Topological analysis of NHE1, the ubiquitous Na\(^+/\)H\(^+\) exchanger using chymotryptic cleavage

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Shrode, Lamara D., Bing Siang Gan, Sudhir J. A. D’Souza, John Orlowski, and Sergio Grinstein. Topological analysis of NHE1, the ubiquitous Na\(^+/\)H\(^+\) exchanger, using chymotryptic cleavage. Am. J. Physiol. 275 (Cell Physiol. 44): C431–C439, 1998.—Proteases, glycosidases, and impermeant biotin derivatives were used in combination with antibodies to analyze the subcellular distribution and transmembrane disposition of the Na\(^+/\)H\(^+\) exchanger NHE1. Both native human NHE1 in platelets and epitope-tagged rat NHE1 transfected into antiport-deficient cells were used for these studies. The results indicated that 1) the entire population of exchangers is present on the surface membrane of unstimulated platelets, ruling out regulation by recruitment of internal stores of NHE1; 2) the putative extracellular loops near the NH2 terminus are exposed to the medium and contain all the N- and O-linked carbohydrates; 3) by contrast, the putative extracellular loops between transmembrane domains 9–10 and 11–12 are not readily accessible from the outside and may be folded within the protein, perhaps contributing to an aqueous ion transport pathway; 4) the extreme COOH terminus of the protein was found to be inaccessible to extracellular proteases, antibodies, and other impermeant reagents, consistent with a cytosolic localization; and 5) detachment of ~150 amino acids from the NH2-terminal end of the protein had little effect on the transport activity of NHE1.

intracellular pH; sodium/hydrogen antiport; amiloride

SODIUM/HYDROGEN EXCHANGERS (NHE) are ubiquitous proteins that play a central role in the regulation of intracellular pH (pHi) and cell volume and contribute to epithelial Na\(^+\) resorption (11, 13). Under physiological conditions, the antiporters exchange extracellular Na\(^+\) for intracellular H\(^+\) by an electroneutral process that is allosterically activated by intracellular acidification (1). In addition, activity is also increased in many cell types by exposure to growth factors (11) and with cell shrinkage (20).

Six distinct isoforms of the NHE have been identified by molecular cloning (14, 23). All isoforms share some basic structural similarities. Analysis of the primary structure predicts the existence of two distinct domains: a transmembrane NH2-terminal region of ~500 amino acids and a hydrophilic COOH-terminal domain of nearly 300 amino acids. The hydrophobic NH2-terminal domain probably spans the bilayer 10–12 times and is thought to encompass the transport and amiloride binding sites. The COOH-terminal region is believed to extend into the cytosol, where it presumably plays a regulatory role (22, 23). This model was derived exclusively from hydropathy analysis of the deduced primary sequence, and there is little biochemical evidence to support the proposed topology. In fact, it is unclear whether the first transmembrane domain is cleaved after serving as a signal sequence (22), and conflicting evidence exists regarding the location of the COOH-terminal domain in different isoforms. Antibodies raised to the terminal 157 residues of NHE1 reacted with this antiporter only after permeabilization of the plasmalemma (19), implying a cytosolic localization. In contrast, monoclonal antibodies raised to the COOH-terminal residues of NHE3 (amino acids 702–832) effectively labeled this isoform when added externally to intact cells or right-side-out vesicles (3). These findings may reflect structural differences between isoforms that are not readily apparent from analysis of hydropathy plots. Indeed, the predicted transmembrane-spanning regions often vary when different programs are used to analyze the hydropathy (9). Clearly, independent verification of the structural disposition of the exchangers would be a useful complement to these theoretical analyses. Using the predicted topology as a cornerstone, we attempted to further elucidate the transmembrane structure of NHE1 by utilizing enzymatic cleavage, impermeable probes, and epitope tagging.

MATERIALS AND METHODS

Materials. NaCl, KCl, CaCl2, MgCl2, sodium pyrophosphate, trisodium citrate, citric acid, NaHCO3, NaH2PO4, and glucose were purchased from Fisher (Pittsburgh, PA). Nigericin, monensin, and 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF)-AM were purchased from Molecular Probes (Eugene, OR). N-glycosidase F, O-glycosidase, and neuraminidase as well as N-0ctyl glucoside were purchased from Boehringer Mannheim (Laval, QB, Canada). Sepharose CL-4B and protein A-Sepharose beads were purchased from Pharmacia Biotech (Baie d’Urfé, QB, Canada). Diisopropyl fluorophosphate was purchased from Aldrich (Milwaukee, WI). HOE694 was a generous gift from Dr. Wolfgang Scholz from Hoechst (Frankfurt, Germany). All other reagents were purchased from Sigma (Oakville, ON, Canada).

