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Secretogranin III Directs Secretory Vesicle Biogenesis in Mast Cells in a Manner Dependent upon Interaction with Chromogranin A

Prerna Prasad,1,8 Angel A. Yanagihara,2‡ Andrea L. Small-Howard,2* Helen Turner,2,3*† and Alexander J. Stokes2*‡†

Mast cells are granular immunocytes that reside in the body’s barrier tissues. These cells orchestrate inflammatory responses. Proinflammatory mediators are stored in granular structures within the mast cell cytosol. Control of mast cell granule exocytosis is a major therapeutic goal for allergic and inflammatory diseases. However, the proteins that control granule biogenesis and abundance in mast cells have not been elucidated. In neuroendocrine cells, whose dense core granules are strikingly similar to mast cell granules, granin proteins regulate granulogenesis. Our studies suggest that the Secretogranin III (SgIII) protein is involved in secretory granule biogenesis in mast cells. SgIII is abundant in mast cells, and is organized into vesicular structures. Our results show that over-expression of SgIII in mast cells is sufficient to cause an expansion of a granular compartment in these cells. These novel granules store immunomodulatory mediators that are released in response to physiological stimuli, indicating that they function as bona fide secretory vesicles. In mast cells, as in neuroendocrine cells, we show that SgIII is complexed with Chromogranin A (CgA). CgA is granulogenic when complexed with SgIII. Our data show that a novel non-granulogenic truncation mutant of SgIII (1–210) lacks the ability to interact with CgA. Thus, in mast cells, a CgA-SgIII complex may play a key role in secretory granule biogenesis. SgIII function in mast cells is unlikely to be limited to its partnership with CgA, as our interaction trap analysis suggests that SgIII has multiple binding partners, including the mast cell ion channel TRPA1. The Journal of Immunology, 2008, 181: 5024–5034.

The granin family (Chromogranin A (CgA),1 Chromogranin B (CgB), Secretogranin (Sg) II, and the less well studied Secretogranins III-VII) comprises a group of acidic proteins that are present in the secretory granules of a wide variety of endocrine and neuro-endocrine cells (1–4). The molecular functions of the granins are poorly understood. There is evidence that granins may be the precursors of biologically active peptides (3, 5–9). However, granins cannot be considered simply as precursors of granule cargo. They may act as helper proteins in the packaging of peptide hormones and neuro-peptides (3, 5–9). However, granins cannot be considered simply as precursors of granule cargo. They may act as helper proteins in the packaging of peptide hormones and neuro-peptides (3, 5–9). The EF-hand containing CgA and CgB may also act as high-calcium, low affinity, calcium binding proteins that are closely associated with calcium-regulated intracellular ion channels (10–13). Moreover, emerging evidence suggests that these proteins play an active role in directing the biosynthesis of secretory granules. This biological role for granins has been proposed on the basis of work in neuroendocrine cells, where several granin proteins are expressed and have been shown to cooperate in the targeting of secretory vesicle biogenesis (14, 15). This cooperation is exemplified by the interaction between SgIII and CgA (15, 16). In this study, an intermolecular interaction between the two granins is needed for their targeting to secretory granules. Courel and coauthors (15) have described a “granulogenic domain” in CgA that promotes the biogenesis of secretory granules probably through exerting control over cholesterol sequestration and the dynamics of intracellular vesicle budding/fusion. The potential contribution of granins to the granularity of secretory immunocytes has not been addressed.

Mast cells are densely granular, secretory leukocytes that reside in tissues (17, 18). In response to a variety of stimuli, including immunological challenge, exposure to secretagogues and exposure to physical stimuli, mast cells release preformed, and de novo synthesized, inflammatory mediators into their surrounding tissues. The preformed mediators are housed within membrane-delimited secretory granules. The contents of mast cell secretory granules include inflammatory mediators such as histamine, serotonin, platelet activating factor, some cytokines (TNF-α), and matrix-active proteases of the chymase and tryptase families (17, 18). Together, these mediators exert diverse proinflammatory effects upon tissue. These include the promotion of vasodilation, induction of inflammatory pain, and recruitment of other immunocytes to the inflammatory site. The central role of these granules in inflammation has led to an intense interest in the signaling

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4 Abbreviations used in this paper: CgA, chromogranin A; CgB, chromogranin B; Sg, secretogranin; w/v, weight-to-volume ratio; RT, room temperature. Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/52.00
mechanisms that control granule exocytosis, because targets for anti-
-inflammatory therapeutics could lie within this system. Moreover, the
mechanisms that control the biogenesis and abundance of these gran-
ules are also of therapeutic interest, but remain poorly studied.

Mast cell granules are structurally, and functionally, related to the
dense core vesicles of neuro-endoctrine cells and neurons.

There has been no published analysis of the expression, or func-
tion, of granins in the mast cell system, although their involvement in
secretory vesicle biogenesis in other neuroendocrine cells and
neurons has been established (6, 7, 14, 19–23). In the current re-
port, we tested the idea that SgIII drives the production of mast cell
secretory granules. Moreover, we propose that its interaction with
CgA is critical to this biological role. Although SgIII and CgA
proteins are abundant in various mast cells, we show that SgIII is
depleted from the cytosol after stimulation of mast cells via either
pharmacological or immunological methods. Moreover, SgIII lev-
els in mast cells recover during the poststimulation refractory phase
during which mast cells replenish their granule population.

