Synaptotagmin I binds intestinal epithelial NHE3 and mediates cAMP- and Ca\textsuperscript{2+}-induced endocytosis by recruitment of AP2 and clathrin

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METHODS

Cell culture. The human colon carcinoma cell line Caco-2BBe was a generous gift from Dr. Mark Mooseker (Yale University, New Haven, CT) (34). Caco-2BBe cells were grown in DMEM supplemented with 10% (vol/vol) FBS, 2 mM l-glutamine, 50 U/ml penicillin, 50 \(\mu\)g/ml streptomycin, and 10 \(\mu\)g/ml transferrin (all from Invitrogen/GIBCO, Grand Island, NY) in a humidified atmosphere with 5% CO\textsubscript{2}. Cells were seeded onto collagen-coated polycarbonate permeable filter supports (0.4-\textmu m pore size, 24.5-mm diameter, 4.7-cm\textsuperscript{2} growth surface; Transwell, catalog no. 3412; Corning Life Sciences, Cambridge, MA) at \((\approx 2.5 \times 10^5 \text{ cells/cm}^2\) and were allowed to differentiate for 14 days before experiments.

Far Western intestinal cDNA library screening. An adult rat small intestinal cDNA library in Lambda Zap II Phage was purchased from Stratagene (San Diego, CA). Phage clones (500,000 total)
were incubated with XL-1 blue MRF bacteria in Lennox LB medium with 0.2% (wt/vol) maltose and 10 mM each of MgSO₄ and CaCl₂ for 15 min at 37°C to allow phage binding, after which 6.5 ml of 1% (wt/vol) NZY agar (−48°C) was added, mixed, and poured onto dry, prewarmed, 150-mm agar plates and allowed to grow for 2.5 h at 37°C. Nitrocellulose filters that had been soaked in 1 mM isopropyl thiogalactoside and air dried were placed on top to induce protein expression and to adsorb expressed proteins. Filters were removed after 3.5 h, and adhering agarose was removed in two washes of TNE buffer [50 mM Tris (pH 7.4), 50 mM NaCl, 1 mM EDTA, and 0.5% (vol/vol) 2-mercaptoethanol]. Filters were blocked in TNE with 5% (wt/vol) nonfat dry milk for 2 h and then were incubated with 3²P-labeled GST fusion protein with amino acids 666-805 of rat NHE3 constructed in pGEX-2TK vector. This protein lacks the final 26 amino acids of the cytoplasmic tail of NHE3, including the COOH-terminal PDZ binding site essentially at the COOH terminus. The GST fusion protein was constructed in pGEX-2TK, allowing efficient phosphorylation of the protein using cAMP-dependent protein kinase. The GST-NHE3 fusion was phosphorylated by cAMP-dependent protein kinase while attached to glutathione-agarose and then was washed extensively to remove unincorporated nucleotide.

3²P-labeled GST-NHE3 666-805 was eluted with 10 mM reduced glutathione in 50 mM Tris, pH 8.0. This probe was added to the nitrocellulose filters at a concentration of 250,000 counts·min⁻¹·ml⁻¹ and was allowed to hybridize overnight. Filters were washed three times in TNE buffer and were exposed overnight at −80°C to film to visualize phage clones that expressed a protein that bound the labeled GST-NHE3 fusion protein. Over 50 clones were initially identified, and, of these, nearly 30 continued to be positive through three rounds of screening. The cDNA inserts were removed from the phage in pBluescript (the manner of construction of this ZAP II library) by using the manufacturer’s instructions, and inserts were sequenced by the Cancer Sequencing Center of the University of Chicago. Of these 30 clones, one clone expressing rat Syt IV was identified.

