Three mammalian SCAMPs (secretory carrier membrane proteins) are highly related products of distinct genes having similar subcellular distributions

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SUMMARY

The primary structures of three human forms of secretory carrier membrane proteins (SCAMPs) have been deduced from full-length clones isolated from a HeLa cell cDNA library and confirmed by a combination of comparison to expressed sequence tags, microsequencing of purified protein, and in vitro transcription and translation. The structures indicated that SCAMPs are highly related products of distinct genes, and that the sequence identity of an individual SCAMP between different mammalian species is almost complete. Analysis of the distribution of SCAMPs among different mammalian tissues and cells indicates parallel expression of polypeptides and cognate mRNAs, and indicates that the three SCAMPs are usually but not always expressed together. The apparent $M_r$s of two SCAMPs (1 and 2) do not vary appreciably among species, while that of the third (SCAMP3) is approximately 2 kDa larger in rodent cells than in humans. Examination of the codistribution of the three forms within individual cells using double label immunofluorescence indicates extensive colocalization of SCAMP2 and SCAMP3 with endogenous SCAMP1, however, subcellular regions enriched for a particular SCAMP are readily visible. These findings suggest that the SCAMPs may largely function at the same sites during vesicular transport rather than in separate post-Golgi recycling pathways.

Key words: Integral membrane protein, Secretory vesicle, Phylogeny
half of the protein, four predicted transmembrane domains, and an unusual alanine-rich repeat at the carboxy terminus.

While nothing is known as yet about the function of SCAMP1, its distribution is quite interesting and suggests that it might serve a role in trafficking of vesicular carriers. First, the intracellular distribution of 7C12 reactivity indicated that SCAMPs were present in virtually all post-Golgi cycling membranes, and colocalized well with endosomal markers in addition to markers of regulated secretory carriers (Brand et al., 1991; Brand and Castle, 1993). SCAMPs are not resident on plasma membrane or lysosomal membranes, so are therefore shuttle components. Second, SCAMPs were readily detectable in many if not all tissues examined in an organism, even in those not having a regulated secretory pathway (Brand and Castle, 1993, and J. D. Castle, unpublished observations), suggesting that SCAMPs might play a role in a general cycling pathway present in all cell types. Third, western blots had previously suggested that SCAMP1 is a member of a family of related proteins, containing two or more peptides of similar molecular masses (Brand et al., 1991), which are quite well conserved among different mammalian species. Identification of SCAMP homologs and paralogs from species other than rat would indicate domains of the protein that are most conserved, and therefore functionally important. Furthermore, comparison of the distribution of the paralogs might help to establish whether individual SCAMPs function in distinct trafficking pathways or potentially cooperate within the same pathways. We report here the molecular cloning of three paralogs of SCAMPs from a human cDNA library, and characterize the relative distribution of SCAMPs in a single cell.

MATERIALS AND METHODS

Cloning of human SCAMP homologs

The predicted SCAMP1 protein sequence (Brand and Castle, 1993; PIR entry S37395) was compared with the Expressed Sequence Tag (EST) database in all 6 potential reading frames using the BLAST (Altschul et al., 1990) server at the National Center for Biotechnology Information (NLM-NIH). The EST clones 135527 and 21840 (Lennon et al., 1996; these clones correspond to the 5' and 3' end, respectively, of the full length SCAMP1 cDNA as a probe) were isolated from a human cDNA library (Stratagene catalog 937216) by hybridization.

The full length SCAMP cDNA was generated by cloning a partial cDNA from the cDNA library. The full length SCAMP cDNA was used to clone human SCAMP homologs by hybridization with the EST clone 154443 (Lennon et al., 1996). The EST clone was sequenced in its entirety and found to have an internal 300 nucleotide deletion of the predicted SCAMP3 open reading frame. The GST-transformed HeLa cDNA library was hybridized with the primers 38-1 (GGGAATTCCTAATGTCGGCTTGCAACC) and 39-2 (GGGAATTCCTCGGGAAGCGATC) for PCR amplification of reverse transcribed human placental mRNA. The resultant product was used as a probe to clone a full-length cDNA for SCAMP2 from a HeLa cDNA library (Stratagene catalog 937216) by hybridization.