Over-expression of SgIII in mast cells causes an increase in the
number of mast cell granules, and thus when SgIII-expressing mast
cells are stimulated we note an increase in the release of proin-
flammatory mediators. Our results also confirm that CgA interacts
with SgIII via a specific granulogenic domain and that the granu-
logenic activity of SgIII in mast cells depends upon its ability to
form a functional interaction with CgA. Although the enigmatic
granin proteins should therefore be considered as components of the
secretory apparatus in mast cells, our data also suggest poten-
tially wider functions as regulators of organelle channels.

Materials and Methods

Cell cultures

RBL2H3, HEK293, and PC12 cells were maintained in DMEM (Mediat-
ich), 10% FBS (Mediatech), and 2 mM glutamine (Invitrogen) in humid-
ified 5% CO2 at 37°C. LAD2 cells were a generous gift from Dr. A. Kir-
shenbaum (National Institute for Allergic and Infectious Diseases,
Bethesda, MD, Ref. 24). LAD2 cells were maintained in DMEM with 2%
FBS, 2 mM Gln and rSDF at 100 ng/ml (PeproTech). Growth medium for
stably transfected cells were supplemented with appropriate antibiotics;
200 μg/ml zeocin (Invigen), 5 μg/ml blasticidin (Invitrogen), 200 μg/ml
hygromycin (Invogen).

cDNA constructs, transient transfection, and stable cell lines

Full-length human Secretogranin III cDNA (Open Biosystems) was ob-
tained by PCR and cloned into the pcDNA4/TO and pcDNA5/TO GFP (A.
Stokes, unpublished observations) vectors using NotI/NotI or EcoRV/NotI
digestion respectively. A V5 epitope tag was incorporated into the carboxy-
terminal of SgIII during this PCR. A truncated SgIII cDNA (residues
1–210) was obtained by PCR and subcloned into pcDNA5/TO empty vec-
tor and pcDNA5/TO GFP vector (A. Stokes, unpublished observations).
All constructs were verified by sequencing. The pcDNA5/TO TRPA1 con-
struct was generated as described previously (25).

For transient transfection, HEK293 cells were seeded and grown until
50% confluent. All cDNA was purified using a QIAfilter Plasmid Maxi Kit
(Qiagen). The cDNA quality was evaluated using a spectrophotometer and
was used in transfection only if O.D. 260/280 > 1.7. HEK293 cells were
transiently transfected using reagent LT1 (Mirus). Serum-free DMEM (100
μl) and LT1 reagent (10 μl) were added together and vortexed for 2 min.
The mixture was then left at room temperature for 15 min. After 15 min, 7.5
μg of V5-SgII cDNA was added together and the mixture was mixed and gently.
After 15 min at room temperature (RT), the mixture was added to the cells in
a dish, the medium was aspirated and swirling the plate. Cells were incubated for
48–72 h and then harvested and lysed for Western blots.

For stable transfection, the RBL2H3 and PC12 cell lines were electro-
porated with V5-SgIII, V5-SgIII-GFP, V5-ΔSgIII, and V5-ΔSgIII-GFP
constructs. Cells were electroporated with 15 μg of DNA in 500 μl of 1×
DMEM, 10% FBS, and 2 mM glutamine at 280 V and 950 μF in a 4 mm
path length cuvette (BioRad). Clonal cell lines were selected by limiting
dilution in 96-well plates. Selection was conducted in zeocin (V5-SgIII
construct) or hygromycin (V5-SgIII-GFP, V5-ΔSgIII, and V5-ΔSgIII-GFP
constructs).

For production of TRex HEK293 cells with inducible expression of
SgIII constructs, parental cells were electroporated with 15 μg of plasmid
DNA in a 500 μl volume of DMEM/10% FBs2/m glutamine. Electro-
porations were performed at 280 V/950 μF in a 4 mm path length cuvette
(BioRad). Clonal cell lines were selected by limiting dilution in 400 μl/ml
zeocin and 5 μg/ml blasticidin. After electroporation, a brief (12–14 h)
recovery period for the cells was used such that no discernable cell division
took place. Recovered cells were trypsinized to a single cell suspension to
disperse any potential daughter cell pairs. The trypsinized cell suspension
was subjected to a limiting dilution (plated in a 96-well plate at less than
one cell per well, i.e., 0.5 cells per 250 μl medium). Each well is there-
fore unlikely to have more than one cell, but of course across the 960 wells
plated there will occasionally be wells where more than one cell is dis-
pensed. After 3 wk, typically 5–15 wells on a given 96-well plate had
visible colonies (i.e., colonies at the 100–200 cell level). Before these
colonies were harvested and transferred to a larger culture vessel, the wells
were inspected microscopically and any wells in which more than one
colony appeared were discarded. After harvesting, great care was taken to
avoid cross-contamination of the clonal cell lines. Depending on the effi-
ciency of the transfection, we observed between five to fifteen clonal col-
onies per 96-well plate. Between five and ten individual clones were se-
lected at random, expanded, and cryopreserved. These clones were
screened for expression of the transfected gene by immunoprecipitation and
Western blot. Experiments were performed with 8 independent, ran-
domly selected clones, but results from one clonal cell line are presented
throughout this article.

