SCAMP2 regulates NHE5 transit through recycling endosomes by dominant-negative Rab11. Together, these results suggest that targeting of NHE5 was reversed by dominant-negative Arf6 but not activity across the plasma membrane. SCAMP2-mediated surface Rab11 positively regulate NHE5 cell-surface targeting and NHE5 fusion, reduced the transporter activity. Although both Arf6 and cytosolic domain, and a mini-gene encoding the N-terminal extension of a deletion mutant lacking the SCAMP2-specific N-terminal cytosolic extensions of SCAMP2. Heterologous expression of SCAMP2 revealed that NHE5 directly binds to the N- and C-terminal cytosolic extensions of SCAMP2. Heterologous expression of SCAMP2 but not SCAMP5 increased cell-surface abundance as well as transporter activity of NHE5 across the plasma membrane. Expression of a deletion mutant lacking the SCAMP2-specific N-terminal cytosolic domain, and a mini-gene encoding the N-terminal extension, reduced the transporter activity. Although both Arf6 and Rab11 positively regulate NHE5 cell-surface targeting and NHE5 activity across the plasma membrane, SCAMP2-mediated surface targeting of NHE5 was reversed by dominant-negative Arf6 but not by dominant-negative Rab11. Together, these results suggest that SCAMP2 regulates NHE5 transit through recycling endosomes and promotes its surface targeting in an Arf6-dependent manner.

NHE5 is a brain-enriched Na\(^+/\)H\(^+\) exchanger that dynamically shuttles between the plasma membrane and recycling endosomes, serving as a mechanism that acutely controls the local pH environment. In the current study we show that secretory carrier membrane proteins (SCAMPs), a group of tetraspanning integral membrane proteins that reside in multiple secretory and endocytic organelles, bind to NHE5 and co-localize predominantly in the recycling endosomes. In vitro protein-protein interaction assays revealed that NHE5 directly binds to the N- and C-terminal cytosolic extensions of SCAMP2. Heterologous expression of SCAMP2 but not SCAMP5 increased cell-surface abundance as well as transporter activity of NHE5 across the plasma membrane. Expression of a deletion mutant lacking the SCAMP2-specific N-terminal cytosolic domain, and a mini-gene encoding the N-terminal extension, reduced the transporter activity. Although both Arf6 and Rab11 positively regulate NHE5 cell-surface targeting and NHE5 activity across the plasma membrane, SCAMP2-mediated surface targeting of NHE5 was reversed by dominant-negative Arf6 but not by dominant-negative Rab11. Together, these results suggest that SCAMP2 regulates NHE5 transit through recycling endosomes and promotes its surface targeting in an Arf6-dependent manner.

Neurons and glial cells in the central and peripheral nervous systems are especially sensitive to perturbations of pH (1). Many voltage- and ligand-gated ion channels that control membrane excitability are sensitive to changes in cellular pH (1–3). Neurotransmitter release and uptake are also influenced by cellular and organelar pH (4, 5). Moreover, the intra- and extracellular pH of both neurons and glia are modulated in a highly transient and localized manner by neuronal activity (6, 7). Thus, neurons and glia require sophisticated mechanisms to finely tune ion and pH homeostasis to maintain their normal functions.

Na\(^+/\)H\(^+\) exchangers (NHEs)\(^3\) were originally identified as a class of plasma membrane-bound ion transporters that exchange extracellular Na\(^+\) for intracellular H\(^+\), and thereby regulate cellular pH and volume. Since the discovery of NHE1 as the first mammalian NHE (8), eight additional isoforms (NHE2–9) that share 25–70% amino acid identity have been isolated in mammals (9, 10). NHE1–5 commonly exhibit transporter activity across the plasma membrane, whereas NHE6–9 are mostly found in organelle membranes and are believed to regulate organelar pH in most cell types at steady state (11). More recently, NHE10 was identified in human and mouse osteoclasts (12, 13). However, the cDNA encoding NHE10 shares only a low degree of sequence similarity with other known members of the NHE gene family, raising the possibility that this sodium-proton exchanger may belong to a separate gene family distantly related to NHE1–9 (see Ref. 9).

NHE gene family members contain 12 putative transmembrane domains at the N terminus followed by a C-terminal cytosolic extension that plays a role in regulation of the transporter activity by protein–protein interactions and phosphorylation. NHEs have been shown to regulate the pH environment of synaptic nerve terminals and to regulate the release of neurotransmitters from multiple neuronal populations (14–16). The importance of NHEs in brain function is further exemplified by the findings that spontaneous or directed mutations of the ubiquitously expressed NHE1 gene lead to the progression of epileptic seizures, ataxia, and increased mortality in mice (17, 18). The progression of the disease phenotype is associated with loss of specific neuron populations and increased neuronal excitability. However, NHE1-null mice appear to develop normally until 2 weeks after birth when symptoms begin to appear. Therefore, other mechanisms may compensate for the loss of NHE1 during early development and play a protective role in the surviving neurons after the onset of the disease phenotype.

NHE5 was identified as a unique member of the NHE gene family whose mRNA is expressed almost exclusively in the brain (19, 20), although more recent studies have suggested that NHE5 might be functional in other cell types such as sperm (21, 22) and osteosarcoma cells (23). Curiously, mutations found in several forms of congenital neurological disorders such as spinocerebellar ataxia type 4 (24–26) and autosomal dominant cerebellar ataxia (27–29) have been mapped to chromosome 16q22.1, a region containing NHE5. However, much remains unknown as to the molecular regulation of NHE5 and its role in brain function.

Very few if any proteins work in isolation. Therefore identification and characterization of binding proteins often reveal novel functions and regulation mechanisms of the protein of interest. To begin to elucidate the biological role of NHE5, we have started to explore NHE5-binding proteins.

