Secretory carrier membrane proteins interact and regulate trafficking of the organellar $(Na^+,K^+)/H^+$ exchanger NHE7

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Summary

The mammalian $(Na^+,K^+)/H^+$ exchanger NHE7 resides chiefly in the trans-Golgi network (TGN) and post-Golgi vesicles where it is thought to contribute to organellar pH homeostasis. However, the mechanisms that underlie the targeting and regulation of NHE7 are unknown. To gain insight into these processes, yeast two-hybrid methodology was used to screen a human brain cDNA library for proteins that interact with the cytoplasmic C-terminus of NHE7. One binding partner we identified was SCAMP2, a member of the secretory carrier membrane protein (SCAMP) gene family. Direct association of these two proteins was further supported by co-immunolocalization and co-immunoprecipitation analyses using transfected cells, by their co-sedimentation in membrane fractions resolved on sucrose density gradients, and by in vitro protein binding assays. Other members of the SCAMP family, such as SCAMP1 and SCAMP5, also associated with NHE7. The majority of the NHE7-SCAMP complexes accumulated at the TGN, but a minor fraction also resided in recycling vesicles. Biochemical analyses indicated that the C-terminal cytoplasmic tail of NHE7 bound preferentially to a highly conserved cytoplasmic loop between the second and the third transmembrane segments (TM2-TM3 loop) of SCAMP2. A deletion mutant of SCAMP2 lacking this region (SCAMP2/Δ184-208) bound weakly to NHE7, but caused a significant fraction of NHE7 and wild-type SCAMP2 to redistribute to a pool of scattered recycling vesicles without noticeably affecting the location of other resident TGN (syntaxin 6) or Golgi cisternae (GM130) proteins. Conversely, a GFP-tagged TM2-TM3 construct of SCAMP2 interacted with NHE7, but also led to the redistribution of NHE7 to dispersed vesicular structures. We propose a model wherein SCAMPs participate in the shuttling of NHE7 between recycling vesicles and the TGN.

Key words: Na^+/H^+ exchanger (NHE), Protein trafficking, Secretory carrier membrane proteins (SCAMPs), Protein-protein interaction, trans-Golgi network (TGN), Recycling endosome

Introduction

The pH of organelles along the secretory and endocytic pathways is acidic and largely determined by the balance between the active accumulation of H^+ driven by the vacuolar H^+-ATPase (V-ATPase) (Nelson, 2003; Nishi and Forgac, 2002) and by the efflux of H^+ through ill-defined pathways (Grabe and Oster, 2001; Schapiro and Grinstein, 2000; Wu et al., 2001). In addition, counterion conductances of Cl^- members of the CLC Cl^- channel family also contribute significantly to organellar acidification (Jentsch et al., 2002). Fine control of organellar pH is crucial for the execution of various physiological/pathological processes such as regulated receptor-ligand interactions (Dautry-Varsat et al., 1983), endocytosis (Aniento et al., 1996; Chapman and Munro, 1994; Maranda et al., 2001; Mellman et al., 1986), post-translational processing and sorting of newly synthesized proteins (Chanat and Huttner, 1991; Halban and Irminger, 1994), release of proteins from eosinophilic granules (Kurashima et al., 1996) and entry of some viruses during infection (Durrer et al., 1996; Huber et al., 2001; Mothes et al., 2000). It has also been suggested that elevation of organellar pH causes apoptosis (Akifusa et al., 1998; Ohta et al., 1998), and that irradiation may trigger the formation of acidic organelles that can protect cancer cells from cell death (Paglin et al., 2001).

A role for Na^+/H^+ exchangers (NHEs) in organellar function has only recently been appreciated. At least nine isoforms have been identified in mammals that are expressed in a tissue-specific manner and are differentially sorted to discrete membrane locations (de Silva et al., 2003; Orlowski and Grinstein, 2004). NHE1 to NHE5 are resident and functional in the plasma membrane, and hence are commonly referred to as 'plasmalemmal-type' isoforms. However, isoforms such as NHE3 (D'Souza et al., 1998) and NHE5 (Szaszi et al., 2002) are not restricted to the cell surface and can internalize into a recycling endosomal pool where they modulate the luminal pH of that compartment and potentially serve as a reservoir of functional transporters. By comparison, NHE6 to NHE9 are more structurally divergent and appear to accumulate primarily

in intracellular compartments (Orlowski and Grinstein, 2004). For instance, NHE7 localizes predominantly to the TGN and to a lesser extent in post-Golgi vesicles (Numata and Orlowski, 2001). More significantly, NHE7 is distinguished from plasmalemmal-type NHEs by its ability to transport either Na^+ or K^+ in a H^+ gradient-dependent manner, and hence was proposed as a contributing factor in organellar pH and possibly volume homeostasis. Isoforms such as NHE7 are evolutionary conserved from yeast to man (Apse et al., 1999; Giannakou and Dow, 2001; Nass et al., 1997; Nehrke and Melvin, 2002; Venema et al., 2002; Yokoi et al., 2002), and yeast mutants devoid of a functional vacuolar NHE ortholog show impaired endosomal protein trafficking (Bowers et al., 2000). Thus, multiple organelle-type NHEs may regulate acidification of organelles, which is an important determinant for protein sorting along the exocytic and endocytic pathways in eukaryotes.

Recent studies of plasmalemmal-type NHEs have begun to identify various interacting proteins and cofactors, including kinases, phosphoinositides, scaffolding proteins and cytoskeletal elements, which modulate their transport activities (Fliegel, 2005; Noel and Pouyssegur, 1995; Orlowski and Grinstein, 2004; Putney et al., 2002). In contrast, the regulation of organellar-type NHEs is largely unknown. To gain insight into factors that govern the targeting and regulation of NHE7, we screened a human brain cDNA library using yeast two-hybrid methodology to identify potential interacting proteins. In this report, we describe secretory carrier membrane proteins (SCAMPs), specifically SCAMP1, -2 and -5, as binding partners of NHE7. SCAMPs are a family of post-Golgi (Brand and Castle, 1993; Brand et al., 1991) and Golgi (Bell et al., 2001) membrane proteins composed of four transmembrane segments flanked by N- and C-terminal cytoplasmic extensions. Five isoforms have been isolated from mammals and they have been implicated in both exocytosis (Fernandez-Chacon et al., 1999; Guo et al., 2002; Liu et al., 2002) and endocytosis (Fernandez-Chacon et al., 2000). Although SCAMPs have been implicated in vesicular trafficking, there is no evidence showing that SCAMPs can directly target other proteins to specific intracellular compartments. Here we show that the cytoplasmic loop between transmembrane segments TM2 and TM3 of SCAMP2 confers binding to NHE7, and that overexpression of dominant-negative mutants of SCAMP2 caused NHE7 to redistribute preferentially to scattered peripheral recycling endosomes rather than concentrate perinuclearly at the TGN. These data implicate SCAMPs as important elements in the trafficking of NHE7 between the TGN and associated recycling vesicles.

Materials and Methods

Antibodies

Mouse monoclonal and rabbit polyclonal anti-HA antibodies, anti-myc antibodies (A-14 and 9E10) and mouse monoclonal anti β-COP (clone maD) were obtained from Covance (Richmond, CA), Santa Cruz Biotechnology (Santa Cruz, CA) and Sigma (Oakville, ON, Canada), respectively. Rabbit anti-GFP (ab290) was obtained from abcam (Cambridge, MA). Antibodies against SCAMP1, -2, and -5 were purchased from Affinity BioReagents (Golden, CO); antibodies against syntaxin 13 and PDI were purchased from StressGen (Victoria, BC, Canada); anti-GM130, EEA1, syntaxin 6, Rab11, and γ-adaptin antibodies were purchased from BD Biosciences (Mississauga, ON, Canada). Anti-synaptophysin (SY38) was from Chemicon (Temecula, CA). Anti-transferrin receptor antibody was from Zymed Laboratories (South San Francisco, CA). The purified mouse monoclonal antibody rho 1D4 against a nine amino acid TETSQVAPA C-terminal epitope (Hodges et al., 1988; MacKenzie and Molday, 1982) was obtained from National Cell Culture Center (Minneapolis, MN). The antibody was coupled to CNBr-activated Sepharose 2B as previously described (Oprian et al., 1987).

Cell culture and transfection

CHO cells, 293-T cells and PC12 cells were maintained in α-MEM with 10% FBS, DMEM with 10% FBS and RPMI with 5% FBS, respectively. CHO and 293-T cells were transiently transfected by either a modified calcium phosphate method (Chen and Okayama, 1987) or Lipofectamine (Invitrogen). NHE7-HA and SCAMP-myc in a pCMV-based vector (0.5 μg of each) were simultaneously transfected into CHO cells grown on glass coverslips and intracellular localization of these proteins were analyzed 8-16 hours after transfection. PC12 cells stably expressing 1D4-tagged NHE7 were established by screening with 200 μg/ml G418 after transfection with pcDNA3 encompassing NHE7 with 1D4 tag at the C-terminal end of the clone. Single colonies were screened on western blots and a clone with an intermediate expression level was further characterized.