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purification of SCAMP2 from bovine liver

Bovine liver was identified as a tissue source in which SCAMP2 is unusually enriched using western blotting, and was therefore used as a source for antigen immunopurification. Liver (500 g) obtained from a single slaughterhouse and maintained on ice (~2 hours) was coarsely minced with razor blades and homogenized (15 seconds, Tekmar Tissuemixer) at 20% (w/v) in ice-cold STKM (0.25 M sucrose, 50 mM Tris-HCl, 25 mM KCl, 10 mM MgCl2, pH 7.5, and freshly added 0.25 mM PMSF). After filtering through three layers of cheesecloth, the homogenate was centrifuged 30 minutes at 10,000 g (Sorvall GSA rotor) to pellet mitochondria and larger organelles and debris. The supernatants were then spun 120 minutes at 98,000 g to pellet crude microsomes. The pellets were resuspended in 1 volume STKM containing freshly added PMSF using a Teflon/glass homogenizer and stored at ~80°C.

For immunoadsorption, 1.5 mg rabbit anti-mouse IgG was bound to 100 mg swollen Protein A-Sepharose, washed with PBS, and coupled overnight at 4°C with 0.03% glutaraldehyde. After washing with PBS, 2 mg anti-SCAMP monoclonal antibody 7C12 was bound to the solid phase and then washed with PBS to complete preparation of the immunoadsorbent. Crude liver microsomes (5.0 g protein) was dissolved at 4°C in 500 ml 1% Triton X-114, 10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.2 μg/ml leupeptin, 0.25 mM PMSF. After centrifuging 30 minutes at 98,000 g to pellet insoluble material, the supernatant was incubated 10 minutes at 25°C to cause phase separation, and then spun 10 minutes at 2,000 g to pellet detergent micelles. The detergent phase was diluted to 280 ml with 10 mM Tris, 150 mM NaCl, 1 mM EDTA (to give approximately 2% Triton X-114 concentration) and then mixed with an equal volume of 2% deoxycholate, 0.2% SDS, 10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.25 mM PMSF. After stirring, this solution was passed twice (~40 ml/hour) over the 0.4 ml immunoadsorbent in a small column at 4°C. Subsequently, the column was washed with 10 ml of detergent-buffer solution having the same composition as used for immunoadsorption. Immunoadsorbed SCAMP (plus unbound 7C12 monoclonal) was then eluted by heating with three 200 μl samples of solubilization buffer for SDS-PAGE made with ultrapure reagents. After electrophoresis on an 11% polyacrylamide gel, the SCAMP2 band (25-30 kDa protein) was located by light Coomassie staining, excised, digested with trypsin-TPCK, and eluted as described previously for SCAMP1 (Brand and Castle, 1993) Tryptic peptides were separated by HPLC and microsequenced by standard procedures in the University of Virginia Biomolecular Research Facility. Sequences of three peptides were obtained; they corresponded exactly to sequences deduced from the human cDNA and are underlined in Fig. 1. Sequences of tryptic peptides from SCAMP3 ( apparent Mr~40,000 in rat) purified from low density microsomes of rat adipocytes were kindly provided by Dr. Gus Lienhard and are underlined in Fig. 1.