Antibodies and stimuli

Affinity-purified goat polyclonal anti-Secretogranin III (Santa Cruz Bio-
technology) was used to study SgIII protein expression. Rabbit polyclonal
anti-Secretogranin I (Abcam) and affinity purified goat polyclonal anti-
Secretogranin II (Santa Cruz Biotechnology) were used to perform compa-
raditive studies. Rabbit monoclonal anti- serotonin (Sigma-Aldrich) and
mouse monoclonal anti-V5 (Serotec) were used for immunostaining and
immunoblotting. Mouse anti-CgA (Spring Bioscience) was used to study
the interaction of SgIII and CgA. Anti-FLAG M2 was from Sigma-Aldrich.
The anti-TRPA1 has been described previously (26). IgE anti-DNP, DNP-
BSA, PMA, and ionomycin were all from Sigma-Aldrich.

Immunoprecipitation and Western blotting

Cells were pelleted (2000 × g, 2 min) and washed once in PBS at room
temperature. Approximately 105 cells were lysed on ice for 20 min in 500
μl of lysis buffer (100 mM HEPES (pH 7.4), 150 mM NaCl, 80 mM NaF,
20 mM iodoacetamide, 50 mM PMSF (phenyl methyl sulfonyl fluoride),
500 μg/ml aprotinin, 1.0 mg/ml leupeptin, and 2.0 mg/ml chymostatin, 1%
(weight-to-volume ratio, w/v) IGEPAL) dissolved in distilled water. Ly-
sates were then clarified (10000 × g, 5 min at 4°C) to remove nuclei. For
preparation of total protein, lysates were acetone precipitated and again
centrifuged at 10 000 × g for 5 min at 4°C. For immunoprecipitation,
supernatants were tumbled (4°C/2 h) with the indicated Ab, followed by
capture of immunocomplexes using ~50 μg Protein A conjugated to agar-
ose beads (Sigma-Aldrich). Excess liquid was aspirated with a Hamilton
syringe. Samples were then boiled (8 min) in reducing sample buffer (20%
(w/v) glycerol, 62.5 mM Tris-HCl (pH 6.8), 0.05% (w/v) b-mercapto-
ethanol, 2 mM 2-mercapto-ethanol) and then resolved by 10% SDS-PAGE
for 16 h.

After being resolved on SDS-PAGE, the proteins were electro-trans-
ferred to Polyvinylidine fluoride membrane (1.4 A for 180 min in 25 mM
Tris base-HCl, 192 mM glycine (pH 8.63)). Membranes were blocked us-
ing 5% (w/v) nonfat milk (1 h at RT). Membranes were incubated with
primary Abs (Ab dissolved in TBS, 0.05% Tween, 0.05% NaN3, and
0.5% (w/v) BSA) for 16 h at 4°C. Membranes were washed with TTBS (4 × 5
min) and incubated with secondary Abs (HRP-conjugated IgG) for 1 h. The
membranes were washed with TTBS (4 × 5 min) and then incubated with
ECL Plus Western Blotting Detection Reagent (Amersham Biosciences).
Emitting light was then detected using autoradiography film (Kodak Sci-
entific Imaging).

Protein determination assay

Protein samples were matched for total protein levels on the basis of a
colorimetric protein determination assay. The D2 Protein Determination Kit
Tritiated serotonin release assay
Adherent RBL2H3 and RBL-SgIII (1.25 × 10^6 cells/cm^2) were incubated with 1 nM IgE-anti-DNP and 0.5 μM [3H]serotonin in 16 h at 37°C. Monolayers were then washed with normal medium at 37°C (2 ×), and cells were incubated with 10 μl of indicated stimuli (1 μM PMA, 10 μM ionomycin, or 10% Triton X-100) or vehicle (PBS) in 190 μl medium for 1 h at 37°C. Reactions were stopped on ice. The supernatant was centrifuged (10,000 × g for 30 s) and 100 μl of supernatant was counted in liquid scintillation mixture (Scintiverse BD mixture, Fisher Scientific). Release was calculated from the supernatant and compared to the Triton X-100 releasable signal. Signals from FceRI stimulation approached 30–50% of the Triton X-100 releasable [3H] serotonin.

Immunofluorescence
Cells were seeded onto glass coverslips. The cells were fixed with pure methanol at −20°C for 5 min. Then they were washed with PBS, and then blocked with 0.7% (w/v) Fish skin gelatin (prepared in PBS) for 20 min. The cells were incubated with primary Ab diluted in the blocking reagent (0.7% (w/v) fish skin gelatin) for 1 h. The cells were washed four times with PBS and then incubated with Alexa fluorophore conjugated Abs (Invitrogen) diluted in the blocking reagent for 20 min. Cells were washed four times with PBS and then finally the cover-slips were dipped in water and mounted on microscope slides using Crystal mount (Biomedia). Imaging was performed with an Olympus IX70 fluorescence inverted microscope with quadruple dichroic filter block and excitation filter set 88000 (Chroma), connected to an F-view monochrome CCD camera.

Toluidine blue staining
Cells were seeded onto glass cover-slips. The cells were first fixed with pure methanol at −20°C for 5 min. Then they were washed with PBS, and then blocked with pure PBS (prepared in PBS) for 20 min. The cells were incubated with primary Ab diluted in the blocking reagent (0.7% (w/v) fish skin gelatin) for 1 h. The cells were washed four times with PBS and then incubated with Alexa fluorophore conjugated Abs (Invitrogen) diluted in the blocking reagent for 20 min. Cells were washed four times with PBS and then finally the cover-slips were dipped in water and mounted on microscope slides using Crystal mount (Biomedia).