Yeast two-hybrid screening

Yeast two-hybrid screening was carried out as described previously (Gietz et al., 1997). The PCR fragment corresponding to H615-A725 (C-terminal end) of human NHE7 was ligated into the pAS2-1 vector (BD Biosciences, Mississauga, ON, Canada) in-frame with the *GAL4* 1-147 DNA binding domain (NHE7 [629-725]/pAS2-1). *Saccharomyces cerevisiae* strain AH109 (*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3, GAL2*$_{UAS}$*-GAL2*$_{TATA}$*-ADE2, ura3::MEL1*$_{UAS}$*-MEL1*$_{TATA}$*-lacZ*) was co-transformed with NHE7[629-725]/pAS2-1, carrying the *Trp* gene and the *GAL4* DNA binding domain and a human brain cDNA library cloned into the pACT2 vector (BD Biosciences), carrying the *LEU2* gene and the *GAL4*-[768-881] activation domain using modified lithium acetate method (Gietz and Schiestl, 1991). Over 2×10^6 clones were screened on the synthetic complete (SC) media containing 0.67% bacto-yeast nitrogen base without amino acids (DIFCO laboratories, Detroit, MI), 2% glucose, 1.5% bacto-agar and 0.2% Leu^-, Trp^-, His^-, Ade^- drop-out mix and subjected to β-galactosidase filter assay. Library-derived cDNA clones in pAC2-1 coding for putative NHE7 interacting proteins were rescued from yeast cells and directly transformed into DH5α cells. The identities of the clones were determined and compared against the National Center for Biotechnology Information (NCBI) database using the BLAST search program.

Molecular cloning of SCAMP cDNAs and expression constructs

To obtain the full-length cDNA encoding SCAMP1 and SCAMP5, first-strand cDNA was synthesized from human brain total RNA by the use of random primers, and subjected to PCR as described previously (Numata et al., 1998). The following primers were used: SCAMP1 forward, 5′-GGG GTA CCA CCA TGT CGG ATT TCG ACA GTA ACC C-3′ (*Kpn*I site is underlined); SCAMP1 reverse, 5′-CTT AAA TCT GGT TAC CCT TGA AAG C-3′; SCAMP5 forward, 5′-CGG GAT CCA CCA TGG CAG AGA AAG TGA ACA ACT TC-3′ (*Bam*HI site is underlined); and SCAMP5 reverse, 5′- TCA CAT CTC ATT GGA GTA CGT GTA ATT GGG-3′. The PCR fragments were verified by restriction digests and subjected to a second round of PCR to introduce a myc- or an HA-tag at the extreme C-terminus, then digested

with appropriate restriction enzymes and ligated into the mammalian expression vector pcDNA3. N-terminal and internal deletion mutations were created according to established protocols (Sambrook and Russell, 2001). To make a GFP fusion construct containing the TM2 to TM3 region of SCAMP2, the PCR fragment corresponding to G181-G246 was ligated into the C-terminal end of the GFP expression construct to create an in-frame fusion protein. The nucleotide sequence of the constructs was verified by automated sequencing.

Expression and purification of GST fusion proteins

For producing GST fusion proteins, PCR fragments corresponding to different regions of SCAMP2 and NHE7 were inserted into the pGEX-2T bacterial expression vector (Amersham Pharmacia Biotech) in-frame with the N-terminal GST tag. Protein expression was induced by incubating transformed BL21 *Escherichia coli* cells with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 30°C for 3 hours. *E. coli* cells were collected by centrifugation, resuspended in lysis buffer containing 1% Triton X-100 and proteinase inhibitor cocktail (Roche Diagnostics, Laval, Canada) in PBS. Cell lysates were then incubated on ice for 30 minutes and sonicated four times for 30 seconds. After cell debris was removed by centrifugation for 20 minutes at 16,000 ***g*** at 4°C, GST fusion proteins were purified by incubation with reduced form glutathione sepharose beads (Amersham Pharmacia Biotech) at 4°C.

Glutathione S-transferase (GST) pull-down

^{35}S-labeled protein was produced by in vitro transcription-translation using the TnT-coupled reticulocyte lysate system (Promega, Madison, WI) according to the manufacturer's instructions. ^{35}S-labeled in vitro translated protein was centrifuged at 100,000 ***g*** for 30 minutes to eliminate insoluble materials. The supernatant (2-5 µl) was incubated with 2 µg GST fusion protein immobilized to the reduced form glutathione sepharose beads for 90 minutes at room temperature. After extensive washing, ^{35}S-labeled in vitro translated protein bound to the GST fusion protein was eluted with SDS sample buffer and detected by SDS-PAGE followed by phosphorimaging. In some experiments, cell lysate was prepared by lysing CHO cells transiently transfected with myc-tagged SCAMPs. This lysate was incubated with GST fusion proteins and the bound SCAMPs were detected on western blots with an anti-myc antibody.

Co-immunoprecipitation

Cells were lysed in PBS containing 0.5% NP40, 1 mM EDTA and proteinase inhibitor cocktail (Roche) on ice for 30 minutes and lysates were cleared for 20 minutes at 16,000 ***g*** at 4°C. A 500 µl volume of cell lysate was incubated with 1.5 µl anti-HA monoclonal antibody at 4°C for 2 hours, followed by overnight incubation with 20 µl protein G sepharose beads (Amersham Pharmacia Biotech). To detect endogenous SCAMP5 protein bound to NHE7, a membrane fraction was isolated as described previously (Numata and Orlowski, 2001) and used for co-immunoprecipitation experiments. The beads were washed with lysis buffer five times and bound proteins were detected by western blot with an anti-myc rabbit polyclonal antibody. In some experiments, the anti-myc rabbit polyclonal antibody was used for immunoprecipitation and the anti-HA mouse monoclonal antibody for detection by western blot. To detect endogenous SCAMP proteins associated with NHE7, anti-SCAMP1, -2, and -5 antibodies were used.

Immunofluorescence microscopy

Cells were transiently transfected with HA-tagged NHE7 and myc-tagged SCAMPs, then fixed with 2% paraformaldehyde in PBS for 20 minutes. Samples were permeabilized with 0.1% Triton X-100 in PBS (PBS-TX) for 15 minutes, incubated with 2% normal goat serum in PBS for blocking, and incubated with 5 µg/ml anti-HA mouse monoclonal antibody and 1 µg/ml rabbit polyclonal anti-myc antibody (A-14) simultaneously for 1 hour. After four washes with PBS-TX, cells were incubated with 2 µg/ml Alexa 568-conjugated anti-mouse IgG and Alexa 488-conjugated anti-rabbit IgG (Invitrogen) for 45 minutes. All the antibodies were diluted in PBS. After extensive washes with PBS-TX, the coverslips were briefly rinsed with distilled water, mounted on glass slides and analyzed by confocal microscopy. To evaluate intracellular localization of endogenous SCAMP1, -2 and -5 proteins, cells were fixed with methanol/acetone 1:1 mixture at –20°C for 20 minutes and stained with anti-SCAMP antibodies as above.

Sucrose equilibrium density centrifugation

Sucrose equilibrium density centrifugation was conducted as described previously (Xu et al., 1997; Yan et al., 2001) with minor modifications. Cell homogenates were prepared by mild disruption through a 26.5-gauge needle in 250 mM sucrose, 10 mM HEPES-NaOH, pH 7.5, 1 mM EDTA, with proteinase inhibitor cocktail (Roche). After centrifugation at 800 ***g*** for 10 minutes, supernatant was applied to the top of a discontinuous sucrose gradient comprising 570 µl of 2 M; 960 µl of 1.3 M; 960 µl of 1.16 M; 770 µl of 0.8 M; 770 µl of 0.5 M; and 380 µl of 0.25 M sucrose prepared in 10 mM Tris-HCl, pH 7.4 in ultra clear 13×51 mm tubes (Beckman). Samples were centrifuged at 100,000 ***g*** for 2.5 hours in an SW50.1 rotor at 4°C. Fifteen 325 µl fractions were collected from the top of the gradient.

Results

Identification of SCAMPs as NHE7 interacting proteins

To identify novel NHE7 interacting proteins, we performed yeast two-hybrid screening of a human brain cDNA library using part of the C-terminal tail of NHE7 (residues 615-725) as bait. Out of 2×10^6 independent clones screened, approximately 300 positive clones were obtained, of which 100 were sequenced. One clone encoding full-length SCAMP2 was isolated as a candidate binding protein of NHE7 (Fig. 1A). To investigate whether NHE7 and SCAMP2 interact in cultured cells, CHO cells were transiently co-transfected with mammalian expression vectors containing HA-tagged NHE7 and myc-tagged SCAMP2. NHE7 was immunoprecipitated with an anti-HA antibody from cell lysates and bound SCAMP2 was detected by western blotting using an anti-myc antibody. A 38 kDa band was detected in immunoprecipitated sample obtained from CHO cells co-transfected with $NHE7_{HA}$ and $SCAMP2_{myc}$ (Fig. 1B). In a reciprocal experiment, $NHE7_{HA}$ was shown to bind to immunoprecipitated $SCAMP2_{myc}$ (data not shown). A control sample immunoprecipitated with pre-immune serum did not yield the corresponding band. Immunoprecipitated $NHE6_{HA}$, a closely related isoform, did not show a detectable level of bound $SCAMP2_{myc}$, further supporting the specificity of NHE7-SCAMP2 binding.