Tissue distribution of SCAMP paralogs

A multiple human tissue northern blot (Clontech) was sequentially probed under high stringency conditions with random-primed radiolabeled probes corresponding to DNA fragments from HeLa cDNA clones of SCAMP1, SCAMP2, and SCAMP3. Size of transcripts was estimated with markers. Probed blots were stripped of detectable signal at 95°C with 1% SDS/0.1x SSC for subsequent reprobing for other transcripts. For analyzing protein antigen distribution among cultured CHO or HeLa cells, post-nuclear supernatants containing total membranes were prepared from cultured cells lysed in 0.5% NP-40/0.1% deoxycholate containing protease inhibitors AEBSP and leupeptin. After clarifying by centrifugation (10 minutes at 10,000 g), the lysates were used for either direct western blotting or sequential immunoprecipitation and
western blotting. For western blotting, samples were adjusted to 2.5-3% SDS in Laemmli solubilization buffer, reduced with 100 mM DTT (2 minutes, 100°C), and subjected to SDS-PAGE on 10% polyacrylamide gels. Following electrophoresis, gels were transferred to nitrocellulose using a semidy unit. Blots were blocked with 4% milk, 0.1% Tween-20 in PBS, and then probed sequentially with primary and peroxidase-conjugated secondary antibodies in the same solution. Bound antibody was visualized by enhanced chemiluminescence. Primary antibodies used for blotting were mouse monoclonal antibody 7C12 and the following affinity-purified rabbit polyclonal antipeptide antibodies: anti-SCAMP1 (1Ω, Wu and Castle, 1997); anti-SCAMP2 (2Ω, Wu and Castle, 1997; and 2γ, raised against the peptide (C)-QPSVEPTQPTPQ coupled to maleimide-activated keyhole limpet hemocyanin); and anti-SCAMP3 (3γ raised against the peptide (C)-SPTEPKNYGSYSTQ, also coupled to hemocyanin). For sequential immunoprecipitation and western blotting, cell lysates prepared in 1% CHAPS, 10 mM Tris-HCl, protease inhibitors as above, were incubated for 1 hour at 25°C with 10 μg of purified 7C12 antibody, and antigen-antibody complexes were adsorbed to 2 mg of Protein A-Sepharose precoated with a rabbit anti-mouse IgG bridge. After washing 3 times in ice-cold CTEG buffer, and once in ice-cold PBS, samples were eluted in Laemmli sample buffer plus 100 mM DTT and treated for western blotting as above.

Relative intracellular distribution of SCAMP paralogs

The intracellular distribution of SCAMP paralogs was examined in HeLa cells cultured in DMEM containing 5% FBS. Relative intracellular distributions of SCAMP paralogs once in ice-cold PBS, samples were eluted in Laemmli sample buffer and then washed 3 times in ice-cold CTEG buffer, and the lysates were prepared in 1% CHAPS, 10 mM Tris-HCl, protease inhibitors as above. After incubation for 1 hour at 25°C with 10 μg of purified 7C12 antibody, and antigen-antibody complexes were adsorbed to 2 mg of Protein A-Sepharose precoated with a rabbit anti-mouse IgG bridge. After washing 3 times in ice-cold CTEG buffer, and once in ice-cold PBS, samples were eluted in Laemmli sample buffer plus 100 mM DTT and treated for western blotting as above.

Relative affinity of antibody 7C12 for various SCAMP paralogs

cDNA fragments encoding the amino terminus of rat SCAMP1 (codons 1 to 151), and human SCAMP2 (codons 1 to 149) and SCAMP3 (codons 1 to 154) were generated by PCR using 5′ oligonucleotides beginning at the initiator methionine codon in the respective sequences, and appropriate 3′ oligonucleotides to yield fragments ending approximately at the junction between the N-terminal domain and the first predicted transmembrane domain. Specifically, the SCAMP1 fragment utilized a unique HindIII site at codon 151, the SCAMP2 fragment contained a XhoI site introduced adjacent to codon 149 by PCR, and the SCAMP3 fragment utilized a unique NcoI site at codon 154 in the sequence. The amino termini of rat and human SCAMP1 differ by only a single amino acid over a 150 amino acid segment (an alanine for threonine substitution), so it is unlikely that the affinity of 7C12 for this paralog would have species-specific differences. Cloned fragments were then ligated in frame into the EcoRI site of glutathione-S-transferase (GST)-fusion vector pGEX-KG, except for the SCAMP2 fragment, which was cloned into the SalI site. Constructs were expressed in bacteria following transformation into chemically competent strain XL-1Blue (Stratagene). GST-fusion proteins induced in logarithmically growing cells by a 2 hour incubation with 1 mM IPTG were purified from E. coli detergent extracts by binding to glutathione agarose and subsequent elution with glutathione. The purified proteins were freed of glutathione by gel filtration, assayed for protein concentration (BCA, Pierce), and the antigenicity normalized to equal protein was determined by quantitative western blotting using 7C12 primary antibody and 125I-goat anti-mouse secondary antibody.

