SCAMP1 Function in Endocytosis*

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Secretory carrier membrane proteins (SCAMPs) are ubiquitous components of recycling vesicles that shuttle between the plasma membrane, endosomes, and the trans-Golgi complex. SCAMPs contain multiple N-terminal NPF repeats and four highly conserved transmembrane regions. NPF repeats often interact with EH domain proteins that function in budding of transport vesicles from the plasma membrane or the Golgi complex. We now show that the NPF repeats of SCAMP1 bind to two EH domain proteins, intersectin 1, which is involved in endocytic budding at the plasma membrane, and γ-synexin, which may mediate the budding of vesicles in the trans-Golgi complex. Expression of SCAMP1 lacking the N-terminal NPF repeats potententially inhibited transferrin uptake by endocytosis. Our data suggest that one of the functions of SCAMPs is to participate in endocytosis via a mechanism which may involve the recruitment of clathrin coats to the plasma membrane and the trans-Golgi network.

SCAMPs (secretory carrier membrane proteins) are discovered as major components of secretory vesicles in exocrine glands and later shown to be universally present in vesicles that recycle at the plasma membrane (1–4). At least three SCAMPs are expressed in vertebrates, and all cells tested express at least one SCAMP isoform (5). All SCAMPs are composed of four transmembrane regions with cytoplasmic N- and C-terminal regions. The function of SCAMPs is unknown. SCAMPs 1 and 3 are tyrosine-phosphorylated by the epidermal growth factor receptor, suggesting that they are subject to regulation by phosphorylation (6). A knockout of SCAMP1, the most abundant SCAMP isoform, failed to display a major phenotype and exhibited only a moderate defect in membrane recycling by phosphorylation (6). A knockout of SCAMP1, the most abundant SCAMP isoform, failed to display a major phenotype. A knockout of SCAMP1, the most abundant SCAMP isoform, failed to display a major phenotype by phosphorylation (6). A knockout of SCAMP1, the most abundant SCAMP isoform, failed to display a major phenotype by phosphorylation (6).

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MATERIALS AND METHODS

Vectors—Vectors encoding the inserts (from rat) were constructed using polymerase chain reaction or standard subcloning techniques: pGEX-SCAMP1 = residues Met1–Leu151 in the XhoI-HindIII sites of the pGEX-KG; pCMVSCAMP1 = full-length SCAMP1 in the EcoRI site of pCMV5; pCMVSCAMP1NPF = Met53–Met151 in the XhoI site of pCMV5; pCMVSCAMP1-NT = Met1–Leu151 in the EcoRI/HindIII sites of pCMV5; pLexN-SCAMP1 = Met1–Leu151 into the Smal site of pLEXN; pLexN-SCAMP1/NPF = Arg44–Val149 of SCAMP1 in the EcoRI/BamHI sites of pLEXN; pVP16-ESPS15 = Gln6–Gln37 of Eps15 in the BamHI/NotI sites of pVP16; pVP16-ESPS15-EH1 = Gln6–Ala37 of Eps15 in the BamHI/NotI sites of pVP16; pVP16-ESPS15-EH1.2 = Gln6–Leu56 of Eps15 in the BamHI/XbaI sites of pVP16–3; pMalC2-synerg = Val153–Ser147 of γ-synerg. pVP16-ESPS1 (full-length EpsiSH1/insertion 1) and pVP16–3EHS1/5 (truncated insertin 1 without EH domains) were described previously (17). All vectors were confirmed by DNA sequencing.

Yeast Two-hybrid Screens and Interaction Analyses—Screening of a rat brain cDNA library with pLexN-SCAMP1 as described previously.
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RESULTS