In mammals, five SCAMP isoforms (SCAMP1-5) have been identified (Fernandez-Chacon and Sudhof, 2000; Singleton et al., 1997). Of these, SCAMP1 and SCAMP5 are major SCAMP isoforms found in the brain where NHE7 is also highly expressed. SCAMP1 and SCAMP2 represent longer isoforms whereas SCAMP5 lacks a part of the N-terminal cytoplasmic extension (Fig. 1A). In order to determine whether other SCAMP isoforms also interact, full-length cDNAs encoding SCAMP1 and SCAMP5 were cloned by RT-PCR using random-primed first-strand cDNAs synthesized from

brain mRNA as a template. The myc-tagged SCAMPs were simultaneously transfected with $NHE7_{HA}$ into CHO cells. Cell lysates were immunoprecipitated with an anti-HA antibody and bound SCAMP protein was detected by western blotting using an anti-myc antibody. A specific 36 kDa band corresponding to SCAMP1 was detected in the immunoprecipitated samples with anti-HA, but not pre-immune serum (Fig. 1B). Likewise, a distinct 29 kDa band corresponding to SCAMP5 appeared only in the cell lysate immunoprecipitated with anti-HA antibody. A fainter band ~25 kDa in size was observed both in the samples immunoprecipitated with anti-HA and pre-immune serum (asterisk in Fig. 1B), which probably represents non-specific binding of the antibody.

The association of SCAMP1 and SCAMP5 with NHE7 was also investigated by in vitro assays. A GST-fusion protein containing the C-terminal tail of NHE7 (GST-NHE7[542-725]) was expressed in *E. coli* and purified with glutathione beads. The immobilized GST and GST-fusion proteins were incubated separately with PC12 whole-cell lysates and the glutathione beads were subsequently washed with 0.5% NP40 containing PBS to remove unbound proteins and facilitate detection of bound SCAMP1 and SCAMP5. Western blotting revealed binding of both SCAMP1 and SCAMP5 to the GST-NHE7 fusion protein, but not to GST alone (Fig. 1C).

To delineate the region of interaction between SCAMP2 and NHE7, GST pull-down assays were conducted using GST fusion proteins constructed from different segments of the C-terminal tail of NHE7. ^{35}S-labeled SCAMP2 showed specific association with GST-NHE7[542-725], GST-NHE7[615-725], and GST-NHE7[542-665], but not with GST-NHE7[666-725], GST-NHE7[542-614], or GST alone (Fig. 2). These results suggest that the SCAMP2 binding domain lies between residues 615 and 665 of NHE7, which lies within the C-

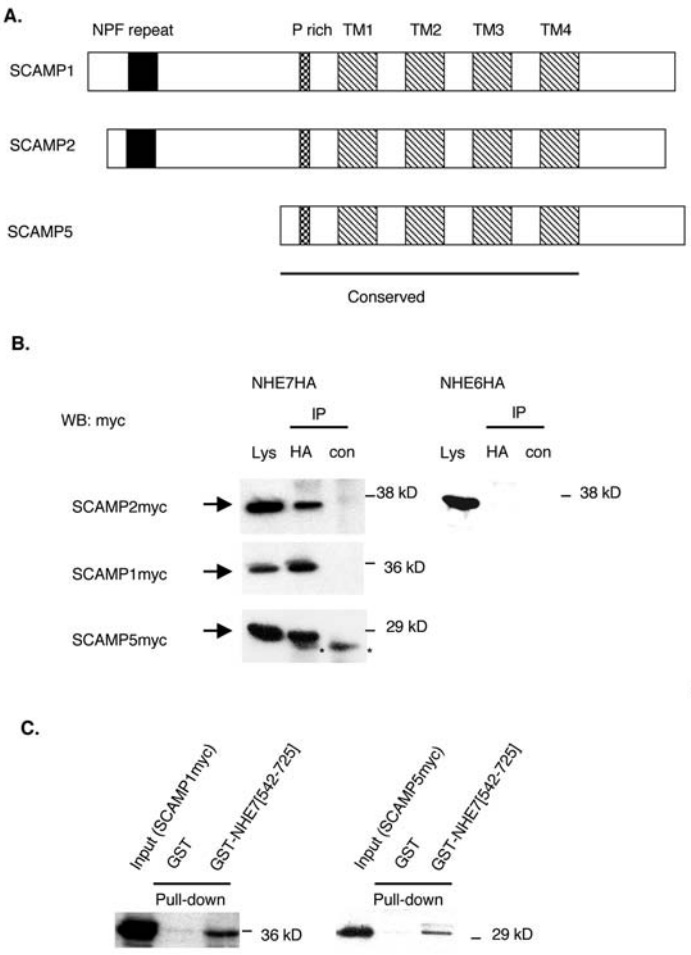

Fig. 1. SCAMPs interact with NHE7. (A) Schematic representation of SCAMP1, 2 and 5 showing the Asn-Pro-Phe (NPF) repeat and a highly conserved region (underlined) containing a Pro-rich (P rich) motif and four transmembrane (TM) segments. (B) CHO cells were transiently transfected with HA-tagged NHE7 or NHE6 and myc-tagged SCAMP1, 2 or 5. Cells were lysed in 0.5% NP40/PBS for 30 minutes on ice and lysates were cleared for 20 minutes at 16,000 ***g*** at 4°C. Cell lysates (Lys) were then immunoprecipitated with mouse anti-HA antibody (HA) or pre-immune serum (con) and bound SCAMPs were detected on western blots probed with rabbit anti-myc antibody. 5% of the total lysate was loaded. (C) GST and GST fused with NHE7 C-terminal tail (GST-NHE7 [542-725]) were expressed in *E. coli* and purified by incubation with glutathione-conjugated sepharose beads. 2 μg immobilized GST or GST-NHE7 C-terminal fusion protein was incubated with PC12 cell lysate, and any SCAMPs bound to the GST fusion protein were detected by western blotting.

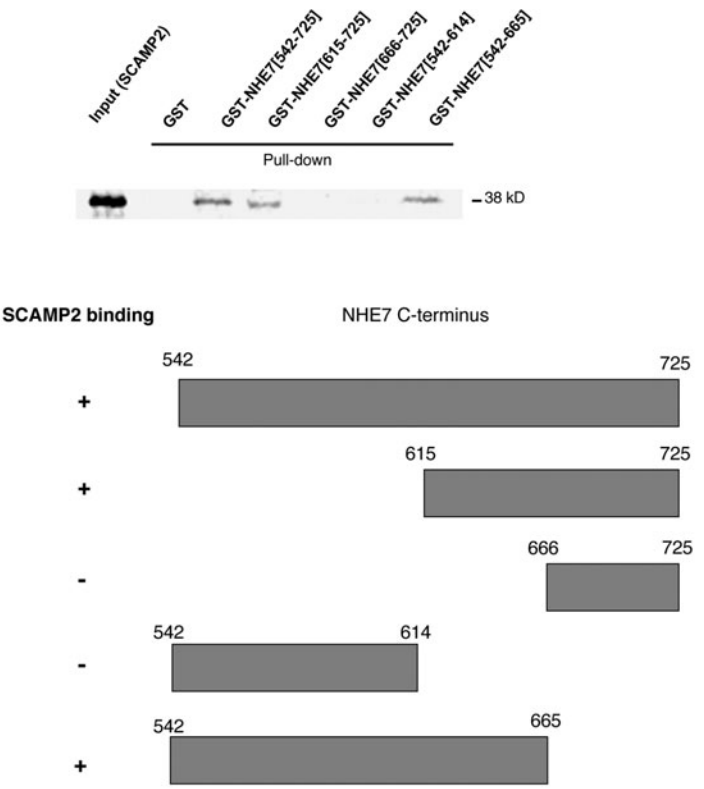

Fig. 2. Identification of SCAMP2 binding domain within NHE7. GST alone and GST fused to various segments of NHE7 (residues 542-725, 615-725, 666-725, 542-614 and 542-665, as illustrated below) were purified by glutathione-sepharose beads and subjected to GST pull-down. The radiolabeled SCAMP2 protein was incubated with 2 μg purified GST or GST fusion proteins immobilized on glutathione sepharose beads. After extensive washing, bound SCAMP2 was eluted with SDS sample buffer, resolved by SDS-PAGE, and visualized using a PhosphorImager.

terminal domain used as the bait for yeast two-hybrid screening, further supporting the specificity of the binding.

Identification of NHE7-binding domain in SCAMPs

To delineate the NHE7-binding domain within SCAMP2, CHO cells were transiently co-transfected with various myc-tagged SCAMP2 deletion mutants and HA-tagged full-length NHE7, immunoprecipitated with an anti-HA antibody, and bound SCAMP deletion mutants were detected in western blotting with an anti-myc antibody. The internal deletion mutant lacking the TM2 domain and part of the cytoplasmic loop (Δ184-208) exhibited a significantly weaker interaction (Fig. 3) when compared with other deletion mutants and the full-length protein (Fig. 1B), implicating this domain in NHE7 binding.

