel with that of POMC (30-fold increase) (14), suggesting that SgIII has a role in the production and release of peptide hormones

In the present study, we investigate the biosynthesis of SgIII

* This work was supported by a PIONIER grant from the Netherlands Organization for Scientific Research (NWO) (to G J M M) The costs of publication of this article were defrayed in part by the payment of page charges This article must therefore be hereby marked "*advertisement*" in accordance with 18 U S C Section 1734 solely to indicate this fact

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[1] The abbreviations used are Cg, chromogranin, BFA, brefeldin A, GABA, γ-aminobutyric acid, NIL, neurointermediate lobe, NPY, neuropeptide Y, PAGE, polyacrylamide gel electrophoresis, PC, prohormone convertase, POMC, proopiomelanocortin, Sg, secretogranin, TGN, *trans*-Golgi network

in *Xenopus* melanotropes, taking advantage of the high metabolic activity of these cells in black-adapted animals We find that SgIII is a sulfated precursor protein and demonstrate that proteolytic processing occurs at two dibasic sites that are recognized by the prohormone convertases PC1 and PC2

MATERIALS AND METHODS

Animals—South-African clawed toads, *Xenopus laevis*, were adapted to a black background by keeping them in black buckets under constant illumination for at least 3 weeks at 22 °C

Production of Recombinant SgIII Protein and Generation of Antiserum—A polyclonal antiserum was raised against recombinant *Xenopus* SgIII produced in *Escherichia coli* using the Qiagen expression system (Qiagen Inc , Chatsworth, CA) For this purpose, a 1 5-kb *Bam*HI fragment of *Xenopus* SgIII cDNA clone X8596 (14) was ligated into the *Bam*HI site of the prokaryotic expression vector pQE-30 This allowed the production of recombinant protein composed of *Xenopus* SgIII residues −8 to 437 with a hexahistidine tail at its amino terminus Following purification of the protein by Ni^{2+}-NTA agarose affinity chromatography, a 500-μg initial dose emulsified with Freund's complete adjuvant was administered to rabbits at 20 subcutaneous sites After 3 and 6 weeks, rabbits were boosted with 250 μg of protein in Freund's incomplete adjuvant The production of specific antibodies was monitored by enzyme-linked immunosorbent assay

Metabolic Labeling of Xenopus NILs and Immunoprecipitation Analysis—NILs from black-adapted *Xenopus* were dissected and preincubated in incubation medium (IM, 112 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 15 mM Hepes, pH 7 4, 0 3 mg/ml bovine serum albumin, 2 mg/ml glucose, pH 7 4) at 22 °C for 20 min Pulse labeling of newly synthesized proteins was performed by incubating lobes in IM containing 1 7 mCi/ml Tran[^{35}S]-label (ICN Radiochemicals) for the indicated periods at 22 °C Subsequent chase incubations were in IM containing 5 mM L-methionine Pulse labeling of sulfated proteins was achieved by incubating lobes in 1 3 mCi/ml $Na_2[^{35}S]SO_4$ (ICN Radiochemicals) Subsequent chase incubations were in IM containing 1 mM Na_2SO_4 Drugs and secretagogues described in the text were added in the 20-min preincubation period and remained present during subsequent pulse and chase incubations Lobes were homogenized on ice in lysis buffer (50 mM Hepes, pH 7 2, 140 mM NaCl, 10 mM EDTA, pH 8 0, 1% Tween 20, 0 1% Triton X 100, 0 1% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and 0 1 mg/ml soybean trypsin inhibitor) Homogenates were cleared by centrifugation (10,000 × *g*, 7 min at 4 °C), supplemented with 0 1 volume of 10% SDS and diluted 10-fold in lysis buffer before addition of anti-SgIII antiserum (1 5000 dilution) Immune complexes were precipitated with protein A Sepharose (LKB-Pharmacia) and resolved by SDS-PAGE Radiolabeled proteins were visualized by fluorography

Eukaryotic Expression Plasmids- A 1 7-kb *Bam*HI fragment of *Xenopus* SgIII cDNA clone X8596–1 encoding the entire protein (14) was subcloned downstream of the cytomegalovirus promotor into the *Bam*HI site of the eukaryotic expression vector pcDNA3 (Invitrogen, San Diego, CA) A 2 7-kb *Hin*dIII-*Eco*RV fragment of plasmid pIP83 covering the entire open reading frame of human PC1 (a generous gift of Dr A Roebroek, University of Leuven) (15) was subcloned in the *Hin*dIII/*Eco*RV sites of pcDNA3 The pcDNA3 vector containing a full-length 2 2-kb human PC2 cDNA was obtained from Dr J Creemers (University of Cambridge, United Kingdom) DNA for transfection studies was isolated using the Qiagen plasmid kit (Qiagen Inc)

Site directed Mutagenesis of SgIII—A 1 7-kb *Bam*HI fragment of *Xenopus* SgIII cDNA clone X8596–1 was subcloned into the *Bam*HI site of the pALTER-1 vector, and oligonucleotide-directed mutagenesis was performed on single-stranded DNA using the pALTER system (Promega, Madison, WI) The desired mutations were checked by restriction enzyme digestion and double-stranded DNA sequencing Correctly mutagenized SgIII DNA inserts were subcloned into pcDNA3 Oligonucleotides used to create amino acid substitutions in dibasic cleavage sites of SgIII were 5'-GGTGATGTGACTAGTAGTGGAAGC-3' ($KR^{69} \rightarrow TS^{69}$) and 5' CTGTGGAGAGCTCGAACGAATTA-3' ($RR^{238} \rightarrow SS^{238}$)

Cell Culture—All cell culture media were obtained from Life Technologies, Inc and supplemented with 10% (v/v) fetal calf serum Green monkey CV-1 kidney fibroblasts were cultured in Iscove's modified Eagle's medium, mouse anterior pituitary-derived AtT20 cells, and mouse glucagon-producing αTC4 cells in high glucose Dulbecco's modified Eagle's medium, and mouse insuloma βTC3 cells in low glucose Dulbecco's modified Eagle's medium

Cell Transfection, Metabolic Labeling, and Immunoprecipitation Analysis—Transfection of CV-1 and AtT20 cells was accomplished by the calcium-phosphate precipitation method (16) AtT20 cells were transfected with the *Xenopus* SgIII-pcDNA3 construct and after 48 h selected for stable expression of SgIII in medium containing 750 μg/ml neomycin (Boehringer Mannheim) For transient expression studies, CV-1 cells were plated in 20 mm culture dishes, grown until 30% confluency, and transfected with 2 5 μg of DNA per construct per dish CV-1 cells (48 h after transfection) and other cell types were starved for 60 min in methionine- and cysteine-free Dulbecco's modified Eagle's medium (ICN Biochemicals) supplemented with 10% (v/v) dialyzed fetal calf serum (Life Technologies, Inc) and subsequently pulsed for 180 min in the same medium with 0 2 mCi/ml Tran[^{35}S]-label Cells were lysed on ice in lysis buffer, and the cell lysates and incubation media were processed for immunoprecipitation analysis as described above

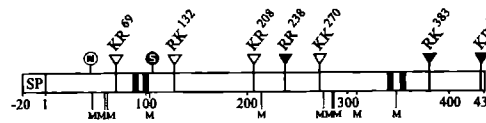

FIG 1 **Structural organization of *Xenopus* SgIII.** *SP*, signal peptide, *filled triangles*, potential dibasic cleavage sites conserved between *Xenopus* and rodent SgIII, *open triangles*, nonconserved dibasic sites, *N* in *open circle*, putative *N* linked glycosylation site (Asn^{47}), *S* in *filled circle*, potential tyrosine sulfation site (Tyr^{110}) *Black boxes* correspond to a conserved internally repeated sequence element, DSTK Arginine, lysine, and methionine residues are indicated by the single-letter codes *R*, *K*, and *M*, respectively This schematic representation is based on the amino acid sequence deduced from a *Xenopus* SgIII cDNA which is available from the EMBL data base under accession number X92872

RESULTS AND DISCUSSION

Biosynthesis and Proteolytic Processing of SgIII in Xenopus Melanotropes—To investigate the biosynthesis of SgIII in the neurointermediate lobe (NIL) of *Xenopus*, we raised a polyclonal antiserum against a recombinant protein comprising *Xenopus* SgIII residues −8 to 437 (Fig 1) In immunofluorescence studies on primary cultures of NILs dissected from black-adapted animals, the antiserum gave a bright staining of the melanotrope cells, whereas no immunostaining above background was detected in other (minor) cell types (*e g* stellate cells, endothelial cells) [2] These findings suggest that the melanotropes constitute the primary site of SgIII production in the NIL When NILs from black animals were pulsed for 15 min with Tran[^{35}S]-label and subjected to immunoprecipitation analysis with the antiserum, two major radiolabeled proteins of 63 and 61 kDa were detected (Fig 2, *lane 2*) These proteins were not immunoprecipitated with preimmune serum (Fig 2, *lane 1*), indicating that they represent newly synthesized SgIII Pulse-chase incubations revealed that these proteins are proteolytically processed, first yielding a 48-kDa product which is then partially cleaved into fragments of 28 and 20 kDa (Fig 2, *lanes 3–5*) Analysis of the chase media showed that only the 48-, 28-, and 20-kDa cleavage products, but not the 63- and 61-kDa precursors, are released into the medium (Fig 2, *lane 9*) Collectively, these data demonstrate that SgIII is a secretory precursor protein and suggest that at least two of the seven potential dibasic cleavage sites present within its sequence (Fig 1) are used by endoproteolytic enzymes

When NILs were preincubated and pulsed in the presence of tunicamycin (a blocker of *N*-linked glycosylation), the mobility of the 63-kDa precursor protein increased by 2 kDa, whereas the migration of the 61-kDa precursor and the three cleavage products remained unchanged [2] This result indicates that SgIII is partially glycosylated at Asn^{47}, the single putative acceptor site for *N*-linked glycosylation found within its primary sequence (Fig 1) Moreover, it can be concluded that

[2] J C M Holthuis, E J R Jansen, and G J M Martens, unpublished observations

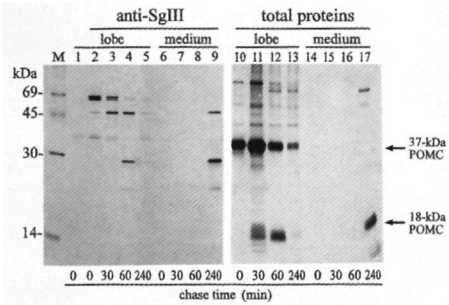

FIG. 2. **Immunoprecipitation analysis of intracellular and secreted forms of SgIII produced by *Xenopus* neurointermediate lobes.** Lobes were pulsed for 15 min with Tran[^{35}S]-label or pulsed for 15 min and chased for 30, 60, or 240 min. Radiolabeled proteins were immunoprecipitated from lobe extracts (two lobes per lane) and chase media using preimmune serum (*lane 1*) or anti-SgIII antiserum (*lanes 2–9*). Immunoprecipitates were resolved by SDS-PAGE and visualized by fluorography. Protein markers for molecular mass (*M*) are indicated on the *left*. Migration positions of newly synthesized POMC and an 18-kDa POMC-derived cleavage product in total protein extracts (*lanes 10–17*; 0.04 lobe per lane) are indicated on the *right*. Note the presence of a nonspecifically reacting 37-kDa POMC band in some of the immunoprecipitates.

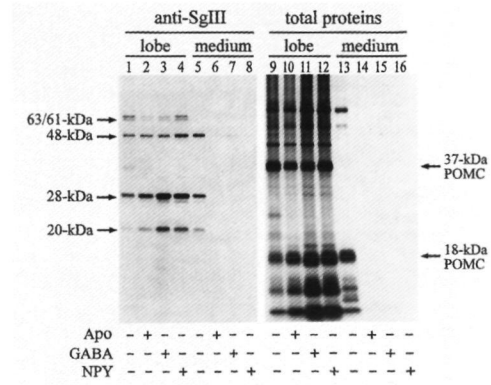

FIG. 3. **Effect of apomorphine, GABA, and NPY on the release of newly synthesized SgIII.** *Xenopus* NILs were pulsed for 60 min with Tran[^{35}S]-label and chased for 210 min in the absence or presence of apomorphine (*Apo*, 10^{-5} M), γ-aminobutyric acid (*GABA*, 10^{-4} M), or neuropeptide Y (*NPY*, 10^{-6} M). Radiolabeled proteins were immunoprecipitated using anti-SgIII antiserum. Migration positions of SgIII precursor forms and cleavage products are indicated on the *left* and those of POMC and an 18-kDa POMC-derived cleavage product on the *right*.

when intact SgIII is converted to the 48-kDa cleavage product, the glycosylated amino-terminal portion of the protein is cleaved.

The mobility of nonglycosylated intact SgIII produced by *Xenopus* melanotropes in SDS-PAGE gels (61 kDa) is considerably slower than one would predict from its amino acid sequence (calculated molecular mass, 49,744 Da). Similar discrepancies were found for other members of the granin family (17, 18). At present, it is unclear to what extent post-translational modifications contribute to this phenomenon (*i.e.* phosphorylation, *O*-linked glycosylation, and sulfation) or whether the highly acidic nature of these proteins causes anomalous behavior on SDS-PAGE in the Laemmli system.

Melanotropes Release SgIII- and POMC-derived Cleavage Products in a Coordinated Fashion—The release of the POMC-derived peptide α-MSH from melanotrope cells is negatively controlled by various factors of hypothalamic origin. These include dopamine, GABA, and neuropeptide Y (NPY) (19–21). To investigate whether secretion of the SgIII-derived cleavage products is a regulated event, NILs were pulsed for 60 min and chased for 210 min in the absence or presence of these secretagogues. Secretion of the SgIII cleavage products was completely blocked by the D_2 dopamine receptor agonist apomorphine, as well as by GABA and NPY (Fig. 3). In all three cases, the inhibition led to an accumulation of SgIII cleavage products in the lobes while the precursor proteins did not accumulate, indicating that these secretagogues had not interfered with SgIII processing. Analysis of the incubation media and lobe extracts showed that the transport of POMC-derived cleavage products was affected in a similar way. These results demonstrate that the secretion of SgIII- and POMC-derived peptides by *Xenopus* melanotropes is regulated in a coordinated manner.

Cellular Compartments Involved in SgIII Processing—To determine the secretory compartment in which processing of SgIII occurs, we conducted pulse-chase experiments on NILs in the presence or absence of the fungal metabolite brefeldin A (BFA) or the sodium ionophore monensin. BFA causes an accumulation of newly synthesized secretory proteins in the endoplasmic reticulum (22), whereas monensin is known to interfere with protein transport between Golgi compartments (23). Treatment of the lobes with these drugs strongly inhibited the generation of SgIII cleavage products and completely blocked their release into the medium, whereas an intracellular accumulation of the SgIII precursor forms was observed (Fig. 4). These findings suggest that the first proteolytic conversion of SgIII does not occur before the protein has reached the distal part of the Golgi apparatus. The minor amount of 48-kDa cleavage product formed in the drug-treated lobes indicates that some SgIII had escaped from the blocks. This was also found for some newly synthesized POMC,[2] a prohormone whose processing is known to occur distal to the site of action of these drugs (24).

Tyrosine sulfation is a post-translational modification mediated by a protein tyrosine-sulfotransferase found in the TGN (25). This modification may affect the biological activity or intracellular transport of a protein (26). We noticed that the *Xenopus* SgIII sequence contains a putative sulfation site on Tyr^{110} (Fig. 1), and we decided to analyze sulfated forms of SgIII in order to further define the compartment where its processing occurs. After a 10-min pulse of NILs with Na_2[^{35}S]SO_4, the majority of immunoprecipitable radioactivity was found associated with the 61- and 63-kDa precursor forms of SgIII (Fig. 5). Following a 20-min pulse, only a small amount of the 48-kDa cleavage product was observed. After an additional chase of 40 min, most of the radioactivity was associated with the 48-kDa cleavage product. A chase of 120 min allowed detection of both the 48- and 20-kDa cleavage products but not the 28-kDa fragment, whereas the radioactivity associated with the 61- and 63-kDa precursors further decreased. Only the 48- and 20-kDa forms could be detected in the chase medium. Together, these data demonstrate that sulfation of SgIII precedes its proteolytic processing and that the protein reaches the TGN in an intact form.

Tooze *et al.* (27) showed that in rat pheochromocytoma PC12 cells the sulfation and subsequent sorting of SgII from the TGN to immature secretory granules occurs within a time interval of 20 min. Our pulse-chase analysis revealed a lag period of about 20 min between SgIII sulfation and the appearance of the first cleavage product (Fig. 5). This finding suggests that SgIII processing in *Xenopus* melanotropes starts in the immature secretory granules.

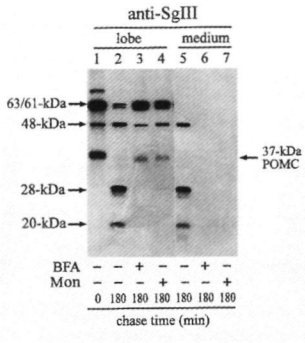

FIG. 4. **Effect of brefeldin A and monensin on processing and release of newly synthesized SgIII.** *Xenopus* NILs were pulsed for 20 min with Tran[^{35}S]-label (*lane 1*) or pulsed for 20 min and chased for 210 min in the absence or presence of brefeldin A (*BFA*, 2.5 μg/ml) or monensin (*Mon*, 100 nM). Immunoprecipitation analysis was with anti-SgIII antiserum. Migration positions of SgIII precursor forms and cleavage products are indicated on the *left* and that of nonspecifically reacting POMC on the *right*.

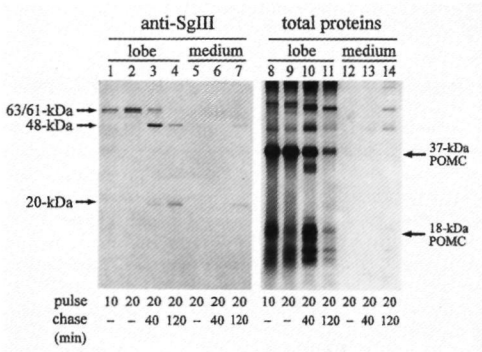

FIG. 5. **Sulfation of SgIII precedes its processing.** *Xenopus* NILs were pulsed for 10 or 20 min with $Na_2[^{35}S]SO_4$ or pulsed for 20 min and chased for 40 or 120 min. Radiolabeled proteins were immunoprecipitated from lobe extracts (three lobes per lane) and chase media using anti-SgIII antiserum. Migration positions of sulfated SgIII precursor forms and cleavage products are indicated on the *left*. Migration positions of sulfated POMC and an 18-kDa POMC-derived cleavage product in total protein extracts (0.1 lobe per lane) are indicated on the *right*.

SgIII Is a Substrate for Prohormone Convertases PC1 and PC2—To determine if the proteolytic system responsible for SgIII processing is restricted to cells of neuroendocrine origin, CV-1 kidney fibroblasts were transfected with a *Xenopus* SgIII cDNA construct and pulsed with Tran[^{35}S]-label for 180 min. Immunoprecipitation analysis of the cell lysate and incubation medium revealed two newly synthesized proteins of 61 and 63 kDa (Fig. 6, *lanes 1* and *2*) whose migrations on SDS-PAGE are identical to those of the intact SgIII precursors produced in *Xenopus* NILs (Fig. 6, *lane 3*). Both lysates and incubation media of transfected cells were devoid of SgIII-derived cleavage products, indicating that CV-1 fibroblasts are not equipped with the proteolytic system by which SgIII is processed in *Xenopus* melanotropes.

The prohormone convertases PC1 (also termed PC3) and PC2 represent two neuroendocrine-specific members of the subtilisin family of endoproteases and are responsible for the proteolytic conversion of a wide range of prohormones and other peptide precursors at pairs of basic amino acids (28, 29). To investigate their possible involvement in SgIII processing,

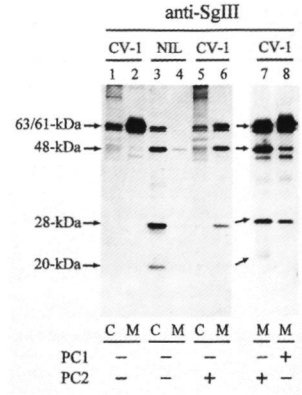

FIG. 6. **Processing of SgIII by prohormone convertase PC1 and PC2 in transfected CV-1 fibroblasts.** PC1 or PC2 was coexpressed with *Xenopus* SgIII in CV-1 fibroblasts by transfection of the respective cDNA constructs. Transfected cells were pulsed for 180 min with Tran[^{35}S]-label, and radiolabeled proteins were immunoprecipitated from cell lysates (*C*) and incubation media (*M*) using anti-SgIII antiserum. Immunoprecipitation analysis of pulse-labeled *Xenopus* NILs (180 min pulse) served as a control. Migration positions of SgIII precursor forms and cleavage products are indicated. Note that some CV-1 immunoprecipitates contain a nonspecifically reacting 45-kDa protein also found in mock-transfected cells.[2]

CV-1 cells were cotransfected with SgIII and PC1 or PC2 cDNA constructs, pulse-labeled, and analyzed for the biosynthesis of SgIII. The incubation medium of cells cotransfected with SgIII and PC2 contained, in addition to the SgIII precursor forms, three smaller immunoreactive proteins whose sizes were indistinguishable from the SgIII-derived cleavage products generated in *Xenopus* NILs (Fig. 6, compare *lanes 3* and *6*). The same set of radiolabeled proteins could be immunoprecipitated from the incubation medium of cells cotransfected with SgIII and PC1 (Fig. 6, *lane 8*). The 20-kDa cleavage product generated in CV-1 cells often appeared as a smear in the gel (*e.g.* see Fig. 6, *lane 7*), hampering its detection in some of our experiments. This smearing, which was also evident for the 63-kDa precursor form, may relate to additional post-translational modifications of SgIII when produced in fibroblasts. Nevertheless, our results demonstrate that both PC1 and PC2 recognize SgIII as a suitable substrate. Unlike PC1, PC2 is a highly abundant protein in *Xenopus* melanotropes (13, 30) and therefore represents the most likely enzyme to be responsible for the processing of SgIII in these cells.

Identification of the Cleavage Sites Involved in SgIII Processing—*Xenopus* SgIII contains seven potential dibasic cleavage sites (Fig. 1). As mentioned above, the first cleavage yielding the 48-kDa product is in the amino-terminal region of the protein, yet carboxyl-terminal of the *N*-linked glycosylation site (Asn^{47}). We noticed that upon cleavage of the 48-kDa product, 2- to 3-times more radioactivity remains associated with the 28-kDa fragment than with the 20-kDa fragment (see Fig. 2 or 3). Combined with the distribution of methionines in the SgIII polypeptide sequence (Fig. 1), these observations led us to predict that the first cleavage of SgIII occurs at the Lys^{68}-Arg^{69} site and the second cleavage at the Arg^{237}-Arg^{238} site. To test this hypothesis, amino acid substitutions were introduced at these sites, and the mutated proteins were coexpressed with PC2 in CV-1 fibroblasts. The mutant in which the Lys^{68}-Arg^{69} site was substituted by Thr^{68}-Ser^{69} gave rise to the SgIII precursor forms and a single cleavage product of 28 kDa (Fig. 7, *lane 3*). Other potential SgIII fragments were undetectable,

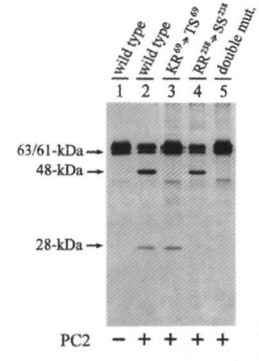

FIG. 7. **Identification of processing sites in SgIII.** Amino acid substitutions at two potential dibasic cleavage sites in *Xenopus* SgIII ($KR^{69} \rightarrow TS^{69}$ and $RR^{238} \rightarrow SS^{238}$) were created by site-directed mutagenesis of wild type cDNA. Wild type protein and mutant proteins carrying substitutions in one or both dibasic sites (*double mut.*) were coexpressed with PC2 in CV-1 fibroblasts by transfection of the respective cDNA constructs. Transfected cells were pulsed for 180 min with Tran[^{35}S]-label, and radiolabeled proteins were immunoprecipitated from the incubation media using anti-SgIII antiserum. Migration positions of SgIII precursor forms and cleavage products are indicated.

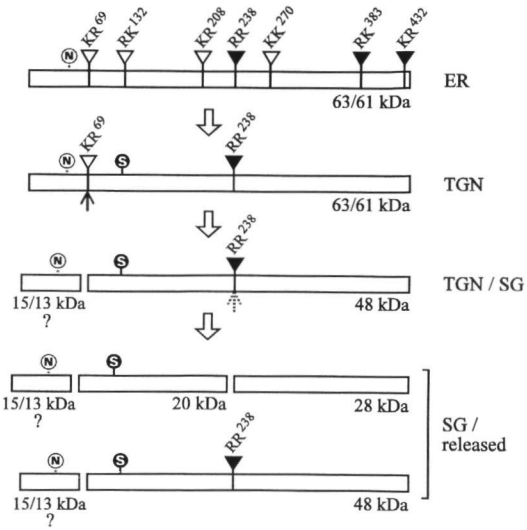

FIG. 8. **Schematic representation of SgIII processing in *Xenopus* melanotrope cells.** Data from Figs. 2, 4, 5, and 7 are summarized. The amino-terminal (partially *N*-glycosylated) fragment formed after cleavage at the KR^{69} site was not detected in our pulse-chase experiments, probably because it is not recognized by the antiserum. *ER*, endoplasmic reticulum; *TGN*, *trans*-Golgi network; *SG*, secretory granules. Other designations are as in Fig. 1. See text for details.

even in overexposed gels. When the Arg^{237}-Arg^{238} site was substituted by Ser^{237}-Ser^{238}, only one cleavage product of 48 kDa was detected (Fig. 7, *lane 4*). The intact precursors but no cleavage products were observed after expressing the mutant that carried substitutions at both sites (Fig. 7, *lane 5*). Together, these results confirm our prediction that the endoproteolytic conversion of SgIII in *Xenopus* melanotropes results from cleavages at Lys^{68}-Arg^{69} and Arg^{237}-Arg^{238}. A schematic representation of SgIII processing in *Xenopus* melanotropes is given in Fig. 8.

SgIII Is Processed in Several Neuronal and Endocrine Cell Types from Various Species—Western blot analysis revealed that *Xenopus* brain and anterior pituitary lobes contain the same set of SgIII-derived cleavage products as those generated in *Xenopus* NILs,[2] indicating that SgIII is processed in a variety of neuroendocrine cell types. Following immunoprecipitation analysis of pulse-labeled NILs and anterior pituitary lobes from rat, a single SgIII precursor of 62 kDa and two cleavage products of 48 and 31 kDa were detected.[2] Radiolabeled proteins of the same sizes were also immunoprecipitated from cell lysates and incubation media of pulsed mouse insuloma βTC3 cells, glucagon-producing αTC4 cells, and anterior pituitary-derived AtT20 cells (Fig. 9, *lanes 1–6*). When AtT20 cells were stably transfected with *Xenopus* SgIII, additional products of 63-, 28-, and 20 kDa were found (Fig. 9, *lanes 7* and *8*). Thus, the dissimilarities in SgIII cleavage patterns observed between *Xenopus* and rodent cells probably result from species-related differences in the primary structures of the proteins. For example, rodent SgIII lacks the functional Lys^{68}-Arg^{69} cleavage site found in the *Xenopus* protein (14). The identification of a bovine chromaffin granule-associated peptide corresponding to the region amino-terminal of the dibasic Arg^{95}-Lys^{96} in rodent SgIII (31), a pair not present in the *Xenopus* protein, further substantiates the notion that SgIII processing in the various species involves dibasic sites that are not conserved during vertebrate evolution.

Ottiger *et al.* (11) previously reported on a single SgIII protein of ~57 kDa which was detected by Western blot analysis in various regions of the rat brain. We did not observe a newly synthesized SgIII-related protein of this size in rat pituitary or mouse endocrine cells. In view of the present data, the possibility should be considered that the protein detected by Ottiger and co-workers (11) does not represent intact SgIII but is a SgIII-derived cleavage product.