RESULTS

Identification and characterization of SCAMP homolog cDNAs

The cloning of SCAMP homologs was facilitated by a combina-

Fig. 1. Relative similarity of SCAMP paralogs. Conceptual translations of SCAMP1, SCAMP2, and SCAMP3 from human were aligned as indicated in the text. Codon numbers of SCAMP1 and SCAMP3 are found above and below the respective sequences. Identity of specific residues between sequences is indicated with lines, highly conserved substitutions are indicated with double dots, and moderately conserved substitutions with single dots. The four putative transmembrane domains are boxed, and microsequenced peptides from purified SCAMP2 (bovine liver) and SCAMP3 (rat adipocytes) are underlined.
ends with a polar segment (residues 284 to 304); and an alanine-rich carboxy terminus of variable length. In the central core, both the putative transmembrane domains and connecting loops are highly conserved in sequence and in length. The most divergent regions in the whole structure are toward the N-terminus just prior to the leucine zipper-like segment (residues 45 to 82 in SCAMP1). Finally, the spacing between each of the conserved regions is also well conserved, suggesting that each of the species might adopt a similar tertiary structure. Several mouse expressed sequence tags have also been identified as encoding SCAMP family members. EST clone 373999 (Lennon et al., 1996) was partially sequenced by the IMAGE consortium. Sequencing of the remainder of the cDNA indicates that the open reading frame encodes a full-length homolog of SCAMP3 which shows 89% identity and 90% similarity to the human protein. The most significant inter-species differences are at the extreme N terminus. Several additional overlapping mouse ESTs encode homologs of SCAMP2, and these show a degree of identity with the human protein comparable with that seen for SCAMP1. As indicated in Table 1, SCAMP3 shows the most significant inter-species differences between the SCAMP paralogs.

In vitro transcription and translation of each of the cDNAs isolated from screening a HeLa cell library was performed to examine the authenticity of the potential open reading frames (Fig. 2). Although the prospective open reading frames encoding SCAMPs 1, 2, and 3, respectively, predict proteins of $M_r=38,000$, $M_r=36,600$, and $M_r=38,300$ and isoelectric points of 7.38, 6.02, and 7.59, the products of in vitro translation migrate at slightly different relative apparent mobilities. Thus human SCAMP paralogs are actually $M_r=36,500-38,000$ polypeptides; however, we will refer to these proteins as SCAMPs 1, 2, and 3, in the order of their molecular characterization. In vitro transcription/translation of the mouse SCAMP3 clone generates a polypeptide of $M_r=40,000$, an apparent mobility higher than either the human SCAMP3 or SCAMP2 proteins (data not shown). Sequencing the mouse SCAMP3 clone indicates that the mouse homolog is slightly larger than the human protein sequence (an insertion of 2 amino acids at codon 56,) and the predicted isoelectric point of the mouse sequence is shifted downwards to 7.18, due to the net loss of positively charged amino acid residues. These differences may contribute to the different apparent mobilities of the mouse and human SCAMP3 homologs. Both the cloning and in vitro transcription/translation studies also confirm that different proteins are not the product of differing post-translational modifications of a single precursor. In view of our earlier demonstration that SCAMP1 and SCAMP2 are not glycosylated (Brand et al., 1991), it is unlikely that any post-translational modifications contribute significantly to the electrophoretic mobility, and thus the mobility differences observed probably reflect different SDS binding among different SCAMPs.

### Recognition of proteins using peptide-specific antibodies

Polyclonal antibodies were raised against synthetic peptides corresponding to deduced sequences from the cDNAs and were used to characterize antigens by western blotting of solubilized membrane samples and immunoprecipitates from HeLa and CHO cells. The monoclonal antibody 7C12 was used as a

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**Table 1. Pairwise comparison of SCAMP protein sequences**

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SCAMP sequences were compared pairwise using the program BESTFIT (GCG) over the entirety of the respective sequences (overall), between the putative leucine zipper and the carboxy terminus (LZ to CT), between the leucine zipper and the first transmembrane domain (LZ to TM), and between the amino terminus and the leucine zipper (NT to LZ). The sequences that were compared were the three human SCAMPs presented in this manuscript (hSC1, hSC2, and hSC3), rat SCAMP1 (rSC1, Brand and Castle, 1993; PIR entry S37395), mouse SCAMP2 (EST clones 423042 and 575301, together cover 43% of the predicted ORF), and mouse SCAMP3 (fully sequenced EST clone 373999).