Macroarray analysis
Total RNA was extracted from adherent RBL2H3 cells that were either left untreated or exposed to IgE (1 μg/ml for 16 h followed by two washes) followed by 250 ng/ml DNP-BSA for the time indicated. Total RNA was purified using a Nucleospin RNA II kit according to the manufacturer’s instructions (BD Biosciences). Before hybridization on the BD Atlas Rat 1.2 Arrays, total RNA samples were labeled with [α-33P]dATP using the Atlas Pure Total RNA Labeling System (BD Biosciences). A set of matched BD Atlas Rat 1.2 Array blots were hybridized (68°C, 16 h). Membranes were washed four times in 2 × SSC/0.1% SDS (68°C, 15 min), then once in 0.1 × SSC/0.5% SDS (68°C, 15 min) and once in 2 × SSC (RT, 5 min). Blots were exposed to storage phosphor screens (Packard Biosciences) for 8 days. Data were captured using a Cyclone System (Packard Biosciences). BD Atlas Image 2.7 (BD Biosciences) software was used to assign genes to specific coordinates on the array membranes, to correct for background counts, to establish the threshold for positive gene expression (2 × background level counts + 10%), and to normalize between-blots hybridization intensity differences based on the median-signal intensity. Per gene differences in mRNA levels were expressed as a fold-change relative to the corresponding unstimulated controls.

Single cell calcium assay
Fura-2 AM ester (Molecular Probes) loading of intact cells was performed by incubating cells for 45 min in a standard modified Ringer’s solution of the following composition (in mM): NaCl 145, KCl 2.8, CaCl2 10, MgCl2, 2.0, glucose 10, HEPES - NaOH 10 (pH 7.4), supplemented with 5 μM fura-2 AM (Molecular Probes). Cytosolic calcium concentration was monitored at a rate of 5 Hz with a photomultiplier-based system using a monochromatic light source that excited fura-2 at 360 and 390 nm for 20 ms each. Emission was detected at 450–550 nm with a photomultiplier whose analog signals were sampled and processed by the X-Chart software package (HEKA Electronics). Fluorescence ratios were translated into free intracellular calcium concentration based on calibration parameters derived from pitch-clamp experiments with calibrated calcium concentrations. Oscillatory was maintained at 300 mM. Local perfusion of individual cells was achieved through a wide-tipped, pressure-controlled application pette (3-μm diameter).

Analysis
Experiments are n = 3 unless otherwise indicated. Results are shown as the mean ± SD. Statistical significance was determined based on a two-way ANOVA (Student’s t test). Adjacent to data points in the respective graphs, significant differences were recorded as follows: *p < 0.05; **p < 0.01; *** p < 0.001; no symbol, p > 0.05.

Results
Secretogranin III and its partner Chromogranin A are expressed in mast cells
SgIII and CgA are considered to be components of the secretory vesicle biogenesis system in neuroendocrine cells. Because the secretory vesicle system in mast cells shares multiple features with the neuroendocrine system we asked whether these proteins are expressed in mast cells. We assayed the presence of SgIII and CgA by Western blot in rodent and human mast cells. The data in Figs. 1, A and B show an expression survey for Secretogranin III and CgA. IGEPAL lysates were prepared from the following mast cell lines RBL2H3 (rat basophilic leukemia), P815 (mouse mastocyteoma), mouse bone marrow derived mast cell, and LAD2 (human mast cell leukemia). LAD2 cells exhibit a high MW (>75 kDa) form of SgIII. This variation in apparent MW of SgIII may reflect an, as yet undefined, posttranslational modification. The size variance is unlikely to be attributable to species differences because HEK are derived from humans, as are the LAD2. The neuroendocrine PC12 cells were used as positive controls for SgIII expression (Fig. 1C). SgIII protein is present in each of the mast cell lines tested. SgIII was also detected in CAD catecholaminergic neurons, RIN5MF pancreatic β cells and the Jurkat T lymphocyte line (data not shown). We also noted that in some cell lines SgIII migrated as a single species on SDS-PAGE (Fig. 1A), supporting the data of Ottiger and coauthors (25) who suggested that SgIII, unlike other granin family members, is not inevitably proteolysed to form bioactive peptides. A high MW (75–80 kDa), uncleaved, version of SgIII that binds to CgA has recently been reported by the Hoshaka laboratory (27).

Secretogranin III is depleted from mast cells following stimulation
We hypothesized that SgIII is a component of the mast cell secretory granule. We reasoned that if so, then SgIII levels in the mast cells may be altered after stimulation of mast cells using agents that evoke secretory responses. We assayed the levels of SgIII protein in mast cells treated with secretion-evoking pharmacological stimuli or antigenic stimuli. The presence of SgIII was assessed by Western blot of matched cytotoxic extracts from unstimulated and stimulated RBL2H3. Our results show that resting cells express SgIII protein, which is markedly depleted at 1–2 h after stimulation with PMA/Ionomycin (Fig. 2A) or via antigenic cross-linking of the FceRI (Fig. 2B). Control experiments suggest that this is not a protein degradation event as secreted SgIII can be detected by Western blot in the extra-cellular milieu of resting mast cells (Fig. 2C). We have also detected full-length endogenous SgIII in the supernatants of resting RBL2H3 but at very low levels (data not shown). SgIII protein starts to reappear at 4 h after stimulation and this expression stabilizes by 24 h after stimulation, and re-attains resting levels (Fig. 2, A and B). The depletion in SgIII levels is consistent with the idea that this protein is a component of the mast cell secretory granule. Other granule components such as chymase and tryptase show a distinctive pattern of depletion and then replenishment in activated mast cell populations (28). SgIII appears to behave in a similar fashion. Moreover, the recovery in...
SgIII protein is apparently due to a transient transcriptional up-regulation of the SgIII gene (Fig. 2D) following mast cell stimulation.