Identification of γ-Synergin as a SCAMP-binding Protein—We used data bank searches to study the evolutionary conservation and domain structure of SCAMPs. Highly conserved SCAMP sequences were detected in plants (Arabidopsis thaliana), nematodes (Caenorhabditis elegans), and insects (Drosophila melanogaster) in addition to vertebrates (data not shown). Sequence alignments demonstrated that all available full-length SCAMP structures contain multiple NPF repeats and a proline-rich sequence in the N-terminal cytoplasmic domain that precedes the four transmembrane regions. The presence of NPF repeats in SCAMPs was intriguing because NPF repeats represent binding sites for EH domains, autonomously folding protein modules that are primarily observed in proteins involved in endocytosis (8, 9). Since SCAMPs appear to be universal membrane components of recycling vesicles in all cells tested (2, 5), their interaction with cytosolic adaptor proteins in endocytosis could provide a mechanism to initiate the assembly of clathrin coats for endocytic budding. To test this idea, and to identify potential EH domain proteins which bind to SCAMP, we screened a rat brain cDNA library by yeast two-hybrid selection for interacting proteins with the N-terminal sequences of SCAMP1.

Sequencing of the prey clones obtained in the yeast two-hybrid screen showed that γ-synergin was the major protein isolated. Two independent clones for γ-synergin were selected multiple times. γ-Synergin is a recently described cytoplasmic protein that contains a central EH domain (28), suggesting that the EH domain could bind to the NPF repeats of SCAMP1. This hypothesis is supported by the fact that the two γ-synergin clones isolated in the yeast two-hybrid screens overlap in the EH domain. The C terminus of γ-synergin binds to α-adaptin, a component of the Golgi clathrin adaptor protein complex AP-1, but the γ-synergin C terminus was not present in the yeast two-hybrid prey clones. To characterize γ-synergin further, we isolated from a rat brain cDNA library multiple independent cDNA clones that covered the N-terminal two-thirds of the protein (data not shown). Sequencing of these clones confirmed the previously described structure of γ-synergin except that at the N terminus, the rat γ-synergin cDNA had an open reading frame that extends 57 residues beyond the reported N terminus of human γ-synergin (28), suggesting that the N terminus of γ-synergin is longer than previously thought. In addition, sequence analyses revealed multiple internal repeats in the C-terminal half of γ-synergin, indicating that γ-synergin is composed of distinct domains (data not shown). RNA blots showed that γ-synergin is expressed in all tissues tested, with at least some tissues synthesizing multiple species of mRNA which appears to be a relatively abundant (data not shown).

SCAMP Binds Selectively to the EH Domains of γ-Synergin and Intersectin 1—The yeast two-hybrid results suggested that the N-terminal NPF repeats of SCAMP1 might bind to the EH domain of γ-synergin. To confirm this hypothesis, we further characterized the interaction of the two different γ-synergin prey clones with the N-terminal region of SCAMP1. Two yeast two-hybrid assays were used, transactivation of histidine auxotrophy (Fig. 1A) or quantitation of β-galactosidase (Fig. 1B). Either the complete N-terminal sequence of SCAMP1 with the NPF repeats or a truncated sequence lacking the NPF repeats were tested. With both assays, SCAMP1 only interacted with γ-synergin when the NPF repeats were present, providing further evidence that the N-terminal NPF repeats bind to the EH domain of γ-synergin.

We next asked how specific the binding of γ-synergin to SCAMPs is and if all EH domains or only a subset of EH domains bind to the NPF repeats of SCAMP1. GenBank™ searches revealed that the EH domain of γ-synergin is most closely related to the two EH domains of intersectin 1, whereas the classical EH domain protein EPS15 is less homologous (data not shown). Therefore we tested in the same yeast two-hybrid assays described above if the EH domains of intersectin 1 and EPS15 interact with SCAMP1 (Fig. 1). Again, a highly specific interaction of SCAMP1 with intersectin was observed. The SCAMP1/intersectin interaction depended on the presence
of NPF repeats in SCAMP1 and of the EH domains in intersectin as would be expected for an EH domain-mediated binding reaction. In contrast to intersectin, Eps15 constructs containing one or two EH domains were inactive in both interaction assays. The Eps15 construct containing all three EH domains showed binds to intersectin (17) was also specifically present immobilized GST-SCAMP1 containing the N-terminal cytoplasmic sequences of SCAMP1 was used as an affinity matrix, with GST alone as a control. Flow-through (lanes 2 and 3) and bound fractions (lanes 4 and 5) were analyzed by immunoblotting for the proteins indicated on the right, with synapsins and amphiphysin which are very sticky proteins used as a control in the bottom panel. Positions of molecular weight markers are indicated on the left.