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**Fig. 2.** In vitro transcription and translation of SCAMP cDNAs. Plasmid DNA isolated from the HeLa cDNA library was linearized at the 3′ end, RNA was synthesized using T3 RNA polymerase, and translated using a rabbit reticulocyte lysate (Promega) in the presence of [35S]methionine (ICN) and canine pancreatic microsomes (Promega). Reaction products were analyzed by electrophoresis in 11% polyacrylamide gels, and fluorography using X-ray film. The gel was loaded as follows; lane 1, SCAMP2; lane 2, SCAMP1, lane 3, SCAMP3; lane 4, no RNA; lane 5, control RNA. The translation product of the control RNA is β-lactamase from *E. coli*, which is processed by the microsomes to yield a 29 kDa product. Size standards are shown at left (kDa).
SCAMPs define a multi-gene family

As can be seen in Fig. 3A and B, 7C12 detects three bands of different apparent Mr in CHO cell extracts and two bands in HeLa cell extracts. Peptide-specific antibody for SCAMP1 (1Ω, lanes 3, 4 in Fig. 3A) and SCAMP2 (2Ω and 2γ, lanes 5-8) recognize bands in rodent and primate samples of 37 and 39 kDa, respectively. Note that 2γ recognizes SCAMP2 in HeLa extract very well (lane 8) but only poorly in CHO extract (lane 7) and that 2Ω recognizes an additional band (~45 kDa) whose nature is not known at present. Anti-SCAMP3 (3γ) recognizes a 40 kDa band from CHO extract having the same mobility as the upmost band detected by 7C12 (Fig. 3A,B) and as the in vitro translation product of mouse cDNA encoding SCAMP3 (data not shown). In HeLa extract, however, 3γ recognizes a band that migrates faster (~38 kDa, Fig. 3A,B) and corresponds in mobility to the in vitro translation product encoded by the HeLa cDNA encoding SCAMP3 (Fig. 2). Finally, antibody 3γ recognizes a second 80 kDa band in the CHO extract (Fig. 3A, lane 9). This band almost certainly represents an SDS-resistant dimer of SCAMP3, as its amount in relation to the 40 kDa monomer varies substantially for different electrophoretic runs of the same sample.

It should be noted that the apparent mobilities of SCAMP1 and SCAMP2 recognized by the monoclonal antibody 7C12 are extremely similar from a variety of mammalian species, such as from rodents (Chinese Hamster ovary cells) and humans (HeLa cells). SCAMP3, however, shows inter-species variability in its apparent mobility, and potentially might co-migrate with SCAMP2 in tissues from human sources, and consequently obscure its relative abundance.

**Tissue distribution of SCAMP paralogs**

To address the possibility that individual SCAMPs might exhibit tissue- or cell-specific expression patterns, their distributions were compared at both the mRNA and protein levels from a variety of sources. Since the cDNAs were all isolated from a human cDNA library, mRNA expression was analyzed by sequentially probing and stripping a human multiple tissue northern blot. SCAMP expression was readily detectable in every tissue examined (Fig. 4), but the relative amounts of mRNA for individual SCAMPs differed widely. SCAMP2 is present in most tissues examined; SCAMP3 expression is greatest in heart and skeletal muscle, and SCAMP1 is especially abundant relative to other SCAMPs in brain. Sizes of...
transcripts were estimated with markers, and the predicted transcript sizes that are indicated in Fig. 4 were identical to those of the cDNAs isolated from the HeLa library. The identity of the 2.4 kb band hybridizing in samples probed for SCAMP2 is unknown, but high stringency washes diminished the intensity of that band to the same degree as the lower mobility band corresponding to the full length transcript (data not shown). This result suggests that the higher band possibly corresponds to an alternatively spliced transcript or one with a longer 3’ untranslated region. Notably, sequential reprobing of the blot with the paralog-specific cDNAs resulted in no apparent cross-hybridization with transcripts encoding the other SCAMPs. Interestingly, previous studies examining the protein expression of SCAMPs in a variety of rat tissues (Brand et al., 1991; Brand and Castle, 1993) correlate well with the mRNA levels from the battery of human tissues depicted in Fig. 4. For instance, SCAMP1 is unusually abundant in brain as assessed by western blotting, but is almost undetectable in kidney. These observations suggest that specific tissues may exhibit a similar composition of paralogs among different mammals.

