The NHE2 isoform of the Na\(^+/H^+\) exchanger (NHE) displays two proline-rich sequences in its C-terminal region that resemble SH3 (Src homology 3)-binding domains. We investigated whether these regions (749PPS-VTPAP, termed Pro-1, and 796YPKPPP, termed Pro-2) can bind to SH3 domains and whether they are essential for NHE2 function and targeting. A fusion protein containing the Pro-1 region showed promiscuous binding to SH3 domains of several proteins in vitro, whereas a Pro-2 fusion bound preferentially to domains derived from kinases. In contrast, cytoplasmic regions of NHE1, NHE3, or NHE4 failed to interact. When expressed in antiporter-deficient cells, truncated NHE2 lacking both Pro-rich regions catalyzed Na\(^+/H^+\) exchange, retained sensitivity to intracellular ATP, and was activated by hyperosmolarity, resembling full-length NHE2. The role of the Pro-rich regions in subcellular targeting was examined by transfection of epitope-tagged forms of NHE2 in porcine renal epithelial LLC-PK\(_1\) cells. Both full-length and Pro-2-truncated NHE2 localized almost exclusively to the apical membrane. By contrast, a mutant devoid of both Pro-1 and Pro-2 was preferentially sorted to the basolateral surface but also accumulated intracellularly. These observations indicate that the region encompassing Pro-1 is essential for appropriate subcellular targeting of NHE2.

The Na\(^+/H^+\) exchangers (NHEs)\(^1\) are a family of proteins found in virtually all mammalian cells, where they catalyze the electroneutral exchange of intracellular H\(^+\) for external Na\(^+\) with a 1:1 stoichiometry (see Refs. 1 and 2 for review). In most cells, NHE activity is important for intracellular pH (pH\(_i\)) homeostasis and also for maintenance of normal cellular volume (3). In addition, in epithelia of the kidney, gastrointestinal tract, and other organs, NHE plays a central role in the absorption of NaCl, bicarbonate, and water. Six distinct NHE isoforms have been identified to date in mammalian cells (1). Although the primary sequence homology between the isoforms is limited (20–60%), they all share the same predicted membrane topology, consisting of 12 membrane-spanning segments at the N terminus and a hydrophilic C-terminal tail thought to extend into the cytoplasm (Ref. 1, but see Ref. 4 for alternative view). Functional characterization of deletion mutants of NHE1 and NHE3 has localized the site of Na\(^+/H^+\) exchange to the N-terminal (transmembrane) region of the protein (1). The C-terminal region is believed to be responsible for modulation of transport by such diverse agents as growth promoters, hormones, and changes in medium osmolarity (1). The cytosolic domain is also thought to encompass the site that confers ATP dependence to Na\(^+/H^+\) exchange, a hallmark of NHE that is common to all the isoforms studied to date (1, 2).

The pattern of expression of individual NHE isoforms varies among tissues. NHE1 and NHE6 are expressed in virtually all cells, whereas other isoforms have a more restricted tissue distribution (1). NHE2 is preferentially expressed in the gastrointestinal tract and, to a lesser extent, in the kidney, uterus, and brain (5, 6). Despite its discovery several years ago, comparatively little is known about the physiology of NHE2. Its selective expression pattern initially suggested a role for this isoform in fluid and electrolyte balance, similar to the function fulfilled by NHE3 in epithelial cells. However, creation of a null mutation of NHE2 in mice caused no significant perturbations of organismal acid-base or salt homeostasis (7). Instead, the only apparent defect was a significant loss of net acid secretion in the stomach due to a severe reduction in the viability of parietal cells of the gastric mucosa. Although the underlying mechanism is unclear, it was proposed that NHE2 is part of the basolateral transport system that maintains parietal cell volume during high acid secretion at its apical surface and that disruption of NHE2 function causes a chronic state of volume depletion, leading to cellular necrosis (7).

Whereas an attractive hypothesis, the subcellular distribution of NHE2 in stomach has yet to be determined and, indeed, remains a controversial issue in other tissues examined. On one hand, NHE2 activity was reported to be restricted to the basolateral membrane of an inner medullary collecting duct cell line, mMCID-3 (8). In contrast, others detected NHE2 activity predominantly on the apical membranes of ileal tissue (9) and in a cortical collecting duct cell line (10). Similar observations were made when NHE2 was transiently expressed in a colon carcinoma cell line (11), and apical localization was also documented immunologically in the medullary thick ascending limb (12). Thus, NHE2 is mainly sorted to the apical surface of most polarized epithelial cells examined, as reported for NHE3.
By contrast, the functional properties of NHE2 differ considerably from NHE3 and more closely resemble those of NHE1 when assessed in transfected mammalian cells; it is activated by agonists of the protein kinase A and C pathways as well as by hyperosmotic-induced cell shrinkage, and its pH sensitivity is moderately reduced by cellular depletion of ATP (3, 13–15).