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\(^3\) The abbreviations used are: NHE, Na\(^+/\)H\(^+\) exchanger; SCAMP, secretory carrier membrane protein; EH, Eps15 homology; GFP, green fluorescent protein; EGFP, enhanced GFP; PBS, phosphate-buffered saline; GST, glutathione S-transferase; CHAPS, 3-[[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; EIPA, 5-[(N-ethyl-N-isopropyl)amiloride; BCECF/ AM, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester.
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β-arrestins, multifunctional scaffold proteins that play a key role in desensitization of G-protein-coupled receptors, were shown to directly bind to NHE5 and promote its endocytosis (30). This study demonstrated that NHE5 trafficking between endosomes and the plasma membrane is regulated by protein-protein interactions with scaffold proteins. More recently, we demonstrated that receptor for activated C-kinase 1 (RACK1), a scaffold protein that links signaling molecules such as activated protein kinase C, integrins, and Src kinase (31), directly interacts with and activates NHE5 via integrin-dependent and independent pathways (32). These results further indicate that NHE5 is partly associated with focal adhesions and that its targeting to the specialized microdomain of the plasma membrane may be regulated by various signaling pathways.

Secretery corymembrane proteins (SCAMPs) are a family of evolutionarily conserved tetra-spanning integral membrane proteins. SCAMPs are found in multiple organelles such as the Golgi apparatus, trans-Golgi network, recycling endosomes, synaptic vesicles, and the plasma membrane (33, 34) and have been shown to play a role in exocytosis (35–38) and endocytosis (39). Currently, five isoforms of SCAMP have been identified in mammals. The extended N terminus of SCAMP1–3 contain multiple Asn-Pro-Phe (NPF) repeats, which may allow these isoforms to participate in clathrin coat assembly and vesicle budding by binding to Eps15 homology (EH)-domain proteins (40, 41). Further, SCAMP2 was shown recently to bind to the small GTPase Arf6 (38), which is believed to participate in traffic between the recycling endosomes and the cell surface (42, 43). More recent studies have suggested that SCAMPs bind to organelar membrane type NHE7 (44) and the serotonin transporter SERT (45) and facilitate targeting of these integral membrane proteins to specific intracellular compartments. We show in the current study that SCAMP2 binds to NHE5, facilitates the cell-surface targeting of NHE5, and elevates Na+/H+ exchange activity at the plasma membrane, whereas expression of a SCAMP2 deletion mutant lacking the N-terminal domain containing the NPF repeats suppresses the effect. Further we show that this activity of SCAMP2 requires an active form of a small GTPase Arf6, but not Rab11. We propose a model in which SCAMPs bind to NHE5 in the endosomal compartment and control its cell-surface abundance via an Arf6-dependent pathway.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Mouse monoclonal anti-HA antibodies were obtained from Covance (Richmond, CA). Rabbit polyclonal anti-Myc (A-14), anti-HA (Y-11), and mouse monoclonal anti-SCAMP2 (8C10) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-Rab11 and anti-Rab4 antibodies were obtained from Zymed Laboratories Inc. (South San Francisco, CA) and Stressgen (Victoria, British Columbia, Canada), respectively. Polyclonal antibodies against SCAMP1, -2, and -5 were purchased from Affinity BioReagents (Golden, CO). The purified mouse monoclonal antibody rho 1D4 against a 9-amino acid TETSQVAPA C-terminal epitope (46, 47) was obtained from the National Cell Culture Center (Minneapolis, MN). The antibody was coupled to CNBr-activated Sepharose beads as previously described (48). Affinity-purified anti-NHE5 rabbit polyclonal antibody raised against human NHE5 (G674-L896), which cross-reacts with rat NHE5 (32), was used for endogenous co-immunoprecipitation experiments. Goat anti-rabbit and goat anti-mouse horseradish-peroxidase fusion secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Alexa 647-coupled goat anti-mouse and Alexa 568 or Alexa 488-coupled goat anti-rabbit secondary antibodies were obtained from Invitrogen.

**Mammalian Expression Constructs**—First strand cDNA was synthesized from human brain RNA (Clontech, Mountain View, CA) by using random hexamers and SuperScript II reverse transcriptase (Invitrogen), and subjected to PCR using Pfu-Turbo (Stratagene, La Jolla, CA) to clone human Arf6 and Rab11. The following primers were used: Arf6 forward (5′-ATG GGG AAG AAG GTA GGA AGA GAA AGA GTC TCA TCC AAA ATC TTC GGT TGC G-3′) and Arf6 reverse (5′-AGA TTT GTA AGA GTT TAA CCA TGT G-3′); Rab11 forward (5′-ATG GGG ACC CGC GAC GAG TAC G-3′) and Rab11 reverse (5′-GAT GTT CTG ACA GCA CTG CAC CTT TG-3′). The identities of the PCR fragments were verified by sequencing and subjected to a second round of PCR to introduce a Myc or HA tag at the extreme C terminus of the clone. The PCR fragment was ligated into mammalian expression vector pcDNA3, and the sequence of the N- and C-terminal constructs was verified subsequently. Arf6T27N and Rab11S25N dominant-negative mutants were generated by using the QuikChange mutagenesis kit (Stratagene) using the HA-tagged Arf6/pcDNA3 or Myc-tagged Rab11/pcDNA3 as a template. The Myc-tagged human SCAMPs were described previously (44). The coding region for SCAMP2 and SCAMP5 was amplified by PCR and ligated into the pEGFP N1-vector in-frame to make GFP fusion constructs (SCAMP2GFP and SCAMP5GFP). GFP-tagged Arf6T27N (49) was a kind gift from Dr. Martin Schwartz (University of Virginia).