This region contains two transmembrane domains (TM2 and TM3) and a cytoplasmic loop (Fig. 4A) (Hubbard et al., 2000). As the bait used for our yeast two-hybrid screen corresponds to part of the C-terminal tail of NHE7 that is predicted to be cytoplasmic (Numata and Orlowski, 2001), we hypothesized that the minimum NHE7-binding domain of SCAMP2 resides in the intracellular loop between the TM2 and TM3. A GST fusion protein comprising the TM2-TM3 loop was expressed, purified and immobilized to glutathione-sepharose beads. This was incubated with in vitro transcribed/translated ^{35}S-labeled NHE7 C-terminus and the bound NHE7 was then visualized by phosphorimaging, revealing ^{35}S-labeled NHE7 C-terminus associated with the TM2-TM3 loop (Fig. 4B). The intensity of the NHE7 band increased proportionally with increasing amounts of GST-SCAMP[201-215]. Together, these results suggest that the TM2-TM3 loop specifically binds to the C-terminal tail of NHE7 by direct protein-protein interaction.

Endogenous SCAMPs bind and colocalize with NHE7

Heterologous expression of certain proteins may cause non-specific aggregation with other proteins owing to overexpression. To minimize this possibility, we next tested whether endogenous SCAMPs and NHE7 interact in cultured cells. Because of the unavailability of antibodies recognizing endogenous NHE7, we stably expressed a 1D4-tagged version of NHE7 in neuroendocrine PC12 cells (NHE7$_{1D4}$/PC12), which endogenously express SCAMP1, -2 and -5. A clone with a moderate expression level of NHE7$_{1D4}$ was selected for further characterization. Cell lysates were incubated with 1D4 antibody-coupled sepharose beads and the immune complexes were eluted with SDS sample buffer. The co-eluted SCAMPs were detected on western blots probed with endogenous anti-SCAMP1 and anti-SCAMP2 antibodies. Immunoprecipitated samples from NHE7$_{1D4}$/PC12 cells, but not from control PC12 cells, contained proteins of approximately 36 kDa and 38 kDa, corresponding to SCAMP1 and SCAMP2 (Fig. 5A). Western blots probed with the anti-SCAMP5 antibody yielded high background signals, which made interpretation of the results

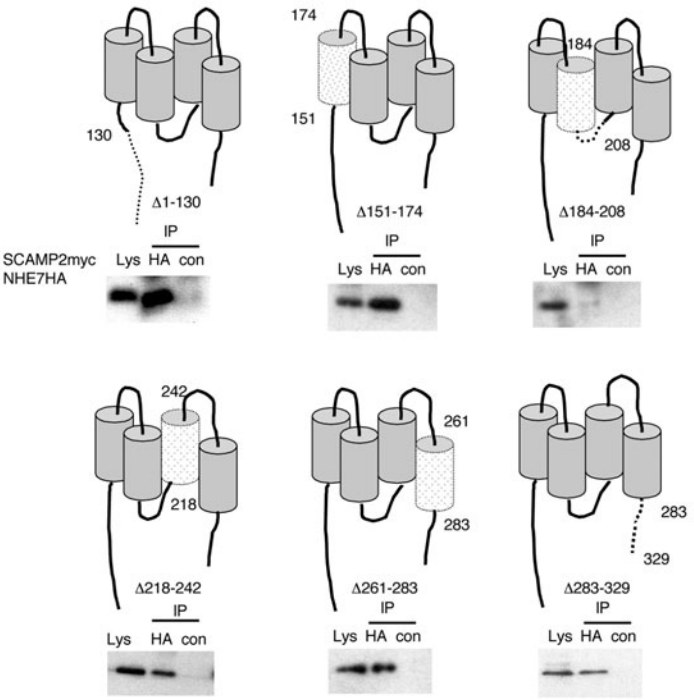

Fig. 3. Co-immunoprecipitation of SCAMP2$_{myc}$ deletion mutants and NHE7$_{HA}$. CHO cells were transiently co-transfected with myc-tagged SCAMP2 deletion mutants as illustrated above the blots, and HA-tagged NHE7. Pre-cleared lysates (Lys) were immunoprecipitated with an anti-HA antibody (HA) or mouse pre-immune serum (con) and bound SCAMP2 was detected on western blot by an anti-myc antibody. 5% of the lysate was run for each sample.

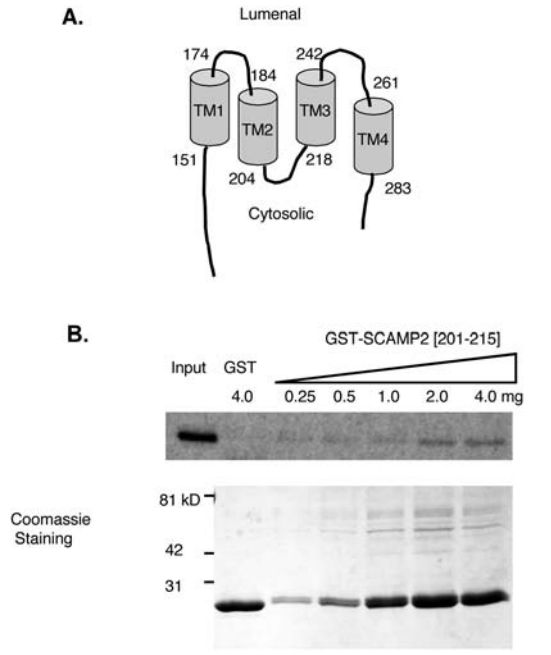

Fig. 4. Dose-dependent binding of SCAMP2[201-215] to NHE7. (A) Predicted membrane topology of SCAMP2. (B) Increasing amounts of GST-SCAMP2[201-215] immobilized to glutathione beads were incubated with radiolabelled NHE7 C-terminus and the bound NHE7 was visualized using a PhosphorImager. 4 μg GST was used as a control.

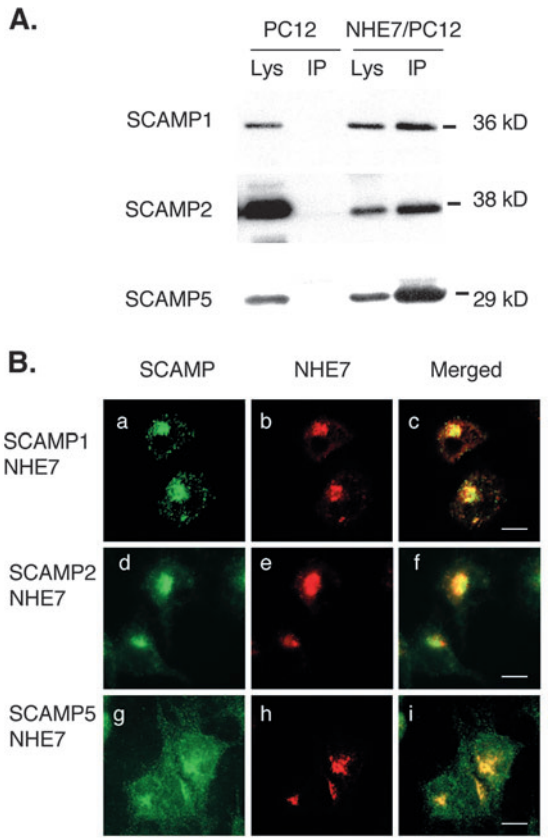

Fig. 5. Endogenous SCAMPs bind and largely colocalize with NHE7. (A) Control PC12 cells (PC12) or PC12 cells stably expressing 1D4-tagged NHE7 (NHE7/PC12) were immunoprecipitated (IP) with the 1D4 antibody conjugated to sepharose beads. Co-immunoprecipitated SCAMP1, 2, or 5 was detected by western blot probed with anti-SCAMP antibodies. (B) Fixed NHE7/PC12 cells on glass coverslips were double stained with anti-SCAMP1, SCAMP2 or SCAMP5 rabbit antibody with anti-1D4 mouse antibody. SCAMP1 (a), SCAMP2 (d) and SCAMP5 (g) were visualized with Alexa 488-conjugated goat anti-rabbit IgG (green) and the corresponding NHE7 by Alexa 568-conjugated anti-mouse IgG (red fluorescence) (b, e and h respectively). Yellow signals in merged images (c, f and i respectively) correspond to colocalized proteins. Bar, 10 μm.