The structural determinants that confer this unique behavior have not been identified. Perusal of the primary structure of NHE2 revealed that the C-terminal region, which is most divergent between isoforms, contains two proline-rich motifs: residues 743–750 (PPSVTPAP), which will be called hereafter Pro-1, and residues 786–792 (VPPKPPP), designated Pro-2. The latter conforms to the consensus sequence of proteins capable of binding Src homology 3 (SH3) domains, ϕpXϕ, where ϕ is a hydrophobic residue, χ is any amino acid, and p tends to be (but is not always) Pro (16–18). Pro-1 approximates but does not match perfectly the SH3 consensus structure. These proline-rich regions are unique to NHE2 and may be important in defining the distinctive behavior of this isoform.

SH3 domains are sequences of 50–75 amino acids found in diverse proteins that include cytoskeletal components, such as spectrin (19), adapter and signaling molecules, such as Grb2 (20, 21) and Ras-GAP (22), and protein and lipid kinases, including Src, Abl, and the p85 subunit of phosphatidylinositol 3'-kinase (23, 24). Coupling of proteins via their SH3 domains has been implicated in a variety of functions, including regulation of cell growth and proliferation (25, 26), endocytosis (27, 28), and activation of the respiratory burst (29, 30). The importance of SH3 domains in other systems prompted us to analyze whether the Pro-rich motifs of NHE2 are capable of interacting with SH3-containing proteins and to assess the functional role of this interaction. To this end, we studied the ability of fusion proteins encoding the Pro-1 and Pro-2 regions of NHE2 to bind SH3 domains in vitro. In addition, we used transfection of epitope-tagged constructs to compare the functional properties and subcellular distribution of the full-length NHE2 with those of mutants lacking either Pro-1 or Pro-2.

**EXPERIMENTAL PROCEDURES**

**Materials and Media**—All polymerase chain reaction (PCR) reagents and Escherichia coli DH5-α were purchased from Life Technologies, Inc. pGEX-2T and *E. coli* HB101 were obtained from Amersham Pharmacia Biotech. Isopropylthio-β-D-galactoside was purchased from Calbiochem. Luria broth (LB) was obtained from the Ontario Cancer Institute (Toronto, Ontario, Canada). Fluorescein-labeled lectins, leupeptin, pepstatin, aprotinin, phenylmethylsulfonyl fluoride, EDTA, antimycin A, reduced glutathione, and glutathione-agarose beads were purchased from Sigma. The NHS-LC biotinyl reagent was purchased from Pierce, and the enhanced chemiluminescence (ECL) detection kit was obtained from Amersham Pharmacia Biotech. Nigerian and 2, 7-bis-(2-carboxethyl)-5-6(5 carbamoylfluorescein (BCECF) acetoxymethylester were purchased from Molecular Probes, Inc.

Monoclonal antibodies to a peptide derived from the influenza virus hemagglutinin (HA) were purchased from BAbCo. Goat anti-mouse Cy3-conjugated IgG and goat peroxidase-coupled anti-mouse secondary antibodies were purchased from Jackson Immunoresearch Laboratories. Horseradish peroxidase coupled to avidin was purchased from Cappel.

Phosphate-buffered saline (PBS) contained 140 mM NaCl, 10 mM KCl, 1.8 mM sodium phosphate, 2 mM potassium phosphate, pH 7.4. The sodium chloride solution contained 117 mM NaCl, 1.66 mM MgSO4, 1.36 mM CaCl2, 5.36 KCl mm, 25 mM Na-HEPES, 5.55 mM glucose, pH 7.4. Sodium-free solutions were made with equimolar substitution of Na+ with choline + or with sucrose. N-ethylmaleimide solution contained 1 mM N-ethylmaleimide and 10 mM cysteine. Sodium-free solutions were made by addition of sufficient NaCl to raise the osmolality to 600 ± 10 mosmol.