**Cell Culture and Transfection**—AP-1 cells stably expressing NHE5 with a triple HA tag inserted after amino acid residue 36 (AP-1/NHE5HA cells) (50) were maintained in α-minimal essential medium with 10% fetal bovine serum, and PC12 and PC12 stably expressing 1D4-tagged NHE5 (PC12/NHE51D4) cells were maintained in RPMI supplemented with 5% fetal bovine serum. Transient transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. NHE51D4 was transfected to PC12 cells using the conventional calcium phosphate method (51), and cells stably expressing NHE51D4 were selected in selection media containing G418 (200 μg/ml). Approximately 20 independent clones were screened to test NHE51D4 expression by Western blot and immunofluorescence microscopy, and several independent clones expressing moderate levels of NHE5 were analyzed.

**Expression and Purification of GST Fusion Proteins**—For producing GST fusion proteins, PCR fragments corresponding to different regions of the SCAMP2 cytoplasmic domains were inserted into a pGEX-2T bacterial expression vector (Amer sham Biosciences) in-frame with the N-terminal GST tag as described previously (44). Protein expression was induced by incubating transformed BL21 Escherichia coli cells with 0.2 mM isopropyl 1-thio-β-D-galactopyranoside at 37 °C for 3 h. E. coli cells were collected by centrifugation and resuspended in lysis buffer containing 1% Triton X-100 and protease inhibitor mixture (Roche Diagnostics, Laval, Canada) in PBS. Cell lysates...
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were then incubated for 30 min on ice and then sonicated four times for 30 s. After sonication, cell debris was cleared by centrifugation for 10 min at 16,000 \( \times g \) at 4 °C. GST fusion proteins were purified by incubation with reduced form glutathione-Sepharose beads (Amersham Biosciences) at 4 °C.

GST Pulldown—A 35S-labeled NHE5 C-terminal domain (Gly491−Leu896) was produced by in vitro transcription-translation using the TNT-coupled reticulocyte lysate system (Promega, Madison, WI) according to the manufacturer's instructions. The 35S-labeled in vitro translated protein was diluted to 1 ml with cold PBS and then centrifuged at 16,000 \( \times g \) for 5 min to remove insoluble materials. The supernatant was then further diluted to 6.2 ml in cold PBS plus protease inhibitor mixture (Roche Applied Science). 750 \( \mu \)l of this diluted solution was incubated with 2 \( \mu \)g of GST fusion protein immobilized to the reduced form glutathione-Sepharose beads for 90 min at room temperature. After extensive washing, 35S-labeled in vitro translated protein bound to the GST fusion protein was eluted with SDS sample buffer, resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and bound 35S-labeled NHE5 C terminus was detected by phosphorimaging. Equal input of the different GST fusion proteins was confirmed by Western blot using Coomassie Blue protein stain.

Co-immunoprecipitation—AP-1/NHE5HA cells were transfected with SCAMP2Myc, SCAMP2ΔNPFMyc (deletion of amino acids 1–55), SCAMP2ΔCMyc, or SCAMP2(1−154)Myc, and the cells were lysed in PBS containing 1% CHAPS and protease inhibitor mixture (Roche Applied Science) on ice for 30 min. Lysates were cleared by centrifugation at 16,000 \( \times g \) for 10 min (two times) at 4 °C. Cell lysates were then incubated with anti-HA monoclonal antibody or pre-immune serum at 4 °C for 4 h, followed by overnight incubation with protein G-Sepharose beads (Amersham Biosciences). After extensive washing, eluted samples were resolved in SDS-PAGE, and the proteins present in the immunoprecipitate were detected by Western blot. To isolate membrane fractions, cells were resuspended in sonication buffer (250 mM sucrose, 10 mM HEPES-NaOH, pH 7.4, 1 mM EDTA, with protease inhibitor mixture (Roche Applied Science), and debris was removed by centrifugation at 16,000 \( \times g \) for 10 min two times at 4 °C. Protein concentration was determined using Bradford assay, and an equal amount of protein was collected from each sample for analysis. A small amount (5%) of lysate was removed and represents the total fraction; the remaining lysate was then incubated with NeutrAvidin-agarose beads (Pierce) overnight to extract biotinylated proteins. Following washing of the beads, biotinylated proteins were then eluted with SDS-sample buffer containing 100 mM dithiothreitol, resolved in SDS-PAGE, and detected by Western blotting. The intensity of the bands was analyzed by densitometry of films exposed in the linear range.

22Na\(^{+}\) Influx Assay—Sub-confluent AP-1/NHE5HA cells were plated into 24-well plates and transfected with SCAMP2Myc, SCAMP5Myc, or empty parental pcDNA3 vector. Transfection efficiency exceeded 50%, as determined by immunofluorescence microscopy. Forty-eight hours post-transfection, cells were acidified using the NH4Cl pre-pulse technique (50). In brief, cells were treated with ammonium choline solution (50 mM NH4Cl, 80 mM choline chloride, 1 mM MgCl2, 2 mM CaCl2, 5 mM glucose, 20 mM HEPES-Tris, pH 7.4) for 20 min at 37 °C followed by a rapid washout with isotonic choline chloride solution (130 mM choline chloride, 1 mM MgCl2, 2 mM CaCl2, 5 mM glucose, 20 mM HEPES-Tris, pH 7.4) to acutely acidify the cytosol. Assays were immediately initiated by adding radioactive 22Na\(^{+}\) (1 \( \mu \)Ci/ml 22NaCl in choline chloride solution) to each well in the absence or presence of 1 mM amiloride. After 5 min, the influx of 22Na\(^{+}\) was terminated by rapidly washing each well three times with ice-cold NaCl-saline solution (130 mM NaCl, 5 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 5 mM glucose, 20 mM HEPES-NaOH, pH 7.4). Cells were then lysed in 0.5 N NaOH to extract the radiolabel. Lysates were neutralized by the addition of an equal volume of 0.5 N HCl, and the radio-
activity was counted by liquid scintillation spectroscopy. Influx values obtained in the presence of amiloride were subtracted from those in the absence of amiloride. The difference represents the "amiloride-sensitive" $^{22}\text{Na}^+$ influx due to NHE. Each experiment was conducted in quadruplicate, and three independent experiments were performed.