Short interfering RNA silencing of Syt I, the AP2 µ-subunit, and the heavy chain of clathrin in Caco-2/BBe cells. Stealth oligonucleotides specific for Syt I, the µ-subunit of AP2, or the heavy chain of clathrin were designed and purchased from Invitrogen (Carlsbad, CA). Sequences were chosen using the BLOCK-IT RNAi Designer software (Invitrogen). With the use of this software, sequences were compared with other human sequences by using a BLAST search obtaining oligonucleotides specific for human Syt (GenBank NM_005639, bases 613–637), the µ-subunit of AP2 (NM_004068, bases 79–103), and the heavy chain of clathrin (NM_004859, bases 3307–3331). In all cases, a second oligonucleotide was used as a control; a number of the oligonucleotide’s bases were altered, but the base composition stayed the same, which should not inhibit expression of these proteins. These control oligonucleotides were designed by the BLOCK-IT RNAi Designer software algorithm and were BLAST searched so as not to inhibit production of other proteins. To achieve sufficient knockdown of these proteins, two treatments with oligonucleotides were required prior to experimental use. Oligonucleotide treatment of cells on Transwells was begun 60 h before harvest. Stealth oligonucleotides were incubated in Opti-MEM media (Life Technologies; 2 µl of a 20 µM Stealth stock solution/100 µl Opti-MEM per well) for a period of 10 min while the lipid transfection reagent SiLentFect (Bio-Rad Laboratories, Hercules, CA) was also incubated at a ratio of 2 µl/100 µl Opti-MEM per well, each in separate polyethylene tubes. After 10 min, the two solutions were mixed to allow complex formation and were allowed to sit for 10–15 min. Fifty microliters of this complexed solution were added to both the basolateral and apical surfaces of Transwells where 30 min before the complete medium had been replaced with 1 ml Opti-MEM on both apical and basolateral sides. The cells were allowed to incubate at 37°C for 30 min to allow for maximal uptake of stealth short interfering RNA (siRNA), after which media containing 30% (vol/vol) FBS was reintroduced to bring volumes to normal levels. This procedure was again repeated at 12 h prior to harvest to achieve knockdown of protein levels. The cells were then harvested as previously described. In each experiment, the level of knockdown was confirmed by immunoblot analysis as described in Immunoprecipitation studies.

²²Na fluxes. Unidirectional apical medium-to-cell uptakes were performed under non-acid-loaded conditions, as previously described (29). Briefly, cells were grown on Transwells and were treated as described for each set of experiments. Immediately prior to flux, Transwells were washed once at room temperature with isotonic choline Cl solution (in mmol/l: 5 KCl, 10 HEPES, pH 7.4, 1 MgCl₂, 2 CaCl₂, and 150 choline Cl). Cells were placed on top of a 2-ml room-temperature flux solution without radioactivity (in mmol/l: 20 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES, pH 7.4, and 150 choline Cl). One milliliter of flux solution containing 1 µCi/ml ²²NaCl was then placed on the apical (top) side of the Transwell. After 10 min, the flux solution was removed and the Transwell was washed rapidly (<7 s) four times in ice-cold saline (composition in mmol/l: 5 KCl, 150 NaCl, and 10 HEPES, pH 7.4). Transwells were placed upside down and were allowed to dry for 10–30 min. Filters containing the cells were cut from the support and placed into vials with scintillation fluid (Budget-Solve; RPI, Mt. Prospect, IL). Samples were solubilized overnight and ²²Na quantified by liquid scintillation spectroscopy.

Immunoprecipitation studies. For all immunoprecipitations, cells were grown on Transwells and were treated with agents as described previously. At the end of incubation with CAMP or thapsigargin, cells were immediately scrapped into ice-cold saline and pelleted (14,000 g for 20 s). Cell pellets were solubilized in immunoprecipitation buffer (IP buffer; 150 mM NaCl, 10 mM HEPES, pH 7.4, 2 mM EDTA, 1 mM PMSF, 0.1 mM vanadate, 1 mM NaF, 1% (vol/vol) Triton X-100, and the complete Protease inhibitor cocktail). Samples were solubilized for 30 min on ice, and an aliquot was removed to measure protein and to run analysis of total cell expression of specific proteins. One-tenth volume Pansorbin (heat-killed protein A bearing Staphylococcus aureus) was added and rotated at 4°C for 60 min. Pansorbin cells were pelleted (2,000 g for 20 s), and the supernatant was removed and added to antibodies conjugated to beads. Antibodies to Syt I (rabbit polyclonal; Strexgen, Victoria, BC, Canada), the α-subunits of AP2 (goat polyclonal; Rockland Immunologics, Gaithersburg, MD), the µ-subunit of AP2 (chicken polyclonal; GeneTex, San Antonio, TX), or the heavy chain of clathrin (Transduction Labs/Pharminingen, Lexington, KY) were conjugated to agarose beads by using the Seize Primary Immunoprecipitation kit (Pierce Chemical, Rockford, IL) using ratios of antibodies to agarose recommended. Incubations of cell lysates were allowed to bind primary antibodies overnight at 4°C. Incubations were washed four times with IP buffer, and then samples were eluted from the beads with IgG elution buffer provided with the Seize kit for 10 min. Samples were spun at 2,000 g for 30 s, and then the supernatants were removed and mixed with one-third volume 3X Laemmli stop solution and were heated to 65°C for 10 min. Samples were analyzed by SDS-PAGE, and Western blots were immediately generated. Resolved proteins were transferred to polyvinylidene difluoride membranes (PolyScreen PVDF; Perkin Elmer Biosciences, Boston, MA) in 1× Towbin’s buffer [25 mM Tris and 192 mM glycine, pH 8.8, with 15% (vol/vol) methanol]. Blots were blocked for 1 h in 5% (wt/vol) nonfat dry milk (Carnation, Solon, OH) in Tris-buffered saline with Tween 20 (T-TBS; 150 mM NaCl, 5 mM HEPES, 10 mM Tris, pH 7.4, 0.05% (vol/vol) Tween 20]. Membranes were incubated overnight with primary antibodies in T-TBS, washed five times (10 min at room temperature), incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) in T-TBS for 1 h at room temperature, washed four times (10 min each) with T-TBS, and given a final wash (10 min) in TBS. Blots were visualized by using an enhanced chemiluminescence system (Supersignal West Pico; Pierce Biochemical, Rockford, IL). The use of the Seize kit was important because the antibodies are
covalently coupled to the agarose beads. Proteins were isolated with a buffer provided with the kit that elutes proteins immunoprecipitated by the antibody but not the antibody itself, which remains coupled to the beads. This results in the lack of appearance of the antibody used for immunoprecipitations in the Western blots made from these reactions.