**Preparation of GST Fusion Proteins**— GST fusion proteins of the Pro-rich regions of NHE2 were prepared by PCR amplification of the appropriate regions of rat NHE2 cDNA (31). The PCR product encompassing the more N-terminal Pro-rich region, which contains amino acids 740–750 (PPSVTPAP) (Pro-1) plus 19 and 16 flanking residues at the 5' and 3'-ends, respectively, was amplified using the primers 5'-cgg-gatecactacagcactc-3' and 5'-gaattggtgctggtc-3'. The primers used to amplify the more C-terminal proline-rich region containing amino acids 774–792 (VPPKPPP) (Pro-2) plus 12 and 21 flanking residues at the 5'- and 3'-ends, respectively, were 5'-cagctgagcgcgcgcgcgcgcgc-5' and 5'-gaattggtgctggtc-3'. The PCR products were cloned into the BamHI site of pGEX-2T (Amersham Pharmacia Biotech). HB101 E. coli transformed with the resulting plasmid were grown at 37 °C and then also a broth supplemented with 50 μg/ml ampicillin to log-phase, and then expression of the fusion protein was induced with 1 μM isopropyl-1-thio-β-D-galactopyranoside for 5 h at 37 °C. Bacteria were lysed in the presence of protease inhibitors (10 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, and 5 mM EDTA), and the fusion proteins were purified from the lysate using glutathione-agarose beads. GST fusion protein encompassing amino acids 560–690 of NHE2 and 667–717 of NHE4 were prepared similarly. A GST fusion protein containing the last 178 residues of the cytosolic tail of rabbit NHE1 was the kind gift of Dr. L. Fliegel (University of Alberta, Edmonton, Alberta, Canada).

Fusion proteins of the SH3 domain of c-Abl, p85, Ras-GAP, N-Src, and c-spectrin were obtained and generated as described previously (31). GST-SH3 domains were subcloned using NHS-LC biotin (Pierce), as described (19). In Vitro Far Western Binding Assays—Fusion proteins (8 μg) of the indicated regions of the exchangers were separated by SDS-PAGE (12% acrylamide), transferred to nitrocellulose, and incubated overnight in blocking buffer (0.25% gelatin, 10% ethanolamine, 0.1 M Tris, pH 9.0) supplemented with 5% powdered milk. The blots were next incubated with 2 μg of the appropriate biotinylated SH3 fusion protein in 10 ml of blocking buffer for 2 h. The blots were then washed and incubated for 2 h at room temperature with streptavidin-peroxidase (12.5 μg/ml) in a buffer containing 0.25% gelatin, 0.05% Nonidet P-40, 0.15 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.5, followed by washing and detection using ECL.

**cDNA Constructs**—The rat NHE2 cDNA was engineered to contain a series of unique restriction endonuclease sites that did not alter the primary structure, but created convenient DNA cassettes for mutagenesis. The modified NHE2 cDNA was inserted into the pCMV mammalian expression vector (plasmid renamed pNHE2) as described previously (6, 33). To allow for immunological detection of the protein, the influenza virus HA epitope YPYDVPDYAS, preceded by a single G amino acid linker (inserted to create peptide flexibility), was inserted at the C terminus of NHE2 (amino acid 1935) using PCR amplification of a C-terminal cDNA fragment. Similarly, the NHE24777HA and NHE23731mu deletion mutants were engineered by inserting the HA epitope plus a stop codon immediately following positions 777 and 731 of the wild type transporter. The PCR fragments were sequenced prior to substitution into NHE2 to confirm the presence of the mutations and to ensure that other random mutations were not introduced. Cells were plated on dishes of endodermal lineages, antimitotic activity that was derived from wild type Chinese hamster ovary (CHO) cells by the “H1-suicide” technique (34). These cells and the transfectants derived thereof were grown in a minimal essential medium (Ontario Cancer Institute, Toronto, Ontario, Canada). HEK-T is a subclone of the transformed human embryonic kidney (HEK) cell line containing the large T antigen. LLC-PK, epithelial cells derived from porcine kidney proximal tubule. HEK-T and LLC-PK cells were grown in Dulbecco’s minimal essential medium (Ontario Cancer Institute, Toronto, ON). Both a minimal essential medium and Dulbecco’s minimal essential medium contained 25 mM NaHCO3 and were supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.). Cells were incubated in humidified environment containing 95% air and 5% CO2 at 37°C. Cultures were re-established from frozen stocks regularly, and cells from passages 3–20 were used for the experiments.

Where indicated, intracellular ATP was depleted by incubating the cells for 10 min in glucose-free medium with 5 mM 2-deoxy-t-glucose and 1 μg/ml antimycin A, to inhibit both glycolysis and oxidative phosphorylation. This protocol has previously been shown to deplete >90% of ATP in CHO cells within 30 min (32). Subsequent fluorescence measurements were performed in glucose-free medium containing 5.5 mM 2-deoxy-t-glucose.

**Transfection and Selection**—AP-1, LLC-PK, and HEK-T cells were transfected with plasmids containing the epoate-tagged wild type or truncated NHE2 constructs by the calcium phosphate-DNA co-precipitation technique of Chen and Okayama (36). For selection of stable
lines, the AP-1 cells were selected for survival by repeated acute acid loads (5–6 times over a 2-week period), in order to discriminate between NHE2 D 777HA, NHE2 D 731HA, and NHE2D731HA. We subcloned NHE2 D 777HA into the pBK vector (Stratagene), which includes the neo gene resistance gene. Stably expressing cells were selected by incubation with 500 μg/ml G418 and then screened by immunofluorescence for expression of the wild type or mutant NHEs.