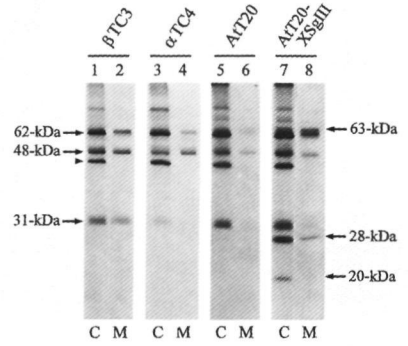

FIG. 9. **SgIII processing in mouse endocrine cell lines.** Mouse insuloma βTC3 cells, glucagon-producing αTC4 cells, anterior pituitary-derived AtT20 cells, and AtT20 cells stably transfected with *Xenopus* SgIII (*AtT20/XSgIII*) were pulsed for 180 min with Tran[^{35}S]-label. Cell lysates (*C*) and incubation media (*M*) were subjected to immunoprecipitation analysis using anti-SgIII antiserum. Migration positions of mouse SgIII precursor and cleavage products are indicated on the *left* and those of *Xenopus* SgIII forms on the *right*. The *arrowhead* indicates a nonspecifically reacting protein also recognized by antisera raised against other His-tagged recombinant proteins.[2]

In this study, we have shown that SgIII is a sulfated precursor protein whose endoproteolytic processing is a wide-spread phenomenon in the neuroendocrine system of vertebrates. At present, the significance of SgIII processing is unclear. The possibility that it serves to liberate functionally important peptides, conforming to what has been proposed for CgA and SgII (3–5), is unlikely. First, the majority of potential dibasic cleavage sites in SgIII is not conserved during vertebrate evolution

(14) Moreover, our present data demonstrate that *Xenopus* SgIII is fully processed at the nonconserved Lys^{68}-Arg^{69} site, whereas only partial cleavage occurs at the conserved Arg^{237}-Arg^{238} site (as diagrammed in Fig 8) Therefore, if SgIII belongs to the group of prohormones and neuropeptide precursors, it would represent a notable exception since the members of this class are generally cleaved at conserved dibasic sites (32) Second, a comparative analysis of SgIII protein sequences from *Xenopus* and rodents showed that regions with the highest degree of sequence identity (over 90%) are not flanked by dibasic sites (14), in contrast to what one would expect for a genuine peptide precursor In fact, the two functional cleavage sites in *Xenopus* SgIII each reside within a poorly conserved region where the degree of sequence identity has dropped below 30%

If not to liberate bioactive peptides, what then is the purpose of SgIII processing? It may terminate a function exerted by the intact protein in the early secretory compartments An interesting example in this respect concerns the neuroendocrine protein 7B2 (SgV) When travelling through the endoplasmic reticulum and Golgi compartments, the uncleaved form of 7B2 is associated with and appears to prevent premature activation of pro-PC2 (7, 8, 10) Upon arrival in the TGN, 7B2 is cleaved and dissociates from pro-PC2, allowing the proenzyme to mature Given the existence of a private chaperone for PC2, it is conceivable that additional helper proteins interact with other enzymes in the secretory pathway If SgIII represents such a helper protein, then its processing could trigger complex dissociation Another possibility is that SgIII promotes the selective aggregation of luminal proteins and their subsequent packaging into secretory granules, whereby its processing serves to dissolve and/or facilitate maturation of the granular content. Alternatively, the proteolytic conversion of SgIII may simply reflect the fate of the protein in the regulated secretory pathway rather than being essential for its mechanism of action

The function of SgIII remains to be established Neither the genetic ablation of its gene in mice (12) nor its overexpression in cultured neuroendocrine cells[2] has provided any clue with respect to the role of this protein in the neuroendocrine system The results of our present study show that SgIII itself is a precursor molecule and can only have an intracellular function, whereas an extracellular role can only be attributed to SgIII-derived peptides.

Acknowledgments – We are grateful to K Janssen and Y Croes for technical assistance, R Engels for animal care, and D Eib and M van Horssen for critically reading the manuscript

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Biosynthesis and Functional Aspects of the Transmembrane Glycoprotein Ac45, a Potential Modulator of Vacuolar H^+-ATPase Activity in the Secretory Pathway

Biosynthesis and Functional Aspects of the Transmembrane Glycoprotein Ac45, a Potential Modulator of Vacuolar H^+-ATPase Activity in the Secretory Pathway

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Vacuolar H^+-ATPases (V-ATPases) mediate the acidification of multiple intracellular compartments, including secretory granules in which an acidic milieu is necessary for prohormone processing. A search for genes coordinately expressed with the prohormone pro-opiomelanocortin in *Xenopus* intermediate pituitary led to the isolation of a cDNA encoding the V-ATPase accessory subunit Ac45. Immunoprecipitation analysis of newly synthesized proteins produced in *Xenopus* neurointermediate lobes (NILs) revealed that Ac45 is an *N*-glycosylated type I transmembrane protein of 60 kDa. In primary cultures of *Xenopus* NILs, the melanotrope cells displayed a strong and punctate Ac45 immunoreactivity in the cytoplasm and a minor staining at the cell surface, while fibroblasts did not react with Ac45 antiserum. When transiently expressed in CV-1 fibroblasts, Ac45 was mainly located in the juxtanuclear region with some punctate labeling throughout the cytoplasm and a moderate staining of the plasma membrane. Antibody internalization experiments revealed that Ac45 is rapidly retrieved from the cell surface and possibly enters a recycling pathway. Co-localization studies with the 115-kDa membrane subunit of V-ATPase indicated that Ac45 is sorted to only a subset of the intracellular compartments harbouring the proton pump. Overproduction of Ac45 in CV-1 cells was found to perturb protein transport to the cell surface. This effect occurs at the level of the Golgi apparatus and involves a vesiculation of membranes reminiscent of that observed in cells exposed to the specific V-ATPase inhibitor bafilomycin A1. Collectively, our findings suggest that, although not a regular subunit of V-ATPases, Ac45 is capable of modulating V-ATPase activity in the secretory pathway.

Acidification of organelles connected with the vacuolar system of eukaryotic cells is of crucial importance in numerous cellular processes, including the targeting of newly synthesized proteins to lysosomes and secretory granules, maintenance of acid hydrolase activity in lysosomes, proteolytic processing of prohormones in secretory granules, coupled transport of biogenic amines across secretory vesicle membranes, and the unloading of cargo during receptor-mediated endocytosis (Mellman et al., 1986). This acidification is accomplished by a unique class of ATP-driven proton pumps called vacuolar H^+-ATPases (V-ATPases) (Forgac, 1989; Harvey and Nelson, 1992). V-ATPases have been purified from clathrin-coated and synaptic vesicles (Forgac et al., 1983; Cidon and Sihra, 1989), chromaffin granules (Percy et al., 1985), the Golgi apparatus (Moriyama and Nelson, 1989), endosomes (Yamashiro et al., 1983) and lysosomes (Harikumar and Reeves, 1983) of higher animal cells as well as from the central vacuoles of plants (Parry et al., 1989), fungi (Bowman et al., 1989) and yeast (Kane et al., 1989). V-ATPases also occur in dense clusters on the plasma membranes of specialized proton-secreting cells like kidney epithelial cells and osteoclasts, where the enzyme participates in urinary acidification and bone resorption, respectively (Al-Awqati, 1986; Blair et al., 1989).

The V-ATPases clarified so far share a remarkable degree of structural similarity and consist of at least nine different subunits which are distributed over two distinct moieties, a hydrophobic membrane sector and a hydrophilic catalytic sector (Forgac, 1989; Harvey and Nelson, 1992). The membrane sector is responsible for proton translocation and contains a hexamer of 16-kDa subunits plus one copy each of ~115-, 38- and 20-kDa subunits. The peripherally-attached catalytic sector faces the cytoplasm and forms the ATP hydrolytic centre of the pump. It comprises a hexamer of three A subunits (65-77 kDa) and three B subunits (55-60 kDa) along with subunits of ~40, 34 and 33 kDa. Genetic studies in yeast suggest that the assembly of a V-ATPase starts with its membrane sector; the disruption of a gene encoding any single transmembrane subunit prevents the catalytic subunits from

associating with the target membrane The membrane sector of the enzyme, however, is correctly assembled and transported to the target membrane independently of the catalytic sector (Doherty and Kane , 1993, Ho et al , 1993) The recent finding that V-ATPase assembly requires several endoplasmic reticulum (ER)-associated proteins indicates that the biogenesis of the pump is initiated in the ER (Hirata et al , 1993, Hill and Stevens, 1995)

How V-ATPases are directed to and acquire their specialized activities in such a large variety of intracellular organelles and membranes is still unknown One possible mechanism for generating this topological and functional diversity may arise from V-ATPases assembled with isoforms of one or more subunits Consistent with this idea is the identification of several tissue-specific subtypes of the V-ATPase subunits A (Van Hille et al, 1993) and B (Sudhof et al , 1989, Nelson et al , 1992, Puopolo et al., 1992) Moreover, subtle changes in the lipid composition of the membrane and/or organelle-specific membrane proteins may also play a role in the sorting and regulation of the pump (Nelson, 1992)

While looking for organelle-specific proteins in purified preparations of V-ATPase from bovine chro-maffin granules, Supek et al (1994) identified a novel 45-kDa protein named Ac45 Cold-inactivation experiments revealed that this protein is associated with the membrane sector of the pump Besides in bovine adrenal medulla, Ac45 was detected in various parts of the brain and in the pituitary gland Compared with V-ATPase in chromaffin granules, the pump purified from kidney microsomes contained reduced amounts of Ac45, whereas kidney membrane V-ATPase preparations were devoid of the protein (Supek et al , 1994) Recently, we isolated a partial cDNA encoding an Ac45 homolog from the amphibian *Xenopus laevis* (Holthuis et al , 1995) This was achieved by a differential screening strategy designed to identify genes coexpressed with the prohormone proopiomelanocortin (POMC) in the melanotrope cells of *Xenopus* intermediate pituitary These neuroendocrine cells can be stimulated to produce and release large amounts of POMC-derived, melanophore-stimulating peptides by placing the toad on a black background (Jenks et al , 1993) Thus, melanotropes of animals adapted to a black background contain ~10 times more Ac45 mRNA and ~30 times more POMC transcripts than those of white-adapted animals (Holthuis et al , 1995)

In the present study, we determined the complete primary structure of *Xenopus* Ac45 and raised a polyclonal antiserum to monitor its biosynthesis in *Xenopus* melanotropes To investigate the functional significance of the protein, Ac45 was ectopically expressed in cultured cells and the effects of overproduction were compared with those evoked by the V-ATPase inhibitor bafilomycin A1

MATERIALS AND METHODS

Animals — South-African clawed toads, *Xenopus laevis*, were adapted to a black background by keeping them in black buckets under constant illumination for at least three weeks at 22°C

cDNA isolation — A *Xenopus* neurointermediate lobe (NIL) cDNA library from black background-adapted toads was differentially screened with cDNA probes derived from NIL mRNA of black- and white-adapted animals as described previously (Holthuis et al , 1995) This screening included the isolation of a partial 1 3-kb cDNA (clone X1311) encoding *Xenopus* Ac45 To isolate a full-length cDNA, the X1311 cDNA insert was random prime-labeled with [α-^{32}P]dATP (3,000 Ci/mmol, ICN Radiochemicals, Costa Mesa, CA, USA) and used to screen $6x10^5$ plaque-forming units from an amplified *Xenopus* hypo-thalamus cDNA library in lambda uni-ZAP XR (Van Riel et al , 1993) Seven positively hybridizing plaques were purified and the pBluescript SK phagemids were rescued by *in vivo* excision according to the manufacturers instructions (Stratagene, La Jolla, CA, USA) One of these clones, X1311-4, contained a 2 0-kb cDNA insert encoding the entire Ac45 open reading frame Sequencing on both strands and with pBluescript subclones or specific primers was performed with single- and double-stranded DNA using T7 DNA polymerase (LKB-Pharmacia, Uppsala, Sweden) and the dideoxy chain termination method (Sanger et al , 1977)

Production of Ac45 recombinant protein and generation of antiserum — Polyclonal antiserum was raised in rabbits against recombinant Ac45 protein produced in *Escherichia coli* using the Qiagen expression system (Qiagen Inc , Chatsworth, CA, USA) For this purpose, a 1 0 kb *SacI/SmaI* cDNA fragment of *Xenopus* hypothalamus cDNA clone X1311-2 (insert 1 7 kb) was ligated into the *SacI/SmaI* sites of the vector pQE-30 This allowed the production of recombinant protein composed of *Xenopus* Ac45 amino acid residues Gly^{68} to Pro^{390} with a hexahistidine tail at its amino-terminus Following purification by SDS-PAGE, the protein was electroeluted from the gel and a 500 μg initial dose emulsified with Freund's complete adjuvant was administered to rabbits at several intradermal sites Four weeks later, and at three-weeks intervals thereafter, rabbits were boosted with 250 μg recombinant protein in Freund's incomplete adjuvant The production of specific antibodies was monitored by enzyme-linked immunosorbant assay

Metabolic labeling of Xenopus NILs and immunoprecipitation analysis — NILs from black adapted *Xenopus* were dissected and incubated overnight in *Xenopus* culture medium (6 7 ml L-15 medium [Gibco-BRL, Gaithersburg, MD, USA], 3 ml milli Q water, 100 μl

kanamycin and 100 µl antibiotic-antimycotic solution [Gibco-BRL], 8 mg $CaCl_2H_20$, 3 mg bovine serum albumin and 2 mg glucose) supplemented with 10% fetal calf serum (Gibco-BRL) in the absence or presence of 10 µg/ml tunicamycin Pulse labeling of newly synthesized proteins was performed by incubating lobes in methionine- and cysteine-free culture medium containing 1 7 mCi/ml Tran[^{35}S]-label (ICN Radiochemicals) for the indicated time periods at 22°C Subsequent chase incubations were in culture medium supplemented with 5 mM L-methionine and 2,5 mM L-cysteine Pulse- and pulse-chase incubations were in the absence or presence of tunicamycin Lobes were homogenized on ice in lysis buffer (50 mM Hepes pH 7 2, 140 mM NaCl, 1% Tween 20, 0 1% Triton X-100, 0 1% deoxycholate, 1 mM phenylmethylsulfonyl fluoride and 0 1 mg/ml soybean trypsin inhibitor) Homogenates were cleared by centrifugation (10,000 x g, 7 min at 4°C), supplemented with 0 1 volume of 10% SDS and diluted 10-fold in lysis buffer before addition of anti-Ac45 antiserum (1 500 dilution) Immune complexes were precipitated with protein-A Sepharose (LKB-Pharmacia) and resolved by SDS-PAGE Radiolabeled proteins were visualized by fluorography

Eukaryotic expression plasmids — A 1 7-kb *Eco*RI fragment of *Xenopus* hypothalamus cDNA clone X1311-4 encoding the entire Ac45 protein, was subcloned downstream of the cytomegalo virus (CMV) promotor into the *Eco*RI site of the eukaryotic expression vector pcDNA3 (Invitrogen, San Diego, CA, USA) The Ac45/ICAM expression construct, encoding the lumenal domain of *Xenopus* Ac45 (ending QGFNV at residue 371, Fig 1) followed by the residues TS and then the entire transmembrane- and cytoplasmic domains of human ICAM-1 (starting NVLSP at residue 446, Staunton et al , 1988), was obtained by creating *Spe*I sites in the Ac45 and ICAM-1 cDNAs through oligonucleotide-directed mutagenesis on single-stranded DNA using the pALTER system (Promega, Madison, WI, USA) The presence of the desired mutations was confirmed by restriction enzyme digestion and double-stranded DNA sequencing Appropriate fragments of correctly mutagenized cDNAs were subcloned downstream of the CMV promoter into pcDNA3 Oligonucleotides used to create the *Spe*I sites in Ac45 and ICAM-1 were 5'-CAATGTGACTAGTATGGCATTTTCA-3' and 5'-CACCCGCGAGGTGACTAGTAATGTGCTCTCCCCC-3', respectively The human ICAM 1 cDNA was obtained from Dr C Figdor (Department of Tumor Immunology, University of Nijmegen) An expression construct encoding *Xenopus* SgIII has been described previously (Holt-huis et al , 1996, Chapter 5) DNA for transfection studies was isolated using the Qiagen plasmid kit (Qiagen Inc)

Cell transfection, metabolic labeling and immunoprecipitation analysis — Cell culture media were obtained from Gibco-BRL and supplemented with 10% (v/v) fetal calf serum Green monkey CV-1 kidney fibroblasts were cultured in Iscoves-modified Eagle's medium and mouse anterior pituitary-derived AtT20 cells in high glucose Dulbecco's modified Eagle's medium Transfection of CV-1 and AtT20 cells was accomplished by the calcium phosphate precipitation method (Graham and Van der Eb, 1973) AtT20 cells were transfected with the *Xenopus* Ac45-pcDNA3 construct and after 48 hr selected for stable expression of Ac45 in medium containing 750 µg/ml neomycin (Boehringer, Mann-heim, Germany) For transient expression studies, CV-1 cells were plated in 20 mm culture dishes, grown until 30% confluency and transfected with 2 5 µg DNA per construct per dish CV-1 cells (48 hr after transfection) and stably transfected At20 cell lines were starved for 60 min in methionine- and cysteine-free Dulbecco's modified Eagle's medium (ICN Biochemicals) supplemented with 10% dialyzed fetal calf serum (Gibco-BRL) and subsequently pulsed for 180 min in the same medium with 0 2 mCi/ml Tran[^{35}S]-label Cells were lysed on ice in lysis buffer and the cell lysates and incubation media were processed for immunoprecipitation analysis as described above

Primary cultures of Xenopus intermediate pituitary cells — Primary cultures of intermediate pituitary cells were established from NILs of black-adapted *Xenopus* To eliminate blood cells from the lobes, animals were perfused intracardially with a Ringer's solution containing 112 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 15 mM HEPES, pH 7 4, and 2 mg/ml glucose for 10 min Lobes were dissected, washed in sterile *Xenopus* culture medium, transferred to Ringer's solution containing 0 25% (w/v) trypsin and incubated for 30 min at 20°C Lobes were suspended by 10 passes through a siliconized Pasteur's pipet, transferred to a syringe and filtered through a nylon filter (pore size 150 µm) Cells were washed in *Xenopus* culture medium supplemented with 10% fetal calf serum, collected by centrifugation and cultured on poly-L lysine coated coverslips at 20°C for three days before using them in experiments

Immunofluorescence microscopy — For steady-state immunofluorescence localization of Ac45, *Xenopus* intermediate pituitary cells (for cell preparation see above) were fixed in 2% paraformaldehyde/XPBS, pH 7 4 (XPBS 67% PBS) for 1 hr at 4°C, incubated in 100 mM glycine/XPBS for 30 min at 4°C, permeabilized in ice-cold 0 1% Triton X-100/XPBS (XPBS-TX), and incubated with rabbit anti-Ac45 antiserum (1 300) in XPBS-TX containing 2% (w/v) bovine serum albumin (XPBS-TXB) for 2 hr at 4°C For immunolabeling of cell surface- expressed and internalized Ac45, cells were incubated with anti-Ac45 antiserum (1 300) in culture medium for 20 min at 4°C, washed and fixed immediately, or returned to antibody-free culture medium at 20°C for the indicated time periods prior to fixation and permeabilization To visualize bound anti-Ac45 antibodies, permeabilized cells were incubated with FITC-conjugated goat anti-rabbit antibodies (1 100, Boehringer) in XPBS-TXB for 2 hr at 4°C Immunolocalization studies on transfected CV-1 fibroblasts were performed essentially as described above, except that undiluted PBS was used in the protocol and that after surface labeling cells were returned to 37°C Bafilomycin A1 (Wako Pure Chemical Industries, Osaka, Japan) was

dissolved in methanol (1 mM stock solution) and added to culture media of transfected CV-1 fibroblasts at a final concentration of 1 μM at the indicated time periods before cells were processed for immunofluorescence microscopy Rabbit anti-secretogranin III antiserum (Holthuis et al , 1996, Chapter 5) was applied at a dilution of 1 1000 For simultaneous immunolocalization of Ac45 and endogenously expressed 115-kDa V-ATPase subunit, transfected CV-1 cells were fixed, permeabilized and consecutively incubated with mouse monoclonal antibody OSW2 (directed against 115-kDa subunit, Sato and Toyama, 1994), FITC-conjugated goat anti-mouse antibodies (1 100, Boehringer), anti-Ac45 antibodies (1 300) and TRITC conjugated goat anti-rabbit antibodies (1 100, Boehringer) Each incubation was in PBS TXB for 1 hr at 4°C, and followed by extensive washing with ice-cold PBS-TXB Immunostained cells were mounted in Citifluor (Agar Scientific Ltd, Stanstedt Essex, UK) and viewed under epifluorescence optics with a Leica DMRB/E microscope (Leica, Heerbrugg, Switzerland) and a vario orthomat camera system

RESULTS

Cloning and sequence analysis of a Xenopus homolog of Ac45 — While searching for genes coordinately expressed with POMC in *Xenopus* melanotrope cells (Holthuis et al , 1995), we isolated a 1 3-kb cDNA (clone X1311) representing a highly regulated tran-script of ~2 3 kb Northern blot- and RNase protection analysis revealed that this transcript is particularly abundant in neuroendocrine tissues with over ten-fold higher expression levels in brain and pituitary than in liver, heart or kidney (Holthuis et al , 1995 and data not shown) In order to obtain the complete nucleotide sequence of this transcript, a *Xenopus* hypothalamus cDNA library was screened with X1311 cDNA as a probe The largest of the hybridization-positive clones isolated, clone X1311-4, contained a cDNA insert of 2002 bp (excluding the poly(A) tail) whose nucleotide and deduced amino acid sequence is presented in Fig 1 The 621-bp 3'-untranslated region carries two polyadenylation signals, 15- and 54-bp upstream of the poly(A) tail A potential translation initiation site was found at nucleotide positions 3-11 (CAGAGATGG, Kozak, 1991) Translation from this site would produce a protein of 458 amino acids with a calculated molecular weight (M_r) of 50,214 The putative initiator methionine precedes a hydrophobic signal sequence of 24 amino acids with a cleavage site conforming to the -1, -3 rule (Von Heijne, 1986) When this signal sequence is cleaved off, a protein with a calculated M_r of 47,756 remains A hydrophobicity plot indicated the presence of a potential transmembrane segment close to the carboxy-terminus The region between the signal peptide and the transmembrane domain contains seven consensus sites for *N*-linked glycosylation A database search revealed a high degree of structural similarity with the bovine protein Ac45, an accessory subunit of V-ATPase from adrenal medulla chromaffin granules (Supek et al , 1994) An additional match was found with a human homolog of Ac45 whose partial cDNA (clone CF2) was isolated during a search for expressed sequences encoded by the Xq terminal region of the human X-chromosome (Yokoi et al , 1994) No similarities were found with other sequences in the database We therefore conclude that the protein encoded by clone X1311 is the *Xenopus* homolog of Ac45

Evolutionary conservation of Ac45 — Fig 2 shows an alignment of the amino acid sequences of *Xenopus*, bovine and human Ac45, at present, only a partial amino acid sequence of human Ac45 is available With the signal peptide sequences excluded, the *Xenopus* and bovine proteins share 60% amino acid sequence identity over 428 matched residues and a similarity of 78% The degree of sequence identity between *Xenopus* and human Ac45 is 59% over 378 matched residues with a similarity of 77% Bovine and human Ac45 share 84% identity and 90% similarity over 379 matched residues The positions of the transmembrane domains and four of the potential *N*-linked glycosylation sites are conserved among the three species Two of the four cysteine residues in *Xenopus* Ac45 are also present in bovine and human Ac45

Biosynthesis of Ac45 in Xenopus melanotropes In order to study the biosynthesis of Ac45 in *Xenopus* melanotropes, we raised a polyclonal antiserum against a recombinant protein comprising *Xenopus* Ac45 residues 68-388 When the antiserum was applied in immunoprecipitation studies on Tran[^{35}S]-labeled neurointermediate lobes (NILs) from black-adapted animals, a newly synthesized protein of 60 kDa was detected (Fig 3a, lane 2) This protein was not recognized by the preimmune serum (Fig 3a, lane 1) Pretreatment of NILs with tunicamycin prior to pulse labeling and immunoprecipitation analysis resulted into a dramatic increase in the mobility of the protein from 60 kDa to 46 kDa (Fig 3a, lane 3) These results indicate that (i) the protein detected by our antiserum represents newly-synthesized Ac45, (ii) Ac45 undergoes *N*-linked glycosylation at multiple sites, (iii) the amino-terminal and major portion of Ac45 containing the potential *N*-linked glycosylation sites is translocated into the ER lumen where this type of glycosylation is known to occur Since the protein,

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                                                                                        5'-AACAGAG     7
                                                                                                  ↓+1
Met Ala Ala Met Ala Glu Trp Ala Leu Leu Ser Leu Leu Phe Leu Ala Gly Pro Phe Pro Ala Ala Pro Ser Gln     1
ATG GCA GCG ATG GCG GAA TGG GCT CTT CTC TCT CTT CTG TTC TTG GCG GGC CCC TTT CCT GCC GCC CCG TCC CAG    82

Gln Val Pro Val Leu Leu Trp Ser Ser Glu Thr Ser Leu Trp Asn Phe Gln Pro Ser Ile His Ser Gly His Ile    26
CAA GTG CCC GTG CTG CTG TGG TCT AGC GAG ACG TCC CTG TGG AAT TTC CAG CCT AGT ATA CAC AGT GGA CAC ATT   157

Thr Thr Asp Ile Gln Leu Gly His Tyr Leu Asp Pro Ala Leu Met Lys Gly Pro Arg Asn Ile Leu Leu Phe Leu    51
ACT ACA GAT ATT CAG CTT GGA CAC TAC CTT GAC CCA GCT CTC ATG AAG GGT CCC AGA AAT ATT CTG TTA TTC CTC   232

Gln Asp Lys Leu Ser Ile Glu Asp Phe Thr Ala Phe Gly Gly Val Tyr Gly Asn Lys Gln Asp Ser Ala Phe Pro    76
CAG GAT AAG TTA AGT ATC GAA GAC TTT ACA GCA TTT GGG GGT GTC TAT GGC AAC AAA CAA GAC AGT GCA TTT CCT   307

Asn Leu Asp Ser Ile Val Glu Ser Ser Pro Ser Ser Leu Val Leu Pro Ala Val Asp Trp Tyr Ala Ala Asn Ile   101
AAT CTA CAG AGC ATT GTG GAG TCA TCC CCT TCC TCC CTG GTT CTG CCT GCA GTG GAC TGG TAC GCT GCT AAC ATA   382

Leu Pro Thr Tyr Leu Lys Glu Lys Leu Gly Val Ser Pro Leu His Val Asp Gln Ser Thr Leu Leu Glu Leu Lys   126
TTG CCC ACA TAC CTG AAG GAG AAG CTT GGA GTC AGC CCC CTG CAT GTA GAC CAG TCC ACT CTC CTG GAG CTG AAG   457
     ●
Leu Asn Glu Ser Val Pro Ser Leu Leu Val Val Arg Leu Pro Tyr Ala Ser Ser Thr Gly Leu Leu Ala Ala Lys   151
CTG AAT GAA AGT GTC CCC TCT CTG CTC GTT GTC CGG TTG CCT TAT GCA AGC AGT ACT GGT TTG CTG GCA GCT AAA   532

Asp Val Leu Arg Ala Asn Asp Gln Ala Ile Gly Gln Val Leu Ser Thr Leu Lys Ser Glu Gly Val Pro Tyr Thr   176
GAT GTT CTG AGA GCT AAT GAC CAA GCA ATT GGA CAA GTT CTG AGC ACA TTA AAG TCA GAG GGT GTG CCC TAT ACT   607