**Surface biotinylation.** NHE3 present on the luminal membrane of confluent cell monolayers grown on Transwells was labeled by using surface biotinylation. Following various treatments, cells were washed once in ice cold HEPES-buffered saline (HBS; in mmol/l: 150 NaCl, 4 KCl, and 10 HEPES, pH 7.4) and placed into ice cold HBS. Proteins in the apical membrane were labeled by using the cell-impermeant biotin Sulfo-NHS-Biotin (1 mg/ml; Pierce Chemical, Rockford, IL) for 30 min in the cold HBS. As a control, basalosolateral proteins were biotinylated in two experiments by adding the Sulfo-NHS-Biotin to the basolateral side. Biotinylation was terminated by the addition of 10 μl of 1 M Tris buffer containing a free amine that reacts with the free biotin. Cell pellets were resuspended in 500 μl RIPA immunoprecipitation buffer [150 mmol/l NaCl, 2 mmol/l EDTA, 0.1% (wt/vol) SDS, 0.5% (wt/vol) Na deoxycholate, and 1% (vol/vol) Triton X-100]. Ten microliters were removed to determine protein and to assess equivalence of total NHE3 expression per Transwell. To the remainder, 50 μl of a 50% (wt/vol) slurry of immobilized streapavidin (Pierce Chemical) were added and rotated in the cold for 120 min. The beads were pelleted (14,000 g for 10 s at 4°C) and were washed five times with RIPA buffer. Samples were eluted from the beads by addition of 50 μl Laemmli stop buffer and by heating to 65°C. Samples were immediately analyzed by Western blot analysis for NHE3 using a polyclonal antisera developed in our laboratory or anti-intestinal alkaline phosphatase (Affinity BioReagents, Boulder, CO) as a control for an apical surface biotinylated protein that was not endocytosed following cAMP- or Ca2+-induced endocytosis of NHE3, using the protocol described above. As controls, apical and basolateral surface biotinylations were analyzed for the intracellular proteins villin (using a monoclonal antibody from BD Pharmingen) and the α- and β-subunits of basolateral Na+/K+-ATPase (both mouse monoclonal antibodies from Upstate Biotechnology). The α-subunit extends to the outside of the basolateral membrane where it can be biotinylated, whereas the intracellular β-subunit cannot be biotinylated using cell-impermeant biotin.