Antibodies. Mouse monoclonal antibodies that recognize the influenza virus hemagglutinin (HA) peptide (YPDYVPDYA) were obtained from BabCo (Berkeley, CA). Polyclonal antibodies to the human homologue of NHE1 were raised by injecting rabbits with a fusion protein encompassing the COOH-terminal 157 residues (658–815) of the exchanger and subsequently affinity purified as described (17). Both anti-rabbit and anti-mouse secondary antibodies were purchased from Jackson Immunochemicals (West Grove, PA).

Platelet isolation. Fresh blood from healthy volunteers (80 ml) was collected into 15-ml conical tubes. Each tube contained 1.5 ml of an acid-citrate-dextrose (ACD) solution containing (in mM) 100 trisodium citrate, 70 citric acid, and 110 dextrose. To prevent platelet activation, blood was collected by gravity with a 19-gauge needle. After gentle inver-
sion, blood was sedimented for 15 min at 250 g to separate the plasma. The platelet-rich plasma from several tubes was combined and sedimented at 2,300 g for 12 min. The supernatant was removed, and the pellet was gently resuspended in 5 ml of Ca²⁺-free Tyrode solution (CFT) containing (in mM) 137 NaCl, 12 NaHCO₃, 5 dextrose, 2.7 KCl, 2 MgCl₂, and 0.4 NaH₂PO₄ (pH 6.5) with 2% EGTA and 0.35% BSA. After a 10-min incubation at 37°C, 357 ml of ACD were added, and the cells were sedimented at 1,000 g for 10 min. The supernatant was removed, and the pellet was resuspended in 5 ml of CFT with 0.35% BSA but without EGTA. The cells were further incubated at 37°C for 10 min, and 357 µl of ACD were added. After centrifugation, the platelets were resuspended in 3 ml of Tyrode solution, consisting of CFT solution plus 0.5 mM CaCl₂. Cells were counted with a Coulter counter and kept at 37°C until used.

Stable transfection and tissue culture. Complementary DNA of the full-length rat NHE1, previously engineered to contain a series of unique restriction sites (to facilitate mutagenic manipulations), was subcloned into a modified eukaryotic expression vector pCMV (for construction details, see Ref. 15). One copy of an influenza virus HA peptide (YPYDVPDYA) was appended to the carboxy cytoplasmic tail (16), and cultured in a 5% CO₂-humidified environment for 10 min with 1 mM BCECF-AM in a NaHEPES-buffered solution (NHB) containing (in mM) 117 NaCl, 25 NaHCO₃, 12 NaH₂PO₄, 2.67 Na₂HPO₄, 5 glucose, 2 MgCl₂, 2 CaCl₂, and 10 mM nigericin. A calibration curve was prepared using low K⁺/high Na⁺, high K⁺/low Na⁺, and high K⁺/high Na⁺ solutions. The pH was measured at 10°C and adjusted to 7.4 by addition of NaOH solution.

For measurement of pH in AP-1-NHE1-HA cells, the samples were resuspended in 1 ml of Tyrode solution and were incubated at 37°C with 50 mM NH₄Cl isosmotically replacing NaCl for 10 min at 37°C and then rapidly transferred to NH₄Cl-free, Na⁺-free solution in which Na⁺ was isosmotically replaced by N-methyl-d-glucammonium ion (NMDG). At the end of each experiment, the cells were equilibrated in KCl solutions of varying pH containing (in mM) 140 KCl, 25 NMDG-HEPES, 5 glucose, 2 MgCl₂, 2 CaCl₂, and 10 mM nigericin. A calibration curve was prepared using low K⁺/high Na⁺, high K⁺/low Na⁺, and high K⁺/high Na⁺ solutions. The pH was measured at 10°C and adjusted to 7.4 by addition of NaOH solution.

