Perturbation of a Very Late Step of Regulated Exocytosis by a Secretory Carrier Membrane Protein (SCAMP2)-derived Peptide*

Zhenheng Guo‡, Lixia Liu‡, David Cafiso§, and David Castle‡†

From the ‡Department of Cell Biology, University of Virginia Health Sciences Center and the §Department of Chemistry, University of Virginia, Charlottesville, Virginia 22908

Secretory carrier membrane proteins (SCAMPs) are conserved four transmembrane-spanning proteins associated with recycling vesicular carriers. In mast cells, as in other cell types, SCAMPs 1 and 2 are present in secretory granule membranes and other intracellular membranes. We now demonstrate a population of these SCAMPs in plasma membranes. Although small, this population partially colocalizes with SNARE proteins SNAP-23 and syntaxin 4. A fraction of SCAMPs 1 and 2 also coimmunoprecipitates with SNAP-23. An oligopeptide, E peptide, within the cytoplasmic segment linking the second and third transmembrane spans, particularly of SCAMP2, potently inhibits exocytosis in streptolysin O-permeabilized mast cells. The E peptide is unique to SCAMPs and highly conserved among SCAMP isoforms, and minor changes in its sequence abrogate inhibition. It blocks fusion beyond the putative docking step where granules contact the cell surface and each other during compound exocytosis. Blockade is also beyond Ca²⁺/ATP-dependent relocation of SNAP-23, which regulates compound exocytosis, and beyond ATP-dependent priming of fusion. Kinetic ordering of exocytotic inhibitors has shown that E peptide acts later than other perturbants at a stage closely associated with membrane fusion. These findings identify a new reagent for analyzing the final stage of exocytosis and point to the likely action of SCAMP2 in this process.

Membrane fusion is a ubiquitous and fundamental process used for joining cells during fertilization and synectia formation, virus internalization, and intracellular membrane trafficking including exocytosis. Although fusion involving extracellular surfaces, as exemplified by hemagglutinin-mediated entry of influenza virus, has been well characterized, intracellular membrane fusion is less well understood. Much progress has pointed to the SNARE (SNAP receptor) proteins as the probable intracellular counterparts of hemagglutinin, and studies have identified many homologs of neuronal SNAREs and demonstrated their interactions in a variety of cells and tissues. Furthermore, recombinant SNARE proteins reconstituted in liposomes can cause membrane fusion, suggesting that SNAREs represent the minimum essential machinery (e.g. Refs. 1 and 2). This contention is supported by structural analyses (3, 4) and by functional studies of SNARE complexes in situ (5–7). However, intracellular fusion is regulated by hierarchies of interactions that control SNARE function (e.g. Refs. 8–11) and by other proteins that facilitate fusion (e.g. Refs. 12 and 13). Also, SNAREs may be insufficient for efficient fusion of physiological membranes (14, 15) and other proteins, e.g. synaptotagmins or Munc18 may be final mediators of fusion pore formation and expansion (16–18).

Secretory carrier membrane proteins (SCAMPs) are four transmembrane-spanning proteins found in Golgi and post-Golgi recycling carriers (e.g. Refs. 19–22). They are conserved across the plant and animal kingdoms, and to date, five distinct SCAMPs have been reported in mammals with SCAMPs 1–4 being ubiquitously expressed and SCAMP5 being neuron-specific (22–24). SCAMPs 1–3 are ~38 kDa, whereas SCAMPs 4 and 5 are ~25 kDa. The larger SCAMPs (SCAMPs 1–3) have an extended cytoplasmic N terminus that is lacking in the smaller ones and contains domains that have been implicated as sites of interactions with other proteins (22, 23, 25). Local structural differences within the N termini of SCAMPs 1–3 and at the extreme C termini of all SCAMPs distinguish the isoforms. Although their function has remained elusive, recent studies have suggested their possible action in membrane fusion. Sequence and topological analysis has revealed that the likely functional domain of SCAMPs is its membrane core, which features three highly conserved, cytoplasmically oriented amphipathic segments closely linking the transmembrane spans and positioned at the membrane surface (22). Like other fusion-supporting proteins, SCAMPs oligomerize in situ and in vitro (26, 27), and assembly of multiple transmembrane spans and amphipathic segments might promote bilayer reorganization leading to fusion pore formation, stabilization, and/or expansion. Indeed, a gene ablation study has shown that mast cells of SCAMP1-null mice exhibit increased exocytotic reversal, suggesting reduced stabilization of fusion pores (28).