Secretogranin III over-expression causes expansion of the secretory vesicle compartment in mast cells

The granulogenic properties of granins have been established in neuroendocrine cells on the basis that over-expression of these proteins is necessary and sufficient to cause the appearance of a novel secretory vesicle compartment. We evaluated the secretory compartment of RBL2H3 mast cells with stable over-expression of SgIII using several methods. First, we used an immunofluorescence approach in RBL2H3 mast cells, HEK cells, and PC12 neuroendocrine cells. We compared each of these cell types with a matched clonal cell line in which V5-SgIII is over-expressed (Fig. 3A). Interestingly, we observed that V5-SgIII over-expression in each cell type was accompanied by the appearance of a prominent vesicle population that could be immunodecorated with anti-V5 (Fig. 3A). This vesicular organelle was most prominent in the secretory RBL2H3 and PC12 cells, while the SgIII-associated structures in HEK293 were relatively smaller and less numerous.

The vesicular structures associated with SgIII over-expression in RBL2H3 cells did not immunodecorate with Abs toward markers of the Golgi apparatus (anti-Golgi-58K), lysosomes (anti-LAMP2), endoplasmic reticulum (anti-calreticulin), or peroxisomes (anti-PMP70) (data not shown). We therefore hypothesized that these structures may represent secretory granules in mast cells. Because serotonin is a marker for secretory granules (29, 30) we asked whether SgIII-associated vesicles could be counterstained with an anti-serotonin Ab. We noted that these vesicles contained significant serotonin, which colocalized with V5-SgIII, indicating that they may be bona fide granules capable of packaging inflammatory mediators in mast cells (Fig. 3B). We also detected some subplasma membrane staining with anti-serotonin, which may reflect its presence in a subpopulation of small, endogenous, exocytotic vesicles. Further, we performed staining on mast cells with toluidine blue, a metachromatic dye that detects the numerous sulfated groups in mast cell granules. RBL2H3 with over-expression of SgIII displayed a large number of toluidine blue-positive granules that were not observed in control cells (Fig. 3C), again suggesting that SgIII over-expression leads to an increase in the secretory vesicle load of mast cells.

SgIII over-expression confers a functional increase in the intensity of mast cell proinflammatory responses

With the idea that V5-SgIII over-expressing granules could package inflammatory mediators such as serotonin, we hypothesized that these V5-SgIII over-expressing mast cells may exhibit enhanced secretion of proinflammatory mediators. In V5-SgIII over-expressing RBL2H3 resting cells, there is a significant increase in basal efflux of [3H]serotonin (Fig. 3D). Together with the fact that the detergent-releasable amount of [3H]serotonin is also enhanced in V5-SgIII over-expressing cells (Fig. 3E), these data suggest that there may be an expansion of the compartment storing [3H]serotonin in these cells. Indeed, the V5-SgIII cells exhibit enhanced secretory responses to pharmacological stimulation (Fig. 3F), suggesting that the vesicles arising from V5-SgIII over-expression may be functionally equivalent to mast cell granules. The potential for V5-SgIII over-expression to induce apparent granulogenesis in

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**FIGURE 1.** SgIII and CgA protein levels in mast cells. A, SgIII protein in mast cells. IGEPAL lysates were prepared from RBL2H3 (rat mast cell), P815 (mouse mast cell), BMMC (mouse bone marrow derived mast cell), HEK (human endothelial cells), and LAD2 (human mast cells). Total protein was recovered by acetone precipitation and was resolved by 10% SDS-PAGE. After electrotransfer and blocking, Western blotting was performed with 0.26 μg/ml anti-SgIII. Molecular mass shown in kDa. Predicted molecular mass of SgIII is 68 kDa. B, CgA protein levels in mast cells. IGEPAL lysates were prepared as described above and Western blotting was performed with 0.4 μg/ml anti-CgA. Molecular mass shown in kDa. Predicted molecular mass of CgA is 68–75 kDa. C, Positive control Western blots for CgA and SgIII in the neuroendocrine cell line PC12. IGEPAL lysates were prepared as described above and Western blotting was performed with 0.26 μg/ml anti-SgIII or 0.4 μg/ml anti-CgA.
mast cells may speak to a role for endogenous SgIII in directing this process physiologically.