**Relative affinity of 7C12 for SCAMP paralogs**

Although Fig. 3 indicates that each of the SCAMP paralogs can be immunoprecipitated using monoclonal antibody 7C12, it is frequently difficult to detect SCAMP3 polypeptide in

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**Fig. 5.** Relative intracellular distribution of SCAMP paralogs. HeLa cells were double labeled with either anti-SCAMP1 polyclonal 37Ω (A), anti-SCAMP2 polyclonal 39Ω (C) or anti-SCAMP3 polyclonal 38γ (E), and the anti-SCAMP monoclonal 7C12 (B,D,F), and examined by immunofluorescence. Bar, 10 μm.
rodent tissues (apparent mobility 40 kDa) that were expected to express abundant amounts of the cognate mRNA. This raised the possibility that the monoclonal antibody 7C12 was much less efficient in detecting SCAMP3 as compared to the other paralogs. To determine the relative affinity of 7C12 for the various SCAMP paralogs, we constructed fusion proteins encoding the N-terminal ~150 amino acids of each SCAMP linked to glutathione S-transferase (GST) in the bacterial expression vector pGEX-KG. The recombinant proteins were purified on glutathione-agarose, protein concentration was determined, and the relative affinity of antibody 7C12 for each SCAMP was compared by quantitative western blotting using an 125I-labeled secondary antibody (data not shown). Quantitation of bound, labeled antibody with normalization to the amount of recombinant protein demonstrates that 7C12 is able to recognize SCAMP1 almost twofold better than SCAMP2, and sevenfold better than SCAMP3.

In vivo colocalization of SCAMP paralogs

To determine whether SCAMPs colocalize within cells or have different patterns of distribution, double label immunofluorescence (Fig. 5) was carried out using HeLa cells. The distributions of individual SCAMPs were assessed using the paralog-specific anti-peptide antibodies, and were each compared to staining with monoclonal antibody 7C12. As can be seen in Fig. 5, all the SCAMPs show extensive codistribution with 7C12 staining. While the staining patterns are practically identical for SCAMPs 1 and 2 as compared to those observed with the monoclonal, the staining for SCAMP3 shows minor differences suggesting that this paralog may be unusually enriched in selected compartments, particularly towards the cell periphery. Notably, these distributions are also reiterated by myc epitope-tagged SCAMPs 2 and 3 when expressed in CHO cells and compared to the staining of endogenous SCAMP1 (data not shown).

DISCUSSION

The present study describes the identification and initial characterization of three distinct SCAMPs from humans and documents that the three paralogs that are now known form a family of highly related gene products. The availability of this new information has enabled us to appreciate the impressive conservation of a single SCAMP homolog among different mammals and the structural similarities among paralogs from a single species. These features also help to develop a picture of the domains that prospectively serve as regulatory and functional elements within the protein.

We have found that the sequences of rat and human SCAMP1 are 98% identical, and this level compares favorably with the identities that have been observed previously for other proteins that comprise the post-Golgi vesicular trafficking machinery, e.g. NSF, 97%; αSNAP, 95%; syntaxin, 98%; synaptobrevin, 96%; synaptotagmin, 98%. Furthermore, expressed sequence tags from various mouse tissues corresponding to the three SCAMPs presented here demonstrate the same high level of conceptual protein conservation with their respective human homologs. It may be of special significance that individual SCAMPs exhibit such high identity even though they reside and presumably function in both constitutive and regulated recycling pathways (Brand et al., 1991; Brand and Castle, 1993; Wu and Castle, 1997). For example, the identity is higher than for proteins like synaptobrevin and cellubrevin (70% identical) and the yeast syntaxin relatives SSO1/SSO2 and PEP12 (20% identical, 50% similar), which are considered to be analogs that function in different post-Golgi pathways. In this respect, SCAMPs may operate more generically among the various post-Golgi pathways. It is not yet clear whether SCAMPs individually encode targeting information that distinguishes between transport routes.