We have been examining the roles of both SNAREs and SCAMPs in exocytosis in mast cells. Previously, we have shown that relocation of the SNARE, SNAP-23, along the cell surface and to granule membranes is required for exocytosis (27). In the present study, we have begun to consider the function of SCAMPs. We have focused on SCAMP2, which like SCAMP1 is prominent in mast cells and is localized to intracellular or-
ganelles including secretory granules and in part to plasma membranes where the concentration of SCAMPs has not been recognized previously. Our findings demonstrate partial colocalization of plasma membrane SCAMPs with SNAP-23 and syntaxin4 and their coimmunoprecipitation with SNAP-23. They also identify a cytoplasmically oriented segment linking transmembrane spans 2 and 3 of SCAMP2 as a potent, sequence-specific and late-acting inhibitor of exocytosis when examined as a synthetic peptide. Inhibition is an order of magnitude stronger than for the corresponding peptide of SCAMP1. We suggest that SCAMP2 may play a critical role in completing exocytosis, which can be competed by the peptide and that the peptide may be a useful new tool for examining the molecular composition of granule-to-plasma membrane and granule-to-granule fusion sites.

**EXPERIMENTAL PROCEDURES**

**Materials**—Sprague-Dawley retired breeder male rats were from Hilltop Inc. Streptolysin O (SLO) was from Murex Diagnostics Ltd. (Dartford, UK). GTP-S and GDP-S were from Roche Molecular Biochemicals. All SCAMP peptides were synthesized and characterized at the University of Virginia Biomolecular Research Facility. Protein kinase C inhibitory peptide 19–31 (RFARKRALRKNV) was from Calbiochem. Mouse monoclonal anti-SCAMP antibody 7C12, having an avidity within the N termini of SCAMPs 1–3 was characterized previously (19, 24). Anti-SCAMP antibody 1o was described previously (26); rabbit antibodies 2r and 3β were raised against peptides (CFSQGHPS- SRTTFHR of SCAMP2 and CQHRPSQYATLDDY of SCAMP3. The antibodies of anti-SCAMP antibodies were compared by using them on Western blots of known amounts of recombinant polypeptides encoding the relevant epitopes. The bound antibodies were detected and quantitated using 125I-labeled secondary antibody and PhosphorImaging. Anti-SNAP-23 (C-terminal peptide) antibody was described (30), and rabbit anti-syntaxin 4 was the gift of Drs. Pam Tuma and Ann Hubbard (The Medical School). Avidity of the antibody against PtdIns(4,5)-diphosphate was obtained from Perspective Biosystems (Framingham, MA) and was reconstituted in phosphate-buffered saline according to the manufacturer’s instructions. Mouse monoclonal antibody against Thy1 was from Serotec (Oxford, UK). Fluorescent-labeled antibodies were from Molecular Probes (Eugene, OR); and 125I-goat anti-rabbit IgG was from PerkinElmer Life Sciences. Recombinant guanidinium thiocyanate dissociation inhibitor for Rho GTPase was from cytosekeleton Inc. (Denver, CO). The protease inhibitor disopropyl fluorophosphate was obtained from Sigma.

**Perturbation of Regulated Exocytosis in SLO-permeabilized Mast Cells**—Mast cells were purified and permeabilized with SLO as described (30). Amounts of SLO used are specified in the figure legends. Aliquots (10 μl) of cell suspension (1 × 10⁶ cells) were added to 40 μl of K-G buffer (137 m M potassium glutamate, 2 m M MgCl₂, 20 m M PIPES, pH 6.8, 1 m M bovine serum albumin) containing various additives: 3 m M EGTA (for control samples) or EGTA-Ca (pCa = 5 or 5.5 for Ca²⁺-stimulated samples), 100 μ M GTP-S (for GTP-S-stimulated samples), and 1 or 3 mATP (for GTP-S and Ca²⁺-stimulated samples, respectively). Peptides or antibodies were also added at this step as noted.