Confirmation of the Interaction of SCAMP1 with \( \gamma \)-Synergin and Intersectin 1 Using Pulldown Experiments—To confirm the yeast two-hybrids results with an independent protein-based assay, we performed pulldown experiments with rat brain proteins. First, we used a MBP fusion with the EH domain of \( \gamma \)-synergin to affinity purify rat brain proteins (Fig. 2A). Immunoblotting revealed that SCAMP1 was bound only to the MBP-synergin fusion protein but not to MBP alone, suggesting that the EH domain of \( \gamma \)-synergin can capture SCAMP1 from the brain extract. Next we used a fusion protein of GST with the N-terminal domain of SCAMP1 in similar pulldown experiments, with GST alone as a control. Immunoblotting revealed that intersectin is highly enriched in the bound protein fraction; in fact, it was the most enriched protein (Fig. 2B). Because we did not have an antibody to \( \gamma \)-synergin, we could not test its binding in these experiments. However, immunoblotting also showed that \( \alpha \)-adaptin of the AP-2 adaptor complex was enriched in the pellet. We currently do not know if AP-2 binds directly to a sequence in SCAMP1 or if it binds indirectly via intersectin. Furthermore, SNAP-25 (which we previously showed binds to intersectin (17)) was also specifically present in the bound proteins. Two controls indicate that these observations reflect specific binding reactions. First, GST alone was unable to capture either intersectin 1, AP-2, or SNAP-25 (lane 4, Fig. 2B). Second, synapsins and amphiphysin, which are relatively abundant and sticky proline-rich proteins, were not bound to GST-SCAMP1 (Fig. 2B).
SCAMP1 Functions in Endocytosis—Because SCAMPs are universally present in all cells tested, their binding to E1H domain proteins could mediate the membrane recruitment of clathrin coats for budding, as occurs for example during receptor-mediated endocytosis. To test directly if SCAMP1 is involved in endocytosis, we transfected full-length SCAMP1, SCAMP1 with a deletion of the N-terminal NPF repeats, and the N-terminal NPF repeats of SCAMP1 without transmembrane regions into COS cells. Transfected cells were then incubated with fluorescently labeled transferrin, fixed, and stained for SCAMP1 to identify transfected cells. An observer who was blind to the nature of the transfected proteins quantitated the transfection results by scoring the percentage of cells that had taken up transferrin by endocytosis (Fig. 3).

COS cells transfected with full-length SCAMP1 display strong labeling of recycling vesicle compartments with SCAMP1 antibodies (cell examples identified by asterisks in panels A–C of Fig. 3); labeling is absent from adjacent nontransfected cells (example identified by a diamond). Endocytic transferrin uptake is normal in most nontransfected cells (Fig. 3B; 92.0 ± 0.7% of untransfected cells contain endocytosed transferrin (mean ± S.E.)). By contrast, cells expressing full-length SCAMP1 exhibit a partial block of endocytosis (53.7 ± 2.3% of transfected cells contain endocytosed transferrin (mean ± S.E.); see diamonds in panels A–C). In cells expressing an N-terminally truncated form of SCAMP1 lacking the NPF repeats (SCAMP1Δ-NPF, exemplary cells identified by asterisks in panels D–F of Fig. 3), endocytic transferrin uptake is almost completely inhibited (15.2 ± 2.1% of transfected cells contain endocytosed transferrin (mean ± S.E.)). Again, adjacent control cells are normal. In cells expressing only the N-terminal SCAMP1 fragment containing the NPF repeats (SCAMP1 N-terminal), the SCAMP1 fragment is diffusely localized throughout the cytoplasm (panels G–I), and no effect of the overexpression of the fragment on endocytosis is observed (89.0 ± 5.2% of transfected cells contain endocytosed transferrin (mean ± S.E.)).