$p\text{H}_i$ Measurements—AP-1/NHE$_5^{HA}$ cells were transfected with GFP-tagged SCAMP2, SCAMP5, SCAMP2ΔNPF, SCAMP2ΔC, SCAMP2-(1–154), or empty parental pEGFP expression vector (Clontech) or co-transfected with Rab11$_{Myc}^{HA}$ or Arf6$_{HA}^{HA}$ (either wild-type or dominant-negative) and SCAMP2$_{GFP}$ or pEGFP vector. Cells were then plated onto glass coverslips and grown for 48 h prior to $p\text{H}_i$ measurements. Coverslips with cells attached were mounted in a temperature-controlled recording chamber filled with NaCl-saline solution, placed on the microscope stage, and GFP-expressing cells were identified by viewing GFP fluorescence during excitation at 488 nm. Subsequently, cells were loaded with BCECF by adding 2 μM BCECF acetoxyethyl ester to the NaCl-saline solution for 10 min at room temperature and then superflused with 2 ml/min with NaCl-saline solution (without dye) at 34 °C for the remainder of the experiment. BCECF-derived fluorescence emission intensities during excitation at 488 nm and 452 nm were at least 20-fold higher than the original GFP fluorescence signal. The dual excitation ratio method was used to estimate $p\text{H}_i$, employing a fluorescence ratio imaging system (Atto Biosciences, Rockville, MD); full details of the methods employed have been presented previously (52, 53). The high-[K$^+$]/nigericin technique was employed to convert background-corrected BCECF emission ratios into $p\text{H}_i$ values. Intracellular acid loads were imposed by exposing the cells for 2 min to NH$_4^+$-choline solution. The recovery of $p\text{H}_i$ following an NH$_4^+$ pre-pulse was fitted to a single exponential function, and the first derivative of this function was used to determine the rate of change of $p\text{H}_i$ (d$p\text{H}_i$/dt) at 0.05 $p\text{H}_i$ unit increments from the point of maximum acidification (52, 53). Proton efflux was calculated by multiplying the measured d$p\text{H}_i$/dt at a given $p\text{H}_i$ value by the intrinsic intracellular buffering capacity (βi) at the same $p\text{H}_i$ value. We calculated βi in AP-1/NHE$_5^{HA}$ cells by measuring the changes in $p\text{H}_i$ elicited by changing the extracellular concentration of NH$_4$Cl as described previously by Roos and Boron (54) and Boyarsky et al. (55). Instantaneous proton efflux was then plotted against absolute $p\text{H}_i$ values, and results from different experiments were compared statistically (Student’s unpaired two-tailed t test) at corresponding values of $p\text{H}_i$.

To confirm the identity of the acid-extrusion mechanism, the NHE inhibitor 5-($N^\text{ethyl}-N^\text{isopropyl})amiloride (EIPA, 10 μM) was added to the perfusion solution for 2.5 min during the $p\text{H}_i$ recovery phase followed by a return to NaCl-saline solution. The compositions of the NaCl-saline and NH$_4^+$-choline solutions were the same as those used for the $^{22}\text{Na}^+$-influx assays.

**Immunofluorescence Microscopy**—PC12/NHE5$_{154}^{HA}$ cells grown on glass coverslips coated with poly-l-lysine and laminin (10 μg/ml, Sigma) were transfected with SCAMP2$_{GFP}$ or co-transfected with SCAMP2$_{GFP}$ and Arf6$_{HA}^{HA}$. 72 h post-transfection, cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature and permeabilized with 0.1% Triton X-100 in PBS for 5 min. Fixed cells were then treated with rabbit polyclonal anti-Rab4, anti-Rab11, or anti-HA antibodies and mouse monoclonal 1D4 antibodies followed by Alexa 488- and Alexa 568-conjugated secondary antibodies (Molecular Probes). To visualize recycling endosomes, AP-1/NHE$_5^{HA}$ cells were serum-starved for 2 h and then incubated with Alexa 568-conjugated transferrin (25 μg/ml, Molecular Probes) for 30 min at 37 °C. Cells were rinsed with PBS, fixed with pre-chilled methanol at −20 °C for 5 min, permeabilized with 0.1% Triton X-100/PBS for 5 min, and internalized transferrin, NHE$_5^{HA}$, and endogenous SCAMP1 or SCAMP2 were visualized by immunofluorescence microscopy as described above, using anti-SCAMP1 or anti-SCAMP2 rabbit polyclonal and anti-HA monoclonal primary antibodies, followed by Alexa 488-conjugated goat anti-rabbit IgG and Alexa 647-conjugated goat anti-mouse IgG secondary antibodies. Preparied coverslips were then analyzed by triple immunofluorescence confocal microscopy.

**RESULTS**

**SCAMPS Are Novel Binding Partners of NHE5**—We showed previously that SCAMPS directly bind the cytosolic C-terminal extension of the organelle-enriched NHE7 isoform and govern its intracellular trafficking between the trans-Golgi network and recycling endosomes (44). NHE7 shuttles between the trans-Golgi network, plasma membrane, and recycling endosomes via the clathrin-dependent pathway (56). Similarly, NHE5 is internalized through the clathrin-dependent pathway and is predominantly associated with recycling endosomes following endocytosis (50). Thus, we reasoned that SCAMPS might also bind to NHE5 and regulate its targeting. To test this possibility, we first carried out co-immunoprecipitation using rat PC12 cells stably expressing 1D4-tagged NHE5 (PC12/NHE5$_{154}^{HA}$). PC12 cells are widely used as a neuronal model system and endogenous expression of SCAMP1, -2, and -5 was observed by Western blot (Fig. 1A (44)).