**Laser scanning confocal imaging.** Caco-2BBE cells were grown as described on PET track-etched membrane inserts (0.4-μm pore size, Falcon 3090; BD Biosciences, Franklin Lakes, NJ) and were used 10–14 days after confluence. Cells were untreated or were exposed to either 8-(4-chlorophenylthio)-cAMP (100 μM) or thapsigargin (100 ng/ml) for 10 min and washed with K-PIPES buffer (80 mM) containing 1.5 mM CaCl2 and 1.5 mM MgCl2, pH 6.5 (warmed to 37°C). With the use of a pH-shift method to preserve cellular three-dimensional structure, fixation was performed using the K-PIPES buffer described above with 3.75% formaldehyde for 5 min at room temperature followed by Na-borate buffer (100 mM) with 3.75% formaldehyde, pH 11.0, for 10 min at room temperature. The fixed monolayers were washed with PBS containing 1.5 mM CaCl2 and MgCl2 (rinse buffer), and the cells were permeabilized by using rinse buffer with 0.2% (vol/vol) Triton X-100 for 15 min at room temperature. The fixed monolayers were exposed to 2% (wt/vol) BSA in PBS for 1 h at room temperature and were washed three times with rinse buffer and affinity-purified anti-NHE3 (rabbit polyclonal at 2 mg/ml) and anti-Syt I (mouse monoclonal 41.1 at 1 mg/ml; Synaptic Systems, Göttingen, Germany) diluted 1:100 in rinse buffer for 18 h at 4°C in a humidified chamber for dual labeling. The cells were then washed with rinse buffer two times and were incubated with a 1/1,000 dilution of Cy2-conjugated AffiniPure F(ab’)2 fragment donkey anti-rabbit IgG and Cy5-conjugated AffiniPure donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. The cells were washed five times for 5 min at room temperature with wash buffer, and the membrane inserts were cut out and mounted on glass slides using 38 μl of 5% (wt/vol) n-propyl gallate (Sigma, St. Louis, MO)/70% glycerol mounting solution, sealed, and kept in the dark at 4°C until use. Imaging of NHE3 and Syt I was performed with a Fluoview 200 laser scanning confocal microscope equipped with a 488-nm argon and a 633-nm HeNe laser using a ×60 objective and a ×4 digital zoom. Images (12 bit, 800 × 600 pixels) were obtained on the x-y or x-z plane (0.2-μm sections). Raw images were processed by deconvolution using the ImageJ unmask and noise reduction algorithm programs, and colorization was performed using the ImageJ colocalization plug-in.

**NHE3-Syt I ELISA assay.** The GST-NHE3 666-815 fusion protein used to screen the rat intestinal cDNA library was subsequently used in an ELISA assay to determine if Syt I would bind. Human Syt I was subcloned into pR-SET (Invitrogen), and a six-His-tagged Syt I was produced in BL21(DE3) E. coli using the manufacturer’s protocol. The His-Syt I was purified by using the HisTag Affinity Resin from Novagen (Madison, WI) according the manufacturer’s instructions. GST-NHE3 or GST alone [made using pGEX-2TK in BL21(DE3) bacteria] or the vehicle (10 mM reduced glutathione in 50 mM HEPES, pH 7.4) were linked to Reacti-Bind glutathione-coated 96-well plates (Pierce Biochemical) at a concentration of 0.1 μg/ml for 1 h at room temperature in a humidified chamber. Plates had been prewashed once in PBS with 0.05% (vol/vol) Tween 20 (wash buffer). Plates were rinsed once with wash buffer, and His-tagged Syt I was added at varying concentrations (0–1 μg/ml), incubated for 1 h as previously described, washed three times with wash buffer, and then incubated with 0.1 μg/ml rabbit polyclonal anti-His antibody (Upstate Biotechnology). After 1 h at room temperature, plates were washed three times with wash buffer and then were incubated with peroxidase-conjugated donkey anti-rabbit IgG (0.1 μg/ml) for 1 h as before. Plates were washed three times with wash buffer and were developed for 30 min in the dark using a TMB Substrate Reagent set (BD Pharmingen, San Diego, CA). Sulfuric acid was added to stop the reactions, and the color was read on a Molecular Dynamics ELISA plate reader at 450 and 570 nm. Assay values were obtained by subtracting the absorbance at 570 nm from the value at 450 nm.

**RESULTS**

Identification of Syt I as a candidate binding protein to NHE3. To identify potential candidate binding proteins to the COOH-terminal domain of NHE3, a rat small intestinal cDNA expression library was probed with the 32P-labeled GST-NHE3 666-805. Of 500,000 clones plated in the library screening, ~30 clones were identified that consistently bound GST-NHE3 666-805 through three rounds of screening. Sequencing of the clones revealed a number of proteins, including rat Syt IV. Since the synaptotagmins have been demonstrated to regulate synaptic vesicle fusion, the role of this isoform in regulation of NHE3 was further investigated.