DISCUSSION

Clathrin coats are assembled on plasma membranes, membranes of the trans-Golgi network, and other intracellular membranes in preparation to budding of transport vesicles from these membranes. Clathrin coats are assembled in all cells, at all times, in a highly regulated and localized manner. One of the unsolved questions surrounding the assembly of clathrin coats, for example during endocytosis, is how clathrin-coat assembly is nucleated. Biochemical studies showed that a proteinaceous receptor on the plasma membrane may be involved in recruiting clathrin coats to specific plasma membrane domains that are destined to become endocytic vesicles (20, 22). Since the receptor is endocytosed together with the clathrin coats, it is presumably a component of recycling vesicles. An alternative hypothesis is that the nucleation point is provided by the localized synthesis of certain lipids, such as phosphatidylinositol phosphates (see for example Refs. 33 and 34). The two hypotheses are not mutually exclusive but could potentially explain different characteristics of the clathrin assembly process. The proteinaceous receptor would provide a co-localization of the coat with the proteins of recycling vesicles, whereas lipid synthesis could explain the regulated assembly of the coat.

It has been suggested that synaptotagmin serves as an AP-2 receptor in endocytosis because it binds AP-2 with very high affinity (24, 25). Indeed, based on a number of experiments it seems likely that synaptotagmin functions as an endocytotic receptor at synapses (e.g., see Ref. 35). However, in non-neuronal cells, the levels of the ubiquitous synaptotagmins (e.g., synaptotagmins III, VI, VII, and IX) are so low that they are barely detectable with sensitive methods, making it unlikely that these synaptotagmins represent the long sought after receptors for endocytosis (25). The situation is different for SCAMPs. These proteins, although also abundant components of synaptic vesicles, are universally expressed at relatively high levels in all cells tested where they are components of the recycling vesicle population (2, 5). Thus based on their distribution, SCAMPs perform functions that are universally shared by all recycling vesicles.

We propose that SCAMPs function in endocytosis and may perform this function by directing the assembly of clathrin
coats at the plasma membrane via intersectin and possibly other interacting EH domain proteins and at the trans-Golgi network via \( \gamma \)-synergin. The following evidence supports this hypothesis: 1) SCAMPs contain N-terminal NPF repeats that are evolutionarily conserved. Since NPF repeats bind to EH domain proteins that in turn are often involved in clathrin-related functions, the presence of NPF repeats is indicative of a function related to the assembly and budding of clathrin coats. 2) Transfection of SCAMP1 with a deletion in the N-terminal NPF repeats inhibits endocytosis, consistent with a function in endocytosis that involves coupling of the N-terminal sequences to the transmembrane regions. 3) Two proteins with a known function in clathrin coats are specifically bound to the N-terminal sequences of SCAMP1, namely intersectin and \( \gamma \)-synergin. In the absence of the NPF repeats, this coupling is disrupted, and the rest of SCAMP is a dominant negative. This result suggests a model whereby the N-terminal NPF repeats of SCAMP1, namely intersectin and \( \gamma \)-synergin, for both proteins, a specific interaction was demonstrated by yeast-two hybrid assays and pulldowns. The large number of proteins with putative functions in clathrin-mediated endocytosis is staggering (for reviews, see Refs. 36–38). It appears that all of these proteins (by the last count more than 20) directly or indirectly interact with each other. Strikingly, all of these proteins are cytosolic, with no structural link to the membranes on which they are supposed to act. In this regard, the identification of SCAMPs as intrinsic membrane proteins that are capable of recruiting components of the clathrin-dependent membrane budding machinery is of potential significance. These results also suggest a possible explanation for the findings that in synaptotagmin I and SCAMP1 knockout studies, no obvious defects in synaptic vesicle endocytosis were detected (7, 39). Because SCAMP1 and synaptotagmin I are both abundant in synaptic vesicles, they could potentially be mutually redundant in synaptic vesicle endocytosis.

In the transfection studies, overexpression of full-length SCAMP1 only had a mild inhibitory effect while the most severe inhibition was observed with truncated SCAMP1 from which the NPF repeats had been deleted (Fig. 3). This result suggests a model whereby the N-terminal NPF repeats of SCAMPs mediate the coupling of the transmembrane regions of SCAMP to the EH domain proteins intersectin and \( \gamma \)-synergin. In the absence of the NPF repeats, this coupling is disrupted, and the rest of SCAMP is a dominant negative. This model implies that the highly conserved transmembrane regions of SCAMPs perform a separate additional function in membrane budding, which is currently unknown.

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