![Image](http://example.com/fig1.png)

**FIGURE 1.** NHE5 interacts with SCAMPS. A, membrane fractions from control PC12 cells or PC12 cells stably expressing NHE5$_{154}^{HA}$ (PC12/NHE5$_{154}^{HA}$) were immunoprecipitated with 1D4 antibody conjugated to Sepharose beads. Bound endogenous SCAMP1, SCAMP2, and SCAMPS found in the immunoprecipitate fraction (IP) were detected by Western blot using SCAMP-specific antibodies. Five percent of the membrane lysate (lys.) was resolved as a positive control. B, 2.5, 5, and 10 μg of protein from rat brain lysate was probed by Western blot to assess the endogenous expression of NHE5 and SCAMP2 protein in brain tissue. C, NHE5 was immunoprecipitated from rat brain lysate using an anti-NHE5 antibody (IP) or pre-immune serum control (Con.), and bound endogenous SCAMP2 was detected by Western blot. One percent of the rat brain lysate (lys.) was probed as a positive control. Western blots shown in A–C are representative of three independent experiments in each case.
A fraction was prepared from PC12 or PC12/NHE5D4 cells and subjected to immunoprecipitation using 1D4 antibody coupled to Sepharose beads. SCAMP1 and SCAMP2, but not SCAMP5, were readily detectable in the immunoprecipitate from PC12/NHE5D4 cells (Fig. 1A). Although equivalent levels of SCAMP expression were seen in lysates from both PC12 and PC12/NHE5D4 cells, SCAMPs were undetectable in the immunoprecipitated samples from untransfected PC12 cells.

Next we examined whether the SCAMP-NHE5 interaction was also found in brain tissue. Endogenous expression of NHE5 and SCAMP2 protein in rat brain was first confirmed by Western blot (Fig. 1B). Rat brain lysate was then subjected to immunoprecipitation using anti-NHE5 antibodies or pre-immune serum. A distinct band of ~37 kDa in size corresponding to SCAMP2 was detected by Western blot in the lysate immunoprecipitated with anti-NHE5 antibody (IP, Fig. 1C), but not the lysate incubated with pre-immune serum (Con, Fig. 1C) suggesting the existence of a SCAMP2-NHE5 complex in brain.

**Determination of the NHE5-binding Site of SCAMP2**—The C-terminal cytosolic extension of NHE proteins serves as a major protein-protein interaction domain, and most of the previously identified NHE-binding proteins were shown to bind to this domain (10). SCAMPs contain possible protein-protein interaction interfaces in the N-terminal and C-terminal cytosolic extensions as well as the cytoplasmic loop between the second and third transmembrane domains (36, 57). To test whether these cytosolic domains of SCAMP2 and NHE5 directly interact, we performed in vitro GST pulldown protein-binding experiments. Immobilized GST alone or GST-SCAMP2 fusion proteins were incubated with [35S]labeled in vitro transcribed/translated NHE5 C terminus (amino acids 492–896). After washing the beads, bound [35S]labeled NHE5 C terminus was eluted, resolved by SDS-PAGE, and detected by phosphorimaging. A small amount of the NHE5 C terminus input (3%), not subjected to pulldown assay, was also included as a control. B and C, two additional GST pulldown experiments were performed using GST fused to fragments of the SCAMP2 N terminus (amino acids 1–154, 1–88, 45–88, 45–154, 75–117, 75–134, and 75–154) to determine the minimum NHE5-binding sites within the SCAMP2 N-terminal tail. Each pulldown experiment was performed three times; representative results are shown. D, a schematic representation showing the membrane topology of SCAMP2. NHE5-binding sites are highlighted with black rectangles, and the N-terminal NPF repeats are labeled with black circles. Numbers indicate amino acid residues.
cytosolic C terminus, and amino acids 45–75 and 117–134 within the cytosolic N terminus, of SCAMP2 (Fig. 2D).

Heterologous Expression of SCAMP2 Affects NHE5 Surface Localization, but Not NHE5 Internalization—SCAMPs have been suggested to play roles in both secretion (36) and endocytosis (39). We postulated that SCAMP2 might modulate the targeting of NHE5 between endosomes and the plasma membrane and thereby regulate transporter activity across the plasma membrane. To address the functional significance of the SCAMP-NHE5 interaction, we used Chinese hamster ovary AP-1 cells devoid of intrinsic NHE activity (58) stably expressing HA-tagged NHE5 (AP-1/NHE5HA, see “Experimental Procedures”). This is a widely used model system to measure the activity of different NHE isoforms in the absence of intrinsic NHE activity (30, 32, 50, 59). It was previously shown that NHE5 cycles between the plasma membrane and recycling endosomes (50). SCAMPs are also localized to the eukaryotic cell-surface recycling system (33). Therefore, we next tested whether NHE5 and SCAMPs co-localize in recycling endosomes. AP-1/NHE5HA cells grown on glass coverslips were incubated at 37 °C for 30 min in media containing fluorescently labeled transferrin to visualize recycling endosomes. In agreement with the previous study, NHE5HA was associated with internalized transferrin in a perinuclear location (Fig. 3). Some of the NHE5HA signal appeared dispersed. This is likely due to partial localization to the endoplasmic reticulum resulting from heterologous overexpression as noted earlier (50). As observed in the three color overlay picture, both SCAMP1 and SCAMP2 co-localized with NHE5 predominantly in the perinuclear location positive for fluorescently labeled transferrin (Fig. 3), suggesting that recycling endosomes are the site of the SCAMP-NHE5 interaction.