Ala Leu Leu Thr Ala Leu Arg Pro Ser Arg Val Ile Lys Glu Ala Ser Phe Ala Val Gly Asn Leu Gly Arg Gln   201
GCT TTG CTG ACT GCA CTA CGT CCC TCA CGG GTT ATC AAA GAG GCC TCA TTT GCT GTG GGA AAC CTT GGG CGC CAG   682
                                                                 ●
Leu Leu Ala Thr Glu Gln Pro Met Pro Ser Tyr Pro Pro Val Ala Tyr Asn Ser Ser Gln Asn Arg Pro Cys Ile   226
TTA CTT GCC ACT GAG CAG CCT ATG CCA AGC TAT CCT CCC GTG GCC TAT AAC AGT AGC CAG AAC AGA CCA TGC ATC   757
                         ●
Leu Phe Trp Ala Thr Asn Val Ser Val Thr Val Asp Asp Val Gln Val Asp Leu Thr Ser Gln Thr Phe Thr Gly   251
CTA TTT TGG GCC ACC AAT GTC TCT GTA ACA GTG GAT GAC GTG CAG GTG GAT CTA ACT AGC CAA ACC TTT ACG GGC   832
             ●
Ser Asp Leu Asn Leu Thr Gly Ser Leu Cys Asn Asn Leu Asn Ala Val Leu Ala Leu Thr Tyr Lys Asp Ala Val   276
AGC GAC CTC AAT CTG ACT GGG TCT CTC TGT AAC AAC CTC AAT GCA GTA CTG GCG CTA ACG TAC AAA GAT GCA GTG   907

Lys Gly Leu Pro Leu Thr Leu Arg Phe Leu Leu Gln Arg Arg Phe Tyr Pro Val Ser Gly Arg Phe Trp Phe Ile   301
AAA GGA CTG CCC CTG ACT TTG CGG TTC CTC CTT CAG CGA AGA TTT TAC CCT GTC TCT GGC CGC TTC TGG TTC ATT   982
                                                                                                 ●
Leu Ser His Val Glu Met Ile His Gly Gln Asn Thr Ala Val Phe Leu Ala Pro Gln Val Asn Ala Pro Ser Asn   326
CTG AGC CAC GTG GAG ATG ATC CAC GGT CAA AAC ACG GCT GTT TTT CTT GCT CCT CAA GTG AAT GCT CCC AGC AAC  1057
                                                                             ●
Tyr Ser Phe Thr Cys Gln Tyr Ile Ser Ser Trp Gln Thr Phe Gly Ser Leu Leu Val Ser Asn Thr Ser Lys Asp   351
TAC TCC TTC CAC TGC CAG TAC ATC AGT AGT TGG CAG ACC TTT GGT TCT CTT CTT GTG AGC AAC ACC AGC AAG GAT  1132
                                                                 ●
Leu Pro Ser Ser Thr Trp Gln Leu His Ile Ala Asp Phe Gln Ile Gln Gly Phe Asn Val Thr Gly Met Ala Phe   376
TTG CCC TCC TCT CGA TGG CAG CTT CAC ATT GCT GAC TTC CAG ATC CAG GGT TTC AAT GTG ACT GGG ATG GCA TTT  1207

Ser Tyr Ala Ser Asp Cys Ala Gly Phe Phe Ser Pro Gly Ile Trp Met Gly Leu Ile Thr Thr Leu Leu Phe Val   401
TCA TAC GCC AGT GAC TGT GCA GGC TTC TTC TCC CCG GGC ATC TGG ATG GGG CTC ATC ACC ACC TTG CTC TTT GTC  1282

Phe Ile Leu Thr Tyr Gly Leu His Met Val Met Ser Leu Lys Thr Met Asp Arg Phe Asp Asp Pro Lys Gly Pro   426
TTT ATC CTG ACC TAT GGA CTG CAC ATG GTT ATG AGC CTG AAG ACT ATG GAT CGT TTT GAT GAT CCC AAG GGC CCA  1357

Ser Ile Ala Val Pro Gln Thr Glu ***                                                                   434
AGC ATT GCT GTG CCC CAG ACA GAG TGA TTGGCATAGGGCAGTTTCCAAACATTGTTGTGCCCCAGACAGCAGTTAAAGTAGGGACAATTC  1447

TCTGATCTGTGATCAAGTACAGCAGTGATTATGTGTATGTGTTCCTGGAAAATAAATGGAGCCAGATTAGTAGGTGGCTTAATCCCGTGTCCATACCCA  1546
GACTGCCATTGTGTTCTGGGTACAGGCTTAGGCTCCACTGTACAGTTCCATCTGGAACCCCTAATTTATTGCCCTTCATGTGTGTTGAGTTGTCAGTTC  1645
ACTGGATATTGATTCCTTCTATTTCCGTAGCCTGAATTCATTCCTTTGCTTTGCATGCAGTCCCACAAGGAGGCCTTATGTCCATAGGTCCAGTACCCA  1744
GGCTGGCTGGAGGGGGCAGCAATCCATGTGATGTATATTTCTCTCTGCGTATATAGTACCAGGCAATGGCTTGTATTTATTTCTTGTTGTGTGATGTCC  1843
AGCTAGAGCTCATGAATGTAAAGTCAGTGTATTTGCCCAAGCACTGTAGCTCATTACCCTCCAGGGCAGTGGGTTCAGCCAGCATCTCCAGGGATAGCA  1942
AATAAATGTTGCTGTGTAACCTTTCACTGTATAACCGCAAATAAAGTGTTTATGTGCTTT(A)n                                      2002
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FIG 1 Nucleotide sequence and deduced amino acid sequence of cDNA clone X1311-4 encoding *Xenopus* Ac45. The signal peptide sequence is *underlined* and the putative signal peptide cleavage site is indicated by an *arrow* Positive numbering of amino acids starts in the mature protein *Doubly underlined* amino acids are predicted to span the membrane Potential Asn linked glycosylation sites are marked by *black dots* *Overlined* is the consensus for polyadenylation The nucleotide sequence of cDNA clone X1311-4 is available from the EMBL database under accession number X82421

or fragments thereof, was never detected in the chase media of pulse-incubated NILs (Fig 3a, lanes 4 and 5), we expect that Ac45 is anchored in the membrane by the hydrophobic segment found in its carboxy terminal region. The mobility of Ac45 did not change when the protein was electrophoresed under denaturing or non-denaturing conditions (data not shown), suggesting that no intramolecular disulfide bonds are formed during its biosynthesis

Supek et al. (1994) demonstrated that Ac45 puri-

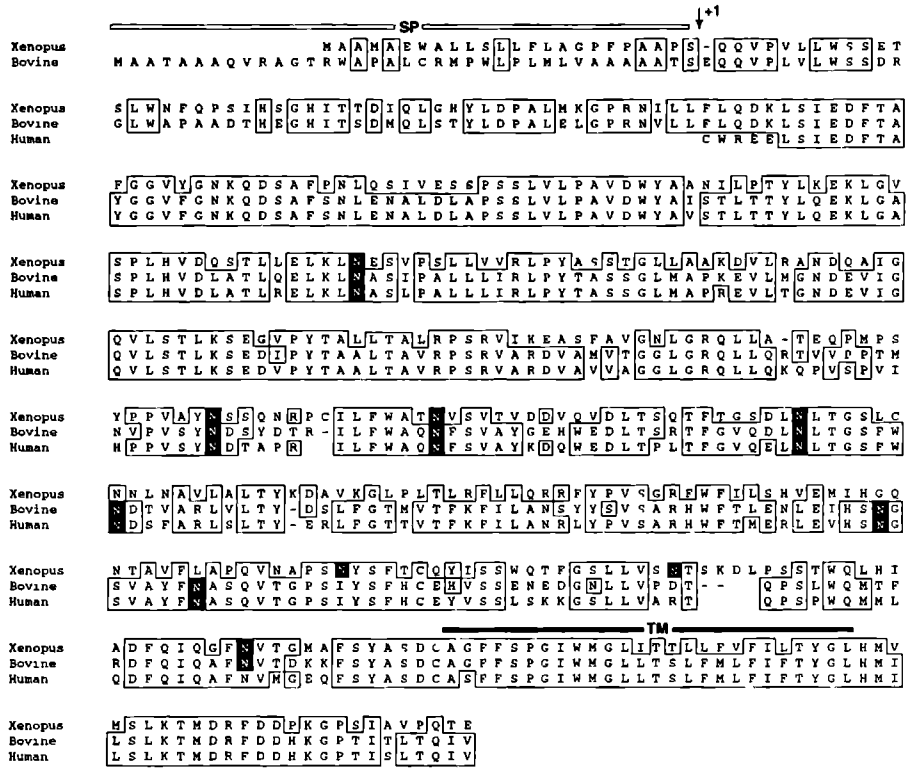


FIG. 2 **Alignment of the amino acid sequences of *Xenopus*, bovine and human Ac45.** The single-letter amino acid code is used and identical residues are boxed Gaps are introduced for optimal alignment Signal peptide (*SP*) and transmembrane (*TM*) regions are *overlined* Positive numbering of residues starts at the first residue following the predicted signal peptide cleavage site (*arrow*) Potential Asn-linked glycosylation sites are shown in *white letters* The bovine Ac45 amino acid sequence was taken from Supek et al (1994) while the human Ac45 amino acid sequence was deduced from the nucleotide sequence of a partial cDNA clone (CF2, Genbank accession number D16469)

fied from bovine chromaffin granules migrates on SDS gels as a protein of 45 kDa To investigate if Ac45 is processed during its intracellular transport, we monitored the fate of the newly synthesized protein in *Xenopus* NILs by pulse-chase analysis As shown in Fig 3b, the radiolabeled Ac45 produced in pulse-incubated NILs (60 min pulse) remained quite stable during subsequent chase incubations No evidence for proteolytic processing was found within the 6-hr chase period Similar results were obtained with Ac45 synthesized in tunicamycin-treated NILs (Fig 3b) In contrast, in both tunicamycin-treated and control lobes, virtually all radiolabeled POMC produced during the pulse-incubation was processed and the POMC-derived peptides were released into the medium following a 2-hr chase period (Fig 3b) These findings indicate that neither Ac45, nor its unglycosylated counterpart, represents a substrate for the proteolytic enzymes operating in the secretory pathway of melanotrope cells

Localization of Ac45 in Xenopus melanotropes When applied in immunofluorescence studies on primary cultures of NILs dissected from black-adapted animals, the Ac45 antiserum gave a bright punctate staining in the cytoplasm of the melanotrope cells (Fig 4a) This labeling pattern was similar to that observed when melanotropes were stained with antisera against the POMC-derived, melanophore-stimulating hormone α-MSH or against the secretory granule-associated protein secretogranin III (data not shown) Other cell types encountered in cultured NILs (e g fibroblasts, endothelial cells, stellate cells) were devoid of Ac45 immunostaining These results demonstrate that melanotrope cells constitute the primary site of

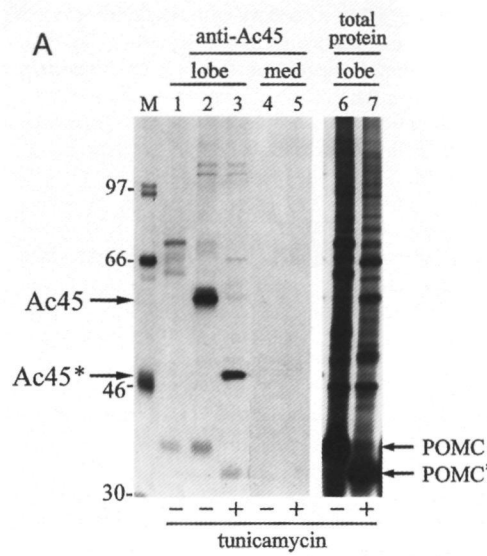
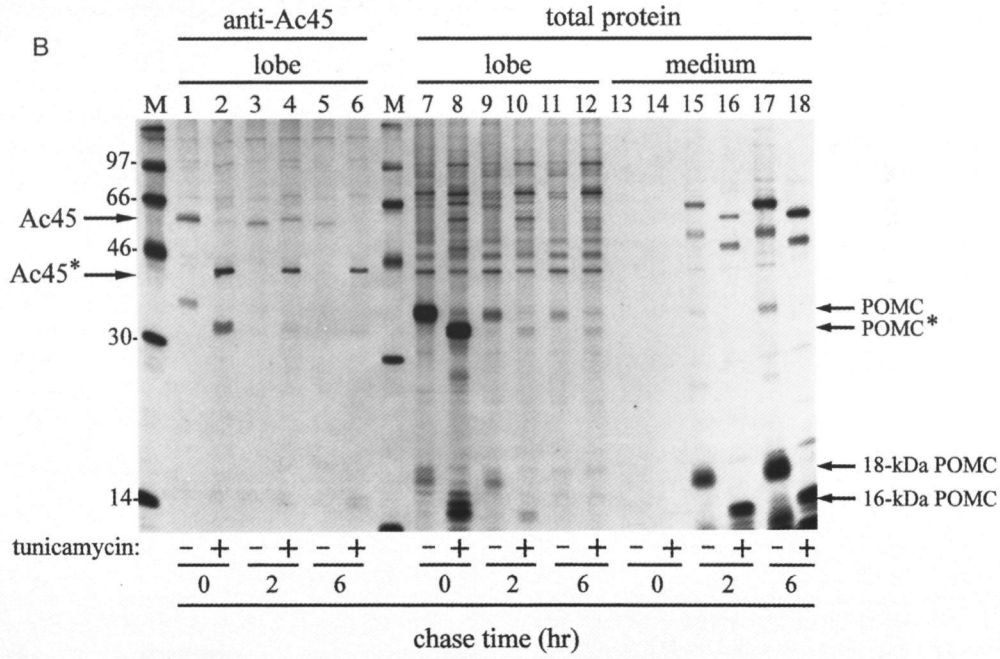


FIG. 3. **Biosynthesis of Ac45 in *Xenopus* neurointermediate lobes.** (**A**) Lobes dissected from black-adapted animals were preincubated overnight in the absence (–) or presence (+) of 10 μg/ml tunicamycin and subsequently pulsed for 3 hr with Tran[^{35}S]-label. Radiolabeled proteins were immunoprecipitated from lobe extracts (3 lobes per lane) and incubation media using preimmune serum (lane 1) or anti-Ac45 antiserum (lanes 2-5). Immuno-precipitates were resolved by SDS-PAGE and visualized by fluorography. (**B**) as in (A), except that lobes were pulsed for 1 hr with Tran[^{35}S]-label and then chased for 0, 2 or 6 hr before immunoprecipitation analysis with anti-Ac45 anti-serum (lanes 1-6). Protein markers for molecular mass (*M*) and migration positions of immunoprecipitated Ac45 are indicated on the *left*. Migration positions of POMC and a POMC-derived cleavage product in total protein extracts (0.04 lobe per lane) are indicated on the *right*. *Asterisks* indicate migration postitions of non-glycosylated proteins.



Ac45 production in the NIL, whereas non-neuroendocrine cells contain, if any, only minor amounts of the protein.

Since the above results suggest that Ac45 resides in the secretory granules of *Xenopus* melanotropes, we wondered if the protein also appears on the surface of these cells. When live melanotrope cells were incubated with antibodies (Ab) against Ac45 for 15 min at 4°C and then fixed and stained with FITC-conjugated secondary Ab, a speckled labelling was observed on their surface (Fig. 4b). When cells exposed to Ac45 Ab for 15 min at 4°C were chased

for 1 hr at 22°C in Ab-free medium prior to fixation and staining with secondary Ab, fluorescent spots were detected throughout the cytoplasm (Fig. 4c). These findings suggest that Ac45 is transiently expressed on the surface of *Xenopus* melanotropes, and that the protein is internalized and sequestred into intracellular compartments shortly after having reached the plasma membrane.

Sato and Toyama (1994) previously reported on a monoclonal Ab (OSW2) directed towards the 115-kDa membrane subunit of V-ATPase which, when incubated with living cells, readily interferes with endosomal acidification. We reasoned that if Ac45 has an essential role in V-ATPase-mediated acidification of the secretory organelles in neuroendocrine cells, a continuous uptake of Ac45 Ab by *Xenopus* melanotropes might eventually affect pH-dependent steps in the sorting and processing of POMC. To evaluate this possibility, primary cultures of NILs from black-adapted animals were incubated overnight in medium containing a purified IgG fraction of the Ac45 antiserum (final concentration of IgGs: ~100 μg/ml). Cells were then pulsed with Tran[^{35}S]-label for 90 min and chased for 180 min (both in the presence of Ab), and the radiolabeled proteins in cell lysates and chase media were analysed by SDS-PAGE. POMC processing patterns and the release of POMC-derived cleavage products into the media of Ab-treated cells were indistinguishable from that of non-treated cells (data not shown). When primary cultures were treated with 1 μM bafilomycin A1, a specific inhibitor of V-ATPases (Bowman et al., 1988), an intracellular accumulation of unprocessed POMC was observed (data not shown). Hence, although these results demonstrate that the generation and release of POMC-derived cleavage products relies on a proper functioning of V-ATPases, they did not provide a clue with respect to the functional significance of Ac45 in this process.

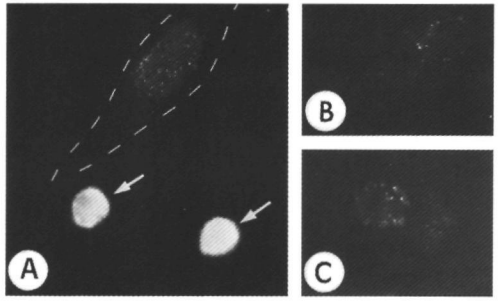


FIG. 4. **Immunofluorescence localization of Ac45 in cultured *Xenopus* neurointermediate pituitary cells.** Neurointermediate lobes dissected from black-adapted animals were dispersed and cultured as described in Materials and Methods. (**A**) Cells were fixed, permeabilized and incubated with anti-Ac45 antiserum; *arrows* indicate two immunolabeled melanotrope cells while the *broken line* marks the position of a non-labeled fibroblast. (**B**) Melano-trope cells were incubated with anti-Ac45 antiserum at 4°C, washed and immediately fixed. (**C**) Melanotrope cells were incubated with anti-Ac45 antiserum at 4°C, washed and then returned to 22°C for 60 min before fixation and permeabilization. Bound anti-Ac45 antibodies were visualized with FITC-conjugated secondary antibodies. Cells incubated with preimmune serum did not stain above background (not shown).

Biosynthesis and localization of Ac45 in transfected cells — As an alternative approach to investigate the functional significance of Ac45, we decided to overexpress the protein in cultured cells and to analyse the effects of overproduction on pH-dependent processes in the secretory pathway. First, we transfected anterior pituitary-derived AtT20 cells with a vector encoding *Xenopus* Ac45 under control of the cytomegalovirus promoter. Ten stable cell lines expressing *Xenopus* Ac45 were selected by immunoprecipitation analysis; these cell lines produced an *N*-glycosylated protein of 60 kDa not observed in control (untransfected) AtT20 cells. Because our antiserum did not recognize endogenous Ac45 in AtT20 cells, the degree of overexpression in the transfected cell lines could not be determined. Nevertheless, the immunoprecipitation data revealed that the production rate of the 60-kDa glycoprotein in all ten cell lines remained below 10% of that measured in the NILs of black-adapted *Xenopus*. The processing patterns of newly synthesized POMC in the transfected cell lines were indistinguishable from those observed in control cells. Moreover, using an ACTH-radioimmunoassay (Sweep et al., 1992), no significant differences were detected between the amounts of ACTH-related products produced in transfected and control cells (data not shown). In view of our previous results regarding successfull stable overexpression of a number of proteins in AtT20 cells (e.g. Holthuis et al., 1996; Chapter 5) and the poor heterologous expression of Ac45 observed in the present study (while using the same transfection technique, expression vector and cell line), we anticipate that this neuroendocrine cell type can not handle the continuous production of high quantities of Ac45.

In the following experiments, we transiently expressed *Xenopus* Ac45 in CV-1 fibroblasts. Since

this non-neuroendocrine cell type is expected to synthesize no or only small quantities of endogenous Ac45, it could provide a usefull model system to study effects of Ac45 overproduction. When CV-1 cells were transfected with the *Xenopus* Ac45 expression construct and subjected to immunoprecipitation analysis, a newly synthesized protein of 60 kDa was detected (data not shown). The mobility of this protein shifted to 46 kDa when transfected cells were pretreated with tunicamycin. When analysed by immunofluorescence microscopy, transfected CV-1 cells gave a strong and punctate Ac45 staining in the juxtanuclear region with numerous labeled vesicular structures distributing in the cell periphery (Fig. 5a). In contrast, tunicamycin-treated cells displayed a reticular staining, typical for the endoplasmic reticulum, throughout the cytoplasmic compartment (Fig. 5b). These results indicate that *N*-glycosylation of Ac45 is indispensible for its proper export out of the endoplasmic reticulum, a phenomenon which applies to many other membrane glycoproteins (Fiedler and Simons, 1995).

Previous immunocytochemical studies on cultured fibroblasts have shown that the 115-kDa membrane subunit of V-ATPase is mainly localized in acidic compartments such as endosomes and lysosomes (Sato and Toyama, 1994). To compare the intracellular dis-tribution of Ac45 with that of the 115-kDa subunit, CV-1 cells transfected with Ac45 were processed for double labeling immunocyto-chemistry. These experiments revealed some co-localization of Ac45 and the 115-kDa subunit in small puncta within the juxtanuclear region (data not shown). However, the numerous perinuclear organelles positive for the 115-kDa subunit were devoid of Ac45 immunoreactivity. Likewise, the Ac45-positive vesicles in the cell periphery displayed only weak, if any, labeling for the 115-kDa subunit. These findings indicate that Ac45 expressed in CV-1 fibroblasts is sorted to only a subset of the intracellular compartments harbouring V-ATPases.

Cell surface expression and internalization of Ac45 — As demonstrated above, *Xenopus* melanotrope cells express significant amounts of Ac45 on their surface. To investigate if Ac45 is capable of reaching the cell surface when expressed in a non-neuroendocrine cell type, transfected CV-1 cells were incubated with anti-Ac45 Ab at 4°C, and then fixed and stained with FITC-conjugated secondary Ab. As shown in Fig. 6b (left pannel, "0 min" micrograph), transfected CV-1 cells display considerable Ac45 immunoreactivity on their surface. Ab-uptake experiments revealed that this cell surface-expressed Ac45 is rapidly internalized. Following a 10-min chase period in Ab-free medium at 37°C, the Ac45-Ab complexes formed on the surface of live cells appeared in numerous small intracellular vesicles the majority of which had a juxtanuclear position (Fig. 6b, "10 min" micrograph). By 30 min of chase, most of these immune complexes were associated with larger vesicles that had a more wide-spread distribution (Fig. 6b, "30 min" micrograph). To find out if the binding of Ab to cell surface-expressed Ac45 by itself is sufficient to trigger internalization, we decided to compare the trafficking of Ac45 with that of a chimeric protein (Ac45/ICAM) in which the lumenal domain of Ac45 is fused to the transmembrane- and cytoplasmic portion of the plasma membrane protein ICAM-1 (Staunton et al., 1988). Immunostaining of permeabilized transfected CV-1 cells showed that, unlike Ac45, the Ac45/ICAM fusion protein accum-

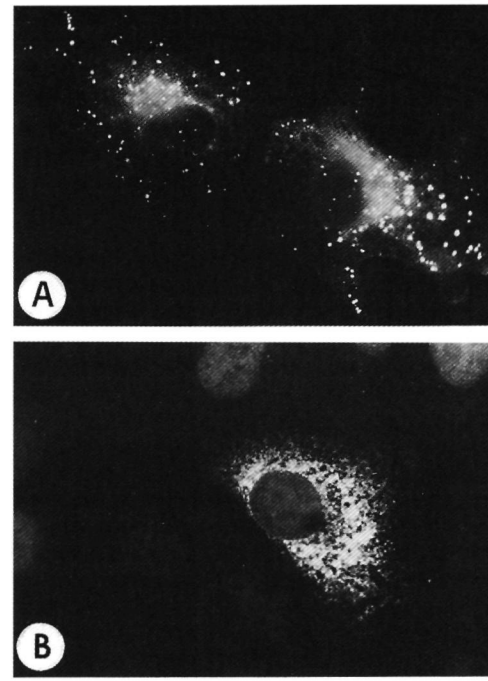


FIG. 5. **Immunofluorescence localization of Ac45 in transfected CV-1 fibroblasts.** Cells were transiently transfected with a *Xenopus* Ac45 expression construct and then incubated overnight in the absence (**A**) or presence of 10 μg/ml tunicamycin (**B**). After fixation and permeabilization, cells were incubated with anti-Ac45 antiserum. Bound anti-Ac45 antibodies were visualized with FITC-conjugated secondary antibodies.

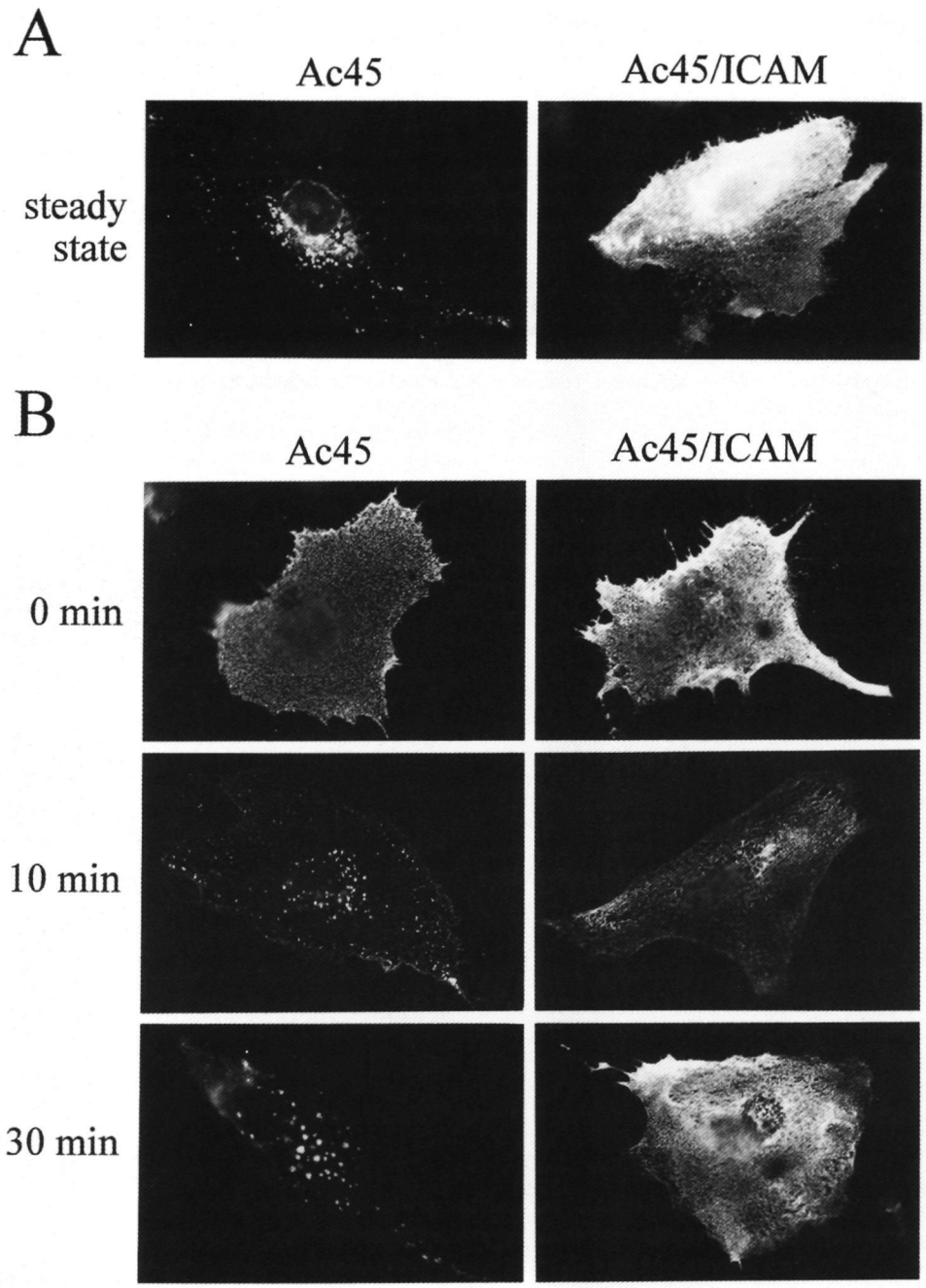


FIG. 6. **Ac45 contains sorting information recognized by CV-1 fibroblasts.** Cells were transiently transfected with an expression construct encoding Ac45 or a chimeric protein (Ac45/ICAM) in which the lumenal domain of Ac45 was fused to the transmembrane- and cytoplasmic domains of ICAM-1. Cells were either fixed, permeabilized and incubated with anti-Ac45 antiserum (**A**) or first incubated with the antiserum at 4°C, washed and then returned to 37°C for the indicated time periods before fixation and permeabilization (**B**). Bound anti-Ac45 antibodies were visualized with FITC-conjugated secondary antibodies.

ulated on the cell surface (Fig. 6a, compare "steady state" micrographs). When live cells were decorated with anti-Ac45 Ab and then chased in Ab-free media for upto 30 min, hardly any internalization of the fusion protein-Ab complexes was observed (Fig. 6b, right panel). In addition to providing a control for Ab-triggered internalization, these data indicate that the transmembrane and/or cytoplasmic domain of Ac45 contains sorting information recognized by CV-1 cells.