After 10 min on ice, samples were transferred to 37 °C for specified times to trigger secretion and then returned to ice. Following centrifugation, 10 μl of each supernatant was removed for assaying hexosaminidase (30), and stimulated secretion was expressed as percent of total activity (cells + supernatant) present in the supernatant. Results were from at least three independent experiments and are expressed as means ± S.E. All peptides were stored at −20 °C either desiccated or for 1 week as stocks in 0.3 M Pipes, pH 6.8. One peptide (CVRPYPIYKAF) derived from SCAMP2 was used most frequently and was stored in solution over longer periods without change in its inhibitory potency. In desiccated form, its structure has remained unaltered over 3 years as determined by mass spectroscopy.

**Immunocytochemistry and Electron Microscopy of Mast Cells**—Immunolocalization of SCAMPs was performed on mast cells that were attached in suspension in protein-free medium, fixed in 3% formaldehyde on ice, and permeabilized with 0.05% saponin. Alternatively, antibodies were internalized by SLO-permeabilized cells and examined without fixation as described previously (30). Images were recorded with a Zeiss 410 confocal microscope. For immunoelectron microscopic studies, anti-SCAMP and 2° antibody conjugates (1.4 nm gold; Nanoprobes, Inc.) were internalized by unfixed SLO-permeabilized mast cells and examined following fixation, silver intensification (4–7 min), embedding, and sectioning (30). Micrographs were taken of unstained specimens. Isolated mast cell granules (30) were washed by centrifugation in CB (137 m M NaCl, 2.7 M KCl, 20 M Pipes, 5.6 M glucose, 1 m M mg bovine serum albumin, pH 6.8), blocked (0% goat serum in CB), and labeled with monoclonal, individual sections were collected at 20 μm (>1 cell diameter) intervals with one section per grid. Grids were scanned, and each cell with a full profile and central nucleus was photographed at fixed magnification. Evaluations on 36 images each of control and peptide-treated/stimulated cells were made as indicated in Fig. 4.

**Immunoprecipitation Studies**—To test for interactions of SCAMPs with SNAP-23 in mast cells, samples of 3 × 10⁶ cells were treated 5 min at room temperature with 0.5 m M disopropyl fluorophosphate, sedi-
mented, and then solubilized 30 min on ice in 1 M of 0.2% Triton X-100, 50 M MOPs (pH 7.2), 1 m M EDTA, and proteinase inhibitors (4-(2-aminooxy)benzenesulfonyl fluoride, phenylmethanesulfonyl fluoride, and leupeptin). After sedimenting insoluble material (15 min, 11,000 × g), the clarified lysate was incubated overnight at 4 °C with 20 μg of anti-SNAP-23 or nonimmune rabbit IgG. The beads were washed three times with 1 M of the solubilization solution and once with PBS (20 m M sodium phosphate, 150 m M sodium chloride). Adsorbed proteins were eluted in sample buffer containing 4% SDS, Tris-Cl (pH 8.5), and 50 m M dithiothreitol and subjected to SDS-PAGE and Western blotting.

**RESULTS**

**Presence of SCAMPs in Secretory Granules and Plasma Membranes in Mast Cells**—We carried out a variety of localization studies using Western blotting, immunocytochemistry, and cell fractionation to assess the distribution of SCAMPs in mast cells. As shown in Fig. 1A, mast cells contained SCAMPs 1–3. We have focused on SCAMPs 1 and 2 because they are much more abundant than SCAMP3, which was detected using an antibody, 3β, having an avidity that is higher than the avidities of the other two SCAMP antibodies (avidities were compared by quantitative Western blotting; see “Experimental Procedures”). Immunofluorescence on fixed and permeabilized cells showed that both of these SCAMPs detected together (monoclonal antibody 7C12) or individually (antibodies 1o, 2r) exhibit punctate labeling throughout the cytoplasm to the border of the cell (Fig. 1B). SCAMPs were evident in purified granules and were directly localized on granule membranes (Fig. 1, A and C). From the recovery of purified granules determined by hexosaminidase assay (30), we estimate that ~25% of mast cell SCAMPs are associated with granules.