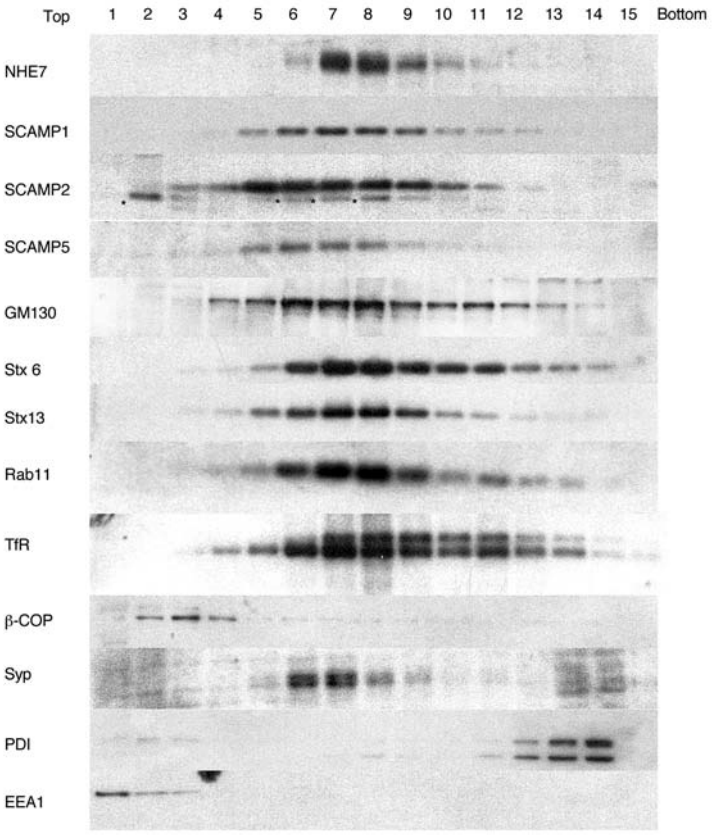

Fig. 6. NHE7 and SCAMPs co-fractionate by sucrose equilibrium density centrifugation. Homogenate was prepared from PC12 cells stably transfected with 1D4-tagged NHE7 and analyzed by sucrose equilibrium density centrifugation. Fifteen fractions were taken from the top and an equal volume of each fraction was analyzed on a western blot probed with anti-1D4 and different antibodies that recognize endogenous proteins. The following organellar markers were used: GM130 (cis/medial-Golgi), syntaxin 6 (Stx6; TGN/TGN-derived vesicles), syntaxin 13 (Stx13; recycling endosome), Rab 11 (recycling endosome), transferrin receptor (TfR; recycling endosome), β-COP (cis-Golgi), synaptophysin (Syp; synaptic-like microvesicles), PDI (ER) and EEA1 (early endosomes). Note that NHE7 and SCAMPs showed similar peaks in fractions 7, 8 and 9, but SCAMPs have broader distribution (fractions 5 and 6). In SCAMP2, a less prominent 35 kDa band (labelled with asterisks) was observed in addition to the expected 38 kDa band. This minor band probably represents a non-specific reaction, but could also reflect a proteolytically cleaved form.

difficult. Therefore, membrane-enriched fractions were prepared and used instead of whole cell lysates for the co-immunoprecipitation experiments. Immunoprecipitated samples from $NHE7_{1D4}$/PC12 cells exhibited a specific 29 kDa band corresponding to SCAMP5, suggesting that endogenous SCAMP5 also interacts with NHE7. The association of $NHE7_{1D4}$ with endogenous SCAMPs was further examined in intact PC12 cells by immunofluorescence confocal microscopy. NHE7 (red) largely colocalized with SCAMP1, -2, and -5 (green), the majority of which accumulated in a juxtanuclear compartment (Fig. 5B).

NHE7 and SCAMPs associate with TGN and recycling endosomes

To define the membrane compartmentalization of NHE7 and SCAMPs, PC12 cell homogenates were layered on sucrose gradients and centrifuged to separate the various organelles according to their buoyant densities (Xu et al., 1997; Yan et al., 2001). Following centrifugation, fractions were taken from the top of the gradients and an equal volume of each fraction was resolved by SDS-PAGE and analyzed by western blot.

NHE7 was detected in fractions 6-11, with major accumulation in fractions 7-9, whereas SCAMP1, -2 and -5 showed a slightly broader, but overlapping, distribution that peaked between fractions 5-9 (Fig. 6). This pattern is largely consistent with their distribution in intact cells as visualized by immunofluorescence confocal microscopy (Fig. 5B). To

establish the identity of the membrane fractions, the samples were probed with a series of organellar markers. The distribution of the TGN marker syntaxin 6 (Bock et al., 1997) and various recycling vesicle markers, such as the transferrin receptor, Rab11 (Ullrich et al., 1996), and syntaxin 13 (Prekeris et al., 1998), most closely matched the pattern of NHE7 and the SCAMPs. Likewise, synaptophysin, a marker for synaptic vesicles/synaptic-like microvesicles (Thomas-Reetz and De Camilli, 1994), also showed close association with NHE7 and the SCAMPs, especially SCAMP1 and SCAMP5, which are highly enriched in synaptic vesicles (Brand et al., 1991; Fernandez-Chacon and Sudhof, 2000). In contrast, markers for the cis-Golgi (β-COP), endoplasmic reticulum (PDI) and early endosomes (EEA1) showed little overlap with NHE7 or the SCAMPs.

To visualize the subcellular distribution of the NHE7-SCAMP complexes more precisely, immunofluorescence studies were conducted using recognized markers for the TGN (syntaxin 6, Stx6) and recycling endosomes (transferrin receptor, TfR). Heterologously expressed $NHE7_{myc}$ closely colocalized with Stx6 (Fig. 7A), consistent with earlier findings that $NHE7_{myc}$ localizes predominantly to the TGN (Numata and Orlowski, 2001). The signal for $NHE7_{myc}$ also overlapped that of the TfR in the recycling endosomal compartment, although the morphology of the latter was more compact than the distribution of NHE7 (Fig. 7B). These data are consistent with the distribution of NHE7 to both the TGN and adjacent recycling vesicles. Likewise, the signals for SCAMPs closely overlapped those of Stx6, but significant portions also colocalized with the TfR. More careful inspection of the signals for the SCAMPs indicated that they are distributed more widely than NHE7, Stx6, or TfR, consistent with previous reports that the SCAMPs are present in a broader range of organelles including the Golgi, post-Golgi recycling pathways, secretory granules and the plasma membrane (Bell et al., 2001; Fernandez-Chacon and Sudhof, 2000; Wu and Castle, 1997).

Overexpression of SCAMP2/Δ184-208 causes redistribution of NHE7

Both GST pull-down and co-immunoprecipitation experiments indicate that the TM2-TM3 loop of SCAMP2 is important for binding to NHE7. Although SCAMPs have been suggested to be involved with vesicular trafficking, there are no reports showing that SCAMPs directly influence the sorting of specific vesicle-associated proteins. Hence, we hypothesized that SCAMPs might modulate the steady-state accumulation of NHE7 in the TGN by binding to NHE7 through the TM2-TM3 region. To address this possibility, CHO cells were co-transfected with $NHE7_{1D4}$ and either full-length $SCAMP2_{myc}$ or an internal deletion mutant of $SCAMP2_{myc}$ lacking this region (SCAMP2/Δ184-208) and visualized their relative distributions by immunofluorescence confocal microscopy. The majority of full-length $SCAMP2_{myc}$ tightly colocalized with NHE7 in a compact juxtanuclear structure (Fig. 8A). In contrast, the SCAMP2/Δ184-208 mutant displayed more scattered staining. Moreover, the $NHE7_{1D4}$ signal also showed a more dispersed vesicular appearance that closely, but not completely, overlapped that of SCAMP2/Δ184-208.

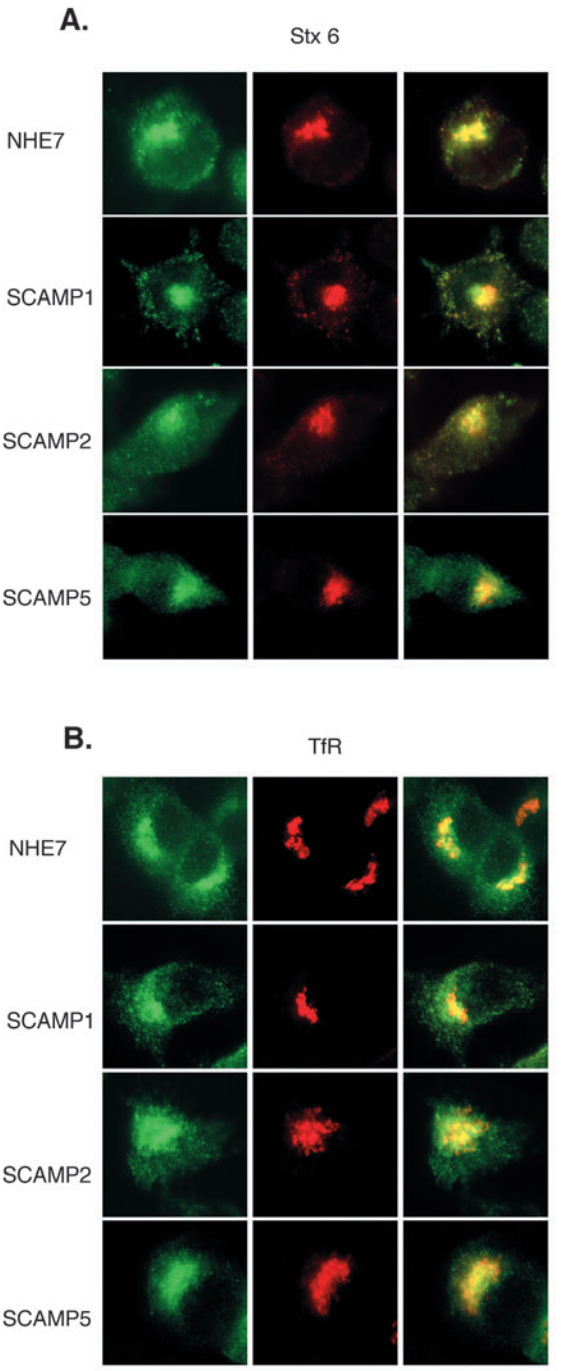

Fig. 7. NHE7 and SCAMPs colocalize with syntaxin 6 and transferrin receptor. Endogenous SCAMP1, -2, and -5 were visualized in PC12 cells using anti-SCAMP polyclonal rabbit antibodies. To determine intracellular localization of NHE7, PC12 cells were transiently transfected with myc-tagged NHE7 and an anti-myc polyclonal rabbit antibody was utilized. Anti-syntaxin 6 (TGN marker) and transferrin receptor (recycling endosomal marker) monoclonal mouse antibodies were used as organellar markers. NHE7 or SCAMPs were visualized with Alexa 488-conjugated goat anti-rabbit IgG (green) and syntaxin 6 or transferrin receptor was visualized with Alexa 568-conjugated anti-mouse IgG (red). NHE7 exhibited the best colocalization with syntaxin 6, whereas partial colocalization with transferrin receptor was also observed. SCAMPs showed close association with both syntaxin 6 and transferrin receptor, suggesting a more widespread distribution.