CgA is a functional partner of SgIII in mast cells

Taken together, our data indicate similarities in the role of SgIII in two major physiological secretory systems: the neuroendocrine cells and mast cells. In neuroendocrine cells, over-expression of CgA has been shown to be granulogenic, as is over-expression of SgIII. Moreover, in neuroendocrine cells the granulogenic properties of CgA are critically dependent upon its interaction with SgIII. Hosaka and coauthors (16) showed that the SgIII (214–373) and CgA (48–111) domains mediate...
FIGURE 3. A, Immunofluorescence analysis of SgIII over-expressing cells and wild-type cells. i, RBL2H3 cells were grown on cover slips and fixed with methanol. After blocking, the cells were stained with anti-V5 (0.2 μg/ml) and an Alexa 568 nm-conjugated secondary Ab (pseudo-colored red). Nuclei were stained using Hoechst 33342 (100 μg/ml, pseudo-colored blue). ii, Overexpression of V5-SgIII in RBL cells. iii, HEK-SgIII cells without induction and iv with tetracycline induction. v, Represents PC12 cells and vi, PC12-SgIII. Cells in panels iv were induced with tetracycline (1 μg/ml) overnight. The left panels are the nuclei, middle panels represent the V5 stained vesicles, and the right panels are the overlaid images. B, SgIII over-expression results in vesicle formation in mast cells. Control RBL2H3 (top panel) and RBL2H3 with constitutive overexpression of V5-SgIII (bottom panel) were grown on glass cover slips and fixed in methanol. After blocking, V5-tagged proteins were visualized using anti-V5 (0.2 μg/ml) and an Alexa 568 nm-conjugated secondary Ab. Serotonin was labeled using anti-serotonin (84 μg/ml) and an Alexa 488 nm-conjugated secondary Ab. Nuclei were stained using Hoechst 33342 (100 μg/ml). Merged image with anti-V5 (pseudo-colored red), anti-serotonin (pseudo-colored green), and Hoechst 33342 (pseudo-colored blue). C, SgIII overexpression results in an increased number of toluidine blue-positive granules in mast cells. Left panels, Control RBL2H3 (a), RBL2H3 with constitutive over-expression of V5-SgIII (b). Right panels, Control HEK (a) and HEK with constitutive over-expression of V5-SgIII (b). Cells were grown on glass cover slips and fixed in methanol. After washing, the cells were flooded with 0.5% toluidine blue (pH 3.14) for 30 min. The cells grown on coverslips were washed and mounted to be observed under the microscope. The RBL2H3-V5-SgIII cells show the presence of an increased number of granules as compared with control RBL2H3 cells. D, Basal secretion of [3H]serotonin from V5-SgIII overexpressing mast cells. The y-axis represents the average scintillation counts per min in 100 μl of supernatant from a culture of 1 × 10⁵ mast cells and SgIII over-expressing cells. Counts from triplicate wells were averaged. Error bars represent SD around the mean. A Student’s t test was performed to calculate the p values. +, p > 0.5; **, p < 0.01; ***, p < 0.001. E, Enhanced [3H]serotonin content in V5-SgIII overexpressing mast cells. The y-axis represents the average scintillation counts per min in 100 μl of supernatant from a culture of 1 × 10⁵ mast cells and SgIII over-expressing cells. Counts from triplicate wells were averaged. Error bars represent SD around the mean. A Student’s t test was done to calculate the p values. +, p > 0.5; **, p < 0.01; ***, p < 0.001. F, Enhanced degranulation of [3H]serotonin in V5-SgIII over-expressing mast cells. The cells were stimulated with PMA (1 μM, 1 h) and iono (ionomycin, 10 μM, 1 h). The y-axis represents the average scintillation counts per min in 100 μl of supernatant from a culture of 1 × 10⁵ mast cells and SgIII over-expressing cells. Counts from triplicate wells were averaged. Error bars represent SD around the mean. A Student’s t test was performed to calculate the p values. +, p > 0.5; **, p < 0.01; ***, p < 0.001.
the functional interaction between CgA and SgIII in mouse corticotrope-derived AtT-20 cells. We asked whether SgIII and CgA interact functionally in the mast cell system, and we produced a truncated form of SgIII that lacks the putative CgA-interacting (and granulogenic) domain (Fig. 4A). We were able to coimmunoprecipitate CgA and SgIII in mast cells (Fig. 4B). Our data indicate that removal of residues 211–468 of SgIII (Fig. 4A) resulted in a loss of interaction with CgA (Fig. 4B).

We asked whether full-length and the SgIII (1–210) truncation mutant were equivalent in their ability to induce the formation of a novel granule population in mast cells. RBL2H3 were transfected with either V5-SgIII or V5-SgIII (1–210). The presence of SgIII-associated vesicle population in the cytosol of these cells was assessed by anti-V5 immunofluorescence. These data (Fig. 5) show that the over-expression of the truncated V5-SgIII (1–210) did not result in the appearance of a prominent vesicular compartment. Diffuse, faint staining of the truncated V5-SgIII was observed, which quenched rapidly. Moreover, a GFP-tagged version of SgIII (1–210) with truncation of CgA binding domain showed a lack of vesicular organization (data not shown). Thus, the granulogenic potential of SgIII in mast cells appears to be dependent upon an intact SgIII C terminus (residues 211–468), which is the region that mediates interaction between SgIII and CgA.