Immunolabeling by antibody uptake into SLO-permeabilized unfixed cells, a procedure applied extensively for studies of SNAP-23 (30), detected SCAMPs deep in the cytoplasm poorly but was especially useful for highlighting SCAMPs near the cell periphery. Limited internal labeling appeared to be due to low antigen accessibility as it could be improved (although at the expense of granule integrity over the lengthy labeling protocol) either by substituting digitonin for SLO during permeabilization or by performing antibody uptake in the presence of Ca²⁺/ATP² SCAMPs at the periphery appeared in foci at the cell surface in both fixed/permeabilized and SLO-permeabilized unfixed specimens. Many of these foci exhibited overlap with SNAP-23 (Fig. 1D), and the codistribution with plasma membrane SNAREs, SNAP-23, and syntaxin 4 was much more apparent in the SLO-permeabilized unfixed cells (Fig. 1, E and F). For the latter observation, we cannot rule out the possibility that the SLO treatment itself might have contributed to an enhanced colocalization. However, we believe that plasma membrane SCAMPs are likely to be associated with the
SNAREs because it is possible to detect specific coimmunoprecipitation of SCAMPs 1 and 2 with SNAP-23 under conditions where no binding is detected using nonimmune IgG, and the mast cell plasma membrane protein Thy1 is not appreciably associated with the immune complexes (Fig. 1G).

We confirmed the presence of SCAMPs in both granules and plasma membrane by immunogold labeling, which is illustrated for SCAMP2 (Fig. 2). Notably, labeling was observed on granule membranes and plasma membranes often at sites where granules are close to the cell surface. Together, these studies verify granule association of SCAMPs and document a previously unreported low level incidence in the plasma membrane where they colocalize at least in part and associate with exocytotic machinery near prospective fusion sites.

The Peptide Linking Transmembrane Spans 2 and 3 Is Highly Conserved—We have sought structural similarities among 11 SCAMPs spanning the plant and animal kingdoms. Comparison has revealed conservation in overall length (except for SCAMP 4 (22) and SCAMP5 (23)) and sequence, both focally within the N-terminal one-third and broadly within the C-terminal two-thirds of the structure (22). Significantly, SCAMPs 4 and 5 lack the N-terminal third and mainly compose the C-terminal two-thirds of other SCAMPs. Thus we assume that the functional domain resides within this common portion, which by similarity analysis contains conserved transmem-
brane spans and three highly conserved amphiphilic segments D–F (Fig. 3A). The latter all face the cytoplasm and are immediately proximal to the first transmembrane span (D), between spans 2 and 3 (E), and immediately distal to the fourth span (F) (22). E is most highly conserved (Fig. 3B), and we have given peptides within this segment (called E peptides) highest priority in addressing SCAMP function.

SCAMP E Peptide Inhibits Exocytosis in Mast Cells Triggered by Ca\(^{2+}\) or GTP\(_S\)—Initially, we have examined a potential role in exocytosis using SLO-permeabilized mast cells. Synthetic peptides corresponding to different segments of SCAMP were screened as secretory inhibitors by adding them to permeabilized cells and then assay Ca\(^{2+}\)– or GTP\(_S\)-stimulated release of hexosaminidase. E peptide inhibited exocytosis by both stimuli and was strongest for the peptides from mammalian SCAMPs 2 and 3, as compared with mammalian SCAMP1 (Gly replacing Lys, residue 8) and Caenorhabditis elegans SCAMP (Phe replacing Cys, residue 1). Fig. 3C illustrates this selectivity. Here we used concentrations of 30 and 10 \(\mu M\) of the peptides during GTP\(_S\) (100 \(\mu M\)) and Ca\(^{2+}\) (10 \(\mu M\)) stimulation (30), respectively, as we have consistently observed distinct sensitivities to inhibition when the two secretory stimuli are examined comparatively (see below). The SCAMP1 and nematode versions of E peptide exhibited increased inhibition at a concentration of 100 \(\mu M\). E peptide of SCAMP1 reduced Ca\(^{2+}\)-stimulated secretion to 24 ± 1% of control whereas the nematode version reduced secretion to 44 ± 12% (mean ± range, two experiments for each).