To investigate whether SCAMPs regulate the subcellular distribution of NHE5, we performed biotin-labeling and internalization assays to monitor the trafficking of NHE5. AP-1/NHE5HA cells were transiently transfected with either SCAMP2Myc or SCAMP5Myc, or empty pcDNA3 vector as a control. Cell-surface-exposed proteins were labeled with a membrane-impermeable protein-reactive biotinylation reagent containing a cleavable disulfide bond at 4 °C. Labeled cells were then incubated in culture media at 37 °C for 0–30 min to facilitate internalization of labeled proteins through endocytosis. Cells were then treated with reduced glutathione (cleavage buffer) to cleave the remaining surface biotin tags and allow for evaluation of the remaining internalized, biotinylated NHE5 population, or left untreated to assess the total surface-exposed and biotinylated NHE5 population. Biotinylated proteins were affinity-purified with avidin-coupled agarose beads and analyzed by SDS-PAGE and Western blot. NHE5 was efficiently biotinylated on the cell surface (Surface NHE5HA, Fig. 4, A and B). The cell-surface biotin tags of NHE5 were efficiently removed by incubation with cleavage buffer (time 0 in Fig. 4, A and B), whereas internalized NHE5 protected from cleavage after chase incubation was detectable by Western blot (time 15 and 30 min in Fig. 4, A and B). The rates of NHE5 endocytosis appeared to be unaffected by overexpression of either SCAMP2Myc or SCAMP5Myc as compared with vector-transfected controls (Fig. 4C). However, SCAMP2Myc, but not SCAMP5Myc, increased the surface abundance of NHE5HA by ∼50% relative to the control (p < 0.01, Fig. 4D). To further define whether SCAMP2 regulates the surface targeting of NHE5, we measured cell-surface Na+/(H+)/ exchange activity in transfected AP-1/NHE5HA cells using the 22Na i-influx assay. Forced expression of SCAMP2 into these cells increased the amiloride-sensitive, acidic H+-,activated influx of 22Na i typically by 50% or greater (p < 0.01), whereas expression of SCAMP5 caused only a slight increase that was not statistically significant (Fig. 4E).

Heterologous Expression of SCAMPs Affects NHE5 Activity at the Cell Surface—The results of cell-surface biotinylation experiments and 22NaCl influx assays suggest the involvement of SCAMP2, but not SCAMP5, in controlling NHE5-surface expression. However, these assays involve measurements from a pool of transiently transfected cells, and the data could be affected by experimental conditions such as transfection efficiency. To address this possibility, pH i measurements were employed to assess Na+/H+ exchange activity in individual AP-1/NHE5HA cells. AP-1/NHE5HA cells were loaded with BCECF, the cytosol was acified by the ammonium pre-pulse technique, and rates of Na+/-dependent pH i recovery were measured (see “Experimental Procedures”). pH i recovery was undetectable when cells were superfused with a Na+-free solution (not shown). Similarly, treatment with the NHE inhibitors EIIPA (10 μM) (Fig. 5A) or amiloride (1 mM) (not shown, see Ref. 57) effectively blocked pH i recovery in a reversible manner. No recovery of pH i was observed in parental AP-1 cells following an intracellular acid load under HEPES-buffered conditions (not shown). Altogether, these results indicate that the recovery of pH i in AP-1/NHE5HA cells is mediated by NHE5. To determine NHE5-dependent proton efflux, we measured the intrinsic intracellular buffering capacity (βi) of AP-1/NHE5HA cells over the pH i range studied in the present experiments (see “Experimental Procedures”). Consistent with previous reports in AP-1 cell transfectants (50, 60), βi in AP-1/NHE5HA cells was 28.4 ± 4.6 mEq/pH unit and was not significantly altered in cells transfected with GFP-tagged SCAMP2, SCAMP5, SCAMP2ΔC, SCAMP2-(1–154), or SCAMP2ΔNPF (see below).
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To define the role of SCAMPs on NHE5 activity across the plasma membrane, we expressed GFP or GFP-tagged SCAMP2 (SCAMP2GFP) or GFP-tagged SCAMP5 (SCAMP5GFP) into AP-1/NHE5HA cells and Na⁺-dependent, EIPA-sensitive pH_{i} recovery following an ammonium pre-pulse was examined using single cell imaging. Cells transfected with SCAMP2GFP exhibited significantly faster (p < 0.05 at all absolute values of pH_{i}) proton efflux than GFP-transfected controls (Fig. 5, B and C). In contrast, SCAMP5GFP expression failed to significantly affect (p > 0.05 at all absolute values of pH_{i}) proton efflux, suggesting the specificity of SCAMP2GFP overexpression.

In vitro protein-protein interaction assays indicated that both the N- and C-terminal cytosolic extensions of SCAMP2 contribute to NHE5 binding (Fig. 2). To investigate the involvement of these domains of SCAMP2 in NHE5 targeting in the cell, we generated serial N-terminal and C-terminal deletion mutants. Because some of the mutants were either poorly expressed or exhibited cell toxicity during pH_{i} measurements, the following three mutants were further characterized: SCAMP2ΔC, which lacks the cytosolic C-terminal tail, SCAMP2-(1–154), the soluble SCAMP2 N terminus alone, and SCAMP2ΔNPF lacking the N-terminal 55 amino acids containing multiple Asn-Pro-Phe (NPF) repeats. We first tested whether the mutants bind to NHE5 in a cellular context. When expressed in AP-1/NHE5HA cells, Myc-tagged SCAMP2 was co-immunoprecipitated with HA-tagged NHE5, while SCAMP2ΔC and SCAMP2-(1–154) showed little or no binding (Fig. 5D). In contrast, SCAMP2ΔNPF was co-immunoprecipitated with NHE5 as efficiently as full-length SCAMP2 (Fig. 5D). If SCAMP2 serves as a scaffold protein then some of the mutants lacking either NHE5 binding domains or binding motifs with other molecules might show dominant-negative effects by competing with intrinsic protein-protein interactions. To test this possibility, we transfected GFP-tagged SCAMP2 mutants and assessed their effects on NHE5 activity. AP1/NHE5HA cells transfected with SCAMP2ΔC recovered visualization of internalized protein or left uncleaved (Cleavage: + or –). Cells were then lysed and biotinylated proteins were purified by incubation with avidin-conjugated agarose beads, resolved by SDS-PAGE, and surface-labeled and -internalized NHE5HA was detected by Western blotting using an anti-HA monoclonal antibody (Surface NHE5_{HA}). A small amount of total lysate (5%) was analyzed as a loading control and probed for SCAMP2Myc or SCAMP5Myc and NHE5HA (Total NHE5_{HA}, SCAMP2Myc or SCAMP5Myc). The Western blots shown are representative of three independent experiments. C, the percentage of labeled NHE5_{HA} internalized after 15 or 30 min of chase was calculated by comparing the signal in the cleaved samples (Cleavage: +) to the corresponding uncleaved samples (Cleavage: –). The amount of NHE5_{HA} internalized at each time point in SCAMP2Myc or SCAMP5Myc-transfected cells is expressed relative to control cells (pcDNA3) and are averaged from three independent experiments ± S.D. Each experiment was performed in quadruplicate. ** and NS, p < 0.01 and not significant, respectively (unpaired Student’s t test).