Immunofluorescence—For immunoblot analysis, the cells were grown to confluence on 10-cm² plastic dishes. The cells were washed three times in PBS, fixed in 10 mM hypotonic buffer (10 mM HEPES, 15 mM potassium acetate, 1 mM EDTA, pH 7.2, 50 mM) and solubilized at 4°C in radioimmune precipitation buffer (150 mM NaCl, 20 mM Tris HCl, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, pH 8.0) containing 1 mM iodoacetamide, 1 mM pepstatin, and 1 mM phenylmethylsulfonyl fluoride. The soluble fraction was subjected to SDS-PAGE (10% acrylamide) and transferred to nitrocellulose. The blot was incubated overnight in blocking buffer (above) and then incubated with anti-HA antibody (1:5000) for 1 h in 0.25% gelatin, 0.05% Nonidet P-40, 0.15 M NaCl, 5 mM EDTA, 50 mM Tris, pH 7.5, supplemented with 5% milk, prior to labeling with peroxidase-coupled anti-mouse secondary antibody (1:1000). Chemiluminescence was then detected using the Amersham Pharmacia Biotech ECL detection kit.

Immunofluorescence—Cells were grown to confluence on sterile 18 mm glass coverslips (Thomas Scientific, Swedesboro, NJ), washed with PBS, and fixed for 20 min with 4% paraformaldehyde, and the excess formaldehyde was quenched with 100 mM glycine for 15 min. Where indicated, the fixed cells were stained with a mixture of fluorescein isothiocyanate-labeled lectins (peanut, orange, wheat germ, and pea, 2 μg/ml each) for 45 min at 4°C. The cells were then permeabilized with 0.1% Triton X-100 supplemented with 5% bovine serum albumin and incubated with anti-HA antibody (1:1000) at room temperature for 45 min. The cells were washed again with PBS and incubated with Cy3-conjugated anti-mouse antibody (1:5000) for 45 min. The coverslips were mounted using Dako mounting reagent and visualized using a Leica fluorescence microscope. Images were obtained using the Winview program and processed using Adobe Photoshop.

Results

Interaction of Proline-rich Regions with SH3 Domains in Vitro—To assess whether SH3 domains are capable of binding specifically to the Pro-rich motifs of the cytosolic domain of NHE2 we initially tested the ability of the Src-SH3 domain to bind to this region in an in vitro overlay assay. A GST fusion protein encompassing residues 724–766 and including the Pro-1 region (743PPSVTPAP750) of NHE2 was subjected to SDS-PAGE, transferred to nitrocellulose, and overlaid with the biotinylated Src-SH3 domain of Src, and binding was detected with streptavidin-peroxidase and ECL. Double bands in some of the Ponceau-stained lanes reflect proteolysis of the fusion proteins. Shown is a representative of four similar experiments. The coverslip was calibrated at the end of the experiment using at least three pH values. Quantification of cell-associated fluorescence was performed using the Felix software package (Photon Technologies, Inc., South Brunswick, NJ). The rate of pH change was derived by linear regression of the pH versus time curve over 4 s intervals using the Origin software (MicroCal Software Inc., Northampton, MA).

Fig. 1. Interaction between NHE fusion proteins and the SH3 domain of src. GST fusion proteins of regions of the C-terminal domain of NHE1, NHE2, NHE3, and NHE4, as well as uncharged GST (8 μg each), were separated by SDS-PAGE (12% acrylamide) and blotted onto nitrocellulose. The blot was initially stained with Ponceau S to reveal the proteins (top panel) and then overlaid with biotinylated GST-Src-SH3 (bottom panel). After extensive washing, bound GST-Src-SH3 was detected using streptavidin-peroxidase and ECL. Double bands in some of the Ponceau-stained lanes reflect proteolysis of the fusion proteins. Shown is a representative of four similar experiments.
or neuronal Src (Fig. 1. Shown is a representative of five similar experiments. Domains were detected using streptavidin-peroxidase and ECL, as in unconjugated GST (8 kDa). GST fusion proteins of the Pro-1 and Pro-2 regions of NHE2 GAP, the p85 subunit of phosphatidylinositol 3-kinase, a spectrin, Src, or neuronal Src (N-Src). After extensive washing, bound GST-SH3 domains were detected using streptavidin-peroxidase and ECL, as in Fig. 1. Shown is a representative of five similar experiments.