SgIII binding partners in mast cells include the ion channel TRPA1

The data presented above suggest that SgIII in mast cells participates in an interaction with CgA, and that the CgA-SgIII complex plays an, as yet poorly understood, role in directing granule biosynthesis. However, there are precedents for the interactions of granin proteins with nongranin binding partners, including the inositol (1, 4, 5) trisphosphate receptor, and intracellular calcium release channel (10–13, 31). We performed an interaction trap analysis (a Sos-Recruitment Screen yeast two-hybrid (Stratagene) performed using the amino-terminal cytoplasmic tail of rat TRPA1 as bait and a rat brain cDNA library a target, data not shown), which suggested that the ion channel TRPA1 may form a protein complex with SgIII. Because the work of several groups does suggest that ion channels in intracellular membranes may interact with certain granins, we asked whether this is a physiological interaction in mast cells. Fig. 6, A and B show that TRPA1 and SgIII can be coimmunoprecipitated in both an HEK over-expression system and in RBL2H3 mast cells. It is clear that a small proportion of the over-expressed, or endogenous, TRPA1 can be immunoprecipitated in a complex with SgIII. Similarly, we find that of the multiple mobility forms of SgIII that are present in cells over-expressing V5-SgIII, only the lowest apparent m.w. (perhaps corresponding to the least processed, immature form of SgIII) participates in an interaction with TRPA1 (Fig. 6C), although these immuno-decorate only weakly with anti-SgIII (data not shown). The interaction between TRPA1 and SgIII clearly has functional implications for the channel however, because intracellular calcium release responses evoked by the TRPA1 ligand icilin are compromised in cells...
FIGURE 6. Secretogranin III is a binding partner for TRPA1 in mast cells. A, Coimmunoprecipitation of V5-SgIII and FLAG-TRPA1. HEK cells stably expressing FLAG-tagged TRPA1 were transiently retransfected with V5-SgIII. Lysates were immunoprecipitated with 1 μg of the indicated Ab and duplicated membranes were Western-blotted for the presence of FLAG- and V5-tagged proteins. B, Coimmunoprecipitation of TRPA1 and SgIII. RBL2H3 mast cells were lysed and immunoprecipitated with 5 μg of the indicated Abs. Immunocomplexes were resolved by SDS-PAGE and Western blotted with anti-TRPA1 (left panel) or anti-SgIII (right panel). C, TRPA1 subcellular localization in resting mast cells. RBL2H3 were grown on glass coverslips and fixed in methanol, before immunofluorescence analysis using anti-TRPA1 (decorated with Alexa-488 nm conjugated goat ant-rabbit IgG) and anti-Golgi S5K mAb (decorated with rabbit anti-mouse Alexa 568 conjugated IgG). Nuclear material was stained (blue pseudo-color) using Hoechst 33342. Scale bar, 10 microns. D, TRPA1-mediated calcium responses in the absence and presence of over-expressed SgIII. HEK cells (lower gray trace), HEK stably expressing TRPA1 (black trace), and TRexTRPA1 cells transiently retransfected with V5SgIII (upper gray trace, offset by +0.1 μM for clarity) were loaded with fura-2 and a single cell calcium assay was performed as described. After establishing a baseline (white bar), individual cells were perfused with nominally calcium-free external buffer containing 12 μM icilin (gray bar).
over-expressing SgIII (Fig. 6D). These data present the first hint of a wider role for SgIII in mast cells, and their implications will be discussed below.

Discussion
Grain family members are present in the secretory granules of a wide variety of endocrine and neuroendocrine cells (1, 3, 4, 32). Granins are involved in granulogenesis in neuroendocrine cells (6, 7) but there has been no published analysis of expression or function of granins in the mast cell system. Our data demonstrate the expression of granins in mast cells, and imply an important role for these proteins in this secretory immunocyte.

The biological roles of granin proteins are not well understood. Several ideas, which are not mutually exclusive, have been proposed for the molecular functions of granins. It is clear that CgA and CgB act as cargo precursors that are proteolytically cleaved to form bioactive peptides. In addition to being sources of granule content in the form of proteolytically derived peptides, Chromogranins have also been described to chaperone secretory granule components (1, 3, 9, 33, 34). Chromogranins have also been described as cholesterol binding proteins, and a model for their role in the cholesterol sequestration events that participate in granulogenesis has been derived from several studies (35). One common observation for several granins suggests that their over-expression result in expansion of the granule compartment (6, 15, 20, 22). Both their proposed roles as precursors of granule cargo, and as regulators of granule membrane dynamics, would be consistent with these observations of granulogenic capacity. In a further, intriguing, set of publications, the CgA and CgB proteins have been described as low affinity, high capacity, calcium buffering proteins that associate with intracellular calcium channels in the ER and Golgi apparatus. In this context, the calcium reservoir that is provided by CgA appears to play a feedback role in regulating the open status of these calcium permeant channels (10–13, 31).

Secretogranin III has not been extensively investigated, and is only weakly similar to CgA/CgB at the sequence level. Our data, and that from other laboratories, allow us to make a number of statements about SgIII. First, it is heavily sulfated, which is consistent with it being a component of the mast cell secretory granule (Ref. 36 and Prerna Prasad, unpublished observation). Second, SgIII is not extensively proteolyzed to form peptides in resting mast cells (this article and Ref. 25). These data suggest that its major physiological role in this system is not to act as a precursor for bioactive peptides. Although Holthuis and coauthors (36) have reported proteolytic fragments that derive from SgIII, no biological activity has been assigned to any of the resulting peptides, in contrast with the situation for the CgA and CgB (37–39). It remains to be seen whether any SgIII-derived fragments act as signaling peptides in neuroendocrine cells, and we have not seen any evidence that pharmacological or immunological activation of mast cells can induce proteolysis of SgIII. In the mast cell system at least, we can argue that the granulogenic capacity of transfected SgIII does not simply reflect an adaptive response to the sudden need to package large quantities of SgIII-derived bioactive peptides.