**FIGURE 4. SCAMP2 controls NHE5 cell-surface abundance.** A and B, AP-1/NHE5_{HA} cells were transiently transfected with Myc-tagged SCAMP2 (SCAMP2Myc, A) or Myc-tagged SCAMP5 (SCAMP5Myc, B), or with empty pcDNA3 vector control. Transfected cells were incubated with a biotinylation reagent followed by chase incubation in the culture media for 0, 15, or 30 min (Chase) to permit endocytosis of labeled proteins. Following the chase period, surface biotin was removed by incubation with a cleavage reagent allowing for detection of NHE5_{HA} surface abundance. Total NHE5_{HA} was used to measure the relative surface abundance of NHE5_{HA}, Total surface NHE5_{HA} in SCAMP2Myc or SCAMP5Myc-transfected cells was compared directly to pcDNA3-transfected control cells from the same experiment and is expressed as a percentage relative to pcDNA3 transfected control. Values represent the mean of three independent experiments ± S.D. ** and NS, p < 0.01 and not significant, respectively (unpaired Student’s t test). C, AP-1/NHE5_{HA} cells were transfected with SCAMP2ΔC (C), SCAMP2ΔNPF (D), or empty pcDNA3 vector, and NHE activity was measured by the amiloride-inhibitable 22Na⁺ influx technique (see “Experimental Procedures”). Results are expressed as a percentage relative to pcDNA3-transfected control. Data from a representative of three independent experiments are shown here ± S.D. Each experiment was performed in quadruplicate. ** and NS, p < 0.01 and not significant, respectively (unpaired Student’s t test).
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FIGURE 5. Effects of overexpression of SCAMPs on rates of pH recovery from cytosolic acid loads. A, AP-1/NHE5HA cells grown on a glass coverslip were loaded with the pH-sensitive dye BCECF and acidified by exposure to 50 mM NH₄Cl for 2 min followed by wash-out with H⁺-containing HEPES-buffered saline solution. The recovery of intracellular pH (pH½) following the wash-out of NH₄Cl was monitored in individual cells. Partway through the recovery phase of the experiment, cells were exposed to the NHE inhibitor EIPA (10 μM) for 2.5 min. The record is the mean of data obtained simultaneously from 17 cells on a single coverslip and is representative of four independent experiments.

B, AP-1/NHE5HA cells were transfected with pEGFP, GFP-tagged SCAMP2 or SCAMP5. pH recoveries in the transfected cells were monitored 48 h following transfection. Records are means of data obtained simultaneously from 10, 7, 7, and 4 cells transfected with pEGFP, GFP-tagged SCAMP2 or SCAMP5, respectively, which exhibited similar peak acidifications. Each experiment was performed on a separate coverslip, and each record is representative of four to six independent experiments in each case. Continuous lines represent the weighted non-linear least-squares regression fits to the data points (mean ± S.E.) indicated for each experimental condition. In each case, data points were obtained from at least three experiments of the type illustrated in A.

C, the pH½ recoveries (see “Experimental Procedures”). SCAMP2GFP and Arf6HA in PC12/NHE51D4 cells in a perinuclear region (Fig. 5E). In contrast, the distribution of endogenous Rab4 was clearly distinct from that of NHE5 and SCAMP2.

Arf6 and Rab11 Control the Cell-Surface Abundance and Activity of NHE5—The immunofluorescence microscopic results showing that Arf6 and Rab11, but not Rab4, associate with NHE5 and SCAMP2 prompted us to test whether Arf6 and Rab11 influence the endosome-plasma membrane targeting of NHE5. AP-1/NHE5HA cells were transfected with wild-type or GTP-binding-deficient dominant-nega-
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Figure 6. NHE5 and SCAMP2 co-localize with the small GTPases Arf6 and Rab11. PC12 cells stably expressing 1D4-tagged NHE5 (PC12/NHE51D4) grown on glass coverslips were transfected with GFP-tagged SCAMP2 (SCAMP2GFP) together with HA-tagged Arf6 (Arf6HA), and the localization of SCAMP2GFP, NHE51D4, and endogenous Rab11 or Rab4 was assessed. White foci in the merged images result from co-localization of the three proteins. Bars, 10 μm.

Discussion

Secretory carrier membrane proteins (SCAMPs) are a group of integral membrane proteins that cycle between multiple organelles and regulate membrane dynamics. In the current study, we have shown that SCAMP2 directly binds to NHE5 and facilitates its cell-surface targeting. SCAMP2 contains an N-terminal cytosolic extension, four transmembrane spans, and a C-terminal cytosolic tail (57). Using an in vitro protein binding assay, we have identified NHE5-binding sites within the cytosolic C terminus, and amino acids 45–75 and 117–134 within the cytosolic N terminus of SCAMP2. Further, we used a co-immunoprecipitation approach to show that NHE5 and SCAMP2 form a complex both in tissue culture cells and in brain tissue.