SH3 domains in vitro, albeit with different specificity. Functional Role of Pro-rich Regions of NHE2—To assess the functional role of the Pro-rich regions of NHE2, we compared the behavior of the full-length protein with that of truncated mutants lacking one or both Pro-rich regions. To this end, vectors encoding epitope-tagged intact or truncated constructs were transfected into mammalian cells and their expression and transport properties were evaluated. As a preliminary experiment to evaluate the immunodetectability of the epitope-tagged constructs, the cDNAs were transiently transfected into HEK-T cells. As illustrated in Fig. 3, transfection of the full-length NHE2 (NHE2HA) construct resulted in expression of a 95–105-kDa polypeptide that was recognized by an antibody directed to the HA epitope, which was attached at the C terminus of the protein. Transfection of constructs truncated at position 777 (NHE2777HA), lacking the C-terminal Pro-2, or at position 731 (NHE2731HA), lacking both Pro-1 and Pro-2, reduced the expression of polypeptides of ~90–95 and ~85–92 kDa. The levels of expression of the full-length and truncated molecules were comparable and the observed differences in molecular mass are consistent with the deletion of 36 and 82 residues, respectively.

The functional properties of the wild type and mutant NHE2s were studied next. To quantify the ion exchange mediated by the intact and truncated forms of NHE2, it was imperative to dissociate their activity from that of endogenous Na+/H+ exchangers present in the cells used for transfection. This was accomplished by stably expressing the cDNAs in AP-1 cells, a mutant line of CHO cells devoid of NHE activity (34). HEK-T cells were transfected with either NHE2HA, NHE2777HA, NHE2731HA or with the empty pCMV vector alone (Sham). After 48 h, the cells were lysed hypotonically and resolved in Laemmli sample buffer for SDS-PAGE analysis. Following electrophoresis, the samples were transferred to nitrocellulose and blotted with monoclonal anti-HA antibody (1:5,000), followed by goat peroxidase-coupled anti-mouse (1:5,000) and detection by ECL. Identical amounts of protein were loaded on each lane. Shown is a representative of at least three experiments.

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The activity of the full-length and truncated forms of NHE2 was initially compared in cells that were acutely acid-loaded by preincubation with 25 mM NH4Cl for 10 min, followed by rapid removal of the weak base. When bathed in Na+-free, N-methyl-D-glucammonium+-rich medium, the cells remained acidic, indicating that Na+-independent systems contribute negligibly to pHi restoration in the time frame studied. As reported earlier, re-addition of Na+ elicited a rapid cytosolic alkalization in cells expressing the original unmodified full-length NHE2, and a comparable pH change was obtained when NHE2 was epitope-tagged, indicating that addition of the C-terminal HA nonapeptide did not interfere with the basic function of the antipporter (Fig. 4). Similar recoveries from the acid load were obtained in cells transfected with NHE2777HA or with NHE2731HA. Comparison of the relative rates of recovery from the acid load indicates that the pHi dependence of the exchange process was not markedly affected by truncation of either one or both Pro-rich domains. The full-length NHE2, as well as the truncated versions, became minimally active between pH 7.0 and 7.5, and their activity increased steeply at more acidic pHi (Figs. 4 and 5). NHE2777HA appeared to become quiescent at a somewhat more acidic pHi than the other constructs. We did not quantify the absolute rates of transport because this parameter is not informative when comparing expression levels in different stable transfecants.

Although the basal operation of NHE2 is not grossly affected by deletion of the Pro-rich regions, it is possible that its regulation may be altered. Intracellular ATP is an important regulator of NHE2 activity (39). Although the precise mechanism of action of ATP is not well defined, depletion of the nucleotide is known to depress the activity of NHE2, shifting its activation threshold to more acidic pH values. We analyzed whether the Pro-rich motifs influenced this regulation. As illustrated in Fig. 5A, insertion of the HA epitope at the C terminus of the full-length NHE2 did not modify the responsiveness of the exchanger to metabolic depletion: the rate of recovery from an acid load was greatly reduced by preincubation for 10 min in glucose-free medium with 5 mM 2-deoxy-d-glucose and 1 μg/ml antimycin A, conditions shown earlier to deplete ATP in CHO cells by >90% within 10 min (35). A comparable inhibition of exchange was noted in NHE2777HA and in NHE2731HA.
transfected cells (Fig. 5, C and D). Therefore, the presence of the Pro-rich sequences is not essential for regulation of NHE2 by ATP. This result is not entirely unexpected because other NHE isoforms are also regulated by ATP, albeit to differing extents, but lack obvious SH3-binding domains.