Overexpression of Ac45 mimicks bafilomycin A1-induced effects on the intracellular transport of proteins to the cell surface — Inspection of a large number of Ac45-expressing CV-1 cells by immuno-fluorescence microscopy revealed that in 5-10% of the cells the protein was distributed in elaborate clusters of swollen spherical structures which seemed to emanate from the Golgi area and often filled a major part of the cytoplasmic compartment (Fig. 7b). Many of these Ac45-containing structures had a 'doughnut-like' appearance with a fluorescent halo enclosing a relativ-ely darker center. A number of studies have shown that pH-neutralizing drugs or V-ATPase inhibitors interfere with protein transport to the cell surface and trigger a vesiculation of the Golgi apparatus (Tougard et al., 1983; Wagner et al., 1986; Henomatsu et al., 1993; Yilla et al, 1993). We wondered, therefore, if the appearance of Ac45 in 'doughnut-like' structures within a subpopulation of transfected CV-1 cells is due to a malfunctioning of V-ATPases. If so, an incubation with bafilomycin A1 should induce similar structures in the entire population of Ac45-expressing cells. Indeed, after a 4-hr treatment with 1 μM bafilomycin A1, virtually all transfected CV-1 cells had accumu-lated Ac45 in clusters of 'doughnut-like' vesicles whose morphological features and intracellular distri-bution were indistinguishable from those found in a subpopulation of untreated transfected cells (Fig. 7c). These results suggest that overexpression of Ac45 in CV-1 cells affects the proper functioning of V-ATP-ases.

To investigate if Ac45 overexpression in CV-1 cells also perturbs protein transport to the cell surface, we decided to coexpress Ac45 with the neuroendocrine-specific secretory protein secretogranin III (SgIII). When transfected into CV-1 cells, SgIII is readily released into the medium (Holthuis et al., 1996; Chapter 5). Immunofluorescence microscopy of transfected CV-1 cells revealed a strong SgIII immunostaining of the Golgi apparatus and a fine punctate labeling throughout the cytoplasm (Fig. 8a). Following co-expression with Ac45, this intracellular distribution of SgIII changed dramatically in 5-15% of the transfected cells. In-

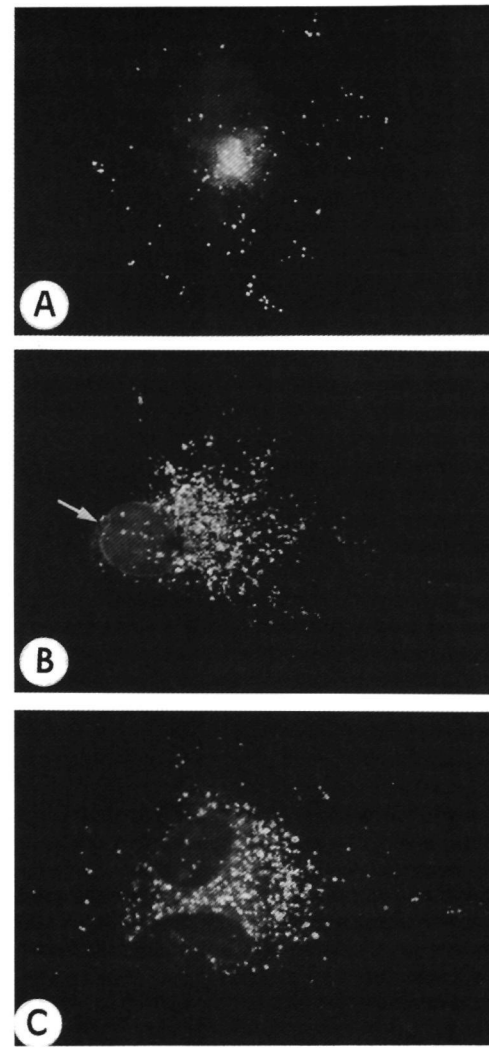


FIG. 7. **Overexpression of Ac45 mimicks bafilomycin A1-induced effects on its intracellular distribution.** CV-1 fibroblasts were transiently transfected with a *Xenopus* Ac45 expression construct and then incubated in the absence (**A**,**B**) or presence of 1 μM bafilomycin A1 for 4 hr (**C**). Note that the cells in (**A**) and (**C**) express moderate levels of Ac45 whereas the cell in (**B**) displays high expression levels (as evidenced by the marked staining of the nuclear envelope; *arrow*). After fixation and permeabilization, cells were incubated with anti-Ac45 antiserum. Bound anti-Ac45 antibodies were visualized with FITC-conjugated secondary antibodies.

stead of exhibiting the discrete Golgi staining pattern, these cells contained numerous labeled vacuolar structures which occupied large portions of the cytoplasmic compartment (Fig 8b) An incubation of cells with bafilomycin A1 for 2 hr was sufficient to evoke a similar redistribution of SgIII in most transfected cells (Fig 8c) These findings lend further support to the notion that overexpression of Ac45 leads to a deregulation of V-ATPases in the secretory pathway

DISCUSSION

V-ATPases are responsible for the acidification of endosomes, lysosomes, the Golgi complex and several classes of secretory vesicles, and consequently participate in a diverse range of cellular processes Since the principal subunits of V-ATPases in the various organelles are highly similar, if not identical, it is conceivable that accessory polypeptides are necessary to coordinate the assembly of the pump and to regulate its activity according to the specific needs of each organelle. In the present study, we have examined the biosynthesis and functional significance of Ac45, a membrane protein initially co-purified with V-ATPase from bovine chromaffin granules (Supek et al , 1994) Our findings indicate that Ac45 has many of the characteristics expected for an accessory subunit capable of modifying V-ATPase activity in the secretory pathway First, Ac45 is predominantly produced in neuroendocrine cells where its level of expression is coupled to the rate of peptide hormone biosynthesis (Holthuis et al , 1995) Second, Ac45 does not co-localize with the 115-kDa subunit of V-ATPase in a number of endosomal- and lysosomal organelles, suggesting that its association with the pump occurs in only a subset of vacuolar compartments Third, overproduction of Ac45 perturbs protein transport from the Golgi apparatus to the cell surface in a manner closely mimicked by the specific V-ATPase inhibitor bafilomycin A1

Our biosynthetic labeling experiments with *Xenopus* melanotropes showed that Ac45 is a type I transmembrane protein of 60 kDa whose lumenal domain is *N*-glycosylated at multiple sites Supek et al (1994) reported that Ac45 from bovine neuroendocrine tissues (brain, pituitary gland, adrenal medulla) migrates on SDS gels as a diffuse band at a position of about 45 kDa, while *in vitro* translation of its mRNA in the presence of dog pancreatic microsomes yields a protein of ~70 kDa These authors suggested that Ac45 is subject to post-translational processing by proteases situated in the Golgi compartment and/or secretory granules of neuroendocrine cells Our pulse-chase studies on newly synthesized Ac45 in *Xenopus* melanotropes did not provide any evidence for such modifications and we consistently immunoprecipitated a single 60-kDa glycoprotein over a chase period of up to 6 hr Moreover, when *Xenopus* Ac45 was expressed in mouse pituitary AtT20 cells or monkey CV-1 fibroblasts, again a 60-kDa glycoprotein was produced Whether these conflicting results on the size of Ac45 can be ascribed to species-related differences in the primary structure of the protein or to the application of different experimental techniques (immunoprecipitation analysis versus Western blot analysis) remains to be established

Our immunofluorescence studies on primary cultures of *Xenopus* melanotropes suggest that Ac45 occurs in the secretory granules of these cells and that low but significant amounts of the protein are present on the cell surface Moreover, anti-Ac45 Ab bound to the surface of live melanotropes were readily internalized and accumulated in numerous intracellular compartments Fibroblasts transfected with an Ac45 expression construct were also capable of binding and internalizing anti-Ac45 Ab In contrast, fibroblasts expressing a chimeric protein in which the lumenal domain of Ac45 was fused to the transmembrane- and cytosolic domains of a plasma membrane protein (ICAM-1) failed to take up the Ab Collectively, these data indicate that Ac45 is actively retrieved from the cell surface and that internalization of the protein is dependent upon signals contained within its transmembrane and/or cytoplasmic domain, apparently, these signals are recognized by the sorting machinery of both neuroendocrine and non-neuroendocrine cell types Ac45 retrieved from the surface of transfected fibroblasts was rapidly transported to the Golgi area where it accumulated in vesicular structures similar to those containing the protein in fixed permeabilized cells It therefore appears that internalized Ac45 is responsible for much of the steady-state immunofluorescence staining observed in the juxtanuclear region of the cells This cellular trafficking of Ac45 is strikingly similar to that described for the integral membrane form of the processing enzyme peptidylglycine α-amidating monooxygenase (PAM) PAM occurs in the secretory granules of neuroendocrine cells where it catalyzes the carboxy-terminal amidation of bioactive peptides (Eipper and Mains, 1988) Both neuroendocrine cells and transfected fibroblasts are capable of internalizing membrane PAM from their surface and subsequently accumulate the protein in what is believed to be a recycling compartment in the vicinity of the Golgi apparatus

(Milgram et al., 1993; Tausk et al., 1992). The trafficking of Ac45 and its relatively slow turnover in *Xenopus* melanotropes raise the possibility that upon reaching the plasma membrane, Ac45 is directed to a recycling pathway which allows its reincorporation into nascent secretory vesicles.

The 115-kDa membrane subunit of V-ATPase is required for the assembly and activity of the pump (Manolson et al., 1992) and is included in V-ATPase preparations derived from many different cellular organelles and species (Perin et al., 1991 and references therein). By means of immunofluorescence microscopy, this protein can be readily visualized in the acidic compartments of cultured fibroblasts (Sato and Toyama, 1994; this study). We found that Ac45 is mainly produced in neuroendocrine cell types and failed to detect its presence in fibroblasts. When transiently expressed in fibroblasts, the intracellular distribution of Ac45 was clearly distinct from that of the endogenous 115-kDa V-ATPase subunit; most of the endosomal and lysosomal compartments positive for the 115-kDa subunit did not stain for Ac45 and co-localization of the two proteins was restricted to some small vesicular structures in the Golgi area. These findings suggest that, unlike the 115-kDa subunit, Ac45 is not a common subunit of V-ATPases but contains sorting signals which direct the protein to only a subset of the intracellular organelles harbouring a V-type proton pump.

In this study, we provide evidence that overproduction of Ac45 interferes with a proper functioning of V-ATPases in the secretory pathway. When Ac45 was transiently expressed in fibroblasts, a subpopulation of the immunoreactive cells was found to accumulate the protein in swollen vesicular structures which appeared first in the Golgi area, and then in the rest of the cytoplasmic compartment. Upon exposure to the V-ATPase inhibitor bafilomycin A1, virtually all immunoreactive cells were found to accumulate Ac45 in such struc-tures. Moreover, the transient expression of Ac45 in fibroblasts had a profound effect on the steady state localization of the secretory protein secretogranin III (SgIII); the immunofluorescence distribution of SgIII changed from a discrete Golgi-type pattern to a diffuse punctate pattern which filled most of the cytoplasmic compartment. Incubation of cells with bafilomycin A1 caused a redistribution of SgIII indistinguishable from that observed in Ac45-overproducing cells. The trans-Golgi network (TGN) is mildly acidic, and generally considered to be the sorting station for delivery of proteins to other organelles or to the cell exterior (Griffiths and Simons, 1986; Mellman and Simons, 1992). It has

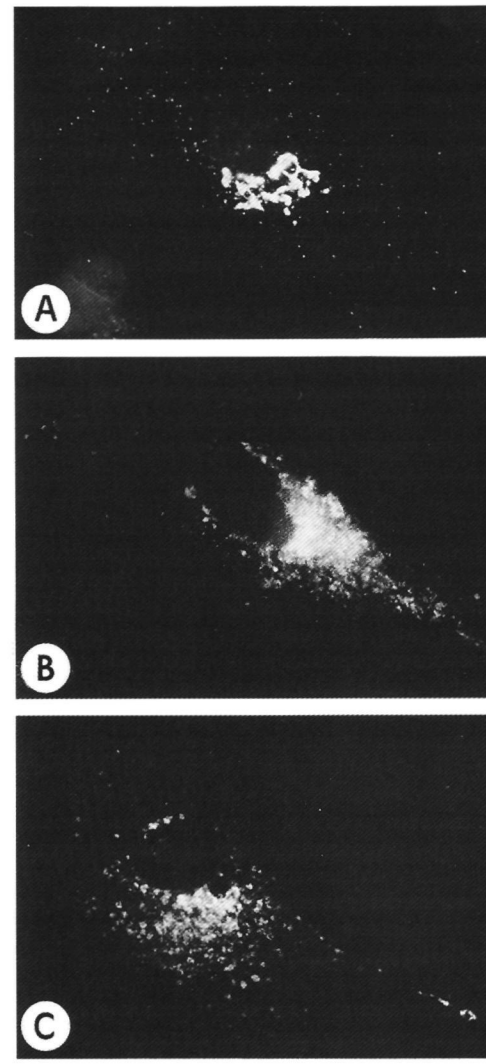


FIG. 8. **Ac45-overexpression mimicks bafilomycin A1-induced effects on protein transport to the cell surface.** (**A**) CV-1 fibroblast transfected with a *Xenopus* secretogranin III expression construct. (**B**) CV-1 fibroblast co-transfected with *Xenopus* secretogranin III and *Xenopus* Ac45 expression constructs. (**C**) CV-1 fibroblast transfected with a *Xenopus* secretogranin III expression construct and then incubated with 1 μM bafilomycin A1 for 2 hr. Cells were fixed, permeabilized and incubated with anti-secretogranin III antiserum. Bound anti-secretogranin III antibodies were visualized with FITC-conjugated secondary anti-bodies.

been inferred from previous work that inadequate acidification of the TGN affects protein sorting and packaging Studies in yeast have shown that strains carrying mutations in V-ATPases accumulate precursor forms of vacuolar hydrolases at some point before delivery to the vacuole but after transit to the Golgi complex (Yaver et al , 1993) Mammalian cells exposed to specific V-ATPase inhibitors (concanamycin B, bafilomycin A1) show a delay in the delivery of proteins from the Golgi to the plasma membrane, accompanied by alterations in Golgi morphology (Yilla et al , 1993, Henamatsu et al , 1993) Ultrastructural studies have indicated that treatment of cells with bafilomycin A1 triggers the formation of abnormal large vacuoles at the trans-site of the Golgi complex (Henomatsu et al , 1993, Chapter 8) Our present data show that overproduction of Ac45 in fibroblasts induces a similar vesiculation of the Golgi apparatus, presumably by perturbing V-ATPase activity in this organelle These findings, together with our previous demonstration that Ac45 expression in *Xenopus* melanotropes is regulated in parallel with the biosynthesis and release of POMC-derived peptides (Holthuis et al , 1995, Chapter 2), are consistent with a critical role for Ac45 in V-ATPase-mediated acidification of the secretory pathway

If, as we argue, Ac45 is a modulator of V-ATPase activity, what could be its mechanism of action? When compared with other V-ATPase subunits, Ac45 takes a unique position in that the major portion of the protein is facing the vacuolar lumen This topography may reflect a role in the communication between the pump and the interior of the vacuolar system Alternatively, Ac45 may help direct V-ATPases to, or assist in the assembly of a functional enzyme complex on the membranes of particular organelles (e g TGN, secretory granules)

Acknowledgements — We gratefully acknowledge Dr S Sato (Department of Biophysics, Kyoto University, Japan) for the monoclonal antibody against the 115-kDa V-ATPase subunit, Dr C Figdor (Department of Tumor Immunology, University of Nijmegen) for the human ICAM-1 cDNA clone, and G Pesman (Department of Experimental and Chemical Endocrinology, University of Nijmegen) for performing the ACTH radioimmunoassays

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Evidence for a Novel Type of Sorting Motif in the Cytoplasmic Tail of the Vacuolar H^+-ATPase Accessory Membrane Subunit Ac45

Evidence for a Novel Type of Sorting Motif in the Cytoplasmic Tail of the Vacuolar H^+-ATPase Accessory Membrane Subunit Ac45

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Ac45 is a type I transmembrane protein associated with vacuolar H^+-ATPase in neuroendocrine secretory granules. By exploring its transient expression in CV-1 fibroblasts, we found that the protein contains sorting information which mediates a rapid internalization from the cell surface and targeting to a vesicular compartment in the juxtanuclear region. Immunolocalization studies on cells expressing carboxy-terminal truncation mutants of Ac45 revealed the presence of essential routing information in the amino-terminal half of the cytoplasmic tail (residues 412-423); deletion of this region abrogated internalization of Ac45 from the cell surface and caused an accumulation of the protein on the plasma membrane. Furthermore, the cytoplasmic tail of Ac45 was intrinsically capable of mediating endocytosis and transport to the juxtanuclear region of the cell surface protein Tac, albeit with a reduced efficiency. Collectively, these results led us to conclude that in Ac45, cytoplasmic residues 412-423 contain autonomous routing information. This region lacks the well described tyrosine-based or di-leucine-based sorting motifs. However, the critical region in Ac45 contains a putative motif with the consensus sequence S/T*XXX*FD which is also found in the cytoplasmic tails of two proteins localized in the storage organelles of regulated secretory cells, namely P-selectin and the transmembrane form of peptidylglycine α-amidating monooxygenase. Our findings provide evidence for the existence of a sorting motif distinct from routing determinants described previously.

Trafficking of integral membrane proteins along the secretory and endocytic pathways in eukaryotic cells is often specified by information contained within the cytoplasmic domains of these proteins (Trowbridge et al , 1993, Sandoval and Bakke, 1994) This information primarily consists of short, linear arrays of amino acid residues that function as sorting signals Three well characterized types of sorting signals are tyrosine-based, di-leucine-based and di-lysine-based signals

Tyrosine-based signals generally conform to the motifs N*XXY* or Y*XXZ* (where Z corresponds to any amino acid with a bulky hydrophobic side chain) (Sandoval and Bakke, 1994), although there are also other contexts in which tyrosine residues appear to be active in sorting (Thomas and Roth, 1994) Di-leucine-based signals, on the other hand, consist of critical LL or LI sequences (Letourneur and Klausner, 1992) Both types of signals can mediate endocytosis of transmembrane proteins through clathrin-coated pits This process involves at least three different steps association of a clathrin adaptor complex (AP-2) with the plasma membrane, recognition of sorting signals in the cytoplasmic tails of transmembrane proteins and assembly of the adaptor complexes with clathrin to form coated pits (Pearse and Robinson, 1990) Recent *in vitro* studies have demonstrated a direct interaction between the medium chains of adaptor complexes and a number of tyrosine-based signals, suggesting that the latter function as discrete autonomous ligands for the clathrin-dependent sorting machinery (Ohno et al , 1995) It is assumed that di-leucine-based signals act in a similar way, although a molecular interaction between these signals and clathrin adaptor proteins remains to be demonstrated

Tyrosine-based and di-leucine-based signals also participate in sorting at the TGN The presence of either of the two signals in lysosomal membrane proteins and mannose 6-phosphate receptors promotes the incorporation of these proteins into TGN-derived transport vesicles destined for late endosomes (Sandoval and Bakke, 1994) Similar to the mechanism at the plasma membrane, sorting at the TGN involves membrane binding of a Golgi-clathrin adaptor complex (AP-1) and the subsequent formation of clathrin-coated vesicles (Pearse and Robinson, 1990) Thus, tyrosine-based and di-leucine-based signals can interact with two distinct adaptor complexes, one associated with the TGN

(AP-1) and the other with the plasma membrane (AP-2) However, not all signals are recognized by the two adaptor complexes with equal efficiency For example, the tyrosine-based signal in the transferrin receptor promotes endocytosis but not sorting at the TGN (Trowbridge et al , 1993) Conversely, one of the two tyrosine-based signals in the LDL receptor is active at the TGN, but does not mediate clustering into coated pits at the plasma membrane (Matter et al , 1992) An increasing body of evidence indicates that subtle changes in the cytoplasmic signal can favour interaction with one of the two adaptor complexes and hence determine its site of action Le Borgne et al (1993) demonstrated that phosphorylation of a serine residue in the cytoplasmic tail of the mannose 6-phosphate receptor is crucial for its interaction with Golgi AP-1 adaptor proteins Phosphorylation of cytoplasmic serine- and threonine residues has also been implicated in the trafficking of the epidermal growth factor receptor (Lin et al , 1986), the polymeric immunoglobulin receptor (Hirt et al , 1993), the proprotein cleavage enzyme furin (Jones et al , 1995) and the peptide-amidating enzyme PAM (Yun et al , 1995)

Di-lysine-based signals (consensus sequence KK*XX* or K*X*K*XX*) are found at the extreme carboxy termini of many type I transmembrane proteins of the endoplasmic reticulum (ER) (Nilsson et al , 1989, Jackson et al , 1990) Proteins bearing such signals are continuously retrieved from post-ER compartments back to the ER (Jackson et al , 1993) Di-lysine-based signals bind to coatomer (Cosson and Letourneur, 1994), a complex of proteins that forms a non-clathrin coat around vesicles budding from the Golgi apparatus (Rothman and Orci, 1992) Mutation of coatomer subunits in yeast leads to a loss in the retrieval of ER transmembrane proteins, an effect which can be attributed to an impaired binding of coatomer to di-lysine motifs (Letourner et al , 1994) These observations have led to the proposal that di-lysine-based signals mediate a coatomer-dependent sorting of transmembrane proteins into vesicles destined for return to the ER Recently, a novel carboxy-terminal sequence related to the di-lysine motif (KKFF) has been shown to mediate internalization of membrane proteins through clathrin-coated pits (Itin et al , 1995) Together with the fact that coatomer and clathrin coats share some structural similarities (Duden et al , 1991, Cosson et al , 1996), these findings suggest that coatomer-mediated retrieval of transmembrane proteins is mechanistically related to clathrin-dependent sorting

We and others recently reported the identification of a novel type I transmembrane protein, Ac45, which is associated with the vacuolar H^+-ATPase in secretory granules (Supek et al 1994, Holthuis et al , 1995, Chapter 6) While exploring its expression in fibroblasts, we found that Ac45 contains routing information which mediates a rapid internalization from the cell surface and accumulation in the juxtanuclear region (Chapter 6) Since Ac45 does not contain any of the sorting signals identified in previous studies, we decided to investigate whether defined regions in the protein were responsible for its localization pattern in fibroblasts By means of mutational analysis, we show that a 12-residue sequence in the cytoplasmic tail of Ac45 contains critical information for the endocytosis and steady state localization of the protein A survey for structurally similar regions in the cytoplasmic domains of other type I transmembrane proteins indicated the presence of a putative motif with the consensus sequence S/T*XXX*FD Interestingly, this motif defines a small group of membrane proteins localized in the storage organelles of regulated secretory cells

MATERIALS AND METHODS

Recombinant DNA procedures — A pcDNA3 expression construct encoding the *Xenopus* Ac45 protein has been described previously (Chapter 6) Carboxy-terminal truncation mutants were constructed by introducing stop codons immediately downstream of the codons for the desired carboxy terminal amino acid This was accomplished by oligonucleotide-directed mutagenesis on single-stranded DNA using the pALTER system (Promega, Madison, USA) Oligonucleotides used to introduce stop codons behind *Xenopus* Ac45 residues 423 and 411 were 5' ATCGTTTTGATGATCTCTAGAGCCCAAGCATTG-CTG 3' and 5'-GGACTGCACATGGTCTAGAGCCTGA-AGACTATG-3', respectively A pCDM8 expression construct encoding the human Tac antigen (interleukin-2 receptor α chain, Leonard et al , 1985) was obtained from Dr J S Bonifacino (National Institute of Health, Bethesda, USA) The Tac45 expression construct, encoding the lumenal and transmembrane domains of Tac (ending GLTWQ at residue 262, Leonard et al , 1985) followed by the residues TS and then the entire cytoplasmic domain of *Xenopus* Ac45 (starting HMVMS at residue 409, Chapter 6), was obtained by creating *Spe*I sites in the Tac and Ac45 cDNAs through oligonucleotide-directed mutagenesis on single-stranded DNA using the pALTER system The desired mutations were checked by restriction enzyme digestion and double-stranded DNA sequencing Appropriate fragments of correctly mutagenized cDNAs were subcloned downstream of the CMV promotor into pcDNA3 Oligonucleo-

tides used to introduce *SpeI* sites in Ac45 and Tac cDNAs were 5'-CTGACCTATGGACTAGTCACATGGTTATG-AG-3' and 5'-GCTCACCTGGCAGACTAGTCAGAGG-AAGAGTAGA-3', respectively DNA for transfection studies was isolated using the Qiagen plasmid kit (Qiagen Inc , Chatsworth, CA)

Cell transfection and immunofluorescence microscopy — Green monkey CV-1 kidney fibroblasts were cultured in Iscoves-modified Eagle's medium (Gibco-BRL, Gaithersburg, USA) supplemented with 10% fetal calf serum For transient expression studies, CV-1 cells were plated on glass coverslips in 20 mm culture dishes, grown until 30% confluency and transfected with 2 5 μg DNA per construct per dish using the calcium phosphate precipitation method (Graham and Van der Eb, 1973) For steady-state immunofluorescence localization of Ac45 and the Ac45 truncation mutants, transfected CV-1 cells were fixed in 2% paraformaldehyde/PBS pH 7 4 for 1 h at 4°C, incubated in 100 mM glycine/PBS for 30 min at 4°C, permeabilized in ice-cold 0 1% Triton X-100/PBS (PBS TX), and incubated with rabbit anti-Ac45 antiserum (1 300, Chapter 6) in PBS-TX containing 2% BSA (PBS-TXB) overnight at 4°C For immunolabeling of cell surface-expressed and internalized protein, cells were incubated with anti-Ac45 antiserum (1 300) in culture medium for 20 min at 4°C, washed and then fixed immediately, or returned to antibody-free culture medium at 37°C for the indicated time periods prior to fixation and permeabilization Immunolocalization of Tac and the Tac45 fusion protein in transfected CV-1 cells was performed with a mouse monoclonal antibody (7G7) recognizing a lumenal epitope on Tac (Rubin et al , 1985) To visualize bound anti-Ac45 antibodies and 7G7 antibodies, permeabilized cells were incubated with FITC-conjugated goat anti-rabbit antibodies (1 100, Boehringer Mannheim, Germany) and FITC-conjugated goat anti-mouse antibodies (1 100, Boehringer Mannheim), respectively, in PBS-TXB for 2 h at 4°C Following extensive washing with ice-cold PBS-TXB, the immunostained cells were mounted in Citifluor (Agar Scientific Ltd, Stanstedt Essex, UK) and viewed under epifluorescence optics with a Leica DMRB/E microscope (Leica, Heerbrugg, Switzerland) equipped with a vario orthomat camera system