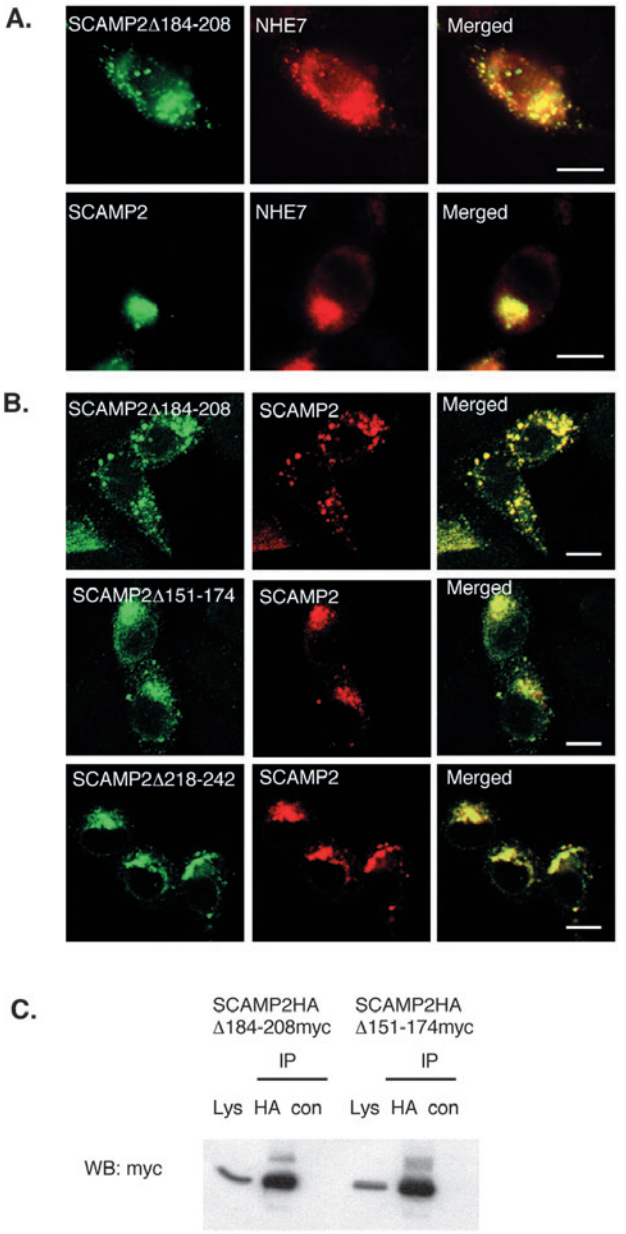

Fig. 8. Expression of SCAMP2Δ184-208 shows scattered vesicular appearance with NHE7 and full-length SCAMP. (A) CHO cells were transfected with myc-tagged Δ184-208 or full-length SCAMP2 and 1D4-tagged NHE7 (NHE7) and viewed by immunofluorescence microscopy. SCAMP2/Δ184-208 had a more scattered vesicular distribution than full-length wild-type SCAMP2. Co-transfected NHE7 was redistributed to scattered vesicular structures mostly in the same compartment as SCAMP2/Δ184-208. (B) CHO cells were simultaneously transfected with myc-tagged SCAMP2 deletion mutants and full-length HA-tagged SCAMP2, and their intracellular localization was visualized by immunofluorescence confocal microscopy. Expression of SCAMP2/Δ184-208, but not other mutants, redistributed full-length SCAMP2 to the same scattered vesicular structure. (C) CHO cells were transiently co-transfected with full-length HA-tagged SCAMP2 and myc-tagged SCAMP2/Δ184-208 or SCAMP2/Δ151-174. Cell lysates (Lys) were immunoprecipitated with a mouse anti-HA antibody (HA) or pre-immune serum (con) and co-precipitated SCAMP2/Δ184-208 and SCAMP2/Δ151-174 were detected in a western blot by rabbit anti-myc antibody. 5% of total cell lysate was loaded. Bar, 10 μm.

Overexpression of SCAMP2/Δ184-208 causes dispersion of full-length SCAMP2

SCAMPs are expressed ubiquitously in most eukaryotic cells and can form homo- or heteromultimeric structures with different isoforms (Wu and Castle, 1997). We speculated that SCAMP2/Δ184-208 might form a complex with endogenously expressed SCAMPs and act as a dominant-negative mutant, which could account for the impaired distribution of NHE7. If this were the case, overexpression of this mutant should also disturb wild-type SCAMPs. To test this possibility, CHO cells were co-transfected with various deletion mutants of myc-tagged SCAMP2 (Δ151-174, Δ184-208 or Δ218-242) and full-length HA-tagged SCAMP2 ($SCAMP2_{HA}$), and their intracellular localizations were visualized by dual-labeled immunofluorescence confocal microscopy. Full-length $SCAMP2_{HA}$ alone exhibited a perinuclear distribution that was identical to exogenous $SCAMP2_{myc}$ or endogenous SCAMP2 (data not shown). However, $SCAMP2_{HA}$ exhibited a more scattered vesicular appearance when co-expressed with SCAMP2/Δ184-208, but not with other deletion mutants lacking either TM1 (Δ151-174) or TM3 (Δ218-242) (Fig. 8B). Moreover, myc-tagged SCAMP2/Δ184-208 was co-immunoprecipitated with full-length $SCAMP2_{HA}$ with similar affinity as SCAMP2/Δ151-174 in transiently co-transfected CHO cells (Fig. 8C), suggesting a physical interaction between SCAMP2/Δ184-208 and full-length SCAMP2 in the cell. The scattered vesicular localization of SCAMP2/Δ184-208 largely coincided with the recycling endosomal marker TfR (Fig. 9A) in the peripheral region of the cell. Thus, residues 184-208 may play a role in directing SCAMPs from peripheral recycling endosomes to the TGN. We next asked whether expression of SCAMP2/Δ184-208 influences intracellular localizations of other organellar markers such as Stx6 to the TGN and GM130 to the Golgi apparatus. There was no noticeable alteration in their localization under our experimental conditions, suggesting the specificity of this effect (Fig. 9B). However, we cannot exclude the involvement of SCAMPs in targeting other TGN resident or cell surface proteins. These possibilities will need to be tested in the future.

GFP-fusion of SCAMP2 TM2-TM3 associates with NHE7

To address whether the TM2-TM3 region (G181-G246) of SCAMP2 is sufficient for both binding and targeting of NHE7 to the TGN, $NHE7_{HA}$ was co-transfected with either GFP or a GFP-tagged TM2-TM3 construct of SCAMP2 (GFP-TM2-3) into CHO cells. The resulting cell lysate was incubated with an anti-HA antibody and any bound GFP or GFP-TM2-3 in the immunoprecipitated fraction was detected by western blotting using an anti-GFP antibody. GFP-TM2-3, but not GFP, was co-immunoprecipitated with HA-tagged NHE7 (Fig. 10A). GST pull-down assays further demonstrated direct association of in vitro transcribed/translated ^{35}S-labeled GFP-TM2-3 protein to the GST-NHE7 C-terminal fusion protein (Fig. 10B). These findings are consistent with the GST pull-down of in vitro translated NHE7 C-terminus and the GST-tagged SCAMP2 TM2-TM3 loop (Fig. 4). Thus, the TM2-TM3 region is sufficient for binding to NHE7. To visually demonstrate their association in the cell, CHO cells transiently co-transfected with $NHE7_{HA}$ and GFP-TM2-3 were analyzed by

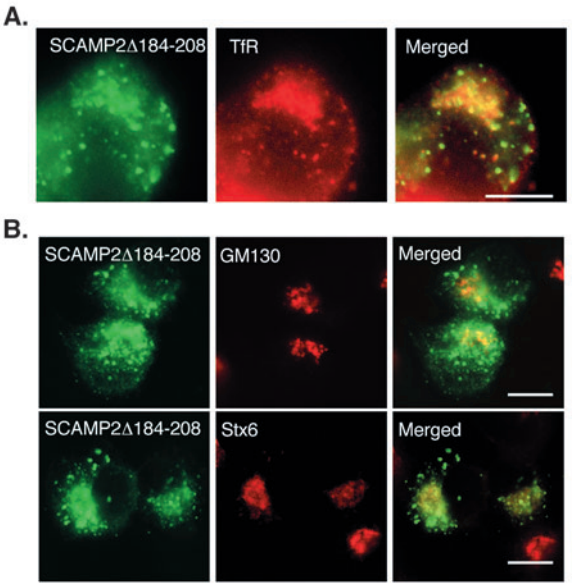

Fig. 9. SCAMP2/Δ184-208 colocalizes with the transferrin receptor at the peripheral region of cells. PC12 cells were transiently transfected with myc-tagged SCAMP2/Δ184-208 and its intracellular localization was analyzed by double-labelled immunofluorescence confocal microscopy with different organellar markers. (A) SCAMP2/Δ184-208 (green) colocalized with transferrin receptor (TfR, red) at the peripheral region of cells. (B) Neither syntaxin 6 (Stx6, red) nor GM130 (red) showed significant association with SCAMP2/Δ184-208 (green). Note that the expression of SCAMP2/Δ184-208 did not appreciably alter localization of these markers (see Fig. 4). Bar, 10 μm.