Osmotic Activation of NHE2—In addition to its important role in maintaining pH homeostasis, the NHE family of proteins plays an important role in volume regulation and H2O transport. In response to hypertonic stress, NHE2 is activated in transfected CHO and porcine proximal tubule LLC-PK1 cells.

In glucose-free medium with 5.5 mM 2-deoxy-D-glucose, the pH of transfected CHO and porcine proximal tubule LLC-PK1 cells was determined by ratio fluorometry and calibrated using nigericin/K+.

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To re-assess the localization of full-length NHE2 and to define the role of the Pro-rich motifs in the establishment of polarity, we transfected HA-tagged constructs into LLC-PK1 cells. Stably transfected cells were obtained by subcloning the constructs into the pBK plasmid that encodes for neomycin resistance, and selected using G418. Stable transfectants were grown to confluence and analyzed by immunofluorescence and confocal laser scanning microscopy using anti-HA antibodies followed by Cy3-conjugated secondary antibodies. Prior to fixation, the apical membrane was counter-stained by overlaying the cells with a mixture of fluoresceinated lectins (see under "Experimental Procedures"). As illustrated in Fig. 7, both the full-length (NHE2HA) and the NHE2Δ777HA, which lacks the C-terminal Pro-rich domain (Pro-2), displayed a punctate appearance that followed the contour of the apical membrane. Accordingly, the distribution of these proteins matched closely the distribution of apically bound lectins (cf. Fig. 7, B and C). There was little evidence of intracellular retention of either the full-length NHE2HA or NHE2Δ777HA, despite wide variation in the level of expression among individual clones.

By contrast, a large fraction of the cells expressing NHE2Δ731HA appeared to retain the construct in the endoplasmic reticulum and/or Golgi complex (Fig. 7D). This was not due to clonal variation or differences in the level of expression, because similar results were obtained in multiple clones with varying degrees of expression. The fraction of cells that successfully exported NHE2Δ731HA to the plasma membrane displayed a distinct distribution pattern. When viewed en face, the truncated exchangers appeared to delineate the cell borders, a pattern characteristic of basolateral proteins. Accordingly, this distribution differed from that of the apical lectins (cf. Fig. 7, E and F) and was instead similar to that of ZO-1, a tight-junctional protein (not illustrated). Only a small fraction of NHE2Δ731HA appeared to be present at the apical membrane.

A quantitative summary of these observations, obtained from 10 separate observations made in polyclonal populations, is presented in Fig. 8. The data confirm that whereas NHE2HA and NHE2Δ777HA are predominantly apical, a large fraction of NHE2Δ731HA is retained intracellularly, and those truncated exchangers that reach the membrane localize predominantly to the basolateral side of the cell. We conclude that the Pro-1 motif is important for effective processing and targeting of NHE2 to the apical membrane of epithelial cells.

**DISCUSSION**

The observations presented in this report provide evidence that the cytoplasmically oriented Pro-rich regions of NHE2 can bind to SH3 domains. Pro-2 bound preferentially to SH3 domains derived from tyrosine kinases and from the regulatory domain of phosphatidylinositol 3’-kinase, whereas Pro-1 was more promiscuous, interacting with a wider variety of SH3 domains. Although promiscuous, this interaction was nevertheless specific to SH3 domains, inasmuch as it was not observed for GST alone, nor for fusion proteins derived from the cytosolic domains of other NHE isoforms, which lack Pro-rich sequences.

The functional significance of these interactions was evaluated by comparing the behavior of intact and truncated forms of NHE2, using heterologous expression systems. Truncation of the C-terminal Pro-2 region (NHE2Δ777HA) had subtle yet reproducible functional consequences. The pHi dependence of

**Fig. 6.** Osmotic responsiveness of full-length and truncated NHE2. pHi was measured fluorometrically in AP-1 cells transfected with either full-length or truncated forms of epitope-tagged NHE2, as in Fig. 4. The cells were initially suspended in isotonic, Na+-rich medium (iso). Where specified, the osmolarity of the medium was increased to 600 ± 10 mosM (Hyper). Traces are representative of at least four experiments.