In exploring the functional role of Syt IV in regulation of NHE3, we were unable to find any changes in basal or in cAMP- or Ca2+-mediated 22Na influx in Caco-2BBE monolayers where Syt IV protein expression had been reduced by nearly 90% by silencing RNA, as determined by Western blot analysis of whole cell lysates. There were also no significant differences in apical membrane expression of NHE3 after cAMP or thapsigargin, as assessed by apical surface biotinylation (data not shown). Thus, Syt IV did not appear to be involved in cAMP or Ca2+ regulation of NHE3 activity or membrane trafficking. However, because of the striking sequence and functional similarities among members of the synaptotagmin family, we explored the possibility that Syt I is associated with NHE3 and is involved in its regulation by second messenger pathways.
Syt I is well expressed intestinal epithelial cells in the native mouse small intestine as well as in Caco-2BBe cells. Immunohistochemical staining of the mouse jejunum demonstrated the greatest expression of Syt I in epithelial cells of the middle to upper villus regions and high expression in the brush border (Fig. 1A). To determine if Syt I and NHE3 interaction occurs, coimmunoprecipitations were performed. Analyses of the immunoprecipitations confirmed expression of a 65-kDa band of Syt I in both the mouse jejunum and Caco-2BBe cells. Association of NHE3 with Syt I occurred under unstimulated (control) conditions in the mouse jejunal brush-border membranes (B) and in Caco-2BBe cell lysates (C) but increases on stimulation of jejunal mucosa or Caco-2BBe cells with either 8-(4-chlorophenylthio)-cAMP (100 μM for 10 min) or with the Ca2+ -ATPase inhibitor thapsigargin (100 ng/ml for 10 min). C:AMP and thapsigargin decrease the amount of apical surface membrane NHE3, but not of alkaline phosphatase (AlkPhos), as assessed by apical membrane biotinylation. Caco-2BBe cells were stimulated with cAMP or thapsigargin for 10 min, and medium was changed to ice-cold saline with Sulfo-NHS-Biotin, a cell-impermeant biotin, in the apical solution. D: apical biotin does not react with intracellular or basolateral membrane-associated proteins (villin and β-subunit of Na+–K+ -ATPase, respectively). Unstimulated Caco-2BBe cell monolayers were placed into ice-cold saline, and Sulfo-NHS-Biotin was added to either the apical or basolateral medium and allowed to react for 30 min. Biotinylated proteins were isolated from each condition as well as from a portion of the lysate. Images shown for B–E are representative of 3 experiments. IP, immunoprecipitation.
of Na\(^+\)-K\(^+\)-ATPase, the intracellular and apical-located protein villin, and the β-subunit of Na\(^+\)-K\(^+\)-ATPase, which is not transmembrane but is associated with the cytosolic face of the basolateral membrane. As seen in Fig. 1E, apical and basolateral surface biotinylations resulted in labeling of the apical and basolateral transmembrane proteins NHE3 and the α-subunit of Na\(^+\)-K\(^+\)-ATPase, respectively, and did not biotinylate the surface protein in the membrane of the other side of the cell. However, the intracellular proteins villin and the β-subunit of Na\(^+\)-K\(^+\)-ATPase were not biotinylated.

Syt I is required for cAMP- and Ca\(^{2+}\)-stimulated endocytosis but not for functional inhibition of NHE3-mediated Na uptake. To investigate a potential functional role for Syt I in NHE3 regulation, Caco-2BBBe cells were treated with a silencing oligonucleotide specific for human Syt I or an oligonucleotide with altered bases (control). An aliquot of total cell lysate was removed from each sample generated for coimmunoprecipitation analyses. Protein was measured in the lysates, and 10 μg protein was analyzed by Western blot for Syt I (or later for AP2 μ-subunit or the heavy chain of clathrin; see Figs. 4 and 5). To determine that the treatment with cAMP or thapsigargin did not alter levels of the silenced protein, three samples were analyzed in each experiment: one not stimulated (control), one treated with cAMP, and one treated with thapsigargin for both control and Syt I siRNA. Samples were analyzed on the same blot to permit densitometric comparison. Densitometric values for the three control siRNA- or Syt I siRNA-treated samples (1 control, 1 cAMP, and 1 thapsigargin in each case) were averaged to allow comparison, because cAMP and thapsigargin did not alter Syt I expression.