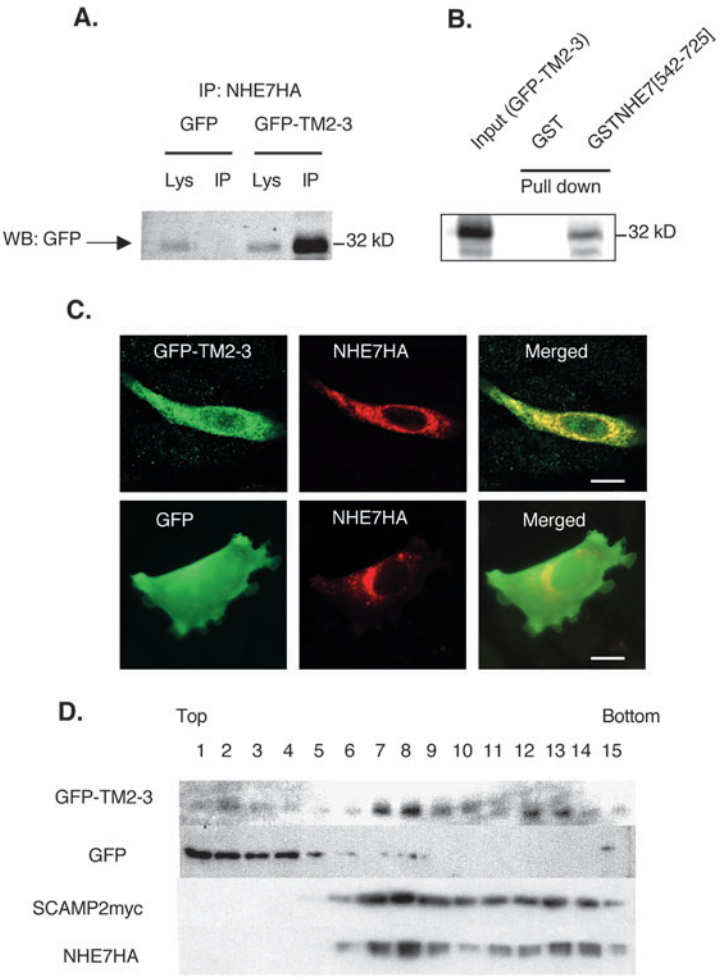

Fig. 10. The TM2-TM3 region is sufficient for NHE7 interaction. (A) CHO cells were transiently co-transfected with HA-tagged NHE7 and GFP-tagged SCAMP2 TM2-TM3 (GFP-TM2-3) or GFP control. Cell lysates (Lys) were immunoprecipitated with anti-HA antibody (IP) and bound GFP fusion proteins were analyzed on western blot. (B) Purified GST fusion protein of NHE7 C-terminus or GST was incubated with radiolabelled GFP-TM2-3 and bound protein was detected using a PhosphorImager. (C) CHO cells were simultaneously transfected with GFP-TM2-3 or GFP alone and NHE7HA and their intracellular localization was visualized by green and red fluorescence, respectively. (D) Homogenate isolated after transient transfection with GFP-TM2-3, GFP, $SCAMP2_{myc}$ or $NHE7_{HA}$ was analyzed by sucrose equilibrium density centrifugation. Bar, 3 μm.

immunofluorescence confocal microscopy. Both green (GFP-TM2-3) and red ($NHE7_{HA}$) fluorescence signals showed considerable overlap in fine vesicular structures (Fig. 10C), whereas untagged GFP was uniformly distributed throughout the cell. To further demonstrate the effect of GFP-TM2-3 on the membrane distribution of NHE7 in other cell types, 293-T cells were transiently transfected with GFP alone or GFP-TM2-3 and either $NHE7_{HA}$ or $SCAMP2_{myc}$, and the resulting cell lysates were fractioned by sucrose equilibrium density centrifugation. The homogenate obtained from GFP-transfected cells showed predominant accumulation of GFP in the upper (light density) fractions, whereas GFP-TM2-3 showed enrichment in the lower denser fractions with a major peak in fractions 7 and 8 and a minor peak in fractions 12 and 13 (Fig. 10D). This biphasic distribution pattern overlaps considerably those of transiently transfected $NHE7_{HA}$ and $SCAMP2_{myc}$, which both showed a broader distribution than cells lacking GFP-TM2-3 (compare with Fig. 6). These results are consistent with the notion that the TM2-3 region of SCAMPs is sufficient for binding to NHE7 and, when overexpressed, can act as a dominant negative that partially impairs the normal predominant accumulation of NHE7 in the TGN.

NHE7 C-terminal construct partly accumulates in perinuclear regions with SCAMP2

To further examine the structural determinants underlying the association of NHE7 and SCAMP2 in intact cells, we assessed whether the cytoplasmic C-terminus of NHE7 was sufficient to target the transporter to SCAMP-containing membranes. To test this hypothesis, CHO cells were co-transfected with a HA-tagged NHE7 C-terminal construct (G525-A725) and full-length $SCAMP2_{myc}$ or $SCAMP2_{myc}$/Δ184-208. Although the NHE7 C-terminal construct was diffusely detected throughout the cytosol when overexpressed, the majority of the protein was concentrated in the juxtanuclear region when expressed in moderate levels (Fig. 11). Substantial colocalization between the NHE7 C-terminus and the full-length SCAMP2 was observed. In contrast, the NHE7 C-terminal construct showed more limited colocalization with SCAMP2/Δ184-208. These

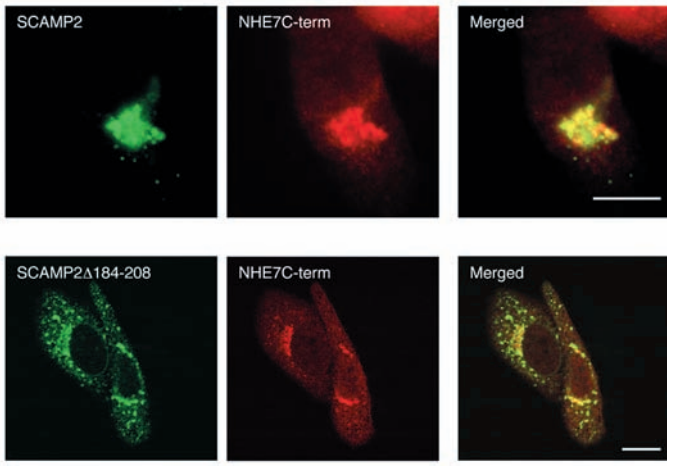

Fig. 11. NHE7 C-terminus partially colocalizes with full-length SCAMP2. CHO cells were co-transfected with myc-tagged SCAMP2 (full-length or Δ184-208) and HA-tagged NHE7 C-terminus (G525-A725) and their localizations in the cell were visualized by immunofluorescence microscopy. NHE7 C-terminus showed significant colocalization with full-length SCAMP2, but only limited association with SCAMP2/Δ184-208. Representative cells with low-to-moderate expression levels are shown. Bar, 10 μm

results further support the notion that the cytoplasmic C-terminal domain of NHE7 plays a pivotal role in protein-protein interactions with SCAMPs.

Discussion

NHE7 is a unique $Na^+(K^+)/H^+$ exchanger isoform that accumulates predominantly in the TGN and to a lesser extent in post-Golgi vesicles where it is thought to play a significant role in organellar pH and volume homeostasis (Numata and Orlowski, 2001). This study has identified SCAMPs as binding partners of NHE7, and implicates them in the shuttling/retrieval of NHE7 from peripheral recycling endosomes to the TGN.

In many cell types, the TGN and recycling endosomes are located in close apposition in the perinuclear region. Many TGN-resident membrane proteins can dynamically cycle to and from the cell surface via endosomes, although the molecular mechanisms underlying this phenomenon are not fully understood. Recent studies have identified several proteins involved in the trafficking of vesicles from the recycling endosomal compartment to the TGN, including certain SNARE complexes and Rab GTPases (Lu et al., 2004; Mallard et al., 2002; Tai et al., 2004; Wilcke et al., 2000). The presence of SCAMPs in the TGN, recycling endosomes, and at the cell surface has also implicated them in the trafficking of vesicles along this pathway. More recently, it was proposed that the recycling endosome might also serve as an intermediate between the TGN and the plasma membrane along the biosynthetic pathway (Ang et al., 2004). These findings suggest that the TGN and recycling endosomal compartments are coupled both spatially and functionally.