Several studies that we performed attested further to the sequence specificity of the inhibitory effect. First, a scrambled sequence of SCAMP2 E peptide was not inhibitory (not shown). Second, an “offset” peptide lacking C Wy at the N terminus and including FR at the C terminus and having the same net charge was not inhibitory at 500 \(\mu M\) (not shown). Third, the inhibitory effect was greatly attenuated by replacing selected amino acids but not by truncating the C terminus, which reduces the amphiphilic character of the peptide (see below). Finally, the MARCKS peptide (KKKRFSPKSSFKLGSPFKKKK), which binds membranes that contain acidic phospholipids (31), is not inhibitory at 500 \(\mu M\) (not shown). In the following studies, we have focused on the E peptide of SCAMP2 because of its potency as an exocytic inhibitor.

Inhibition of secretion stimulated by Ca\(^{2+}\) and by GTP\(_S\) as a function of the concentration of E peptide was assayed biochemically (Fig. 4A) and by phase microscopy (not shown). Ca\(^{2+}\)-stimulated secretion consistently was more sensitive to the peptide than was GTP\(_S\)-stimulated secretion. For example, 10 and 30 \(\mu M\) E peptide reduced Ca\(^{2+}\)-stimulated secretion to −40 and <20%, respectively, of the maximum stimulated secretion observed in the absence of peptide, whereas the same peptide concentrations reduced GTP\(_S\)-stimulated secretion to −80 and 32%, respectively (Fig. 4A). Whereas the difference in sensitivity of the two types of stimulation supports the possibility that the peptide has a specific rather than a nonspecific action, insight regarding the basis of the distinction is lacking at present. C-terminal deletions of 4 and 7 residues from the 15-residue E peptide of SCAMP2 had little effect (Fig. 4B). In contrast, replacement of N-terminal Cys by Ala and of subsequent Wy residues by AA dramatically decreased inhibition (Fig. 4B). Inhibition was also substantially reduced by substituting separately Ile\(^6\) by Ala and Lys\(^8\) by Ala in the 8-residue E peptide (CWYRPYIK) and Arg\(^4\)-Pro\(^5\) together by AA in the 11-residue E peptide (CWYRPYIKAFFR) (not shown). These analyses underscore the sequence specificity of the inhibitory effect and the importance of the peptide’s first eight residues.