Syt I was expressed in cells treated with the control siRNA but was reduced by the Syt I-silencing oligonucleotide (Fig. 2A). Although not shown, the levels with the control oligonucleotide were not different from those of untreated Caco-2BBBe cells. In addition, no changes in brush-border alkaline phosphatase surface biotinylation were observed (data not shown). Silencing Syt I, however, did not alter the ability of cAMP or thapsigargin to inhibit apical NHE3 activity (Fig. 2B). To determine whether apical expression might be altered, apical surface proteins were biotinylated. Both cAMP and thapsigargin decreased apical surface expression of NHE3 (Fig. 2C) in Caco-2BBBe cells, whereas Syt I expression was unchanged (control oligonucleotide). However, when Syt I expression was silenced, NHE3 endocytosis from...
the apical surface after cAMP or thapsigargin stimulation did not occur (Fig. 2C). Because NHE3 endocytosis is dependent on an AP2- and clathrin-mediated process (7, 17, 42), we determined where Syt I might function in the sequence of events. Coimmunoprecipitation studies using antibodies to Syt I, AP2, and the heavy chain of clathrin were performed to determine association with NHE3. Immunoprecipitations for Syt I, the α-subunit of AP2, or the heavy chain of clathrin were analyzed to determine that equivalent amounts of these proteins were indeed isolated (data not shown). After cAMP or thapsigargin stimulation, NHE3 abundance in the Syt I-, AP2-, and clathrin-containing domains increased (Fig. 2D). Silencing of Syt I effectively reduced its expression and concomitantly reduced the amount of coimmunoprecipitated NHE3, implicating an association between the two molecules. In addition, NHE3 association with AP2- and clathrin-containing domains was also decreased by Syt I silencing, suggesting that Syt I association with NHE3 precedes AP2 or clathrin complexes.

Syt I and NHE3 association determined by confocal microscopy. To further determine if Syt I and NHE3 associate, confocal laser-scanning microscopy was used. Cells were treated with cAMP or thapsigargin for 10 min and were fixed to colocalize Syt I and NHE3. Dual immunolabeling was used to visualize NHE3 (Cy2 fluorophore, 492-nm absorption/510-nm emission, green) and Syt I (Cy5 fluorophore, 652-nm absorption/679-nm emission, red) and their colocalization (green and red emissions observed as yellow). The x-z single-slice images (0.2 μm) were analyzed. To determine the basal pole of the cell, reflected light was used (panels labeled RL/NHE3 in all conditions). The red filter was removed from the light path, preventing visualization of Syt I tagged with the Cy5 fluorophore. Reflected light signals were given the pseudocolor red for visualization of the polyethylene terephthalate filter that the cells were grown on. The apical pole of the cells in these panels can be appreciated by the NHE3 staining, which appears green (Fig. 3; RL/NHE3). Images for Syt I were also obtained for the same sections by placing the filter inline. Images for Syt I and NHE3 alone are presented in red and green below the RL/NHE3 panels for each condition in Fig. 3. Before stimulation (control), limited colocalization of NHE3 and Syt I was observed in these x-z sections (Fig. 3). The panels on the right side of Fig. 3 are presented in two different manners. On the top, the red and green signals are merged, and
where colocalization occurs appears yellow. The colocalized pixels from the merged image are presented as a white-on-black grayscale image to more clearly appreciate the colocalization. From the x-z images, it is apparent that both NHE3 and Syt I are well expressed at the apical pole of the cell. On stimulation with cAMP or thapsigargin, colocalization increases (Fig. 3, right). Additionally, the x-z sections demonstrate that some NHE3 and Syt I move away from the apical membrane surface into the cell.

**Synaptotagmin I is required for NHE3 association with AP2 complex in endocytosis.** To further explore the role of Syt I in membrane endocytosis of NHE3, its relationship to two other key proteins involved in membrane trafficking was examined. AP2 is a multisubunit complex that appears to be involved not only in NHE3 endocytosis (17) but also in a number of other proteins, specifically facilitating their recruitment to clathrin-coated pits (32, 36). Therefore, Caco-2BBe cells were treated with silencing oligonucleotides to the $\mu$-subunit of AP2 or the heavy chain of clathrin, and silencing of these proteins was confirmed by Western blot. As shown in Figs. 4A and 5A, silencing RNAs, but not oligonucleotides with altered bases, caused significant and selective inhibition of AP2 and clathrin protein expression. The levels of silencing of the AP2 $\mu$-subunit and the heavy chain of clathrin were determined as described for Syt I silencing for Fig. 2. For the $\mu$-subunit of AP2, siRNA decreased the $\mu$-subunit expression 93 ± 14% ($n=3$, Fig. 4A) and for the heavy chain of clathrin, the reduction was 89 ± 11% ($n=3$, Fig. 5A). Similar to the effect of Syt I siRNA treatment, no changes in cAMP- or Ca$^{2+}$-mediated inhibition of $^{22}$Na influx were observed with these treatments (Figs. 4B and 5B). Silencing of either the AP2 $\mu$-subunit or the heavy chain of clathrin blocked the decrease of surface expression of NHE3 after cAMP or thapsigargin stimulation (Figs. 4C and 5C). Silencing of the AP2 $\mu$-subunit or the heavy chain of clathrin prevented the decrease of surface expression of NHE3 after cAMP or thapsigargin stimulation (Figs. 4C and 5C). Silencing the heavy chain of clathrin prevented NHE3 association into the clathrin complex but not NHE3 association with the Syt I-containing complex (Fig. 4D). Silencing the heavy chain of clathrin prevented NHE3 association into the clathrin complex but not NHE3 association with the Syt I- or AP2-containing complexes (Fig. 5D). This is consistent with a model where Syt I associates with NHE3 first, then later with complexes containing AP2 and subsequently with complexes containing clathrin. The sequence of events from second messenger actions on NHE3 to recruitment as cargo to the AP2-clathrin complex and finally membrane removal by curvature and