Fluorescence was calibrated vs. pH by the addition of nigericin and monensin (10 mM each), followed by titration of the extracellular pH by addition of aliquots of concentrated Tris-Cl to reach 7.4 at 37°C and immediate incubation in a thermostatically controlled holder of a Hitachi F-4000 fluorescence spectrometer. After a baseline recording was obtained, the indicated amounts of Na⁺ were added to elicit NHE activity. Fluorescence was calibrated vs. pHi by the addition of nigericin and monensin (10 mM each), followed by titration of the extracellular pH by addition of aliquots of concentrated Tris-Cl to reach 7.4 at 37°C and immediate incubation in a thermostatically controlled holder of a Hitachi F-4000 fluorescence spectrometer. After a baseline recording was obtained, the indicated amounts of Na⁺ were added to elicit NHE activity. Fluorescence was calibrated vs. pHi by the addition of nigericin and monensin (10 mM each), followed by titration of the extracellular pH by addition of aliquots of concentrated Tris-Cl to reach 7.4 at 37°C and immediate incubation in a thermostatically controlled holder of a Hitachi F-4000 fluorescence spectrometer. After a baseline recording was obtained, the indicated amounts of Na⁺ were added to elicit NHE activity.

Immunofluorescence. AP-1-NHE1-HA cells plated onto glass coverslips were grown to ~60% confluence. They were then washed three times with PBS containing (in mM) 137 NaCl, 7.74 Na₂HPO₄, 2.26 NaH₂PO₄, and 2.7 KCl and fixed for 30 min at room temperature with 4% paraformaldehyde in PBS. After fixation, the cells were washed three to four times with PBS and then incubated with 100 mM glycine in PBS for 10 min. The cells were washed again and, except when indicated, were permeabilized with 0.1% Triton X-100 in PBS for 20 min at room temperature. After blocking with 5% donkey serum for 1 h, the cells were incubated with mouse anti-HA (1:1,000) antibody for 1 h. After this period, cells were washed four to five times with PBS and incubated for 1 h with a donkey anti-mouse antibody conjugated with Cy3 (1:1,000). Cells were washed for the final time and mounted onto glass slides using Dako fluorescent mounting medium.

Biotinylation, immunoprecipitation, and immunoblotting. NHE1. Platelets (10⁹) were suspended in PBS (pH 7.8) with 0.5 mM NHS-LC biotin (Pierce). After a 20-min incubation in the dark at 0°C, the cells were sedimented and resuspended in PBS (pH 7.4) with or without 100 U/ml chymotrypsin for 20 min at room temperature. Platelets were then sedimented and resuspended in 1 ml of immunoprecipitation buffer containing 150 mM NaCl, 50 mM HEPES, 5 mM EDTA, 3 mM KCl, 10 mM ATP, 25 mM sodium pyrophosphate, 1 mM o-phenanthroline, 1 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 100 µM N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 1 µM pepstatin A, 20 µM leupeptin plus 10 µg/ml aprotinin, and 1% Triton X-100. Triton-insoluble material was removed by centrifugation of the cell lysates at 20,000 g for 30 min at 4°C. The Triton-soluble supernatant was then incubated with Sepharose CL-4B for 1 h at 4°C to preclude nonspecific binding to the beads. NHE1 was immunoprecipitated from the lysates by incubation for 3 h at 4°C with a 1:50 dilution of the affinity-purified anti-NHE1 antibody. Immune complexes were collected by addition of 60 µl of a 50% slurry of protein A-Sepharose beads to the lysate and incubation for an additional 1 h at 4°C. The beads were then washed five times with ice-cold immunoprecipitation buffer and finally resuspended in 50 µl of twofold-concentrated Laemmli sample buffer.

The samples were resolved by 10% SDS-PAGE and then transferred to Immobilon. The blots were incubated with horseradish peroxidase-labeled anti-rabbit secondary antibody (1:5,000). Immunoreactive bands were detected by enhanced chemiluminescence (ECL). Blots were stripped and reprobed with horseradish peroxidase-labeled avidin (1:2,500) and detected by ECL.

