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

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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. SgII was synthesized as a 63- 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 SgII 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 SgII is cleaved at two dibasic sites, namely Lyse-Arg62 and Arg237-Arg238. Pulse-chase incubations of lobes with Na2[35S]SO4 showed that SgII is sulfated in the trans-Golgi network before it is processed. Finally, SgII 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) and CgB (secretogranin I, Sg) 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 ACT20 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, SgII 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.
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 convertase 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 protein composed of Xenopus SgIII residues 8 to 437 with a hexahistidine tail at its amino terminus. Following purification of the protein by Ni²⁺-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 Analyses—NILs from black-adapted Xenopus were dissected and preincubated in incubation medium (IM, 112 mM NaCl; 2 mM KCl; 2 mM CaCl₂; 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 μCi/ml [³⁵S]methionine for 15 min at 22 °C. Subsequent chase incubations were in IM containing 5 mM methionine. Pulse labeling of sulfated proteins was achieved by incubating lobes in 1 M containing 1.7 μCi/ml [³⁵S]methionine and 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mg/ml soybean trypsin inhibitor. Homogenates were centrifuged (10,000 × g, 7 min at 4 °C), supplemented with 1 volume of 10% SDS and 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 BamHI fragment of Xenopus SgIII cDNA clone X8596–1 encoding the entire protein (14) was subcloned into the BamHI site of the eukaryotic expression vector pCDNA3 (Invitrogen, San Diego, CA). A 2.7-kb HindIII-EcoRV fragment of plasmid pBP3 covering the entire open reading frame of human PC1 (a generous gift of Dr. A. Roebroeck, University of Leuven) was subcloned into the HindIII/EcoRV 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., Chatsworth, CA). For this purpose, a 1.5-kb BamHI fragment of Xenopus SgIII cDNA clone X8596 (14) was ligated into the BamHI site of the prokaryotic expression vector pQE-30. This allowed the production of recombinant protein comprising of Xenopus SgIII residues 8 to 437 with a hexahistidine tail at its amino terminus. Following purification of the protein by Ni²⁺-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.

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). These findings suggest that the melanotrope constitute the primary site of SgIII production in the NIL. When NILs from black animals were pulsed for 15 min with [³⁵S]methionine 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. This result indicates that SgIII is partially glycosylated at Asn⁶⁰, the single putative acceptor site for N-linked glycosylation found within its primary sequence (Fig. 1). Moreover, it can be concluded that...
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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, 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 Tyr110 (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 NaN3, 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 41- 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.

Touzo et al. (27) showed that in rat pheochromocytoma PC12 cells the sulfation and subsequent sorting of Sgl 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.
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**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[^35S]-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 by Na[^35S]SO₄ 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[^35S]-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 Xenopus 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[^35S]-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.

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 carboxy-terminal of the N-linked glycosylation site (Asn28). We noticed that upon cleavage of the 48-kDa product, 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.

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Fig. 7. Identification of processing sites in SgIII. Amino acid substitutions at two potential dibasic cleavage sites in Xenopus SgIII (KR69 → TS69 and RR238 → SS238) 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[35S] label, and radiolabeled proteins were immunoprecipitated from the incubation media using anti-SgIII antisera. Migration positions of SgIII precursor forms and cleavage products are indicated.

![Diagram of SgIII processing](image)

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 SgI (3–5), is unlikely. First, the majority of potential dibasic cleavage sites in SgIII is 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. 8. Schematic representation of SgIII processing in Xenopus melanotrope cells. Data from Figs. 2, 4, 5, and 7 are summarized. The aminoterminal (partially N-glycosylated) fragment formed after cleavage at the KR69 site was not detected in our pulse-chase experiments, probably because it is not recognized by the antisera. ER, endoplasmic reticulum; TGN, trans-Golgi network; SG, secretory granules. Other designations are as in Fig. 1. See text for details.

![Diagram of SgIII processing](image)

![Diagram of SgIII processing in mouse endocrine cell lines](image)
(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 (9) 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.

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