**Fig. 7.** Localization of full-length and truncated NHE2 in LLC-PK1 cells. LLC-PK1 cells were stably transfected with NHE2HA (A), NHE2Δ777HA (B and C), or NHE2Δ731HA (D–F) using the calcium phosphate method and selected with G418 as described under "Experimental Procedures." The cells were grown to confluence on coverslips, washed with PBS prior to fixation with 4% paraformaldehyde, and subsequently labeled with fluorescein isothiocyanate-labeled lectins. After extensive washing, the cells were permeabilized with 0.1% Triton X-100 in 5% bovine serum albumin and labeled with 1:1000 anti-HA antibody, followed by secondary labeling was with 1:1000 Cy3 labeled anti-mouse IgG (A, C, D, and F). Fluorescence microscopy was used to detect the distribution of the lectin (B and E) or of the HA epitope, indicative of NHE2. Images are representative of at least five experiments. Bar in D is 10 μm, and all panels are the same magnification.
These observations suggest that the cytosolic region between residues 732 and 777, encompassing Pro-1, is necessary for appropriate targeting of NHE2 to the apical surface. In this context, Pro-1 was found to bind to the SH3 domain of α-spectrin, a protein that has previously been reported to participate in the apical targeting of epithelial Na\(^+\) channels (ENaC) (19). Like NHE2, ENaC interacts with the SH3 domain of α-spectrin in vitro. Moreover, microinjection of a fusion protein containing the Pro-rich region of ENaC revealed targeting to the apical membrane of epithelial cells (19). Based on these observations, it was suggested that α-spectrin may function to retain ENaC apically, a mechanism that may also apply in the case of NHE2. Alternatively, interaction with spectrin present in the Golgi (48) may be essential for export of the exchangers and channels to the plasmalemma. Clearly, other SH3-containing proteins may also function in apical delivery and retention of NHE2. A complete understanding of the role of Pro-rich domains awaits identification of all the proteins that interact with the exchanger in vivo.

**REFERENCES**

1. Orlowski, J., and Grinstein, S. (1997) J. Biol. Chem. 272, 22373–22376
2. Wakabayashi, S., Shiogekawa, M., and Pouyssegur, J. (1997) Physiol. Rev. 77, 51–74
3. Kapus, A., Grinstein, S., Wasan, S., Kandasamy, R., and Orlowski, J. (1994) J. Biol. Chem. 269, 23544–23552
4. Bioesmeder, D., DeGray, B., and Aronson, P. S. (1998) J. Biol. Chem. 273, 12391–12396
5. Collins, J. F., Honda, T., Knobel, S., Bulus, N. M., Conarya, J., Dubois, R., and Ghishan, F. K. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 3898–3894
6. Wang, Z., Orlowski, J., and Shall, G. E. (1995) J. Biol. Chem. 266, 11925–11928
7. Schultheis, P. J., Clarke, L. L., Meneton, P., Harline, M., Boivin, G. P., Steemerman, G., Dufy, J. J., Dietrichman, T., Miller, M. L., and Shall, G. E. (1998) J. Clin. Invest. 101, 1243–1253
8. Soleimani, M., Singh, G., Bizal, G. L., Gullans, S. R., and McAteer, J. A. (1994) J. Biol. Chem. 269, 27573–27578
9. Proline-rich Regions of NHE2
10. Guerra, L., Di Sole, F., Valenti, G., Ronco, P. M., Perlino, E., Casavola, V., and Reshkin, S. J. (1996) Kidney Int. 53, 1269–1277
11. Tse, C. M., Levine, S., Yun, C., Montrose, M. H., Little, P. J., Pouyssegur, J., and Donowitz, M. (1995) J. Biol. Chem. 268, 11917–11924
12. Sun, A. M., Liu, Y., Dwarkin, L. D., Tse, C. M., Donowitz, M., and Yip, K. P. (1997) J. Membr. Biol. 166, 85–90
13. Tse, C. M., Levine, S., Yun, C., Brant, S., Counillon, L. T., Pouyssegur, J., and Donowitz, M. (1996) J. Membr. Biol. 153, 93–108
14. Kandasamy, R. A., Yu, F. H., Harris, R., Boucher, A., Hanrahian, J. W., and Orlowski, J. (1995) J. Biol. Chem. 270, 29209–29216
15. Singh, G., Orlowski, J., and Soleimani, M. (1995) J. Membr. Biol. 151, 261–268
16. Ren, R., Mayer, B. J., Cicchetti, P., and Baltimore, D. (1993) Science 259, 1157–1161
17. Viguera, A. R., Arrondo, J. L., Musacchio, A., Saraste, M., and Serrano, L. (1995) EMBO J. 14, 1243–1253
18. Yu, H., Chen, J. K., Feng, S., Dalgarno, D. C., Brauer, A. W., and Schreiber, J. M. (1994) Cell 76, 85–90
19. Takeshige, K., Sakaki, Y., and Ito, T. (1998) EMBO J. 17, 7631–7639
20. Chen, C. A., and Okayama, H. (1988) BioTechniques 6, 632–638
21. Cow, C. W., Kapus, A., Romanek, R., and Grinstein, S. (1997) J. Gen. Physiol. 110, 185–200

**Fig. 8. Quantitative estimation of the subcellular distribution of full-length and truncated NHE2.** LLC-PK1 cells transfected with full-length or truncated forms of NHE2 were stained and analyzed by epifluorescence microscopy using a × 100 objective as in Fig. 7. The subcellular distribution of each one of the tagged exchangers was assessed in 10 random samples. Data summarize the aggregate observations.