Evidently, the different paralogs of SCAMP are less identical to each other than are individual homologs compared between species. Paralog sequences are about 50% identical and 65% similar overall. However, the organization indicates that within particular domains, the sequence conservation is greater than 90%. Because the complete lengths and spacing between conserved domains are so similar between paralogs, we believe that their higher order structures will also be very similar and that their function and regulation (mediated by the conserved domains) will also prove to be very similar. The most divergent feature in the overall structure includes a segment within the amino terminus that is proximal to the highly conserved leucine zipper-like motif. By analogy with known leucine zipper containing proteins (Vinson et al., 1993; Baxevanis and Vinson, 1993), this segment may confer the capability to bind specific targets among the paralogs. Also, the length and sequence of the alanine-rich C terminus differs sufficiently to enable generation of paralog-specific antibodies (Wu and Castle, 1997), and the ways in which this variation might contribute to unique function among SCAMP paralogs are not yet apparent.

One further feature of interest between SCAMPs is the extensive structural conservation between the putative transmembrane domains of the three SCAMP paralogs. The percentage identity and similarity that we have observed considerably exceeds that of synaptobrevin/cellubrevin (25% identity, 70% similar, McMahon et al., 1993) and is comparable to that of synaptophysin/pantophysin (45% identity, 67% similarity, Haass et al., 1996). Although the roles of the physins are not yet known we believe that, as for SCAMPs, these transmembrane segments will prove to be key functional elements.

We have verified by protein immunopurification and microsequencing that the 39 kDa band visible on western blots (Fig. 3) corresponds to the SCAMP2 sequence presented here. We have demonstrated that a peptide-specific polyclonal antibody is able to recognize a band of the correct apparent electrophoretic mobility in lysates of cultured cells, and that the mRNA is abundantly expressed in a variety of tissues. However, we have found that 7C12 recognizes SCAMP3 several-fold more poorly than the other SCAMPs. Also, the apparent similar mobility of in vitro translated proteins in SDS-PAGE gels (Fig. 2) would suggest that SCAMP2 might obscure at least partially the presence of SCAMP3 on blots.

Our findings regarding the distribution of SCAMPs among tissues and within cells tentatively provide some guiding insight as to how SCAMPs might function. While there are some variations in the amounts of individual SCAMPs expressed in certain tissues, e.g. brain, which is unusually enriched in SCAMP1 (Fig. 4; and Brand and Castle, 1993) and polymorphonuclear leukocytes, which are highly enriched in SCAMP2 (Brumell et al., 1995), the general picture seems to
be that most tissues express detectable amounts of each SCAMP. Certainly, the growing number of studies that we are conducting with cultured cell lines suggests that all three SCAMPs are typically expressed in many individual cells. SCAMP distribution in cultured cells has been examined in a recent notable report (Haass et al., 1996) The authors examined the comparative distribution of the broadly expressed synaptophtysin homolog pantophysin in a human epithelial cell line. Of the markers they examined (β-COP, transferrin receptor, LAMP-1, cellubrevin, and SCAMPs,) colocalization of pantophysin was most complete with only the v-SNARE cellubrevin and SCAMPs. Transferrin receptor also showed good but incomplete colocalization with pantophysin, and this was previously shown to be the case as well for SCAMPs and transferrin receptor using 7C12 (Brand and Castle, 1993) and with paralog-specific antibodies (Wu and Castle, 1997). These reports would indicate that SCAMPs, like pantophysin, collectively reside in recycling transport vesicles comprising post-Golgi and endocytic pathways.