RESULTS AND DISCUSSION

Intracellular trafficking of Ac45 is dependent on structural information in its cytoplasmic domain — Integral membrane proteins that lack routing determinants are thought to travel to the plasma membrane via a default pathway (Pfeffer and Rothman, 1987) When Ac45 was transiently expressed in CV-1 fibroblasts and visualized by immunofluorescence staining, much of the protein was found in vesicular structures on one side of the nucleus whereas a minor portion was associated with the plasma membrane (Chapter 6) Antibody-uptake experiments revealed that Ac45 is rapidly retrieved from the cell surface and subsequently accumulates in juxtanuclear vesicles reminiscent of those containing the protein under steady-state conditions In contrast, a chimeric protein in which the lumenal domain of Ac45 was fused to the transmembrane- and cytoplasmic domains of the cell surface protein ICAM-1 failed to internalize and consequently accumulated on the plasma membrane (Chapter 6) These findings led us to conclude that Ac45 contains routing information recognized by the sorting machinery in CV-1 cells

Since the cytoplasmic tails of many type I transmembrane proteins play a critical role in trafficking (Trowbridge et al , 1993, Sandoval and Bakke, 1994), we decided to analyse the effects of carboxy-terminal truncations on the intracellular transport of Ac45 For this purpose, two Ac45 truncation mutants were constructed one lacking the eleven most carboxy-terminal amino acids in the 26-residue cytoplasmic tail (Ac45/423) and the other one lacking all but three of the cytoplasmic residues that follow the transmembrane domain (Ac45/411) (see schematic representation in Fig 1A) When these constructs were transiently expressed in CV-1 cells, Ac45 proteins of the expected sizes could be immunoprecipitated from the cell extracts (data not shown) To determine whether the carboxy-terminal truncations altered the trafficking of Ac45, the transfected CV-1 cells were incubated with anti-Ac45 antibodies and processed for immunofluorescence microscopy Proteins on the cell surface were distinguished from those associated with internal membranes by comparing cells that were first exposed to the antibodies at 4°C and then fixed (Fig 1C, "0 min" micrographs) with cells that were fixed and permeabilized before exposure to the antibodies (Fig 1B, "steady state" micrographs) To monitor internalization of proteins from the cell surface, cells were incubated with antibodies at 4°C, washed and then returned to 37°C for 10 min or 30 min before fixation and permeabilization (Fig 1C, "10 min" and "30 min" micrographs)

The steady state distributions of Ac45 and Ac45/423 were very similar, in both cases the majority of protein was located in the juxtanuclear region whereas only a limited amount was present on the cell surface (Fig 1B) Furthermore, the antibody-uptake experiments revealed that the Ac45/423 mutant was retrieved from the cell surface and transported to the juxtanuclear region with similar kinetics as the full-length protein (Fig 1C) In contrast, cells expressing the Ac45/411 mutant displayed a strong immunostaining of the plasma membrane while hardly any labeling was

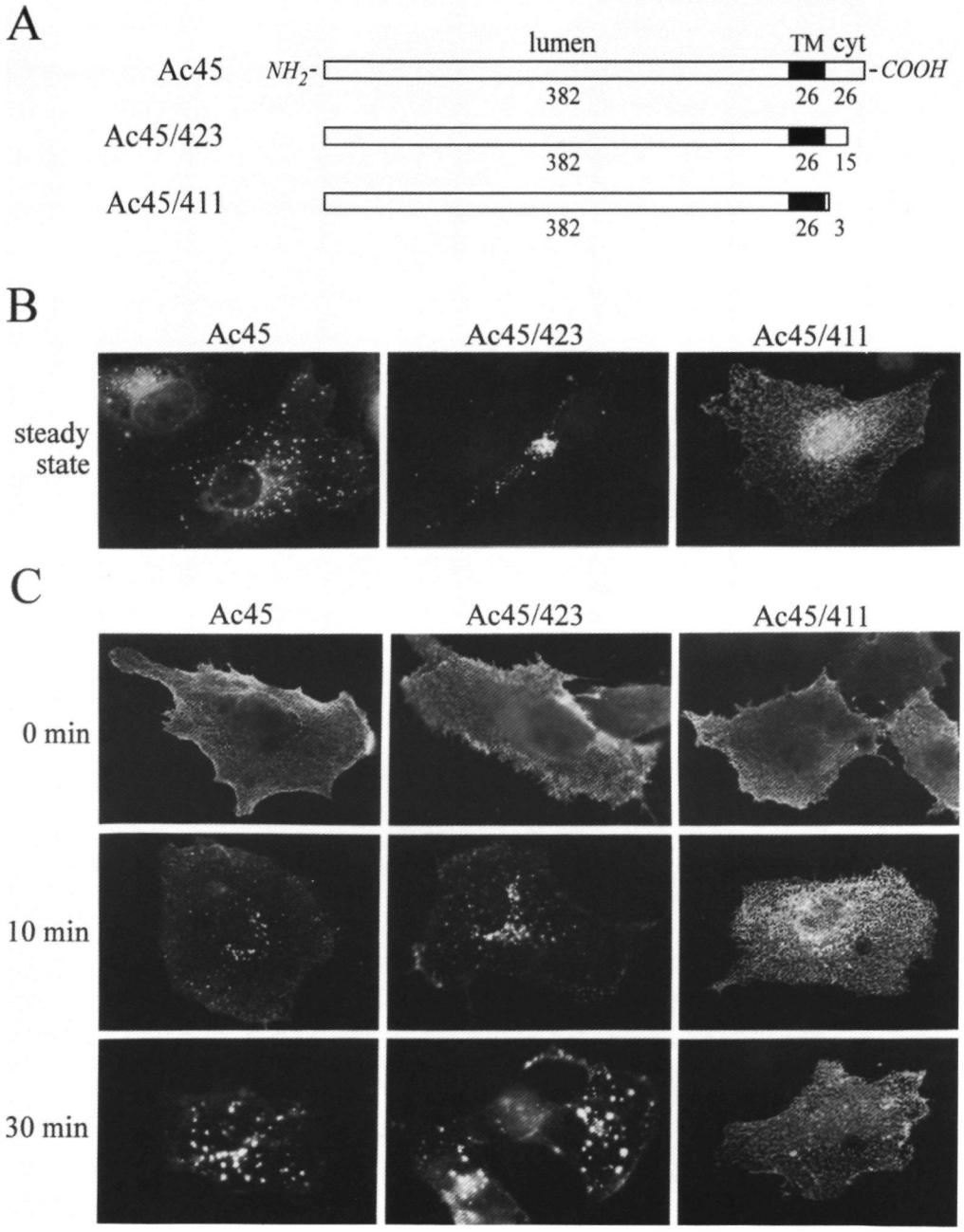
found associated with intracellular compartments (Fig 1B) Moreover, the Ac45/411-expressing cells failed to take up any appreciable amounts of anti-Ac45 antibodies that had bound to their surface (Fig 1C) Thus, deletion of the 23 carboxy-terminal residues of Ac45 causes an accumulation of the protein on the plasma membrane Taken together, the above results indicate that residues 412-423 in the cytoplasmic tail of Ac45 contain at least part of the information necessary for the internalization and intracellular targeting of the protein

The cytoplasmic domain of Ac45 contains autonomous targeting information — The dramatic changes in subcellular localization induced by truncations in the cytoplasmic domain of Ac45 can be explained by either a direct involvement of this segment in trafficking or an indirect effect on other sequences To investigate if the targeting information contained within the cytoplasmic domain of Ac45 can function independently of other regions in the protein, we constructed a fusion protein consisting of the lumenal- and transmembrane domains of the cell surface protein Tac (α chain of the interleukin-2 receptor, Leonard et al , 1984) and the cytoplasmic domain of Ac45 (as schematically represented in Fig 2A) Next, the steady state localization of this fusion protein, Tac45, was compared with that of wild type Tac and Ac45 For this purpose, CV-1 cells were transiently transfected with the appropriate constructs and processed for immunofluorescence microscopy essentially as described above, except that Tac and the Tac45 fusion protein were visualized using a monoclonal antibody (7G7) recognizing an epitope in the lumenal domain of the protein (Ruben et al , 1985)

During steady state conditions, Ac45 was predominantly associated with vesicles in the juxtanuclear region whereas Tac was nearly exclusively located on the plasma membrane of transfected cells (Fig 2B) Cells expressing the Tac45 fusion protein gave a strong immunostaining of the plasma membrane although some labeling of intracellular vesicles was also observed (Fig 2B) Antibody-uptake experiments showed that the Tac45 fusion protein, unlike wild type Tac, was retrieved from the cell surface and accumulated at one side of the nucleus (Fig 2C) However, this internalization was slower and less efficient than that observed for Ac45, whereas a 30-min chase was sufficient for Ac45-expressing cells to take up most of the antibodies that had bound to their surface (Fig 1C), for the Tac45-expressing cells it took a 60- to 120-min chase period (Fig 2C) From these observations it can be concluded that, although probably not containing all sorting information recognized by CV-1 cells, the cytoplasmic domain of Ac45 harbours an autonomous routing determinant which contributes both to internalization and transport of the protein to the juxtanuclear region

Identification of a putative phenylalanine-based sorting motif — Having established that the cytoplasmic domain of Ac45 contains sufficient information to mediate the intracellular sequestration of a plasma membrane protein, we sought to identify sequences or structural motifs responsible for this routing Remarkably, the entire 26-residue cytoplasmic domain of Ac45 is devoid of known targeting signals, such as tyrosine- or di-leucine-based motifs (Sandoval and Bakke, 1994, Letourneur and Klausner, 1992), or the more recently characterized acidic sequences involved in the trafficking of furin (Voorhees et al , 1995) As demonstrated above, critical routing information in the cytoplasmic domain of Ac45 is contained within residues 412-423 This region has a phenylalanine at position 420 Piper et al (1993) previously identified a phenylalanine-based internalization motif (FQQI) in the cytoplasmic amino-terminal region of the glucose transporter GLUT-4 which is reminiscent of a *YXXL* motif Other researchers have demonstrated that phenylalanine can functionally substitute for tyrosine in tyrosine-containing internalization signals (Jadot et al , 1992, Ktistakis et al , 1990) The phenylalanine in the cytoplasmic domain of Ac45, however, is not part of a *YXXL*-like or *NXXY*-like motif Given the prominent role of aromatic residues in sorting motifs identified thus far, we decided to survey the sequences of various type I transmembrane proteins for the presence of cytoplasmic phenylalanines, selecting those which appeared in a structurally similar context as the phenylalanine in the carboxy terminal region of Ac45 This search uncovered a putative motif with the consensus sequence S/T*XXX*FD which occurs in the cytoplasmic tails of two transmembrane proteins

FIG 1 **The cytoplasmic domain of Ac45 contains critical targeting information.** (A) Schematic representation of Ac45 and the carboxy terminal truncation mutants Ac45/423 and Ac45/411 CV-1 fibroblasts transiently expressing Ac45 or Ac45 truncation mutants were fixed, permeabilized and then incubated with anti-Ac45 antiserum (B), or first incubated with anti Ac45 antibodies at 4°C, washed and then returned to 37 C for the indicated time periods before fixation and permeabilization (C) Bound anti Ac45 anti bodies were visualized with FITC-conjugated secondary antibodies


A
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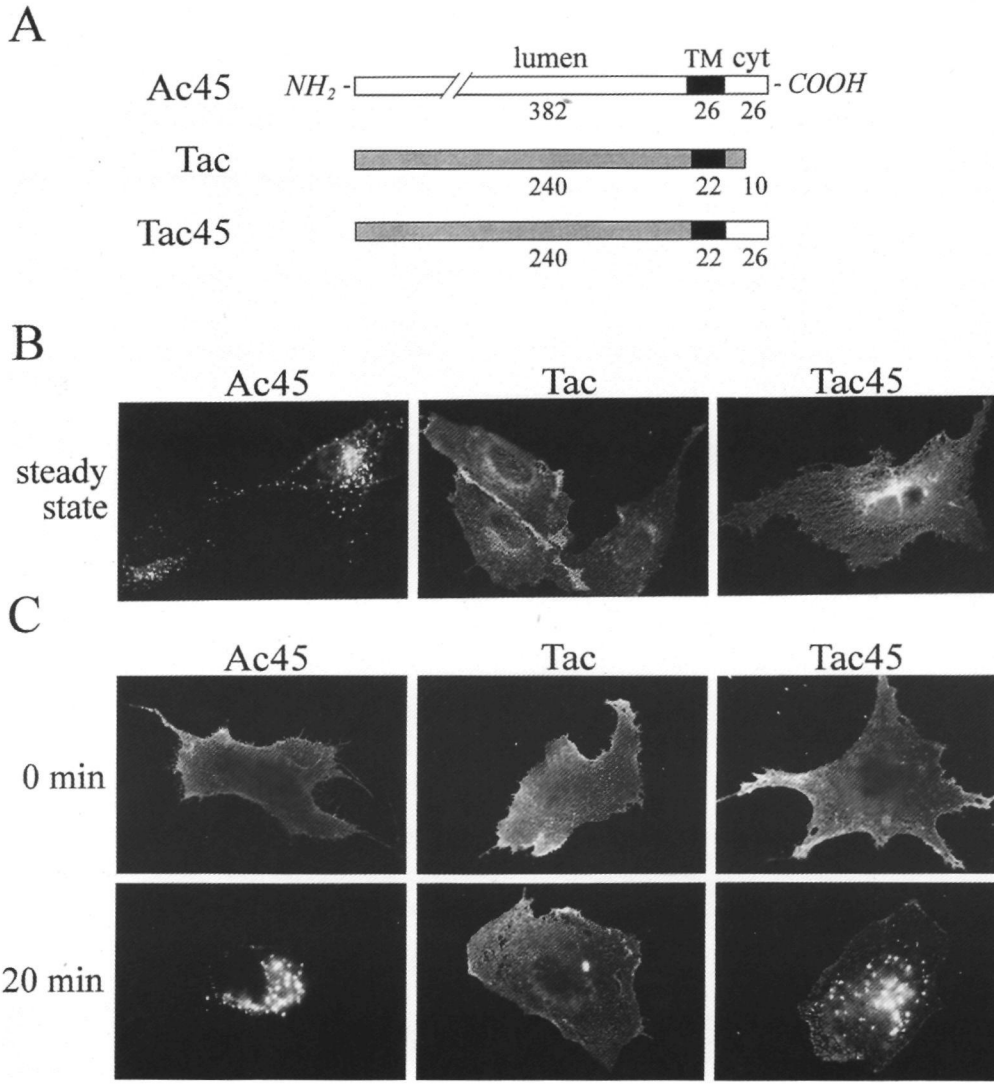

FIG. 2. **The cytoplasmic domain of Ac45 contains autonomous targeting information.** (**A**) Schematic representation of Ac45, Tac and a chimeric protein Tac45 which contains the lumenal and transmembrane regions of Tac and the cytoplasmic region of Ac45. CV-1 fibroblasts transiently expressing Ac45, Tac or Tac45 were fixed, permeabilized and then incubated with antibodies (**B**), or first incubated with antibodies at 4°C, washed and then returned to 37°C for the indicated time periods before fixation and permeabilization (**C**). Ac45-expressing cells were incubated with anti-Ac45 antiserum while cells expressing Tac or Tac45 were incubated with a monoclonal antibody (7G7) recognizing a lumenal epitope on Tac. Bound antibodies were visualized with FITC-conjugated secondary antibodies.

Ac45 (*Xenopus*)	*TM*- H M V M S L K T M D R F D D P K G P S I A V P Q T E -*COOH*
Ac45 (human)	*TM*- H M I L S L K T M D R F D D H K G P T I S L T Q I V -*COOH*
PAM (*Xenopus*)	*TM*-39aa- F A T H K G Y S R K G F D R L S T E G S D Q E K D D -21aa-*COOH*
PAM (human)	*TM*-39aa- F A S R K G Y S R K G F D R L S T E G S D Q E K E D -21aa-*COOH*
P-selectin (bovine)	*TM*-19aa- L G T Y G V F T N A A F D P S P -*COOH*
P-selectin (human)	*TM*-19aa- L G T Y G V F T N A A F D P S P -*COOH*
consensus	S/T *X X X* F D

FIG 3 **Amino acid sequence comparison of the cytoplasmic tails of Ac45, the integral membrane form of peptidylglycine α-amidating monooxygenase (PAM) and P-selectin.** The region in Ac45 containing critical targeting information is *overlined* A consensus sequence deduced from the alignment is shown below the sequence The sequence of *Xenopus* Ac45 was taken from Holthuis et al (Chapter 6), of human Ac45 from Yokoi et al (1994), of *Xenopus* PAM from the GENSEQP database (accession number R14029), of human PAM from Glauder et al (1990), of human P-selectin from Johnston et al (1989) and of bovine P-selectin from Strubel et al (1993)

other than Ac45, namely the integral membrane form of peptidylglycine α-amidating monooxygenase (PAM) and the leukocyte adhesion protein P-selectin (Fig 3) A search in the SwissProt/NBRF databases (release 04/96, search on proteins with cytoplasmic tail < 100 amino acid residues) did not reveal other type I transmembrane proteins with S*XXX*FD con-sensus sequences in their cytoplasmic domains

The presence of the S/T*XXX*FD motif in the above three proteins has several interesting implications First, all three proteins are predominantly or exclusively located in the storage organelles of regulated secretory cells Thus, Ac45 is associated with vacuolar H^+-ATPases in several types of neuroendocrine secretory granules (Supek et al , 1994, Holthuis et al , 1995, Chapters 2 and 6) PAM is selectively present in the secretory granules of peptidergic cells where it catalyses the carboxy-terminal amidation of bioactive peptides (Eipper et al , 1993) P-selectin is stored in the α-granules of platelets and in the Weibel-Pallade bodies of endothelial cells, its stimulus-dependent exposure on the cell surface mediates a recruitment of leukocytes into sites of inflammation (Lasky, 1992) Moreover, when expressed in neuro-endocrine cell lines, P-selectin is packaged into secretory granules (Koedam et al , 1992) Hence, given the striking resemblance in their subcellular distributions, membrane PAM, P-selectin and Ac45 can be expected to share at least part of their routing information Second, the intracellular trafficking of membrane PAM, P-selectin and Ac45 relies on sorting information present in the cytoplasmic domains of these proteins (Milgram et al , 1993, Tausk et al , 1992, Koedam et al , 1992; Disdier et al , 1992, this study) Furthermore, amino acid substitutions in the cytoplasmic tails of membrane PAM and P-selectin have revealed residues in the consensus sequence of the S/T*XXX*FD motif which are critical for routing Thus, membrane PAM in which the serine residue of the motif was changed into alanine exhibited an altered pattern of internalization in transfected neuroendocrine cells with the mutant protein being targeted to lysosomes (Yun et al , 1995) Sub-stitution of the phenylalanine residue to an alanine in the P-selectin motif significantly reduced the internalization rate of the protein in transfected fibroblasts (Setiadi et al , 1995)

Combined with our current finding that deletion of the region containing the S/T*XXX*FD sequence in Ac45 abrogates internalization and intracellular sorting, the above observations reinforce the notion that the S/T*XXX*FD motif represents a novel type of routing determinant and argue against the possibility that its presence in the above set of proteins is based on coincidence The fact that this motif occurs in three distinct secretory granule-associated transmembrane proteins raises the possibility that it has a role in sorting to the regulated secretory pathway The significance of the S/T*XXX*FD motif as a sorting signal for Ac45 is currently being evaluated by site-directed mutagenesis and transfection studies

Acknowledgements — We gratefully acknowledge Dr J S Bonifacino (National Institute of Health, Bethesda, USA) for the human Tac cDNA construct and the anti-Tac monoclonal antibody 7G7

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Inhibition of Vacuolar H^+-ATPase Perturbs Proteolytic Processing and Intracellular Transport of Regulated Secretory Proteins in *Xenopus* Intermediate Pituitary Cells

Inhibition of Vacuolar H^+-ATPase Perturbs Proteolytic Processing and Intracellular Transport of Regulated Secretory Proteins in *Xenopus* Intermediate Pituitary Cells

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To determine if neuroendocrine cells require intraorganellar acidification to fulfill their specialized secretory function, we have examined the effects of the specific vacuolar H^+-ATPase inhibitor bafilomycin A1 on the processing and intracellular transport of three distinct neuroendocrine secretory proteins in *Xenopus* melanotrope cells. Pulse-chase analysis in combination with immunoprecipitation techniques were used to monitor the fates of newly synthesized proopiomelanocortin, prohormone convertase PC2 and secretogranin III (SgIII). When cells were treated with 1 μM bafilomycin A1, the proteolytic processing of all three precursors was inhibited while an intracellular accumulation of intact precursor forms and intermediate cleavage products became apparent. Moreover, bafilomycin A1-treated cells released considerable amounts of unprocessed SgIII and a PC2 processing intermediate, products that under normal conditions do not reach the extracellular environment. This secretion was insensitive to the dopamine D2 receptor agonist apomorphine, a drug which effectively blocks exocytosis of regulated secretory products from untreated melanotrope cells. Electron microscopic analysis of bafilomycin A1-treated melanotropes revealed a reduction in the number of small, dense core secretory granules and the appearence of vacuolar structures in the *trans*-Golgi area. Collectively, our data indicate that bafilomycin A1-sensitive vacuolar H^+-ATPases play a critical role in secretory granule biogenesis and in the correct processing, storage and release of regulated secretory proteins.

Peptide hormone-producing cells concentrate and store their secretory products in dense core secretory granules that bud from the *trans*-Golgi network (TGN) Upon stimulation by an extracellular signal, these secretory granules fuse with the plasma membrane and release their contents into the external milieu by a process referred to as regulated secretion (Kelly, 1985) Work from several laboratories has provided evidence that the TGN, nascent secretory vesicles and mature secretory granules possess an acidic interior, and that acidification of these organelles is necessary for their functions in the sorting, processing and intracellular storage of peptide hormones Using an immunocytochemical approach, Orci et al (1987) and Anderson and Orci (1988) demonstrated that the endoproteolytic conversion of proinsulin to insulin occurs in coordination with a progressive acidification of immature secretory granules Additional support came from studies using acidotropic weak bases which accumulate in acidic compartments and increase the lumenal pH by consuming protons For example, Moore et al (1983) and Stoller and Shields (1989) demonstrated that treatment of cultured neuroendocrine cell lines with chloroquine or NH_4Cl disrupts sorting of peptide hormone precursors to the regulated secretory pathway and inhibits their endoproteolytic processing to mature bioactive peptides In contrast, Mains and May (1988) found that both sorting and processing of the prohormone proopiomelanocortin (POMC) in pituitary-derived AtT20 cells are not affected by chloroquine and NH_4Cl A likely explanation for these conflicting results is that acidotropic drugs are not free of undesirable side effects (Mellman et al , 1986)

Macrolide antibiotics that inhibit vacuolar H^+-ATPases (V-ATPases) form an attractive alternative for exploring the role of the acidic pH in the regulated secretory pathway since, in contrast to acidotropic drugs, the effects of these compounds can be assigned to specific enzymes (Bowman et al , 1988) Bafilomycin A1, for example, is a highly selective inhibitor of V-ATPases and already effective at nM concentrations *in vitro* (Bowman, 1988) Bafilomycin A1 has been reported to perturb intra-

cellular transport of newly synthesized prolactin in pituitary-derived GH3 cells (Henomatsu et al , 1993) and to inhibit prosomatostatin processing in a permeabilized cell system (Xu and Shields, 1994).

When compared with cultured neuroendocrine cell lines, the melanotrope cells in the intermediate pituitary of *Xenopus laevis* offer two important advantages that facilitate the detection of processing and sorting events in the regulated secretory pathway. First, *Xenopus* melanotropes can be readily stimulated to produce large amounts of POMC by placing the animal on a black background (Jenks et al , 1993). Second, *Xenopus* melanotropes are strictly regulated secretory cells. In cultured neuroendocrine cell lines, on the other hand, the mechanism of prohormone sorting to the regulated secretory pathway is in general inefficient in that a considerable proportion of the prohormone is released constitutively To further evaluate the role of V-ATPase-mediated acidification in neuroendocrine secretion, we have analysed the effects of bafilomycin A1 on the processing and storage of POMC and two other regulated secretory proteins produced in *Xenopus* melanotropes.