Our third observation concerning SgIII is that transfection of SgIII cDNA is necessary and sufficient to drive expansion of a vesicular compartment in mast cells, neuroendocrine cells, and even in the nonsecretory HEK cell line. This expansion is accompanied by a concomitant increase in the proinflammatory capacity of the transfected mast cells. The granulogenic potential of SgIII depends on the presence of an intact carboxyl terminal of SgIII that interacts with the amino terminus of CgA. Thus, in mast cells, as in sympathoadrenal AtT-20 cells (16, 35), a functional interaction between SgIII and CgA seems to be required for their granulogenic capacity. Hosaka and coauthors (34, 35) have suggested that SgIII binds to the amino terminus of CgA thereby initiating the secretory granule biogenesis in the AtT-20 cells. This SgIII-CgA complex binds to the cholesterol moieties within the secretory granule membrane, but the exact mechanism by which this binding promotes granulogenesis is not yet fully understood. Several groups have determined that cholesterol depletion blocks the formation of constitutive secretory vesicles from the Golgi apparatus (30), and the formation of microdomains of the “cone-shaped” cholesterol molecules may contribute to the negative curvature of the lumenal leaflet of the vesicle membrane. One key open question regards the localization of endogenous SgIII in untransfected mast cells. We have not been able to produce any convincing immunofluorescence analysis for endogenous SgIII in mast cells, because the available anti-SgIII antibodies only weak, diffuse vesicular staining that may either indicate a vesicular localization or may represent the type of background staining that is typically associated with low affinity Abs in this application.

The data from our SgIII over-expression experiments in mast cells suggest that SgIII drives granule biogenesis. The data concerning an expansion of the granule content that can be released in response to antigenic or pharmacological treatment of mast cells could also reflect an alternate explanation, such as an action of SgIII in promoting expression, or activity, of the transporters that load serotonin into preexisting granules. Similarly, we cannot exclude that SgIII over-expression enhances the activity of serotonin efflux transporters, a caveat to the secretion assays presented in this study. However, our microscopy data are more consistent with the fact that SgIII over-expression simply results in the presence of more mast cell granules. Knockdown or knockout of SgIII would reveal whether this protein is needed for the biosynthesis of endogenous vesicles in a native expression system. We have found SgIII knockdown in mast cells to be associated with suppression in secretory capacity, but these experiments are difficult to interpret in the light of the confounding effects of decreased viability, possibly because this protein may plays central role in membrane trafficking (data not shown). A SgIII knockout mouse has been described by Dopazo and coauthors (Ref. 40; unpublished observations) but no endocrine or immunological phenotype was described and the mice are no longer extant. However, a recent paper by Hendy and coauthors (23) suggests that knockout of granins may not be very informative, due to a compensatory up-regulation in the expression levels of other granin genes.

The data summarized above suggest that the function of SgIII in mast cells closely parallels that described in the neuroendocrine secretory system, where SgIII is involved in regulating the membrane dynamics of secretory vesicles via an interaction with CgA. Intriguingly, our interaction trap and coimmunoprecipitation data suggest that CgA is not the only binding partner for SgIII in mast cells. SgIII clearly interacts with the ligand-gated cation channel TRPA1. These data are strongly reminiscent of the observations of the Ehrlich and Yoo laboratories (10, 11, 13, 31), where CgA and CgB bind to and regulate the inositol (1, 4, 5) trisphosphate receptors, which are intracellular calcium channels. We had initially considered the possibility that interaction between SgIII and TRPA1 might reflect the fact that an SgIII-derived peptide could be a ligand for this channel. However, the clear interaction between TRPA1 and full-length minus, which in turn is the domain of SgIII that interacts with the amino terminus of CgA.
SgIII, and the data from studies of the granin/InsP3R interaction would seem to argue against this model. TRPA1 seems, like TRPV1, to function as an intracellular calcium release channel when supplied with an appropriate membrane-permeant (lipophilic) ligand (41), and it is clearly localized to vesicular intracellular structures. Moreover, channels of the TRP family have been reported to be associated with cholesterol-rich microdomains, at least in the plasma membrane (42). Thus, the association between TRPA1 and SgIII may simply reflect the localization of the latter to lipid membrane microdomains. However, there is also a clear precedent for a regulatory role of gramins that associate with certain intracellular calcium release channels, the InsP3R.

Choe and coauthors (10) demonstrated that CgA and CgB associate with the InsP3R, and that provision of competing concentrations of a peptide derived from the interacting portion of the granin had a dominant inhibitory effect upon Ins(1,4,5)P3-evoked calcium release. Similarly, our over-expression of SgIII seems to suppress calcium release via TRPA1. In the case of the Chromogranin/InsP3R interaction, the calcium-binding activity of the granin, through conserved EF-hand motifs, has been proposed to play a role in shaping the channel’s open probability (10, 43). In the case of SgIII, there is no obvious EF-hand motif (CgA/CgB and SgIII are only weakly similar at the sequence level; Ref. 4) and no calcium-buffering activity has been ascribed to this protein. At this time, it seems likely that the loss of function in TRPA1 that is associated with SgIII over-expression is likely to reflect a disruption of its localization to cholesterol-rich microdomains. Future work will determine whether the activity of TRPA1 in intracellular membranes absolutely depends upon correct localization to SgIII-containing lipid rafts.

In summary, the present report suggests that granins in mast cells recapitulate the varied functional contributions made by these proteins to the neuroendocrine secretory system. Granins such as SgIII can obviously contribute to multiple facets of cell biology, and the reaching of a consensus as to their molecular roles is clearly a high priority. In the mast cell, granins such as SgIII may be critical determinants of granule load, and hence the intensity and duration of inflammatory responses.

Acknowledgments

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