SCAMP 2 E Peptide Inhibits a Late Step of Exocytosis—To clarify where peptide inhibition was occurring along distal signaling pathways emanating from Ca\(^{2+}\) and GTP\(_S\) stimuli,
four different experimental approaches were used. First, unstimulated cells and cells stimulated with and without E peptide were compared by EM. In unstimulated cells, most of the granules were intact with uniform electron dense content (Fig. 5A). In GTPyS-stimulated cells, membrane-bounded sacs connected to the cell surface and filled with partially dispersed contents of multiple granules were indicative of compound exocytosis (Fig. 5B). In contrast, inclusion of E peptide with GTPyS prevented discharge and preserved the appearance of unstimulated cells (Fig. 5C). However, closer examination (Fig. 5D) and quantitative measurements (Fig. 5, E and F) revealed key differences from controls. Although the total number of granules per cross-sectional cell profile and the number of peripheral granules were unchanged (Fig. 5E), peptide-blocked stimulation caused increased membrane contact (granule-to-plasma membrane and granule-to-granule; Fig. 5, D and F), implying accumulation of tethered or docked granules without fusion. In mast cells, we have shown that relocation of SNAP-23 from cell surface folds to putative fusion sites is required for compound exocytosis presumably for trans-SNARE complex formation (30). To reach this conclusion, we incubated permeabilized cells with Ca\(^{2+}\) and ATP, the total secretory response was maintained at a higher concentration and selected 24 °C and 3 μM Ca\(^{2+}\) (in the presence of 3 mM ATP) as conditions leading to increased release over a 30-min period. Accordingly, during the ensuing experiments, inhibitors were added to individual samples after different times and were incubated for a total time of 30 min. E peptide inhibited secretion at every time point it was added indicating that exocytosis remained sensitive to it throughout incubation (Fig. 6C). The time course was identical to that observed when secretion was stopped at each time point by transferring to ice
Fig. 6. E peptide of SCAMP2 inhibits a late step leading to exocytosis. A, peptide inhibits downstream of Ca\(^{2+}\)-dependent relocation of SNAP-23. Cells were permeabilized with SLO (1.6 IU/ml) and incubated on ice (30 min total) in media containing EGTA + ATP, Ca\(^{2+}\) + ATP, or Ca\(^{2+}\) + ATP with E peptide (10 \(\mu\)M) added either before or after, as specified. All samples were then warmed at 37 °C for 5 min to trigger secretion. B, inhibition of secretion by E peptide in the absence and presence of ATP. To remove endogenous ATP, mast cells were treated with 2-deoxyglucose (6 mM) and antimycin A (5 \(\mu\)M) for 5 min at 37 °C. Following SLO permeabilization (0.4 IU/ml), they were washed, incubated with the indicated concentration of E peptide and Ca\(^{2+}\) + GTP-\(\gamma\)S (but no ATP), and then warmed 10 min at 37 °C to test secretion. A second group of cells was not pretreated with ATP synthesis inhibitors, and secretion was triggered with ATP + Ca\(^{2+}\) + GTP-\(\gamma\)S. Secretion is plotted as percent of maximal secretion, which was observed in the presence of ATP. C and D, kinetic assays of inhibitor action on mast cell secretion. Permeabilized mast cells were incubated at 24 °C in medium containing Ca\(^{2+}\) and ATP, and inhibitors were added at the indicated times for 10 min on ice before returning to 24 °C and continuing incubation until the total time at elevated temperature was 30 min. At each time point, one set of samples (ICE) was placed on ice and not incubated further at 24 °C. Secreted hexosaminidase is expressed as a percent of release at the 30-min time point (the maximum).

DISCUSSION

Since discovering that SCAMPs are ubiquitous components of membranes that function in cell surface recycling (20) and are broadly distributed in the animal and plant kingdoms (22), we have been considering the likely possibility that they function in membrane-trafficking processes. Although it has been suggested that SCAMP1 may function in exocytosis and possibly endocytosis, (25, 28), these findings and our own earlier studies have focused on the presence and role of SCAMPs within the recycling carrier membranes. Now, however, we have identified a population of the mast cells SCAMPs in the plasma membrane. This population, as observed in fixed and saponin-permeabilized cells (Fig. 1, B and D), appears to be rather small (estimated as <10% of total cellular SCAMP). By using unfixed and SLO-permeabilized cells, SCAMP staining at the cell periphery is emphasized, and evidence for SCAMPs at the plasma membrane has been supported by clearer colocalization with SNARE proteins SNAP-23 and syntaxin 4 (Fig. 1, E and F) and by immunoelectron microscopic localization of SCAMP2 (Fig. 2). Unfortunately, the latter preparations are not amenable to meaningful quantitation because intracellular antigen is not uniformly accessible. Our studies now in progress indicate that SCAMPs are present in the plasma membranes of several cell types including fibroblasts, exocrine acinar cells, and neuroendocrine cells. In retrospect, plasma membrane-associated SCAMPs were overlooked in our original studies not only because we did not recognize this localization by immunofluorescence but also because the analysis by Western blotting did not distinguish association with plasma membrane fragments or contaminating organelles present in purified subcellular fractions (19, 20).