MATERIALS AND METHODS

Animals — South-African clawed toads, *Xenopus laevis*, were adapted to a black background by keeping them in black buckets under constant illumination for at least three weeks at 22°C

Biochemicals and antibodies — Bafilomycin A1 was purchased from Wako Pure Chemical Industries (Osaka, Japan), dissolved in methanol (stock concentration, 1 mM) and stored at -20°C Apomorphine (stock concentration, 16 3 mM in phosphate buffer, pH 7 4) was obtained from the university hospital of the Free University of Amsterdam and stored at 4°C in the dark Polyclonal antiserum directed against the C-terminus of mouse PC2 (4BF, Shen et al , 1993) was kindly provided by Dr I Lindberg (New Orleans, LO, USA) Polyclonal antiserum directed against *Xenopus* SgIII was raised as described previously (Holthuis et al , 1996)

Acridine orange studies — Primary cultures of intermediate pituitary cells were established from neurointermediate lobes (NILs) of black-background adapted *Xenopus* as described previously (Chapter 6) Cells were cultured on poly-L lysine coated coverslips in XL culture medium (67% L-15 medium [Gibco-BRL, Gaithersburg, MD, USA], 0 8 mg/ml $CaCl_2$, 0 3 mg/ml bovine serum albumin, 2 mg/ml glucose and 0 1% antibiotic-antimycotic solution [v/v, Gibco]) supplemented with 10% fetal calf serum (v/v) at 22°C After 2 days of culture, cells were incubated in XL medium in the absence or presence of 1 μM bafilomycin A1 for 50 min at 22°C Twenty min before termination of the experiment, a 1000-fold concentrated stock solution of acridine orange was added to the cultures so that the final concentration was 2 μM At the end of the incubation period, the coverslips were gently removed from the wells and inverted onto a glass slide Cells were immediately viewed under epifluorescence optics with a Leica DMRB/E microscope (Leica, Heerbrugg, Switzerland)

Electron microscopy — NILs from black-adapted *Xenopus* were dissected out and incubated in XL medium in the absence or presence of 1 μM bafilomycin A1 for 2,5 h at 22°C NILs were fixed with 2% glutaraldehyde, 2% formaldehyde and 0 5% picric acid in 0 1 M cacodylate buffer, pH 7 4, for 2 h at 4°C, washed with cacodylate buffer, and then postfixed in 1% OsO_4 in 0 05 M phosphate buffer, pH 7 4, for 1 h at 4°C Subsequently, the NILs were dehydrated with ethanol and embedded in Spurr's resin Ultrathin sections were double-stained with uranyl acetate and lead citrate, and examined under a Jeol JEM 100CX II electron microscope

Metabolic labeling of Xenopus NILs and immunoprecipitation analysis — NILs from black-adapted *Xenopus* were dissected out and preincubated in incubation medium (IM 112 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 15 mM Hepes pH7 4, 0 3 mg/ml BSA, 2 mg/ml glucose, pH 7 4) for 20 min at 22°C Pulse labeling of newly synthesized proteins was performed by incubating lobes in IM containing 1 7 mCi/ml Tran[^{35}S]-label (ICN Radiochemicals) for 20 min at 22°C Subsequent chase incubations were in IM containing 5 mM L-methionine and 2 5 mM L-cysteine Bafilomycin A1 (1 μM) and apomorphine (10 μM) were added in the 20-min preincubation period and remained present during pulse and chase incubations Lobes were homogenized on ice in lysis buffer (50 mM Hepes pH 7 2, 140 mM NaCl, 10 mM EDTA pH 8 0, 1% Tween 20, 0 1% Triton X-100, 0 1% deoxycholate, 1 mM phenylmethylsulfonyl-fluoride and 0 1 mg/ml soybean trypsin inhibitor) Homogenates were cleared by centrifugation (10,000 x g, 7 min at 4°C), supplemented with 0 1 volume of 10% SDS and diluted ten-fold in lysis buffer before addition of anti-PC2 antiserum (1 300 dilution) or anti-SgIII antiserum (1 5000 dilution) Immune complexes were precipitated with protein A-Sepharose (LKB-Pharmacia) and resolved by SDS-PAGE Radiolabeled proteins were visualized by fluorography

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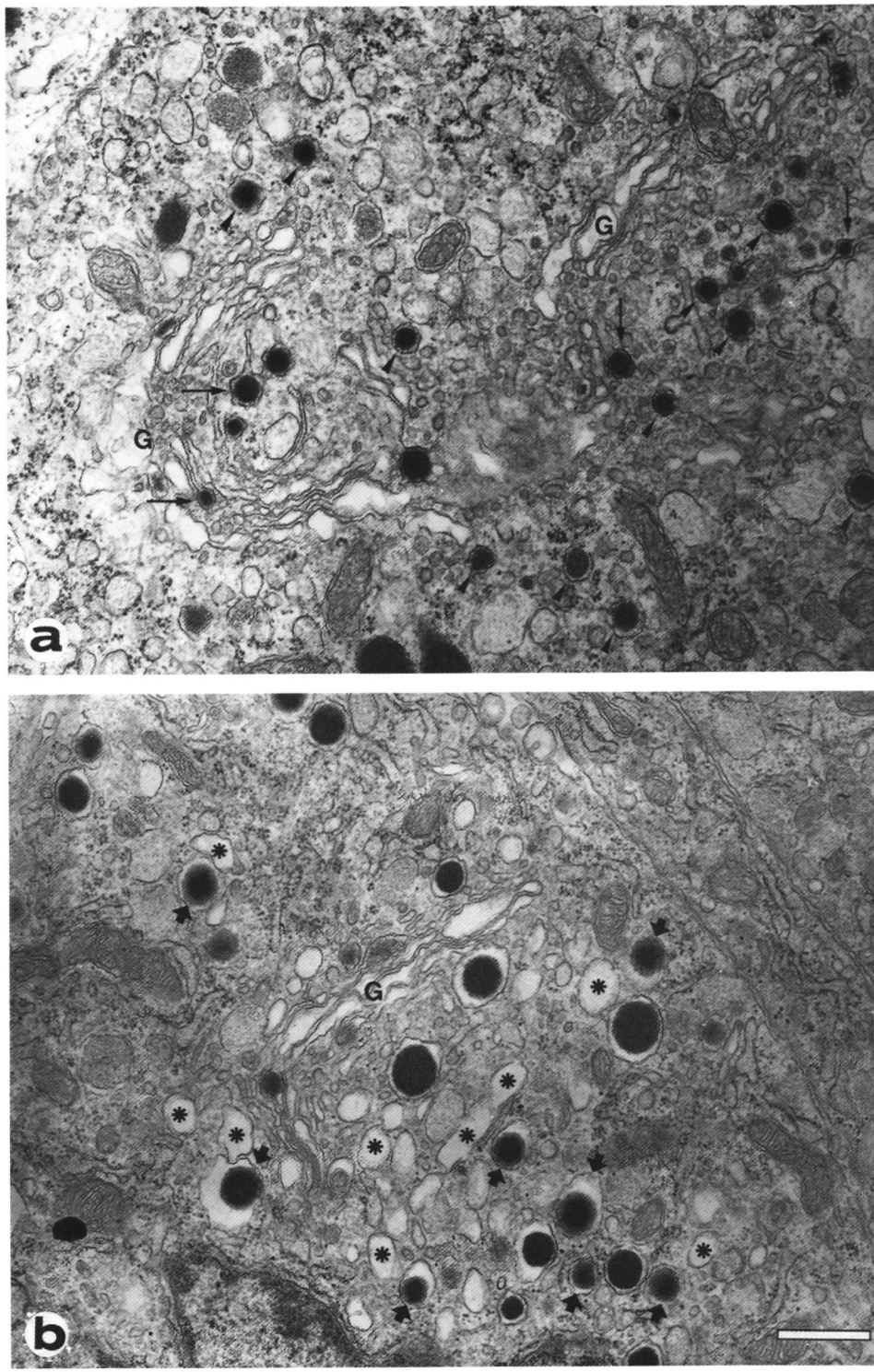
FIG. 1. ***Bafilomycin A1 affects the biogenesis of secretory granules in Xenopus melanotrope cells.*** Neurointermediate lobes dissected from black background-adapted *Xenopus* were incubated for 2,5 h in the absence (**A**) or presence (**B**) of 1 μM bafilomycin A1 and processed for electron microscopy Note the numerous small dense core secretory granules (*arrow heads*) in control cells Occasionally, dense cores are present in tubular structures at the *trans* side of the Golgi complex (*small arrows*) Note the appearance of numerous clear vacuoles (*asterisks*) and swollen dense core-containing vesicles in bafilomycin A1-treated cells, some cores consist of an electron-dense center surrounded by a more electron-translucent ring (*arrows*) G, Golgi complex Bar, 500 nm


G
G
a
G
b

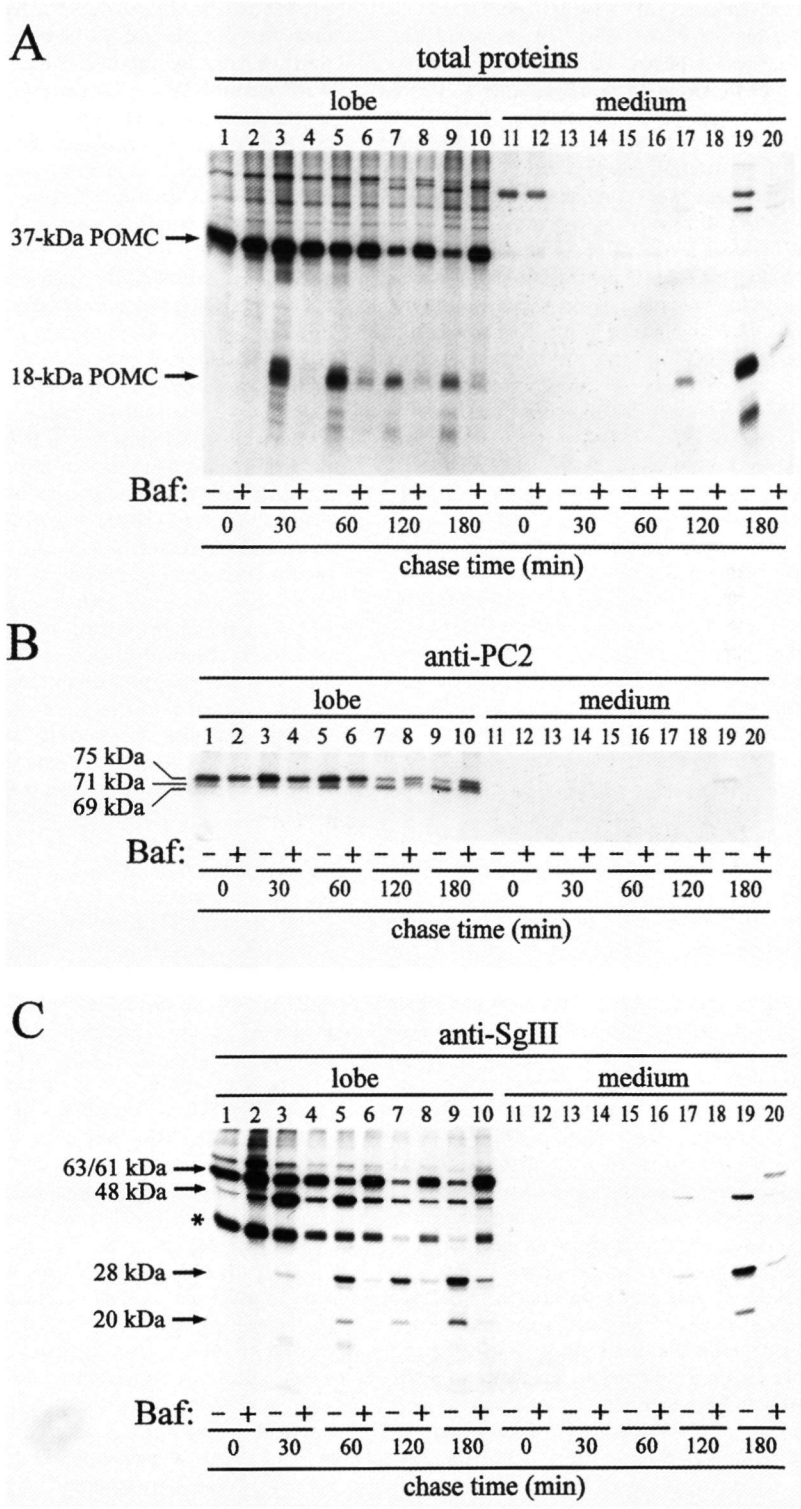
RESULTS

Bafilomycin A1 affects secretory granule biogenesis in Xenopus melanotropes — The effects of bafilomycin A1 on the acidification of intracellular organelles in *Xenopus* melanotropes were tested by vital staining of the cells with acridine orange This dye is an "acidotropic" weak base, which is taken up by living cells and accumulates in acidic compartments (Allison and Young, 1969) Fluorescence of acridine orange is green at low concentrations, whereas at high concentrations the fluoresence changes to orange When primary cultures of NILs dissected from black-adapted *Xenopus* were incubated in the presence of 2 μM acridine orange for 20 min at 22°C, the nuclei and cytoplasms of the melanotrope cells showed green fluorescence whereas a bright orange fluorescence was observed in granular patterns throughout the cytoplasm (data not shown) Incubation of cultures in the presence of 1 μM bafilomycin A1 for 30 min prior to acridine orange-staining caused a complete disappearence of the orange fluorescence in the melanotropes and an overall green fluorescence was observed in their cytoplasmic compartments (data not shown) These results indicate that the drug had effectively blocked *in vivo* acidification of all intracellular organelles in *Xenopus* melanotropes

To determine the effects of bafilomycin A1 on the ultrastructure of *Xenopus* melanotropes, NILs dissected from black-adapted animals were incubated for 2,5 h with or without 1 μM of the drug and processed for electron microscopy Typical Golgi complexes and numerous dense core secretory granules were observed in melanotrope cells of untreated NILs (Fig 1A) Occasionally, dense cores were seen in tubular elements of the TGN (Fig 1A) Some secretory granules displayed buds coated with clathrin-like material, a feature associated with immature granules (Tooze and Tooze, 1986) Small vesicles coated with clathrin-like material were often found in the proximity of the secretory granules (see upper right part of micrograph in Fig 1A) In the melanotrope cells of bafilomycin A1-treated NILs, the number of conventional secretory granules had dramatically dropped Instead, many vacuolar structures appeared at the *trans* side of the Golgi complex (Fig 1B) These vacuoles were generally larger than the secretory granules observed in control cells, some had no apparent content while others contained dense cores surrounded by clear halos of considerable width These cores often had a dark center with a concentric ring consisting of more electron-translucent material (Fig 1B) Although the vacuoles seemed to lack coated buds, small vesicles coated with clathrin-like material were sometimes observed in their vicinity Except for some dilation of the *trans*-Golgi cisternae and the TGN, the overall morphological appearance of the Golgi apparatus in bafilomycin A1-treated cells was similar to that in control cells Furthermore, bafilomycin A1 did not cause any obvious changes in the ultrastructural appearence of other cellular organelles such as the nucleus, the endoplasmic reticulum and mitochondria We therefore conclude that in *Xenopus* melanotropes bafilomycin A1 predominantly interferes with the biogenesis of dense core secretory granules

Bafilomycin A1 inhibits the proteolytic processing and release of regulated secretory proteins in Xenopus melanotropes — To invesigate the effects of bafilomycin A1 on the biosynthesis and release of regulated secretory proteins in *Xenopus* melanotropes, NILs from black-adapted animals were preincubated for 20 min, pulsed with Tran[^{35}S]-label for 20 min, and then chased for various time intervals in the absence or presence of 1 μM bafilomycin A1 The prohormone POMC clearly represents the main radiolabeled protein in pulse-incubated NILs (Fig 2A, lane 1) In the absence of bafilomycin A1, the proteolytic processing of this radiolabeled 37-kDa protein into an ~18-kDa fragment and a range of smaller peptides commenced within the 30-min chase period that followed the pulse incubation (Fig 2A, lane 3) After 120 min of chase, the majority of radiolabeled POMC was processed and its cleavage products started to appear in the medium (Fig 2A, lanes 7 and 17) Incubation of

FIG 2 ***Effect of bafilomycin A1 on the biosynthesis of POMC, PC2 and SgIII in Xenopus neurointermediate lobes.*** Neurointermediate lobes from black-adapted *Xenopus* were pulsed for 20 min with Tran[^{35}S]-label and then chased for the indicated time periods in the absence (—) or presence (+) of 1 μM bafilomycin A1 (*Baf*) Total protein extracts (**A**) or protein extracts immunoprecipitated with anti-PC2 antibodies (**B**) or anti SgIII antibodies (**C**) were resolved by SDS-PAGE and visualized by fluorography Migration positions of newly synthesized POMC and an 18-kDa POMC derived cleavage product in total protein extracts (A), as well as the migration positions of intact and processed forms of PC2 (B), and SgIII (C) are indicated Note that some of the SgIII immunoprecipitates contain a non-specifically reacting 37 kDa POMC band (*asterisk*) The prominent radiolabeled band present in lanes 11 and 12 of the gel containing total protein extracts (A) presumably corresponds to bovine serum albumin that was added to the pulse incubation medium


A
total proteins
lobe
medium
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
37-kDa POMC
18-kDa POMC
Baf: - + - + - + - + - + - + - + - + - + - +
0 30 60 120 180 0 30 60 120 180
chase time (min)
B
anti-PC2
lobe
medium
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
75 kDa
71 kDa
69 kDa
Baf: - + - + - + - + - + - + - + - + - + - +
0 30 60 120 180 0 30 60 120 180
chase time (min)
C
anti-SgIII
lobe
medium
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
63/61 kDa
48 kDa
*
28 kDa
20 kDa
Baf: - + - + - + - + - + - + - + - + - + - +
0 30 60 120 180 0 30 60 120 180
chase time (min)

NILs with bafilomycin A1 did not affect the production rate of POMC (Fig 2A, compare lanes 1 and 2), yet strongly inhibited the proteolytic processing of the prohormone. Even after a 180-min chase period, most of the radiolabeled POMC was still intact and present intracellularly (Fig 2A, lane 10), while only trace amounts of POMC-derived cleavage products were detected in the medium (Fig. 2A, lane 20).

Having established that bafilomycin A1 is a potent inhibitor of POMC processing and causes an accumulation of the intact prohormone in *Xenopus* melanotropes, we wondered if the drug also affects the processing and release of other regulated secretory proteins. Two neuroendocrine precursor proteins with relatively high expression levels in *Xenopus* melanotropes are the prohormone convertase PC2 (Braks et al , 1992, Holthuis et al., 1995; Chapter 2) and the secretory granule-associated protein secretogranin III (SgIII) (Holthuis and Martens, 1996; Holthuis et al. 1996; Chapter 5). Their fates in normal and bafilomycin A1-treated NILs were monitored by immunoprecipitation ana-lysis of radiolabeled proteins produced in pulse- and pulse-chase incubated lobes. Immunoprecipitation of the radiolabeled proteins in control pulse-incubated NILs with anti-PC2 antibodies revealed the 75-kDa proform of PC2 (Fig 2B, lane 1) During subsequent chase incubations, this newly synthesized protein was gradually converted into the mature 69-kDa form of the enzyme, with a short-lived 71-kDa product as the processing intermediate (Fig 2B, lanes 3,5,7 and 9). Only mature 69-kDa PC2 was released into the medium (Fig 2B, lanes 17 and 19). In bafilomycin A1-treated NILs, a clear delay in the proteolytic maturation of PC2 was found (compare lanes 7 and 8 in Fig 2B). After a 180-min chase period, only a minor portion of the 75-kDa proenzyme was converted into the mature form (Fig. 2B, lane 10) In addition, an accumulation of the 71-kDa processing intermediate was observed (Fig. 2B, lanes 8 and 10) and, in contrast to control lobes, bafilomycin A1-treated NILs released both mature PC2 and the 71-kDa intermediate cleavage product into the medium (Fig. 3A, compare lanes 5 and 6, data not shown).

When radiolabeled proteins in control pulse-incubated NILs were immunoprecipitated with anti-SgIII antibodies, two newly synthesized SgIII proteins of 63 kDa (*N*-glycosylated) and 61 kDa (non-glycosylated) were detected. After a 30-min chase period, the majority of newly synthesized 63/61-kDa SgIII was processed to a 48-kDa product (Fig 2C, lane 3) In subsequent chase incubations, this 48-kDa product was partially cleaved into fragments of 28- and 20 kDa (Fig 2C, lanes 5, 7 and 9). Only the 48-, 28- and 20-kDa cleavage products of SgIII, but not the intact 63/61-kD precursor forms, were secreted (Fig 2C, lanes 17 and 19). Bafilomycin A1 strongly inhibited the processing of SgIII. Even after 180 min of chase, the majority of newly synthesized SgIII remained in the unprocessed 63/61-kDa forms (Fig. 2C, lane 10). Furthermore, in addition to a low amount of SgIII-derived cleavage products, the bafilomycin A1-treated NILs released a considerable portion of unprocessed 63/61-kDa SgIII into the medium (Fig. 2C, lane 20).

Bafilomycin A1-treated melanotropes release immature secretory products in a constitutive fashion — The above results demonstrate that bafilomycin A1 inhibits the proteolytic processing of various neuroendocrine precursor proteins in *Xenopus* melanotropes In addition, we found that bafilomycin A1-treated melanotropes release significant amounts of intact precursors and intermediate cleavage products that under normal conditions do not reach the extracellular environment Since sorting of regulated secretory proteins is assumed to be a pH-dependent process and because the proteolytic processing of such proteins often starts after their packaging into secretory granules, we sought to determine whether the release of immature products by bafilomycin A1-treated melanotropes is caused by a missorting event. We previously observed that incubation of *Xenopus* melanotropes with the dopamine D2 receptor agonist apomorphine effectively blocks the release of regulated secretory proteins (Jenks et al., 1993, Holthuis et al. 1996, Chapter 5, data not shown). In the following experiments, we therefore analysed the fates of newly synthesized PC2 and SgIII in *Xenopus* NILs that were treated with bafilomycin A1 in the absence or presence of apomorphine.

Consistent with the results presented above, most of the 75-kDa proform of PC2 produced in control pulse-incubated NILs was converted to mature 69-kDa PC2 and secreted during a subsequent 180-min chase period (Fig. 3A, lanes 1, 2 and 5). In pulse-chased bafilomycin A1-treated NILs, there was a clear accumulation of the 75-kDa proform and the 71-kDa processing intermediate of PC2 (Fig 3A, lane 3) while the incubation medium contained both the mature form and the 71-kDa processing intermediate of the enzyme (Fig 3A, lane 6) However, when NILs were treated with both bafilomycin A1 and apomorphine, only the 71-kDa PC2 processing intermediate but not the mature form of the enzyme was observed in the medium (Fig 3A, lane 7)

Similar results were obtained for SgIII. Whereas bafilomycin A1-treated NILs released both 63/61-kDa precursor forms and 48/28/20-kDa cleavage products of SgIII (Fig. 3B, lane 6), NILs incubated with both bafilomycin A1 and apomorphine released only the precursor forms and a minor portion of the 48-kDa cleavage product, but not the 28/20-kDa cleavage products (Fig. 3B, lane 7). These findings suggest that in bafilomycin A1-treated melanotropes, a considerable portion of the newly synthesized PC2 and SgIII proteins does not enter the regulated (dopamine-sensitive) secretory pathway but instead is delivered to the extracellular environment in a constitutive fashion.

DISCUSSION

The biochemical data described in this study indicate that V-ATPase-mediated acidification plays a critical role in the proper handling of regulated secretory proteins by *Xenopus* melanotrope cells. Upon exposure of these cells to the specific V-ATPase inhibitor bafilomycin A1, the proteolytic processing of newly synthesized POMC, PC2 and SgIII ceased, while an intracellular accumulation of intact precursor forms and intermediate cleavage products became apparent. Based on two lines of information, these bafilomycin A1-induced effects likely result from an inhibition of V-ATPases located in the TGN and/or immature secretory granules. First, morphological analysis of bafilomycin A1-treated melanotropes revealed a decrease in the number of small dense core secretory granules and the appearence of vacuolar structures in the *trans* Golgi area; these vacuolar structures often contained electron-dense material, suggesting that they represent aberrant forms of TGN-derived secretory granules. Second, the proteolytic processing of newly synthesized POMC and SgIII in *Xenopus* melanotropes, which is perturbed by the bafilomycin A1-treatment, normally starts in the TGN/immature secretory granules and is assumed to be primarily mediated by PC2 (Holthuis et al., 1996; Chapter 5), an enzyme which, at least *in vitro*, requires an acidic pH for optimum activity (Davidson et al., 1988; Shennan et al., 1996). The TGN and immature granules indeed provide an acidic microenvironment (Anderson and Orci, 1988).

Although our results demonstrate the necessity of V-ATPase-mediated acidification for the processing of various neuroendocrine precursor proteins, the precise molecular basis of this requirement remains to be established. As mentioned above, the activity of the proteolytic enzymes may depend on an acidic environment. We found that bafilomycin A1 impairs

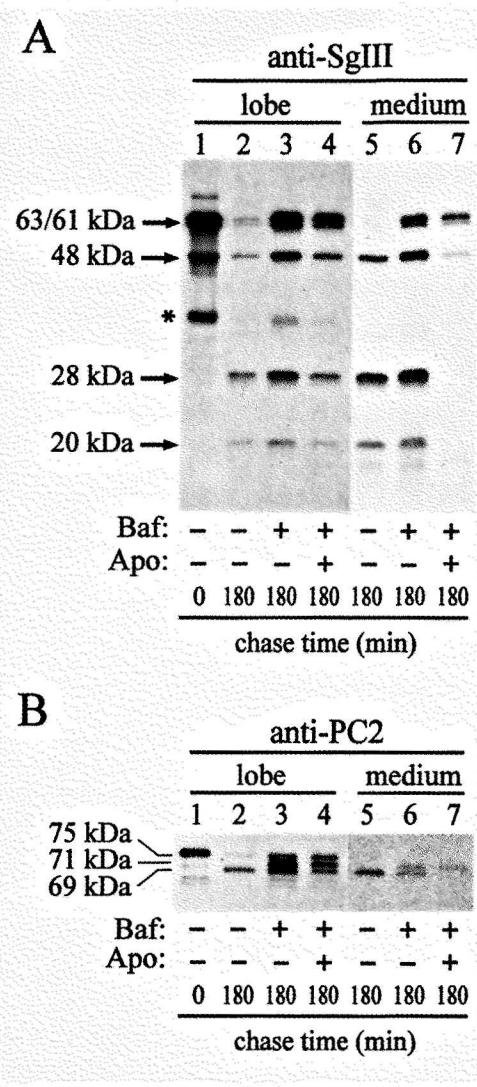


FIG. 3. ***Bafilomycin A1 causes the release of immature forms of PC2 and SgIII via a non-regulated secretory pathway.*** Neurointermediate lobes from black-adapted *Xenopus* were pulsed for 20 min with Tran[^{35}S]-label or pulsed for 20 min and chased for 180 min in the absence (–) or presence (+) of 1 μM bafilomycin A1 (*Baf*) and 10 μM apomorphine (*Apo*). Radiolabeled proteins in lobe extracts and incubation media were immunoprecipitated with anti-PC2 antibodies (**A**) or anti-SgIII antibodies (**B**). Immunoprecipitates were resolved by SDS-PAGE and visualized by fluorography. Migration positions of intact and processed forms of PC2 and SgIII are indicated. Note that some of the SgIII immunoprecipitates contain a non-specifically reacting 37-kDa POMC band (*asterisk*).

the maturation of the proform of PC2, the key processing enzyme in *Xenopus* melanotropes (Braks et al , 1992, Holthuis et al , 1995, Chapter 2) Hence, an alternative explanation for the inhibition of precursor processing in bafilomycin A1-treated melanotropes is the limited availability of functional enzymes Furthermore, it is possible that an acidic pH promotes the interaction between processing enzymes and their substrates, or induces conformational changes in precursor molecules that lead to the exposure of cleavage sites (Xu and Shields, 1994)

Besides affecting processing, bafilomycin A1 also perturbs intracellular transport and secretion of POMC, PC2 and SgIII in *Xenopus* melanotropes Consistent with our findings, Henomatsu et al (1993) reported that bafilomycin A1 inhibits the secretion of newly synthesized prolactin from rat pituitary GH3 cells Their results show that the bafilomycin A1-sensitive step in the intracellular transport of prolactin occurs shortly after the hormone is exported from the TGN Ultrastructural analysis of bafilomycin A1-treated GH3 cells revealed the presence of large vacuoles that were loaded with prolactin and appeared first in the *trans*-Golgi region and then in the rest of the cytoplasm, concomitant with a decrease in the number of small dense core secretory granules (Henomatsu et al , 1993) Our present data allow a generalization of the concept that V-ATPase-mediated acidification is critical for achieving a timely delivery of regulated secretory proteins to the cell exterior This concept raises the possibility that inadequate acidification of the regulated secretory pathway interferes with a proper delivery of secretory granules to the site of exocytosis or, alternatively, with the formation of fusion competent granules, for example by affecting the recruitment of granular membrane components involved in vesicular targeting (e g rab3A, rabphilin, Shirataki et al , 1993) docking (e g syntaxins, Sollner et al , 1993) or fusion (e g synaptotagmin, Debello et al , 1993)

Previous work with neuroendocrine cell lines has indicated that neutralization of acidic organelles by weak bases can cause a missorting of peptide hormone precursors to the constitutive secretory pathway (Moore et al , 1983, Stoller and Shields, 1989) In the present study we have shown that bafilomycin A1-treated *Xenopus* melanotropes release considerable amounts of unprocessed SgIII and a PC2 processing intermediate via a constitutive (dopamine-insensitive) secretory pathway Considering these results, it was remarkable that we detected only mature POMC-derived cleavage products but no intact prohormone in the chase media of bafilomycin A1-treated melanotropes Since the experiments described in this study involved static incubations, it is possible that intact POMC molecules are cleaved once released into the medium Recent superfusion experiments demonstrated that bafilomycin A1-treated melanotropes do release intact POMC via a constitutive (dopamine-insensitive) pathway, however, the relative amount of prohormone secreted in a constitutive fashion was considerably smaller than that observed for intact SgIII and the PC2 processing intermediate (V Th G Schoonderwoert and G J M Martens, unpublished results) These findings indicate that a block in V-ATPase-mediated acidification differentially affects the sorting of regulated secretory proteins in *Xenopus* melanotropes Although the precise mechanism whereby proteins are conveyed to the regulated secretory pathway remains to be clarified, self-condensation of regulated secretory proteins in the acidic microenvironment of the TGN/immature granules is considered to be a main factor in the sorting process (Burgess and Kelly, 1987, Gerdes et al , 1989, Chanat and Huttner, 1991) Self-condensation is thought to be dependent upon local protein concentration, Ca^{2+} levels and pH The morpholog-ical analysis of bafilomycin A1-treated *Xenopus* melanotropes suggests that inactivation of V-ATPases interferes with a proper condensation of regulated secretory proteins, the vacuolar structures observed upon exposure to the drug often contained only partially condensed material This raises the possibility that the differential effects of bafilomycin A1 treatment on the sorting of newly synthesized POMC, PC2 and SgIII in *Xenopus* melanotropes reflect different aggregative properties of these proteins Indeed, *in vitro* experiments performed under ionic conditions similar to those at the sites of granule formation have shown that some regulated secretory proteins can aggregate individually in the absence of other proteins, while others require an aggregating partner to precipitate (Colomer et al , 1996) A propensity of POMC to form aggregates by homotypical interactions in combination with its relatively high concentrations in the secretory pathway of stimulated *Xenopus* melanotropes (Chapter 2) may render sorting of this protein to the regulated secretory pathway less sensitive to pH perturbences in TGN/secretory granules than that of PC2 and SgIII