Exogenous expression of SCAMP2 increased both the cell-surface abundance and the ion-translocation activity of NHE5. The agreement between experiments examining cell-surface NHE5 abundance and NHE5 activity suggest the predominant action of SCAMP2 acts on membrane trafficking. Furthermore, SCAMP2 appeared to have no effect on the rates of endocytosis of NHE5 from the plasma membrane. Thus, SCAMP2 likely regulates the abundance of NHE5 at the cell surface by promoting its delivery from the perinuclear recycling endosomes. However, we cannot rule out the possibility that NHE5 ion-translocation activity may be partially regulated through interaction with SCAMP2 (see below). It is unlikely that the effect of SCAMP2 expression on NHE5 cell-surface targeting is an over-expression artifact, because expressing comparable levels of SCAMP5 or deletion mutants of SCAMP2 did not cause the same change. Interestingly, among the SCAMP2 deletion mutants tested, the N-terminal deletion mutant lacking the NPF repeats (SCAMP2ΔNPF) markedly suppressed NHE5 activity across the plasma membrane. Likewise, expression of a mini-gene encoding the N-terminal fragment of SCAMP2 (SCAMP2-1–154) caused a milder but significant decrease in NHE5 activity despite its weak binding affinity to NHE5. SCAMP2-1–154 may compete with endogenous SCAMP2 for binding to other molecules such as soluble EH-domain proteins. In contrast, the ΔNPF mutant binds to NHE5 but may not be able to recruit necessary cytosolic factors to the NHE5-SCAMP2 complex. NPF repeats commonly interact with the EH-domain and regulate endocytosis and endocytic recycling (41, 64–66). Furthermore, intersections, EH-domain-containing proteins that were reported to bind to the NPF repeats of SCAMP (39), regulate recycling of synaptic vesicles in Drosophila and Caenorhabditis elegans (67–70). Thus, we hypothesize that SCAMP2 recruits cytosolic EH-domain proteins to recycling endosomes via its N-terminal NPF repeats and promotes vesicle formation and the plasma membrane targeting of NHE5. This proposed model is in agreement with a

load. Arf6T27N significantly reduced the ability of concomitantly transfected SCAMP2 to up-regulate NHE5 activity (p < 0.05), whereas Rab11S25N did not influence the SCAMP2-mediated up-regulation of NHE5 (p > 0.05 (Fig. 8, A and B)). These results suggest that the activity of SCAMP2 in controlling NHE5 cell-surface targeting is Arf6-dependent and Rab11-independent.

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previous report showing that newly formed transferrin-containing vesicles are SCAMP-deficient, which suggests that endocytosis of transferrin occurs independently of SCAMPS (33). The internalized transferrin-containing vesicles are fused with a pre-existing internal pool of SCAMP-positive membranes and then accumulate in the SCAMP-rich perinuclear region corresponding to the recycling endosomal compartment. Vesicles leaving this compartment, returning transferrin to the cell surface were again SCAMP-deficient, suggesting that the perinuclear recycling endosomes are a likely site of SCAMP function. Interestingly, SCAMP1 and SCAMP2 and to a lesser extent SCAMP3 showed considerably more overlap with trafficking transferrin than SCAMP4, which lacks a large part of the N-terminal cytosolic tail, including NPF repeats (33). Thus, these findings together with our own suggest that the N-terminal cytosolic extension of SCAMP2 is an important domain for cell-surface targeting through recycling endosomes.

The small GTPases Arf6 and Rab11 have both been implicated as master regulators of membrane traffic from recycling endosomes to the cell surface (43). Overexpression of either Arf6 or Rab11 significantly enhanced NHE5 abundance and activity at the cell surface, whereas expression of dominant-negative Arf6 and Rab11 alone had very little effect on NHE5 activity. Co-expression of both dominant-negative GTPases caused a substantial decrease in NHE5 activity and cell-surface abundance. When concomitantly expressed with SCAMP2, Arf6\textsubscript{T27N} but not Rab11\textsubscript{S25N} impaired the SCAMP2-mediated NHE5 translocation to the plasma membrane. These results indicate that, although both Arf6 and Rab11 participate in controlling the membrane traffic of NHE5, SCAMP2-mediated trafficking is Arf6-dependent and Rab11-independent. Thus we propose that NHE5 accesses the cell surface from the recycling endosomes via at least two distinct pathways: a Rab11-dependent pathway and an Arf6/SCAMP2 pathway. It was previously shown that Arf6 binds to SCAMP2 and regulates fusion pore formation during dense-core vesicle exocytosis in PC12...
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A.

\[ \text{pH}_i \]

SCAMP2
Rab11S25N/SCAMP2
Arf6T27N/SCAMP2

\[ \text{NH}_4^+ \text{ 2 minutes} \]

B.

Proton Efflux (\(\mu\)M/sec)

SCAMP2
Rab11S25N/SCAMP2
Arf6T27N/SCAMP2

\[ \text{pH}_i \]

6.6
6.7
6.8
6.9
7.0
7.1

60
120
180
240
300
360
420
480
540
600

FIGURE 8. SCAMP2 facilitates cell-surface targeting of NHE5 via Arf6. AP-1/NHE5-HA cells were transfected with SCAMP2-GFP (SCAMP2), SCAMP2-GFP plus Rab11S25N-Myc (Rab11S25N/SCAMP2), or SCAMP2-GFP plus Arf6T27N-HA (Arf6T27N/SCAMP2). Transfected cells were loaded with BCECF and examined by fluorescence microscopy. A, example of pH-recovery time course. B, pH dependence of NHE5-dependent acid extrusion in an alkaline direction (e.g. Fig. 5C). Continuous lines represent the weighted non-linear least-squares regression fits to the data points (mean ± S.E.) indicated for each experiment. Each experiment was performed on a separate coverslip, and each record is representative of three independent experiments in each case.

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