Interestingly, the plasma membrane population of SCAMPs may be partially associated, either directly or indirectly, with the SNARE proteins, SNAP-23 and syntaxin 4 (Fig. 1G) that have been implicated in granule exocytosis in mast cells (29, 30). Moreover, SCAMP2 has been identified between closely apposed granule and plasma membranes, which are prospective fusion sites (Fig. 2). These strategic associations have led us to reconsider where SCAMP2 might function during secretion. It may act from the plasma membrane as well as from granules. We can envision at least two scenarios for SCAMP function at these sites. First, plasma membrane and granule SCAMP2 may collaborate during membrane fusion, acting in trans between the two membranes, similar to the SNARE proteins. Second, because mast cells characteristically undergo compound exocytosis involving both granule-to-plasma membrane and granule-to-granule fusion, SCAMP2 may function

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4 We have not attempted to conduct immuno-EM studies of SCAMP localization on cryosections of intact rat peritoneal mast cells, which could be used for quantitative studies of antigen distribution, because we have been unable to identify fixation conditions that simultaneously preserve the integrity of the secretory granules and the antigenic epitopes of anti-SCAMP antibodies.
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from the target membrane at both types of fusion site. Although we are currently unable to distinguish between these scenarios, our thinking about them is influenced by other studies that we have been conducting in neuroendocrine PC12 cells. In these cells, SCAMP2 is present in plasma membranes and appears to function at docking/fusion sites for the large dense core vesicles but is not detected in the vesicles themselves.5 Thus by analogy, we favor the scenario that the function of SCAMP2 in exocytosis in mast cells is from the target membrane in both granule-to-plasma membrane and granule-to-granule fusion. Notably, a correlation between secretion by compound exocytosis and presence of granule-associated SCAMP2 seems to hold for exocrine acinar cells and neuroendocrine cell lines. Acinar cells, another paradigm for compound exocytosis, have abundant granule membrane SCAMP2 (19), whereas PC12 cells and pituitary AtT-20 cells, which are unlikely to employs compound exocytosis, lack SCAMP2 in their granules.3,5 In view of the fact that SCAMP2 is best known for its function in endosomal and other recycling membranes (20, 26), it will be interesting in future studies to examine whether it (or another SCAMP) colocalizes with SNAREs and functions in membrane fusion at these sites.

So far, the E peptide segment, which has been our initial focus in exploring SCAMP function, is unique to SCAMPs (based on data base searching), and our findings generally support the view that its sequence is critical to its ability to block exocytosis as a free oligopeptide (Figs. 3 and 4). Indeed, inhibition is highly sequence-specific such that changes of individual or pairs of residues near the N terminus dramatically diminish inhibition while C-terminal truncation to eight residues, which reduces net charge and amphipathicy, has no corresponding effect (Fig. 4). Thus even though the 11-amino acid version of E peptide at the concentrations used in this study has been shown to bind to phosphatidylserine-containing liposomes (22), the cellular lipid concentrations in the assays of mast cell secretion are ~1 μM or less and should not result in a significant fraction of membrane-bound peptide. These features suggest that the peptide may interact with a specific binding site. If so, this site seems unlikely to support a high affinity interaction in view of the observation that micromolar concentrations of peptide are required to achieve inhibition.

In combination, the unique association of E peptide segment with SCAMP and the structural specificity of the inhibitory effect are suggestive that the peptide is competing with endogenous SCAMP2 for an interaction that occurs in the final stages of exocytosis. Strikingly, the inhibitory effects, including its structural specificity, potency, and action downstream of the calcium requirement (Fig. 6), extend to PC12 cells.5 Thus we believe that the peptide may prove to be generally applicable as a late-acting secretory inhibitor, even in cell types that maintain a population of preocked and ready releasable granules. Also it will be interesting in future studies to examine whether E peptide blocks fusion events at other sites within the cell surface recycling system where SCAMPs reside.

Finally, although we have identified E peptide as a promising tool for interrupting exocytosis in its final stages and probing the organization of membrane fusion machinery in advance of fusion pore formation, our most important mission is to define how SCAMP2 and other SCAMPs function in the exocytotic process. Their localization with exocytotic SNAREs in the plasma membranes of both mast cells and PC12 cells places the SCAMPs at the right location, but their direct interactions and function with other candidate fusion machinery remain to be defined.

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