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**Fig. 4.** Silencing the $\mu$-subunit of the AP2 complex does not prevent cAMP- or thapsigargin-mediated inhibition of NHE3 but prevents NHE3 internalization after these agents and association with the AP2 complex and the clathrin complex, but not with Syt I. A: silencing oligonucleotide of bases 79–103 but not an oligonucleotide with altered bases (control siRNA) decreases Caco-2BBe AP2 $\mu$-subunit protein expression by ~90%. B: $^{22}$Na influx is inhibited by cAMP (100 μM) and thapsigargin (100 nmol/ml) treatment, but this effect is not inhibited by AP2 $\mu$-subunit silencing. C: internalization of NHE3, determined by decreased surface-biotinylated NHE3 by cAMP or thapsigargin, is prevented by silencing AP2 $\mu$-subunit. D: silencing AP2 $\mu$-subunit prevents association of NHE3 with the AP2 complex and the clathrin complex but not with a Syt I-containing compartment (compare left and right co-IPs). Fluxes and images shown are representative of 3 separate experiments. Data are means ± SE for the 3 experiments; ++P < 0.01 compared with untreated controls.
Scission into endocytic vesicles is consistent with the roles of AP2 and clathrin hypothesized in other systems (21). NHE3 binds directly to Syt I. Coimmunoprecipitation does not imply direct binding, because complexes may exist that involve multiple proteins. To determine if NHE3 and Syt I directly interact, the GST construct we made to screen the rat intestinal expression library, rat NHE3 amino acids 666-815, was used to bind His-tagged human Syt I. The GST-NHE3-purified protein (0.1 µg/ml) was bound to glutathione-coated ELISA plates as described in METHODS. Data are means ± SE for 3 separate experiments.

**DISCUSSION**

Our studies demonstrate the pivotal role of Syt I in mediating cAMP- and Ca^{2+}-stimulated endocytosis of intestinal epithelial NHE3 through selective binding of NHE3 followed by recruitment of AP2 and clathrin assembly. Syt I is part of a large family of transmembrane proteins characterized by a short NH_{2}-terminal luminal domain, a single transmembrane-spanning region, and a long cytoplasmic tail that usually contains two C2 domains connected by

Fig. 5. Silencing the heavy chain (HC) of clathrin does not prevent cAMP- or thapsigargin-mediated inhibition of NHE3 but prevents NHE3 internalization after these agents and association with the clathrin complex, but not with Syt I or the AP2 complex. The silencing oligonucleotide with altered bases (control siRNA) decreases Caco-2BBe heavy chain of clathrin expression by ~90%. A and B: 22Na influx is inhibited by cAMP (100 µM for 10 min) or thapsigargin (100 ng/ml for 10 min) treatment, but this effect is not inhibited by silencing the heavy chain of clathrin. C: internalization of NHE3, determined by decreased surface-biotinylated NHE3 by cAMP or thapsigargin, is prevented by silencing the heavy chain of clathrin. D: silencing the heavy chain of clathrin prevents association of NHE3 with the clathrin compartment but not NHE3 association with compartments containing Syt I or AP2 (compare left and right co-IPs). Fluxes and images shown are representative of 3 separate experiments. Data are means ± SE for the 3 experiments; ++P < 0.01 compared with untreated controls.

Fig. 6. The cytoplasmic tail of NHE3 binds to Syt I. Glutathione-coated ELISA wells were coated with a GST fusion protein of amino acids 666-815 of rat NHE3, the parent GST alone (GST proteins at 0.1 µg/ml), or no protein, and His-tagged recombinant human Syt I was allowed to bind (a solution of 0.1 µg/ml). Protein binding was detected by using an anti-His antibody and was developed as described in METHODS. Data are means ± SE for 3 separate experiments.
a short linker region (16). Syt I, IV, and VII are the most highly conserved (8) and are likely involved in membrane trafficking, particularly, Syt I and Syt VII, which mediate exocytic fusion of synaptic and secretory vesicles in neural and exocrine tissues (9). However, several studies have reported that Syt I is also involved in endocytosis (14, 20) and membrane retrieval following kiss-and-run or stimulated exocytosis (2). In Syt I-null mice (30) and Drosophila (35), for instance, the lack of functional Syt I is associated with decreased rates of synaptic vesicle endocytosis. Our findings now show that Syt I has important roles in membrane trafficking in nonneural and nonhormonal cells, exemplified by mediation of second messenger-induced endocytosis of apical membrane NHE3.