In the present study, we show that SCAMP1, -2, and -5 colocalize with NHE7 in the TGN and post-Golgi vesicles of intact cells. By biochemical assays, we identified the highly conserved TM2-TM3 region of SCAMP2 as a critical domain required for binding to NHE7 in vitro and in intact cells. A deletion mutant of SCAMP2 lacking this segment (SCAMP2/Δ184-208) was unable to associate directly with NHE7 and accumulated preferentially in peripheral vesicles that stained positively for TfR, a recognized marker of recycling endosomes. Strikingly, overexpression of SCAMP2/Δ184-208 also caused a significant fraction of NHE7 to accumulate in peripheral endosomes rather than the TGN despite its inability to bind NHE7. Similarly, a sizeable portion of wild-type SCAMP2 redistributed to the peripheral recycling endosomal compartment. As SCAMPs form homo- and heteromultimeric complexes amongst themselves (Wu and Castle, 1997), these data are best explained by the ability of SCAMP2/Δ184-208 to form complexes with wild-type SCAMPs, but act in a dominant-negative manner to disrupt their proper sorting function, thereby leading to a common redistribution of mutant and wild-type SCAMP2 as well as NHE7. Consistent with this hypothesis, myc-tagged SCAMP2/Δ184-208 was efficiently co-immunoprecipitated with full-length $SCAMP2_{HA}$ in transiently transfected CHO cells. Importantly, the effect of overexpression of SCAMP2Δ184-208 on redistribution of NHE7 and SCAMP2 was specific, in as much as other TGN-resident proteins such as Stx6 were unaffected. These data are complemented by other analyses showing that expression of a GFP-tagged TM2-TM3 construct of SCAMP2, which in this case binds to NHE7, but also causes the mistargeting of this transporter. Based on these findings, we propose the following model (Fig. 12) where endogenously expressed SCAMPs facilitate the retrieval of NHE7 from recycling endosomes to the TGN; and heterologously expressed SCAMP2/Δ184-208 causes either structural alterations of endogenous SCAMPs or inhibits oligomer formation between intact SCAMPs, which impairs the targeting capability of endogenous SCAMPs.

The binding module between SCAMPs and NHE7 appears to be complex. Although SCAMPs directly bind to the C-terminal tail of NHE7, we have recently observed that deletion mutants of NHE7 lacking the C-terminal tail can associate with SCAMPs by co-immunoprecipitation (data not shown). The binding between C-terminal deletion mutants of NHE7 and SCAMPs may be mediated by indirect interactions, such as the formation of oligomers between the mutant NHE7 and endogenously expressed wild-type NHE7. This is possible because the plasmalemmal NHEs are known to form homodimers (Fafournoux et al., 1994) and presumably this tertiary structure is conserved in the organellar NHEs. Alternatively, other domains within the transmembranous region of NHE7 may also contribute to interactions with the SCAMPs. Nevertheless, the C-terminal tail of NHE7 seems to play an important role in this protein-protein interaction. Heterologous expression of the NHE7 C-terminal tail lacking the N-terminal transmembrane domains colocalized, at least in part, with concomitantly expressed full-length SCAMP2 or endogenous SCAMPs, but not with the Δ184-208 mutant determined by immunofluorescence microscopy. It is still not clear whether a single motif or two independent, but closely apposing, domains are responsible for NHE7 binding and Golgi targeting. Creation and

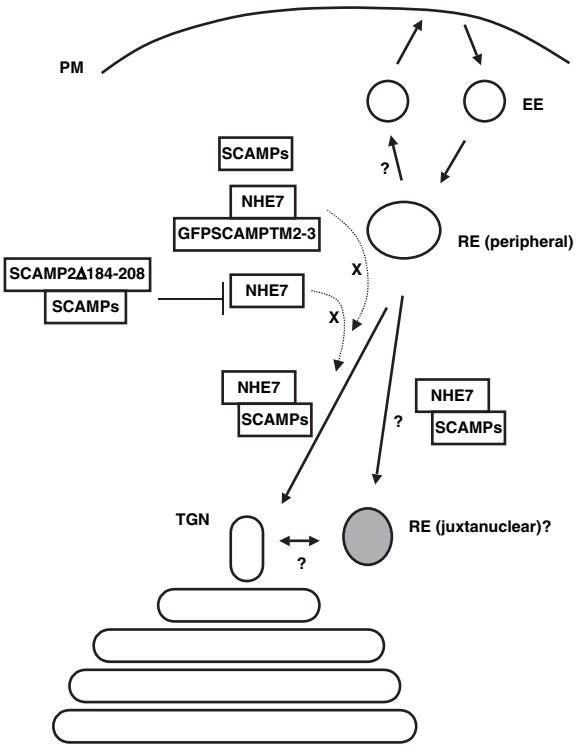

Fig. 12. Proposed model showing that targeting of NHE7 from the recycling endosome to the TGN is facilitated by binding to SCAMPs. Expression of the SCAMP2/Δ184-208 mutant blocks the normal targeting by interfering with full-length SCAMP, whereas GFP-tagged SCAMP2 TM2-TM3 (GFP-TM2-3) blocks normal targeting of NHE7 by competitive inhibition with endogenous SCAMPs. EE, early endosomes; RE, recycling endosomes; PM, plasma membrane; TGN, trans-Golgi network.

characterization of substitution mutants in this region will be required to answer this question.

SCAMPs 1-3 have an extended cytoplasmic N-terminal domain containing protein interaction motifs, whereas SCAMP4 and SCAMP5 lack a major part of the N-terminus (including the NPF repeat). The binding affinity of SCAMP5 to NHE7 appears to be weaker than that of SCAMP1 or SCAMP2. Although the in vitro and in vivo binding assays indicated that the TM2-TM3 loop is the predominant binding domain, the N-terminal cytoplasmic extension may have additional function in protein-protein interaction (e.g. stabilizing the protein interaction). It is of note that overexpression of a deletion mutant of SCAMP1 lacking the N-terminal domain potently inhibited transferrin uptake by endocytosis (Fernandez-Chacon et al., 2000). Longer isoforms and shorter isoforms of SCAMPs may bind differently to NHE7 and regulate such biological functions. SCAMPs are also suggested to transduce various signals by tyrosine phosphorylation and through protein interactions (Fernandez-Chacon et al., 2000; Wu and Castle, 1998). Thus, it is possible that SCAMPs tether NHE7 to signalling molecules and control its transporter function. It will be interesting to search for proteins that bind to both SCAMPs and NHE7 and characterize these binding proteins with respect to the function of the transporter.

In specialized eukaryotic cells, such as neurons, endocrine and exocrine cells, the regulated secretion exists in addition to the constitutive secretion. Constitutive secretory vesicles are targeted from the TGN to the plasma membrane and continuously fused with the plasma membrane to release their contents. A conventional model proposes that regulated secretory proteins such as chromogranins are segregated from constitutive secretory proteins at the TGN, targeted to the secretory granules for maturation, and released from the cell after stimulation (Arvan and Castle, 1998; Tooze et al., 2001). Acidic pH and millimolar Ca^{2+} concentrations trigger the formation of aggregates of regulated secretory proteins in the TGN, which plays a crucial role in sorting regulated secretory proteins and the formation of regulated secretory vesicles (Tooze et al., 2001). A recent study reported that bafilomycin A (a selective V-ATPase inhibitor) perturbed sorting of a chromogranin A chimera protein and decreased the number of dense-core vesicles in PC12 cells (Taupenot et al., 2005), which further support the importance of acidic organellar pH in regulated secretion and secretory granule formation. Hence, it would be interesting to investigate the consequence of overexpression or suppression of NHE7 on regulated secretion and the formation of secretory vesicles.

Sorting of regulated secretory proteins is also proposed to be controlled in immature secretory granules budded from the TGN (Arvan and Castle, 1998). In this case, pH environment within the immature granule appears to play a crucial role in retaining regulated secretory proteins within the granule, whereas other proteins are diverted to the constitutive secretory pathway (Arvan and Castle, 1998). In addition, protein-protein interactions as well as membrane anchoring of secretory proteins may also define their targeting and secretion (Arvan and Castle, 1998). A small fraction of chromogranin B was found to be associated with membranes after stimulation (Pimplikar and Huttner, 1992), although the underlining mechanisms and biological roles are unknown. It is tempting to postulate that the association of secretory proteins with certain granule membrane proteins plays a key role in secretion, and such protein interaction modulates the function of the membrane proteins. In this context, it is intriguing to address whether NHE7 resides in the same vesicular compartment as chromogranin B and, if so, whether NHE7 can bind to chromogranin B.

In summary, we have identified a novel interaction between NHE7 and SCAMPs that appears to be important for the proper sorting of this transporter to the TGN. It remains to be determined whether this association is regulated by phosphorylation-dependent processes and whether it influences the kinetic properties of this transporter. Regulated trafficking of NHE7 may account for a new module of organellar ion homeostasis, which may eventually regulate trafficking of other proteins along exocytic and endocytic pathways. Additional studies are ongoing to test these possibilities.

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