In conclusion, our data indicate that neuroendocrine cells require intraorganellar acidification by bafilomycin A1-sensitive V-ATPases in order to accomplish a correct sorting, processing and release of their regulated secretory products

Aknowledgements — We thank Mr R Engels for animal care and Dr I Lindberg for the anti-PC2 antibodies

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Chapter 9

General Discussion

General Discussion

Xenopus melanotropes and the search for genes involved in neuroendocrine secretion

A general outline for the sequence of events involved in the production, processing, storage and release of peptide hormones by neuroendocrine cells has been known for many years From a mechanistic point of view, however, our current knowledge of how peptide hormones end up properly processed in secretory granules and how these organelles deliver their contents in accordance with the physiological demand is still very limited A comprehensive understanding of the molecular machinery underlying neuroendocrine secretion requires a complete inventory of its individual components

The objective of the investigations described in this thesis was to systematically identify and characterize genes whose products participate in steps of peptide hormone biosynthesis and secretion As an experimental approach, a differential hybridization technique was developed and used to trace genes coordinately expressed with the POMC gene in the melanotrope cells from *Xenopus* intermediate pituitary (Chapter 2) The rationale behind this strategy is that only gene products associated with the specialized secretory function of neuroendocrine cells are regulated in parallel with the prohormone, while others not part of the secretory apparatus need not be regulated In *Xenopus* melanotropes, the expression levels of POMC can be readily manipulated by changing the background colour of the animal On a black background, the POMC gene is actively transcribed, whereas on a white background the gene is virtually inactive As schematically represented in Fig 1, a subset of genes transcribed in *Xenopus* melanotropes was found to display a dynamic coregulation with the POMC gene during background adaptation The proteins encoded by these genes can be divided into a number of categories, namely ER resident proteins, proteins cycling between ER and Golgi, proteins cycling between Golgi and plasma membrane, and secretory proteins (see also Fig 2)

ER resident proteins

Two of the regulated genes identified in this study code for proteins residing in the ER, i e the translocon-associated protein TRAPδ and the cysteine protease ER60 TRAPδ is a subunit of a tetrameric complex of transmembrane proteins located at the site where nascent secretory proteins enter the ER (Hartmann et al , 1993) The TRAP complex is not essential for the translocation process (Migliaccio et al , 1992) and at present its function is unknown Since the complex consists of highly conserved proteins (Chapter 3, Hartmann et al , 1993), a role of general physiological importance can be anticipated Our finding that alternative splicing of the TRAPδ transcript generates two structurally distinct proteins in various tissues of different species (Chapter 3) suggests that the subunits of the TRAP complex occur in more than one assembly state in the ER However, the precise implications of this finding are, as yet, obscure

The cysteine protease ER60 is a soluble protein whose steady state localization in the ER lumen is probably accomplished by the presence of an ER retention signal (KDEL) at its carboxy terminus (Urade et al , 1992) In a recent study, it was shown that ER60 selectively associates with misfolded secretory proteins *in vivo* and effects their degradation *in vitro* (Otsu et al , 1995) Thus, ER60 likely represents a component of the proteolytic system by which the ER is cleared of abnormal secretory proteins

Proteins cycling between ER and Golgi

One of the regulated genes gives rise to a protein initially classified as a novel accessory component of the translocon because of its structural similarity to Gp25L, a glycoprotein previously co-purified with subunits of the TRAP complex (Chapter 3) However, recent work has indicated that both Gp25L and the Gp25L-like protein identified in our study belong to a family of type I transmembrane proteins (the p24 protein family) whose members are found in COPI- and COPII-coated transport vesicles (Schimmoller et al , 1995, Stamnes et al , 1995) These p24 proteins are good candidates for cargo receptors They generally possess a highly variable amino-terminal region and a more conserved, membrane-proximal region with a predicted propensity to form coiled coils in the vesicle lumen, suggestive of a receptor domain mounted on a stalk The short, carboxy-terminal cytoplasmic domains of p24 proteins bind to coatomer, the subunit of the COPI coat (Rothman and Wieland, 1996) In yeast, the deletion of a p24 protein associated with COPII-coated vesicles was found to slow down both vesicle budding and transport of a subset of secretory proteins from the ER to the Golgi (Schimmoller et al ,

1995, Stamnes et al , 1995) It is speculated that p24 proteins preferentially recognize correctly folded proteins in the ER lumen and effectuate their packaging into COP-coated vesicles Such a mechanism of positive selection, when operating in tandem with chaperone-mediated retention of incompletely folded proteins, may constitute an additional layer in the ER quality control system which ensures that only proteins with an appropriate conformation are exported to the Golgi (Rothman and Wieland, 1996) On the other hand, it is possible that the p24 protein family has a more widespread function in the secretory pathway and includes members involved in selecting cargo for transport beyond the ER-Golgi interface, i e across the Golgi stack or to post-Golgi destinations

Proteins cycling between Golgi and plasma membrane

Another regulated gene codes for Ac45, a protein previously co-purified with vacuolar H^+-ATPase (V-ATPase) from chromaffin granules (Supek et al , 1994) V-ATPases are multimeric enzymes responsible for the lumenal acidification of a number of organelles along the endocytic and secretory pathways (Forgac, 1989, Mellman et al , 1986) In neuroendocrine cells, the pH gradients established by V-ATPases are of fundamental importance for the efficient targeting, processing, storage and release of regulated secretory proteins (Henomatsu et al , 1993, Chapter 8) Ac45 is an *N*-glycosylated type I transmembrane protein predominantly expressed in neuroendocrine cell types (Chapters 2 and 6) Immunofluorescence studies on cultured *Xenopus* melanotropes suggest that the protein is associated with secretory granules and enters a recycling pathway upon exocytosis (Chapter 6) The intracellular trafficking of Ac45 is, at least in part, mediated by sorting signals in its cytoplasmic tail Interestingly, the carboxy terminal region of Ac45 contains a putative sorting motif which defines a small group of transmembrane proteins found in the storage organelles of regulated secretory cells (Chapter 7) Ac45 unlikely represents a common subunit of V-ATPases, yet its ectopic expression in fibroblasts seems to perturb V-ATPase activity in the secretory pathway (Chapter 6) One feature which distinguishes Ac45 from all other V-ATPase subunits is that the major portion of the protein is located in the lumen of the vacuolar system Consequently, Ac45 represents an attractive candidate for a protein involved in the communication between V-ATPases and the interior of the vacuolar system Obviously, some form of communication will be necessary to adjust the influx of protons to the specific needs of a particular organelle, such a coupling could be especially relevant in the regulated secretory pathway where an appropriate timing of sorting and processing events likely requires a tight regulation of V-ATPases Perhaps Ac45 acts as a pH sensor in the regulated secretory pathway, allowing for a fine-tuning of the activity of the pump Alternatively, Ac45 may act as a device to convey V-ATPases to secretory granules, for instance by a propensity of its lumenal domain to co-aggregate with regulated secretory proteins at the site of granule formation Colomer et al (1996) reported that the lumenal domains of other granule membrane proteins (e g dopamine ß-hydroxylase, PAM) undergo a selective co-aggregation with soluble granular proteins when incubated under ionic conditions similar to those occuring in the TGN, suggesting that protein aggregation indeed plays a potential role in the segregation of membrane proteins to storage organelles

Secretory proteins

This category includes two enzymes involved in the proteolytic maturation of prohormones, namely the prohormone convertase PC2 and carboxypeptidase H/E (CPH) The three other proteins in this group are members of the chromogranin/secretogranin family, namely secretogranin (Sg) II, SgIII and the neuroendocrine polypeptide 7B2 (also referred to as SgV) All five proteins are stored and released in coordination with peptide hormones (Fricker, 1988, Ayoubi et al , 1990, Kirchmair et al , 1992, Fischer-Colbrie et al , 1995, Chapter 5) The functions of PC2 and CPH in neuroendocrine secretion are firmly established (Steiner et al , 1992, Fricker, 1988) The biological significance of the granins, on the other hand, is only partially understood 7B2 selectively interacts with the proenzyme form of PC2 and is involved in regulating PC2 activity in the regulated secretory pathway (Martens et al , 1994, Braks and Martens, 1994, Zhu and Lindberg, 1995) SgII is the precursor of a bioactive peptide called secretoneurin (Fischer-Colbrie et al , 1995) The alignment of SgII amino acid sequences from *Xenopus* and mammals raises the possibility that this protein gives rise to more than one functionally important peptide (Chapter 4) Whether SgII acts solely as a peptide precursor or if the protein has an additional function in neuroendocrine secretion (e g a helper function

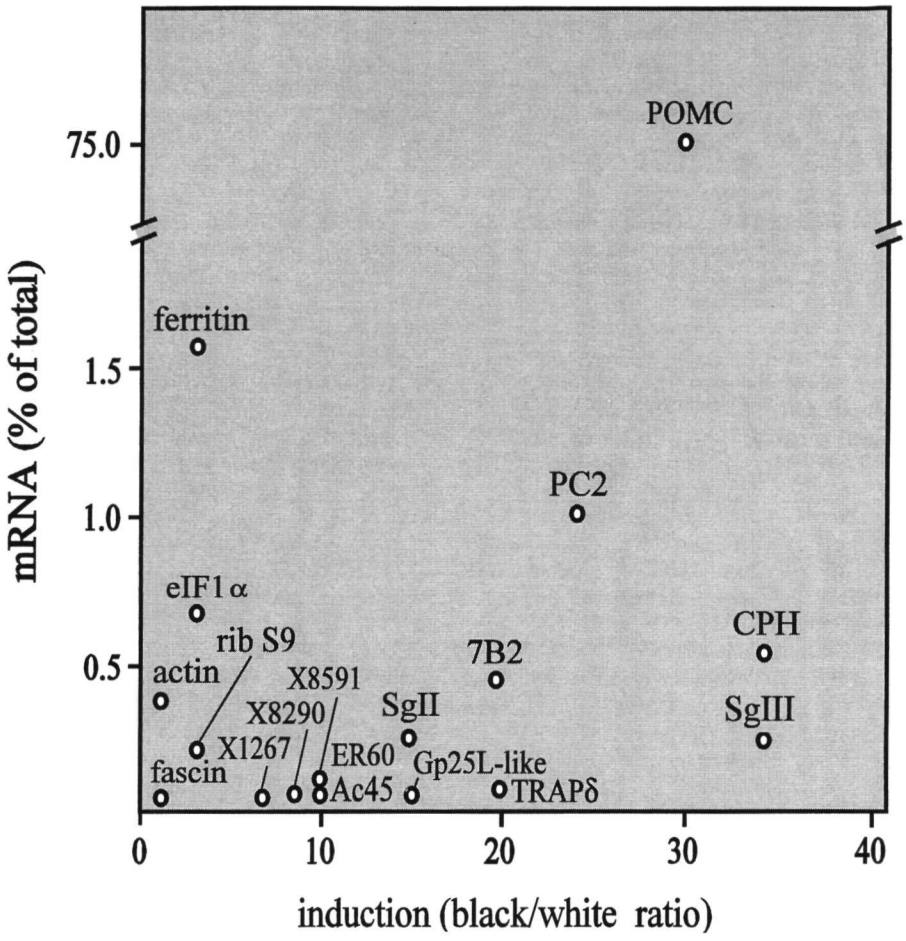

FIG. 1. **Differential expression of genes transcribed in *Xenopus* melanotrope cells.** For the various genes shown, the ratio of transcript levels in the intermediate pituitary between black- and white-adapted animals (black/white ratio) was determined by RNase protection assays, Northern blot analysis and/or dot blot hybridization experiments. The expression level of each transcript in the intermediate pituitary of black-adapted animals relative to the total amount of mRNA (% of total) was estimated on the basis of the frequency by which corresponding cDNA clones were isolated from the primary intermediate pituitary library and by comparing its protection/hybridization signals with that of other messengers analysed. Abbreviations used are as follows: POMC, proopiomelanocortin; PC2, prohormone convertase 2; CPH, carboxypeptidase H; 7B2, neuroendocrine protein 7B2; SgII, secretogranin II; SgIII, secretogranin III; TRAPδ, translocon-associated protein δ; Gp25L-like, glycoprotein 25L-like protein; ER60, endoplasmic reticulum cysteine protease 60; Ac45, V-ATPase accessory subunit 45; eIF1α, translation initiation factor 1α; Rib S9, ribosomal protein S9. X1267, X8290 and X8591 represent genes whose products await further characterization. See text for details.

in the packaging or processing of other regulated secretory proteins) remains to be established

Genetic ablation of the SgIII gene in mice did not provide any clue with respect to the role of this protein in the neuroendocrine system (Kingsley et al , 1990) Our biosynthetic studies in *Xenopus* melanotropes showed that SgIII is a sulfated precursor protein which is cleaved at two dibasic sites before exocytosis (Chapter 5) In view of the comparative analysis of the *Xenopus* and mammalian proteins (Chapter 4), SgIII processing unlikely serves to liberate peptides of general biological importance Processing could terminate an intracellular function of SgIII or simply reflect its fate in the regulated secretory pathway A peculiar feature of SgIII is the presence of a highly conserved motif (DSTK) which is repeated four times in its primary structure (Chapter 4) Similar motifs occur in the lumenal domains of the transmembrane proteins TGN38 and TGN41, two molecules implicated in the packaging of cargo for export from the TGN (Stanley and Howell, 1993) Hence, one possibility is that SgIII represents a type of molecular packing material required for an efficient loading of storage granules, for instance by interacting with those regulated secretory products that lack the inherent ability to form aggregates upon arrival in the TGN

SIGNIFICANCE OF THE COORDINATED GENE EXPRESSION IN *XENOPUS* MELANOTROPES

The physiological relevance of the coregulation between the above gene products and POMC in *Xenopus* melanotropes is in some cases obvious For instance, PC2 and CPH can be directly implicated in POMC processing and a coordinated expression of these proteins with other peptide precursors has previously been demonstrated in neuroendocrine systems other than *Xenopus* melanotropes (Bloomquist et al , 1991, 1994, Shuppin and Rhodes, 1996) PC2 and CPH are not recycled upon secretion and their highly elevated expression levels in stimulated *Xenopus* melanotropes most probably serve to supply for an increased demand of POMC processing For 7B2, the link is indirect, but nevertheless important since this secretory protein acts as a private chaperone for PC2, thus enabling the latter to fulfil its essential task during the generation of POMC-derived peptide hormones The functions of two additional secretory proteins, SgII and SgIII, are not or only poorly understood and the significance of their coexpres-sion with POMC is unclear Although disruption of the SgIII gene in mice did not produce any obvious phenotype (Kingsley et al , 1990), our finding that its expression in *Xenopus* melanotropes is subject to a similar magnitude of regulation as that of the POMC, PC2 and CPH genes (Fig 1) provides a strong indication that SgIII plays a significant role in neuroendocrine secretion

The other gene products identified in this study all represent permanent residents of the secretory pathway Their differential expression is indicative for an involvement in processes that affect, either directly or indirectly, the fate of POMC in *Xenopus* melanotropes POMC represents by far the most abundant (~80%) of all newly synthesized proteins that pass along the secretory pathway in stimulated melanotropes (Chapter 2) It therefore appears reasonable to assume that the TRAP complex participates in an early step of POMC biosynthesis, that is either during, or shortly after the newly synthesized protein emerges in the ER lumen The massive amounts of POMC entering the ER in stimulated melanotropes may lead to a premature condensation event in that organelle It has been well documented that hyperstimulated exocrine and endocrine cells tend to develop intracisternal granules in the ER which consist entirely of regulated secretory products (Farquhar, 1971, Tooze et al , 1989) The upregulation of ER60 may represent a necessary step to prevent the formation of such aggregates and to ensure their degradation once they are formed Of the p24 proteins implicated in ER to Golgi transport, the Gp24L-like protein identified in this study obviously forms an attractive candidate for mediating an efficient export of POMC from the ER Finally, we found that V-ATPase-mediated acidification in *Xenopus* melanotropes is indispensible for POMC processing and the timely delivery of regulated secretory products to the cell surface (Chapter 8) The enhanced expression levels of Ac45 in stimulated melanotropes may therefore reflect an important function of this protein in the adequate acidification of the regulated secretory pathway

CONCLUSIONS REGARDING THE DIFFERENTIAL SCREENING APPROACH

The strategy of black/white screening employed in this study turns out to be a viable approach for a systematic identification of genes involved in neuroendocrine secretion The collection of regulated genes established so far encodes soluble as well as integral membrane proteins that operate at

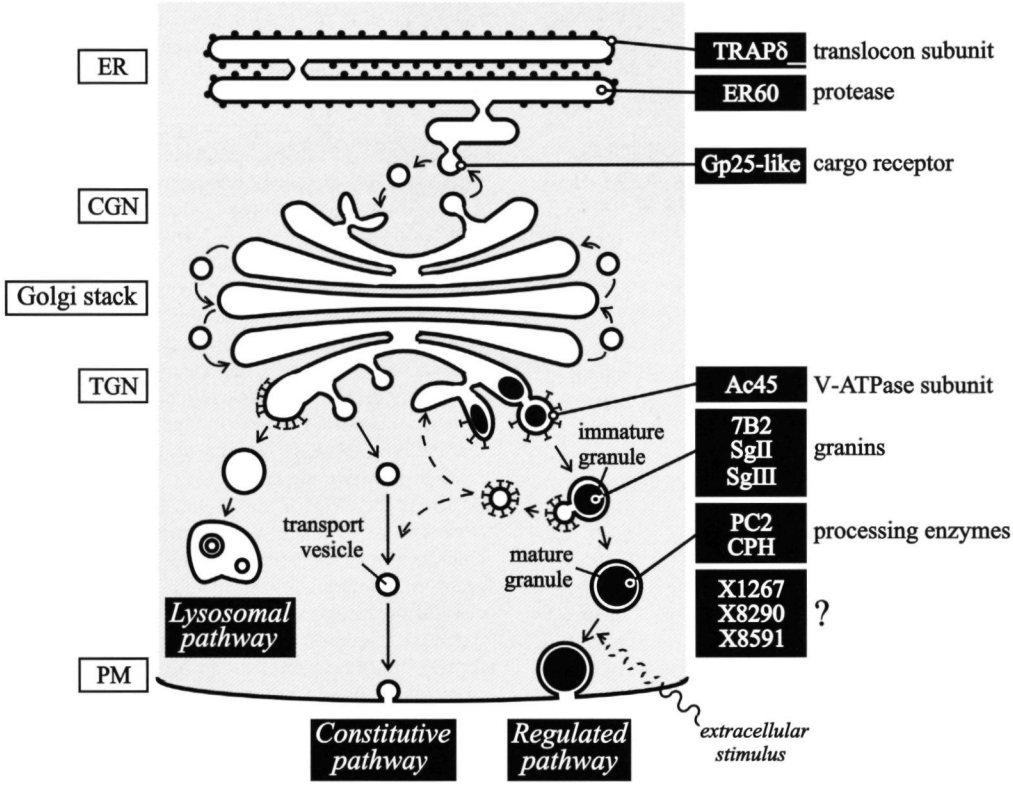

FIG. 2. **Gene products coordinately expressed with POMC in *Xenopus* melanotropes and their presumed sites of operation in the secretory pathway.**

diverse locations in the secretory pathway (see Fig. 2). The products of three regulated genes (X1267, X8290 and X8591) remain obscure. Two of these genes (X8290 and X8591) are of particular interest since their expression is restricted to neuroendocrine tissues (Chapter 2). Unfortunately, the corresponding cDNAs isolated so far all represent 3'-untranslated regions of large transcripts. Classification of the proteins encoded by these genes and prediction of their functions requires the isolation of 5'-extended cDNAs.

During the course of our study, we identified several genes displaying no, or only a moderate co-regulation with the POMC gene (Fig. 1). The majority of these genes codes for mitochondrial enzymes (e.g. subunits of NADH-oxidoreductase, ATP synthase), components of the translational apparatus (ribosomal proteins, translation initiation/elongation factors) or proteins that are part of the cytoskeleton (actin, fascin). The gene for ferritin H, a cytoplasmic protein involved in iron housekeeping, shows a remarkably high degree of expression in *Xenopus* melanotropes, yet displays only a minor fluctuation in expression levels during background adaptation (Fig. 1). These findings underscore the usefullness of the black/white screening approach as a highly selective method for tracking down components of the secretory pathway in neuroendocrine cells.

A considerable number of cytoplasmic proteins and cytoplasmically oriented intergral membrane proteins has been implicated in vesicle-mediated

protein transport along the secretory pathway These include coat proteins (e g COPs), small GTP-binding proteins (e g ARFs, Rabs), receptor-like molecules (v-SNAREs, t-SNAREs), and components of the fusion machinery (e g NSF, SNAPs), each with a fundamental role in the budding, docking or fusion of transport vesicles (Rothman and Wieland, 1996) Genes encoding such proteins have not yet been encountered in our study A likely explanation for this is that these genes are only moderately expressed and therefore may not produce a detectable hybridization signal in the differential screening technique utilized

Future directions and perspectives

Most of the regulated gene products described above remain to be further characterized and their postulated functions evaluated in future experiments One frequently used approach to assess the physiological role of a gene product is to manipulate its *in vivo* expression level This can be accomplished by inducing ectopic expression with appropriate DNA constructs or by targeted disruption of the gene through homologous recombination For instance, our preliminary analysis of the effects of Ac45 overexpression in cultured cells indicates that this protein has a critical role in V-ATPase-mediated acidification of the secretory pathway (see Chapter 6) Overexpression studies with mutated versions of Ac45 could help define the functional domain(s) of this protein and thereby shed more light on its mechanism of action However, altering the *in vivo* expression level of a gene product does not always lead to an easy recognizable phenotype (like in the case of the SgIII knock-out mice, Kingsley et al , 1990), especially when its function overlaps with that of another protein An alternative approach to collect valuable information on the role of a protein is to study its physical interactions with other proteins. Communoprecipitation studies with lysates prepared from metabolically labeled *Xenopus* intermediate pituitaries in the presence of chemical crosslinkers previously revealed that 7B2 binds to the proenzyme form of PC2 *in vivo*, a finding which led to the discovery that the former acts as a novel type of molecular chaperone in the secretory pathway of neuroendocrine cells With the present identification of *Xenopus* SgII and SgIII, and the antibodies raised against these proteins one could now apply similar experimental techniques to investigate if granins, being classified as a family, have comparable roles as helper proteins in neuroendocrine secretion

The collection of regulated genes described in this study was established upon screening only a minor portion of the total number of primary cDNA clones in the *Xenopus* intermediate pituitary library (namely 204 out of 7,500 preselected non-POMC/neuroendocrine-enriched cDNA clones; Chapter 2). Thus, many more regulated genes remain to be identified. A number of these will likely code for previously unrecognized components of the secretory pathway One of the major challenges in the field of neuroendocrine cell physiology is presently the issue of sorting receptors that have been postulated to recognize regulated secretory proteins and to deliver them to nascent secretory granules Efforts to identify such receptors have remained elusive (e g Chung et al , 1989) Moreover, despite extensive structural analysis of numerous regulated secretory proteins, not one clear-cut, transplantable sorting signal for regulated secretion has been identified (for review see Halban and Irminger, 1994) This failure has hampered the establishment of appropriate binding assays that could serve in the detection and identification of sorting receptors It is conceivable that these molecules recognize conformationally determined epitopes and that binding of ligand occurs with low affinity If so, the identification of sorting receptors by means of classical biochemical approaches will be a difficult and time-consuming task As discussed above, the black/white screening approach has enabled us to identify a novel member of a family of putative cargo receptors involved in selective protein transport early in the secretory pathway In view of this result, it is not unlikely that if protein sorting to the regulated secretory pathway is dependent upon binding to receptors, such molecules will eventually become apparent in subsequent rounds of black/white screening

Recent innovations in the experimental techniques available for the isolation of differentially expressed genes (PCR-based differential display/automated DNA sequence analysis) should allow a rapid expansion of the gene catalogue established in this study with regulated genes displaying lower levels of expression The structural and functional analysis of these genes will be aided by the various ongoing genome projects and the deployment of alternative DNA sequencing programmes It is expected that most of the approximately 100,000 different genes expressed in humans will be sequenced and available as expressed sequence tags (ESTs) within a few years

(Adams et al , 1992) Collectively, these developments will facilitate further exploitation of the principle of black/white screening and eventually accelerate a molecular dissection of the secretory pathway in neuroendocrine cells

In conclusion, the data presented in this thesis demonstrate that stimulation of the rate of peptide hormone biosynthesis in neuroendocrine cells involves the activation of a distinct subset of genes Further detailed analysis of the presently identified genes and the study of additional genes that in the future could be selected by the black/white screening approach should contribute to a better understanding of the mechanisms employed by neuroendocrine cells to fulfil their highly specialized secretory function In addition, the systematic identification and characterization of components from the secretory pathway in neuroendocrine cells should provide fresh insight into the pathophysiology of neuroendocrine disorders and may enable the development of novel or improved versions of existing heterologous systems for efficient large scale production of clinically relevant peptide hormones and neuropeptides

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Samenvatting

Voor het goed functioneren van een multicellulair organisme is een intensieve communicatie tussen de verschillende cellen en organen van essentieel belang De overdracht van informatie wordt voor een belangrijk deel verzorgd door peptide-hormonen en neuropeptiden. Deze boodschappers zijn betrokken bij de regulatie van de voortplanting, de groei, de stofwisseling, de water- en ionenhuishouding, en het vermogen van een organisme om zich aan te passen aan zijn omgeving Peptide-hormonen en neuropeptiden worden geproduceerd en uitgescheiden door endocriene cellen en peptiderge neuronen (hier gezamenlijk aangeduid als neuro-endocriene cellen) Teneinde de aanmaak en afgifte van de boodschappers nauwkeurig te kunnen afstemmen op de fysiologische behoeften van het organisme beschikken neuro-endocriene cellen over een unieke secretieroute Het doel van het onderzoek beschreven in dit proefschrift was om op een systematische wijze genen op te sporen wier producten een rol vervullen in deze secretieroute De identificatie en karakterisatie van dergelijke genproducten is noodzakelijk voor een beter begrip van de mechanismen verantwoordelijk voor de aanmaak, opslag en afgifte van biologisch actieve peptiden, en zou bovendien kunnen resulteren in nieuwe inzichten met betrekking tot het onstaan (en eventueel de behandeling) van neuro-endocriene aandoeningen

De secretieroute in neuro-endocriene cellen

De meeste peptide-hormonen en neuropeptiden worden in eerste instantie aangemaakt in de vorm van een niet werkzaam voorloper-eiwit, het zogenaamde prohormoon Direct na hun synthese komen deze prohormonen terecht in de secretieroute een systeem van intracellulaire, door membranen afgescheiden compartimenten waarmee nieuw-geproduceerde eiwitten kunnen worden vervoerd naar het celoppervlak In de secretieroute doorlopen de prohormonen achtereenvolgens het endoplasmatisch reticulum, het Golgi-apparaat en de secretiegranula Tijdens hun tocht door deze compartimenten worden de prohormonen stapsgewijs omgezet in functionele moleculen Het merendeel van de hierbij betrokken enzymen komt alleen voor in neuro-endocriene cellen en opereert uitsluitend in het zure milieu van de secretiegranula Aldaar worden de prohormonen in kleinere stukken geknipt door prohormoon convertases Deze enzymen grijpen aan op de paren van basische aminozuren waarmee een in het prohormoon opgesloten peptide aan beide zijden wordt geflankeerd Na verwijdering van de basische aminozuren door carboxypeptidases wordt een aantal van de vrijgekomen peptiden biochemisch gemodificeerd Deze modificaties, waaronder amidering en acetylering, bepalen in belangrijke mate de biologische activiteit en stabiliteit van een peptide. Aangezien de prohormonen bij het verlaten van het Golgi-apparaat sterk opeen worden gepakt en in een geaggregeerde toestand terechtkomen in de secretiegranula, bevatten deze laatste compartimenten uiteindelijk zeer hoge concentraties aan peptiden. Secretiegranula fungeren niet alleen als reactievaten voor de aanmaak van peptiden, zij doen tevens dienst als opslagorganellen die hun inhoud pas afstaan nadat de cel daarvoor een specifiek extern signaal heeft ontvangen (dat kunnen ionen, metabolieten, hormonen, neuropeptiden of neurotransmitters zijn) Neuro-endocriene cellen zijn aldus gespecialiseerd om, als het nodig is, in een kort tijdsbestek enorme hoeveelheden van hun producten te mobiliseren en uit te scheiden in de extracellulaire ruimte