The rate of exchange was modestly shifted to more acidic values, and the osmotic activation was depressed. However, an even more profound truncation (NHE2731HA) that eliminated both Pro-1 and Pro-2 restored both the normal pH\(_d\) dependence and osmotic responsiveness. This biphasic behavior is not unprecedented. Moderate truncation of the C-terminal domain of NHE1 can produce an alkaline shift in its pH\(_d\) dependence, with loss of responsiveness to a variety of stimuli (47, 49). The shift and loss of responsiveness are reversed by more profound truncations, which can in fact induce an acidic shift in the pH vs. activity relationship (50, 51). Thus, the exchangers can seemingly exist in several conformations that are exquisitely dependent on subdomains of the cytosolic tail.

Despite the subtle effects noted upon deletion of Pro-2, it is clear that neither Pro-1 nor Pro-2 is absolutely essential for the ability of NHE2 to exchange Na\(^+\) for H\(^+\), nor for its dependence on ATP or sensitivity to osmotic activation. This conclusion stems from the observations made using the NHE2731HA construct, which displayed near-normal behavior under these conditions. We therefore considered the possible role of the Pro-rich regions in protein processing and subcellular targeting. This initially required confirmation of the subcellular distribution of NHE2 in epithelial cells, which has been the source of controversy. By transfecting the full-length, epitope-tagged NHE2 into LLC-PK cells, we found that this isoform accumulates in the apical membrane, as reported by the majority of studies (9–12). The source of the discrepancies with the results of Soleimani et al. (8), who detected basolateral NHE2, remains unclear. Neither tissue nor species differences appear to account for the inconsistency, because both apical and basolateral locations have been reported for NHE2 in renal medullary cells of rodents (8, 12).

Despite these uncertainties, comparison of the full-length and truncated constructs in a single expression system provided useful information concerning the role of the Pro-rich regions in targeting. Briefly, we found that whereas deletion of Pro-2 was ineffectual, the additional removal of Pro-1 greatly impaired the ability of NHE2 to reach the apical membrane. A sizable fraction of the truncated exchanger was retained in the secretory pathway, and the exchanger that was exported to the plasmalemma was primarily found in the basolateral membrane. It is tempting to speculate that the existence of splice variants or posttranslational truncation of NHE2 may account for the reports in which this isoform was detected basolaterally.
38. Thomas, J. A., Buchsbaum, R. N., Zimniak, A., and Racker, E. (1979) *Biochemistry* **18**, 2210–2218
39. Demaurex, N., and Grinstein, S. (1994) *J. Exp. Med.* **180**, 389–404
40. Nath, S. K., Hang, C. Y., Levine, S. A., Yun, C. H. C., Montrose, M. H., Donowitz, M., and Tse, C. M. (1996) *Am. J. Physiol.* **270**, G431–G441
41. Boucher, R. C. (1994) *Am. J. Respir. Crit. Care Med.* **150**, 271–281
42. Boucher, R. C. (1994) *Am. J. Respir. Crit. Care Med.* **150**, 501–503
43. Bookstein, C., DePaoli, A., Xie, Y., Niu, P., Musch, M. W., Rao, M. C., and Chang, E. B. (1994) *J. Clin. Invest.* **93**, 106–113
44. Yoshioka, S., Suzuki, T., and Kawakita, M. (1997) *J. Biochem.* **122**, 641–646
45. Noel, J., Roux, D., and Pouyssegur, J. (1996) *J. Cell Sci.* **109**, 929–939

46. Amemiya, M., Loffing, J., Lotescher, M., Kaissling, B., Alpern, R. J., and Moe, O. W. (1995) *Kidney Int.* **48**, 1206–1215
47. Wakabayashi, S., Bertrand, B., Ikeda, T., Pouyssegur, J., and Shigekawa, M. (1994) *J. Biol. Chem.* **269**, 13710–13715
48. Holleran, E. A., and Holzbaur, E. L. (1998) *Trends Cell Biol.* **8**, 26–29
49. Bertrand, B., Wakabayashi, S., Ikeda, T., Pouyssegur, J., and Shigekawa, M. (1994) *J. Biol. Chem.* **269**, 13703–13709
50. Wakabayashi, S., Fafournoux, P., Sardet, C., and Pouyssegur, J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 2424–2428
51. Wakabayashi, S., Bertrand, B., Shigekawa, M., Fafournoux, P., and Pouyssegur, J. (1994) *J. Biol. Chem.* **269**, 5583–5588