Our study further reveals that Syt I is potentially capable of recognizing and binding specific cargo, although the specific recognition site remains to be determined. Other studies have implicated a similar ability of Syt I to recognize specific cargo, as was reported in the Mu2 muscarinic receptor (27). In mouse L cells expressing high levels of transferrin receptor, specific targeting of AP2 to the receptor was observed prior to clathrin recruitment and assembly, although the basis of this interaction was not described (18).

The events that follow Syt I recruitment or binding to NHE3 likely involve interaction and binding between AP2 and Syt I, as has been previously described (14). This interaction occurs through a high-affinity dual interaction of the Syt I C2B domain with the Mu2- and α-subunits of AP2 (15, 41), distinct from that which involves tyrosine-based motifs (14). This interaction is rapidly followed by nucleation of other adaptor proteins (3, 16) and clathrin assembly and coated pit endocytosis (13). Indeed, clathrin has been demonstrated to be pivotal for endocytosis of NHE3 (7). Our study suggests that allostERIC modification of NHE3, either through direct phosphorylation (6, 42) or through interaction with Ca2+- or cAMP-activated modulatory adaptor proteins, results in enhanced recognition and binding by Syt I. Syt I, in turn, recruits AP2 and clathrin assembly at sites of NHE3 location, rather than mobilizing NHE3 to sites of coated pit formation (12, 25). The confocal findings and the fact that clathrin pit formations do not occur when proximal Syt I or AP2 interactions with NHE3 are blocked support this notion.

Because the basal rate of apical NHE3 activity was not decreased by silencing Syt I, the AP2 μ-subunit, or the heavy chain of clathrin, these proteins appear not to be involved in the removal and recycling of NHE3 from the apical membrane of Caco-2BBe cells that has been previously reported (4, 5, 10, 18, 23). The key proteins responsible for NHE3 recycling are yet to be elucidated. NHE3 may exist in the apical membrane in a number of membrane domains and may be associated with different proteins. In OK opossum kidney cells, NHE3 in the apical membrane is mostly in a Triton X-100 (a nonionic detergent)-insoluble membrane fraction and exists in complexes of varying sizes, suggesting potential association with different proteins (1). Studies done in these cells demonstrated that internalized NHE3 was active in certain endosomal compartments. However, it should be noted that this occurred under basal conditions. On second messenger stimulation, such as by cAMP or thapsigargin, we show that NHE3 is internalized through the Syt I–AP2–clathrin pathway, which appears to be independent of constitutive recycling for NHE3.

The modification(s) of NHE3 that regulate its direction into the AP2–clathrin endocytic path may be complex. We hypothesize that a modification of NHE3, or a regulatory accessory protein, by second messengers such as cAMP or thapsigargin inhibits NHE3 activity and endocytosis. Our results demonstrate that inhibition of transport activity can be dissociated from membrane trafficking from the apical membrane. Whether phosphorylation stimulated by cAMP or thapsigargin is responsible or whether there is altered association with an accessory protein (either disruption of a protein required for activity or a protein that associates to inhibit activity) is unknown. NHE3 may be regulated by a number of events, including association with regulatory proteins, phosphorylation, or membrane trafficking, events that may be unique to specific cell types.

In summary, we report a novel role for Syt I in mediating Ca2+- and cAMP-induced endocytosis of NHE3, resulting in acute inhibition of vectorial Na absorption by intestinal epithelial cells. Evidence is provided that NHE3 is recognized and bound by Syt I as preendocytic cargo following stimulation by Ca2+ and cAMP, an event that then serves as a nucleation site for AP2 recruitment, assembly of other adaptor proteins, and eventual clathrin-coated pit formation. The Ca2+- and cAMP-induced changes in membrane trafficking proceed independently of events that cause functional inhibition of apical membrane NHE3. This study therefore not only reveals new insights into the regulation of intestinal Na transport that have relevance to homeostasis of salt and water transport by the gut but also provides a better understanding of the molecular basis of diarrheal diseases.

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