Identificatie van genproducten met een rol in de secretieroute

De melanotrope cellen uit de hypofyse van de Zuidafrikaanse klauwpad *Xenopus laevis* vormen een aantrekkelijk modelsysteem voor het bestuderen van de secretieroute op moleculair niveau Deze neuro-endocriene cellen produceren het hormoon MSH (voor Melanofoor-Stimulerend Hormoon), een peptide dat na afgifte aan het bloed in staat is de verspreiding van een donker pigment in de huid van de pad te stimuleren, waardoor deze zwart kleurt Wanneer het dier zich bevindt op een donkere achtergrond worden de melanotrope cellen aangezet tot de productie van grote hoeveelheden MSH, terwijl op een witte achtergrond het productieniveau sterk afneemt MSH wordt gekliefd uit het prohormoon POMC (voor ProOpioMelanoCortine) In eerdere studies is aangetoond dat een stimulatie van de MSH productie in de melanotrope cellen gepaard gaat met een verhoogde activiteit van het POMC gen Op basis van deze gegevens werd een strategie ontwikkeld voor het opsporen van genen die, zoals het POMC gen, actiever zijn in de melanotrope cellen van zwarte padden dan in die van witte dieren Deze strategie gaat uit van het concept dat alleen genen die van belang zijn voor de aanmaak en afgifte van peptide-hormonen gezamenlijk met

het prohormoon tot expressie zullen worden gebracht, terwijl andere genen, wier producten geen deel uitmaken van de secretieroute, niet verhoogd tot expressie hoeven te komen

In Hoofdstuk 2 van dit proefschrift wordt de identificatie van twaalf verschillende genen beschreven die tijdens het proces van achtergrondadaptatie gezamenlijk met het POMC gen tot expressie komen Deze genen werden achterhaald met behulp van een speciaal ontwikkelde techniek (differentiele hybridisatie) waarmee veranderingen in genactiviteit kunnen worden waargenomen Tenminste negen van de twaalf gereguleerde genen bleken te coderen voor eiwitten die op diverse lokaties in de secretieroute werkzaam zijn (voor een schematisch overzicht, zie Fig 2 op pagina 115) Tot deze groep behoren twee enzymen die betrokken zijn bij de proteolytische omzetting van prohormonen, namelijk het ***prohormoon-convertase PC2*** en het ***carboxypeptidase H*** Een derde enzym, het ***cysteine protease ER60***, bevindt zich in het lumen van het endoplasmatisch reticulum en wordt verondersteld aldaar een rol te vervullen in de kwaliteitscontrole en eventuele afbraak van nieuw-geproduceerde eiwitten De producten van vijf andere gereguleerde genen werden geselecteerd voor aanvullende studies

KARAKTERISATIE VAN DE GEIDENTIFICEERDE GENPRODUCTEN

In Hoofdstuk 3 werd de aminozuurvolgorde bepaald van twee genproducten die vroeg in de secretieroute actief zijn Een van deze producten, het translocon-geassocieerde eiwit ***TRAPδ***, maakt deel uit van een eiwitcomplex dat zich in de directe nabijheid bevindt van het translocon een gespecialiseerd kanaaltje in de membraan van het endoplasmatisch reticulum waarmee nieuw-geproduceerde eiwitten worden opgenomen in de secretieroute De functie van TRAPdelta is niet bekend Een vergelijking van de aminozuurvolgorde van *Xenopus* en humaan TRAPdelta gaf aan dat de structuur van het eiwit tijdens de evolutie nauwelijks is veranderd, hetgeen wijst op een belangrijke functie Verder kon worden vastgesteld dat het transcript afkomstig van het TRAPdelta gen, middels een proces dat bekend staat als alternatieve splicing, aanleiding kan geven tot de productie van twee structureel verschillende eiwitten De betekenis van deze vondst is vooralsnog onduidelijk

Het andere in Hoofdstuk 3 beschreven genproduct, het ***'Gp25L-achtig'*** eiwit, betreft een nieuw lid behorend tot een recent ontdekte familie van cargo-receptoren Een aantal leden van deze familie is betrokken bij het verpakken van eiwitten in transportblaasjes die worden afgesnoerd uit de membraan van het endoplasmatisch reticulum en die bestemd zijn voor het Golgi-apparaat Het POMC gen is veruit het meest actieve gen dat in de melanotrope cellen van zwarte padden tot expressie komt en POMC zelf vertegenwoordigt maar liefst 80% van alle nieuw-aangemaakte eiwitten die de secretieroute doorlopen (zie Hoofdstuk 2) Er bestaat dus een reele mogelijkheid dat het 'Gp25L-achtig' eiwit een sleutelrol vervult in het intracellulaire transport van dit prohormoon, d w z van het endoplasmatisch reticulum naar het Golgi-apparaat, of misschien zelfs door de verschillende Golgi-compartimenten tot in de secretiegranula

De in Hoofdstuk 4 beschreven genproducten, ***secretogranine II*** en ***secretogranine III***, behoren tot een groep van negatief geladen eiwitten die uitsluitend wordt aangetroffen in de secretiegranula van neuro-endocriene cellen (de zogenaamde granines) Secretogranine II is een voorloper-eiwit waaruit het neuropeptide secretoneurine wordt gekliefd Uit een vergelijking tussen de aminozuurvolgorden van *Xenopus* en zoogdier secretogranine II kon worden opgemaakt dat dit eiwit mogelijk meer dan een functioneel belangrijk peptide herbergt Of secretogranine II enkel dienst doet als precursor voor peptiden of daarnaast nog een andere functie uitoefent, is niet bekend

Net als secretogranine II, bevat secretogranine III een opvallend hoog aantal paren van basische aminozuren In Hoofdstuk 5 wordt aangetoond dat secretogranine III een substraat vormt voor prohormoon-convertases en tijdens het transport door de secretieroute wordt gekliefd op paren van basische aminozuren Echter, deze klievingsplaatsen zijn, in tegenstelling tot die men aantreft in prohormonen, slecht bewaard gebleven tijdens de evolutie Bovendien bezit secretogranine III structurele kenmerken die beter in overeenstemming zijn te brengen met een intracellulaire functie dan met een rol als peptide-precursor Daarom is het meer waarschijnlijk dat de proteolytische omzetting van secretogranine III dient voor het beeindigen van een door het intacte eiwit uitgeoefende functie, dan voor het vrijmaken van fysiologisch relevante peptiden

De Hoofdstukken 6 en 7 handelen over de aanmaak, het intracellulaire transport en de functionele betekenis van het transmembraan-eiwit ***Ac45*** Dit eiwit bindt aan de proton-pomp waarmee de zuurgraad in de secretiegranula wordt geregeld Het amino-terminale deel van Ac45 bevindt zich in het lumen van de secretiegranula en ontvangt tijdens de aanmaak van het eiwit meerdere suikergroepen Bij actief-secreterende melanotrope cellen verschijnt

Ac45 tijdelijk aan het celoppervlak Na aankomst in de plasmamembraan wordt Ac45 geïnternaliseerd en mogelijk gerecycleerd aan de hand van structurele informatie die zich bevindt in de korte cytoplasmatische staart van het eiwit Het deel waarin deze internalizering-signalen zijn gelokaliseerd vertoont een opmerkelijke structurele gelijkenis met delen uit de cytoplasmatische staarten van andere, in secretiegranula voorkomende transmembraan-eiwitten Deze informatie is van belang voor toekomstig onderzoek aan de mechanismen waarmee nieuw-geproduceerde transmembraan-eiwitten die bestemd zijn voor de secretiegranula correct kunnen worden afgeleverd

Wanneer de productie van Ac45 in een cel kunstmatig wordt verhoogd, ontstaan er defecten in de secretieroute ter hoogte van het Golgi-apparaat (zie Hoofdstuk 6) Dergelijke defecten treden eveneens op wanneer cellen worden blootgesteld aan de stof bafilomycine A1, een specifieke remmer van de proton-pomp Deze resultaten duiden op een nauwe betrokkenheid van Ac45 bij de assemblage en/of de regulatie van de proton-pomp in de secretieroute

Hoofdstuk 8 gaat in op de fysiologische betekenis van de proton-pomp voor het functioneren van neuro-endocriene cellen Na behandeling van *Xenopus* melanotrope cellen met bafilomycine A1 trad er een sterke remming op van de proteolytische processen die normaal gesproken plaatsvinden in de secretiegranula Bovendien werden verschillende, onvolledig gekliefde secretieproducten (waaronder POMC en secretogranine III) op een ongecontroleerde (d w z stimulus-onafhankelijke) wijze uitgescheiden Deze bevindingen tonen aan dat een optimale werking van de proton-pomp in de secretieroute van neuro-endocriene cellen onontbeerlijk is voor zowel de aanmaak als de gereguleerde afgifte van peptiden

Conclusie

Uit de studies beschreven in dit proefschrift kan worden geconcludeerd dat een stimulatie van de productie van peptide-hormonen in neuro-endocriene cellen gepaard gaat met een verhoogde activiteit van een specifieke groep van genen Deze groep blijkt nagenoeg uitsluitend te coderen voor eiwitten die werkzaam zijn in de secretieroute Door een verdere exploitatie van dit gegeven, met behulp van *Xenopus* melanotrope cellen als modelsysteem en de in dit proefschrift gehanteerde experimentele benaderingen, zal onze huidige kennis over het functioneren van de secretieroute in neuro-endocriene cellen worden vergroot

Résumé

Pour le bon fonctionnement d'un organisme multicellulaire, une communication intensive entre les différentes cellules et les différents organes est d'une importance primordiale La transmission de l'information est en grande partie réalisée par les peptides hormonaux et les neuropeptides. Ces messagers sont impliqués dans la régulation de la procréation, la croissance, le métabolisme, et dans la capacité d'un organisme à s'adapter à son environnement. Les peptides hormonaux et les neuropeptides sont produits et secrétés par des cellules endocrines et des neurones peptidiques (que nous appelons communément cellules neuroendocrines). Pour que la production et la libération des messagers puissent s'adapter de façon précise aux besoins physiologiques de l'organisme, les cellules neuroendocrines disposent d'une voie de sécrétion unique. Le but de la recherche décrite dans cette thèse était de repérer systématiquement les gènes dont les produits jouent un rôle dans cette voie de la sécrétion. L'identification et la caractérisation de tels gènes sont nécessaires à une meilleure compréhension des mécanismes respon-sables de la biosynthèse, du stockage et de la libéra-tion vers le milieu extérieur des peptides biologique-ment actifs. Ainsi de nouvelles approches concernant l'origine (et éventuellement le traitement) des affections neuroendocrines pourraient être ultérieurement envisagées.

La voie de sécrétion neuroendocrine

La plupart des peptides hormonaux et des neuropeptides est tout d'abord produite sous forme d'un précurseur protéique non-actif, la prohormone. Directement après leur synthèse, ces prohormones arrivent dans la voie de la sécrétion un système de compartiments intracellulaires séparés par des membranes par lequel les protéines nouvellement synthétisées sont transportées vers la surface cellulaire. Dans ce parcours les prohormones traversent successivement le réticulum endoplasmique, l'appareil de Golgi et les granules sécrétoires Pendant leur passage à travers ces structures cellulaires, les prohormones sont converties progressivement en molécules fonctionnelles. La plupart des enzymes impliquées dans ce phénomène opèrent dans le milieu acide des granules sécrétoires A cet endroit, les prohormones sont clivées en plus petits morceaux par des convertases prohormonales Ces enzymes s'agrippent aux paires d'acides aminés basiques auxquelles est flanqué un peptide enfermé dans la prohormone Après l'élimination des acides aminés basiques par les carboxypeptidases, une partie de peptides ainsi libérée est modifiée biochimiquement. Ces modifications, comme l'amidation et l'acétylation, déterminent de façon importante l'activité biologique et la stabilité d'un peptide Lorsqu'elles sortent de l'appareil de Golgi, les prohormones sont fortement comprimés, arrivant à l'état d'agrégats dans les granules sécrétoires. En consequence, ces dernières compartiments contiennent finalement de très hautes concentrations en peptides Les granules sécrétoires fonctionnent pas seulement comme des réacteurs pour la production des peptides; ils servent également d'organites de stockage qui déchargent leur contenu dès que la cellule a reçu un signal spécifique du milieu extérieur (qui pourra être des ions, des métabolites, des hormones, des neuropeptides, ou des neurotransmetteurs). De cette manière, les cellules neuroendocrines sont spécialisées pour, si nécessaire, mobiliser et libérer des quantités énormes de leurs produits en très peu de temps.

Identfification des gènes impliqués dans la voie de la sécrétion

Les cellules mélanotropes de l'hypophyse du crapaud d'Afrique du Sud, Xenopus laevis, forment un système modèle attrayant pour l'analyse de la voie de la sécrétion au niveau moléculaire. Ces cellules produisent l'hormone HSM (signifiant Hormone responsable de la Stimulation des Mélanophores), un peptide qui après être libéré dans le sang est capable de stimuler la dispersion d'un pigment foncé dans la peau de l'animal, qui la rend noire Quand le crapaud est placé dans un environnement foncé, les cellules mélanotropes sont stimulées à produire de l'HSM en grande quantité Dès que l'animal est placé sur un arrière-plan blanc, le niveau de production diminue fortement L'HSM est clivée par la prohormone POMC (signifiant ProOpioMélanoCortine). Il a été montré dans de précédentes études qu'une stimulation de la production d'HSM dans les cellules mélanotropes est accompagnée d'une expression plus élevée du gène POMC. A partir de ces données, une stratégie a été développée pour repérer des gènes qui, comme le gène POMC, sont plus actifs dans les cellules mélanotropes des crapauds noirs que dans celles d'animaux blancs Cette stratégie part du principe que seuls les gènes jouant un rôle dans la production et la libération des hormones peptidiques sont exprimés en coordination avec la prohormone, tandis que les autres gènes, dont les produits ne participent aucunement à la voie de la sécrétion n'ont pas besoin d'être plus fortement exprimés.

L'identification de douze gènes activés en parallèle avec le gène POMC pendant l'adaptation du Xénope à un arrière-plan noir a été décrite dans le Chapitre 2 Ces gènes ont été découverts à l'aide d'une technique particulière (l'hybridation différentielle) permettant la détection des changements dans l'activité des gènes Au moins neuf de ces gènes régulés semblaient coder pour les protéines faisant partie de la voie de la sécrétion A ce dernier groupe appartiennent deux enzymes impliquées dans la transformation protéolytique prohormonale· la ***convertase prohormone PC2*** et la ***carboxipeptidase H***. Une troisième enzyme, la cysteine protéase ER60, se trouvant à la lumière du réticulum endoplasmique est supposée remplir un rôle dans le contrôle de qualité des protéines nouvellement synthétisées et dans leur éventuel catabolisme. Les produits de cinq autres gènes corégulés ont été sélectionnés pour des études supplémentaires.

CARACTÉRISATION DES GèNES IDENTIFIÉS

Dans le Chapitre 3, la séquence d'acides aminés a été déterminée pour deux produits de gènes régulés étant actifs très tôt dans la voie de la sécrétion. Un de ces produits, la protéine ***TRAPδ***, fait partie d'un complexe protéique situé juste à côté du translocon un canal spécialisé dans la membrane du réticulum endoplasmique avec lequel les protéines nouvellement synthétisées sont déversées dans la voie de la sécrétion. La fonction de la TRAPδ n'est pas connue Une comparaison de la séquence d'acides aminés TRAPdelta chez le Xénope et chez l'être humain indique que la structure de la protéine a très peu changé au cours de l'évolution, ceci indiquant une fonction importante. Par ailleurs, il a été établi que l'ARN Messager (signifiant Acide RiboNucléique) provenant du gène TRAPδ, par un procédé que l'on nomme épissage alternatif, peut donner lieu à la production de deux protéines structurellement différentes Le sens à donner à cette découverte reste encore à éclaircir

L'autre protéine décrite dans le Chapitre 3, la protéine similaire à la ***Gp25L***, concerne un nouveau membre appartenant à une famille découverte récemment de récepteurs cargo Quelques membres de cette famille sont impliqués dans lors de l'emballage des protéines dans les vésicules de transport garrottées à partir de la membrane du réticulum endoplasmique et destinées à aller vers l'appareil de Golgi Le gène POMC est de loin le gène le plus actif qui s'exprime dans les cellules mélanotropes des crapauds noirs et POMC même représente 80% de toutes les protéines nouvellement synthétisées passant par la voie de la sécrétion (voir Chapitre 2) Il existe donc une réelle possibilité que dans les cellules mélanotropes la protéine similaire à la Gp25L joue un rôle clef dans le transport du POMC, c'est-à-dire du réticulum endoplasmique vers l'appareil de Golgi, ou peut-être même par les compartiments de Golgi direction les granules sécrétoires

Les protéines décrites dans le Chapitre 4, à savoir la ***sécrétogranine II*** et la ***sécrétogranine III***, appartiennent à un groupe de protéines électronégatives trouvées sélectivement dans les granules sécrétoires des cellules neuroendocrines (désignées les granines) La sécrétogranine II est une protéine précurseur dont le neuropeptide sécrétoneurine est clivé A partir de la comparaison faite entre les séquences d'acides aminés de la sécrétogranine II du Xénope et de celle des mammifères, on peut conclure que cette protéine renferme plus qu'un peptide fonctionnel important Il n'a pas été identifié si la sécrétogranine II agit uniquement en tant que précurseur protéique ou si elle exerce une autre fonction Tout comme la sécrétogranine II, la sécrétogranine III contient un nombre remarquablement élevé de paires d'acides aminés Dans le Chapitre 5 il a été montré que pendant le transport dans la voie de la sécrétion la sécrétogranine III est clivé en paires d'acides aminés réconnues par les convertases prohormones. Cependant ces emplacements où se déroule le clivage sont, contrairement à ce qu'on a trouvé dans les prohormones, mal conservés pendant l'évolution. De plus les caractéristiques structurelles de la sécrétogranine III correspondent plus à la fonction intracellulaire qu'à un rôle de peptide précurseur. C'est pourquoi il est plus vraisemblable que la transformation protéolytique de la sécrétogranine III pourrait plutôt servir à mettre fin à une fonction exercée par la protéine intacte qu'à la libération des peptides physiologiquement adéquats

Les Chapitres 6 et 7 traitent de la biosynthèse, du transport intracellulaire et de la signification fonctionnelle de la protéine transmembrannaire ***Ac45*** Cette protéine se lie aux pompes à protons, qui fixent le taux d'acidité dans les granules sécrétoires La partie terminale aminee de l'Ac45 se trouve dans la lumière des granules sécrétoires et reçoit pendant la biosynthèse plusieurs groupes d'oses Chez les cellules mélanotropes sécrétant activement, l'Ac45 apparaît de façon temporaire sur la surface cellulaire Après l'arrivée sur la membrane plasmique, l'Ac45 semble être recyclée par des signaux d'intériorisation situés à l'extrémité cytoplasmatique de la protéine. La région dans laquelle ces signaux sont situés présente une similitude structurelle remarquable avec des zones situées sur les extrémités cytoplasmatiques d'autres protéines transmembrannaires éventuelles localisées dans les granules sécrétoires Cette information forme un point de départ important pour l'étude des

mécanismes au cours desquels les protéines transmembrannaires nouvellement produites, destinées aux granules sécrétoires, peuvent être correctement délivrées.

Quand la production d'Ac45 est augmentée de façon artificielle dans une cellule, des défauts se produisent dans la voie de la sécrétion au niveau de l'appareil de Golgi (voir Chapitre 6) De tels défauts apparaissent également quand les cellules sont exposées au composant chimique bafilomycine A1. un inhibiteur spécifique de la pompe à protons. Ces résultats indiquent une directe implication de l'Ac45 dans l'assemblage et/ou la régulation de la pompe à protons dans la route de la sécretion.

Dans le Chapitre 8 la signification physiologique des pompes à protons pour le fonctionnement des cellules neuroendocrines est abordée Après le traitement des cellules mélanotropes du Xénope à la bafilomycine A1, une forte inhibition se manifeste dans les phénomènes protéolytiques se déroulant normalement dans les granules sécrétoires Par ailleurs les différents produits clivés partiellement (dont le POMC et la sécrétogranine III) sont liberés de façon incontrôlée (stimulus-indépendant) dans le milieu extracellulaire. Ces découvertes montrent qu'un fonctionnement adéquat des pompes à protons dans la voie de la sécrétion est d'un intérêt fondamental pour aussi bien la production que la sécrétion régulée des produits de la sécrétion neuroendocrine.

Conclusion

A partir des études décrites dans cette thèse, on peut conclure qu'une stimulation de la production des peptides hormonaux est accompagnée d'une activité accrue d'un groupe spécifique des gènes. Ce groupe semble presque uniquement codé pour des protéines actives à différents endroits dans la voie de la sécrétion Une exploitation ultérieure de ces données, avec l'aide des cellules mélanotropes du Xénope comme modèle système et de l'approche expérimentale utilisée dans cette thèse, nos connaissances actuelles en matière de fonctionnement de la voie de la sécrétion dans les cellules neuroendocrines pourront augmenter considérablement.

Dankwoord

Wetenschappelijk onderzoek, zo heb ik ooit vernomen, is het speuren naar een speld in de hooiberg in de hoop er de knappe dochter van de boer in aan te treffen. Dat men, terwijl men zo bezig is op het laboratorium, voortdurend placht te stuiten op diens tandeloze schoonmoeder werd er toen niet bijvermeld. Bij deze. Behalve het verkondigen van een foute (want seksistische) visie op het onderzoek wil ik graag een aantal mensen bedanken voor het mee helpen zoeken.

Allereerst zijn dat Carola Driessen, Ton Stoffels, Judith Jeucken, Vincent Schoonderwoert, Frank Detmers, Marc Werten en Yvonne Croes die in het kader van een hoofdvak, bijvak of praktijkstage een indrukwekkende hoeveelheid (vriezers en koelkasten vol) werk hebben verricht. Ik vond het hartstikke leuk om met jullie te mogen samenwerken en het spijt me dat dit proefschrift een onvolledig beeld geeft van alle daadwerkelijk geleverde inspanningen. Vincent, ik wens jou veel succes bij het voortzetten van het onderzoek aan de proton-pomp; ik hoop alleen dat dat wonderbaarlijke enthousiasme van je nimmer zal verzuren.

De assistentie die ik gedurende mijn onderzoek heb gehad van Dr. François van Herp, Karel Janssen, Franklin Boekhorst, Tony Coenen, Peter Cruijssen en Ron Engels was eveneens onontbeerlijk, of het nu ging om een nieuwe immunofluorescentie-microscoop (François), een goede borrel (Franklin), een emmertje padden (Ron), of een geslaagde greep uit de voorraadkasten (Karel).

Collette McGrouther, many thanks for your contributions to Chapter 7 of this thesis and the best of luck for the future. Cheers! Dr. Shigeyasu Tanaka, thank you for sharing with me the pleasures of electron microscopy and good Japanese food.

Maarten van Riel, jou ben ik zeer erkentelijk voor al je hulp en goede raad gedurende de eerste fase van mijn onderzoek. Zonder jou kundigheid was mijn vliegende start wellicht geëindigd in een noodlanding.

Eric Jansen, ik hoop dat onze samenwerking nog een tijdje blijft voortduren. De eerste òòòhs en àààhs die wij hebben geslaakt vanachter de immunofluorescentie-microscoop klinken nog na in mijn oren. Ik ben je oneindig dankbaar voor alle steun, gastvrijheid en vriendschap die je me de afgelopen jaren hebt geboden.

Mijn promotor, Prof. Gerard Martens, ben ik veel dank verschuldigd voor de grote vaardigheid en het geduld waarmee hij deze soms weerspannige onderzoeker heeft weten voor te bereiden op een moeilijke toekomst. De talrijke werkbesprekingen die we hadden waren vaak even woest als inspirerend. De rust en zelfverzekerdheid die jij onder alle omstandigheden placht uit te stralen hadden op mij nogal eens de uitwerking van een rode lap. Maar goed, een discussie die bij geen van de betrokkenen iets losmaakt is wat mij betreft dan ook volslagen waardeloos. Gerard, ik zal de aangename en leerzame tijd die ik onder jou hoede heb doorgebracht in Nijmegen niet licht vergeten.

Mijn kamergenoten (in chronologische volgorde) Anneke Braks, Cees Broers, Doug Eib, Dominique de Kleijn, Martin van Horssen, Frank Coenjaerts, Tjadine Holling, Roland Kuiper, Helma van de Hurk, Louis Penning, Frank van Cuppenveld en Susan Janssen dank ik voor het willen delen in de vreugde en het "leed" dat zich tijdens mijn promotieonderzoek heeft voorgedaan. Gezamenlijk vormen jullie een fraai en luisterrijk gezelschap waarover ik met gemak een tweede boekje had kunnen vol schrijven, ware het niet dat de hoge drukkosten me hiervan hebben weerhouden. Wie weet wordt het ooit nog eens een apart hoofdstuk in mijn memoires. Roland en

Martin, extra bedankt voor het aangename gezelschap in de trein, alwaar we in alle rust de wederwaardigheden van iedere dag nog even konden doornemen.

Mijn vader en moeder dank ik voor hun niet aflatende steun en belangstelling bij het tot stand komen van dit proefschrift. Hetgeen waar jullie regelmatig naar hebben gevraagd, namelijk ontdekkingen die de wereld doen schokken, staan er helaas niet in. Maar zeg nu zelf, wie had ooit kunnen bedenken dat ik tijdens mijn exercities in die hooiberg nog een parel ("une perle rare") op zou duiken. Laurence, onzegbaar veel dank voor al het begrip dat je voor mij hebt weten op te brengen tijdens deze beproeving.

Curriculum vitae

Joost Holthuis werd geboren op 23 februari 1964 te Horst. Na het behalen van het VWO diploma aan de Christelijke Scholengemeenschap Jeruzalem te Venray startte hij in 1983 met de studie Biologie aan de Rijksuniversiteit van Utrecht. In 1989 werd het doctoraalexamen afgelegd met als afstudeervakken Ontwikkelingsbiologie (Dr. E.J.J. van Zoelen, Hubrecht Laboratorium, Utrecht), Electronen Microscopische Structuuranalyse (Dr. J. Boonstra en Prof. Dr. A.J. Verkleij, Rijksuniversiteit Utrecht) en Moleculaire Celbiologie (Dr. G.S. Stein en Dr. J.L. Stein, University of Massachusetts, Worcester, USA). Vanaf januari 1990 tot mei 1991 vervulde hij de militaire dienstplicht als ROAG (reserve officier academisch gevormd) op de afdelingen Toegepaste Humane Fysiologie (Dr. J.C. Bakker) en Bloedstolling (Dr. K. Mertens en Dr. J.A. van Mourik) van het Centraal Laboratorium van de Bloedtransfusiedienst te Amsterdam. Vanaf mei 1991 tot mei 1995 was hij als onderzoeker in opleiding verbonden aan de vakgroep Experimentele Dierkunde van de Katholieke Universiteit van Nijmegen. In deze periode werd onder leiding van Prof. Dr. G.J.M. Martens gewerkt aan een door NWO gefinancierd onderzoeksproject (NWO-PIONIER), hetgeen heeft geresulteerd in dit proefschrift. Sinds augustus 1996 is hij in het kader van een Training and Mobility Research Fellowship van de Europese Commissie werkzaam als post-doctoraal medewerker bij Dr. H.R.B. Pelham, MRC Laboratory of Molecular Biology, Cambridge, Engeland.

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