Within these pathways, a significant issue is whether SCAMP paralogs are functionally redundant, complement one another at the same sites, or perform analogous functions in different pathway segments. The comparative localization studies presented in Fig. 5 and elsewhere (Wu and Castle, 1997) illustrate that individual SCAMPs extensively colocalize with one another in fibroblasts and also in the regulated pathway of specialized secretory cells. While the codistribution of SCAMP1 and SCAMP2 is especially thorough everywhere it has been examined, our findings also point to detectable differences for at least a portion of SCAMP3. We feel that the data argue against the possibility that the SCAMPs are analogs that function in separate pathways, but instead are either complementary or redundant within the same pathway(s). Indeed, the results obtained by immunoprecipitation that are presented elsewhere (Wu and Castle, 1997) raise the interesting possibility that SCAMPs may cooperate as parts of a larger functional complex. However, in view of both the extreme enrichment of particular SCAMPs that are observed in some cell types and the incomplete codistribution of SCAMP3 with other SCAMPs, it also seems possible that the nature of the prospective complexes may vary both within and between cells. Evidently further investigation of these important issues is needed, and future studies may be aided greatly by the availability of the new antibody that binds SCAMP3 selectively.

The ubiquitous presence of SCAMPs in mammalian tissues and in post-Golgi recycling pathways and the high degree of evolutionary conservation argue that SCAMPs perform an essential function. So far, however, this function remains elusive. Structurally, the SCAMPs have no significant relatives in protein databases, although there is a limited resemblance to synaptophtysin/pantophysin, another family of putative four transmembrane pass proteins with a similar localization. Certain conserved structural elements within SCAMPs, e.g. the leucine zipper-like heptad repeat and the succeeding proline-rich segment resembling an SH3 binding site, are likely to interact with other proteins, maybe even those that function in vesicular trafficking. However, to our knowledge, no interactions with more heavily studied trafficking machinery (e.g. SNAREs, rabs) have been detected. Nevertheless, we maintain an interest in the possibility that SCAMPs contribute to the process of vesicle targeting/fusion because the interactions that mediate these events are complex and may be transient. Finally, we note that the putative transmembrane domains have extensively conserved primary structures, arguing that they might perform an essential role in SCAMP function. As these segments are nearly devoid of charged residues, we doubt that SCAMPs function as membrane-bound transporters.

As a final issue, we consider the interesting question of whether there are more SCAMP paralogs to be described. While SCAMPs appear to be confined to post-Golgi recycling pathways, most other proteins that function in post-Golgi trafficking have analogs that function in ER-Golgi and inter-Golgi transport (Ferro-Novick and Jahn, 1994). So far the monoclonal antibody, which has been our most reliable experimental tool, routinely recognizes on western blots from rodent tissues only 37 and 39 kDa bands, and a more slowly migrating 40 kDa band that is apparently a species variant of the SCAMP3 protein (Fig. 3; see also Wu and Castle, 1997). Together with two-dimensional analysis by IEF/SDS-PAGE, we observe surprisingly little diversity of each band (data not shown). We have recognized, however, that monoclonal antibody 7C12 has limitations in that there are variations in the avidity with which it binds to the different SCAMPs that we have characterized. Thus the presence of other SCAMP paralogs is certainly possible. Interestingly, however, all of the SCAMP-related sequences that we have identified by routinely screening the EST databases available for mouse, rat and human clones are readily assigned as relatives of one of the described SCAMP sequences. One intriguing exception is a novel mouse EST clone which we have recently sequenced in its entirety. The conceptual translation of this clone, which is very likely to be full-length based upon Northern analysis of RNA isolated from cultured cell lines, represents a distinct gene with the same conserved domain structure outlined in Fig. 1, except that the first available initiator codon begins to encode an open reading frame which completely lacks the first 100 amino acids of the other SCAMPs, up to and including the putative leucine zipper. From this point on, however, the sequence is as conserved with the other SCAMPs as they are with each other. The putative reading frame has not been characterized further, as none of the peptide-specific antibodies used in this study are able to recognize a protein of the correct electrophoretic mobility on blots. Nevertheless, we keep open the possibility that related proteins may be identified that support other vesicle trafficking events, especially as new antibodies currently being characterized suggest that related antigens with different intracellular distributions may be present.

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Ruedi Aebersold (Department of Molecular Biotechnology, University of Washington) for providing tryptic peptide sequences from SCAMP3. The sequences described in this manuscript have been assigned GenBank accession numbers AF005036, AF005037, AF005038 and AF005039.

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