Deglycosylation of NHE1. AP-1-NHE1-HA cells grown on 60-mm dishes were incubated in the presence or absence of chymotrypsin as specified. The cells were then scraped off the dish with a rubber policeman, sedimented, and subsequently resuspended in PBS containing 1 mM PMSF, 1 mM TPCK, 1 µM pepstatin A, 20 µM leupeptin, and 10 µg/ml aprotinin. The cells were then incubated for 30 min at room temperature.
with 2.5 mM of the protease inhibitor diisopropyl fluorophosphate. After this incubation, cells were sedimented, resuspended, and then incubated for 10 min at room temperature in a hypotonic buffer containing 10 mM HEPES, 18 mM potassium acetate, 1 mM EDTA, 1 mM PMSE, 1 mM benzamidine, 100 µM TPCK, 1 µM pepstatin A, 20 µM leupeptin, and 10 µg/ml aprotinin (pH 7.2). After centrifugation, the cells were resuspended in 25 µl of enzyme buffer A, which contained 38.7 mM Na₂HPO₄, 11.3 mM NaH₂PO₄, 1 mM pepstatin A, 20 mM leupeptin, 10 µg/ml aprotinin, 1% β-mercaptoethanol, and 0.5% SDS. After a 10-min incubation at room temperature, we added 25 µl of enzyme buffer B, which is identical to buffer A, except that SDS was replaced with 3% N-octyl glucoside. As suggested by the manufacturer of O-glycosidase, N-octyl glucoside was added to minimize the denaturing effect of SDS, by making the ratio of nonionic to ionic detergents 10:1. After a further 10-min incubation at room temperature, deglycosylation enzymes were added, and the samples were incubated for 5 h at 37°C. To terminate the enzymatic reactions at the end of the 5-h period, 50 µl of twice-concentrated Laemmli sample buffer were added. After the samples were resolved by 10% SDS-PAGE, proteins were transferred to Immobilon membranes that were subsequently blocked by incubation in PBS containing 0.1% Tween 20 and 5% skim milk for 2 h. The blots were incubated with a monoclonal antibody to HA (1:5,000), followed by a horseradish peroxidase-conjugated anti-mouse antibody (1:5,000) and development by ECL.

RESULTS

Chymotryptic cleavage of human NHE1. Human platelets were used as a model system to investigate the topology of NHE1 because they are readily available and express comparatively large amounts of the exchanger. The degree of exposure of NHE1 to the extracellular space was probed by using chymotrypsin. This protease cleaves the peptide bonds on the carboxy side of accessible tyrosine, tryptophan, and phenylalanine residues. As illustrated in Fig. 2A, human NHE1 has three potential cleavage sites on the first putative extracellular loop and several more on the last three loops. Intact human platelets were incubated for various times with 100 U/ml chymotrypsin. Proteolysis was halted by washing the cells once in PBS containing the chymotrypsin inhibitor TPCK. Whole cell lysates were resolved by SDS-PAGE and subjected to immunoblotting with an antibody to COOH-terminal 157 residues (Fig. 1A). Cleavage of NHE1 by extracellular chymotrypsin was apparent at the earliest time studied, i.e., 5 min. The first detectable cleavage reduced the apparent molecular mass from ~110 to ~90 kDa. Because a comparatively low concentration of chymotrypsin produced quantitative cleavage within 5 min, the susceptible site(s) must be readily accessible. A second product of NHE1 proteolysis of molecular mass of ~70 kDa became apparent by 10 min. The abundance of this polypeptide increased over the next 60 min, and that of the 90-kDa polypeptide intermediate diminished proportionately.

The results of Fig. 1A suggest that NHE1 exposes at least two distinct sites to the extracellular milieu. This conclusion rests on the assumption that the platelet membrane remains intact and impermeant to chymotrypsin during prolonged incubation with the protease. This was ascertained by analyzing the polyepitopic pattern of intact platelets after varying times of incubation with the protease. Whole cell lysates were analyzed by SDS-PAGE and stained with Coomassie blue (Fig. 1B). Of particular interest are intracellular proteins known to be susceptible to proteolysis. In platelet lysates, it is well established that the three heaviest major polypeptides, with molecular masses of 280, 215, and 200 kDa, correspond to actin binding protein (ABP), talin, and myosin, respectively (7). It is noteworthy that these proteins remained unaffected throughout the 60-min incubation period used in Fig. 1A. Incipient degradation of these proteins, especially of ABP, was noted only after 90 min. On the other hand, when platelets were permeabilized with Triton before exposure to chymotrypsin, ABP, talin, and myosin were rapidly and completely degraded (not illustrated). Thus platelets remain intact and impermeable to chymotrypsin for at least 60 min. This conclusion was validated during the course of pH₁ measurements by the intracellular retention of fluorescent dyes and by the stability of transmembrane ionic gradients (see below).

Biotinylation of human NHE1. The previous results suggest that a sizable portion of NHE1 is exposed to the external medium. This notion was confirmed using an impermeant biotin derivative capable of reacting covalently with the susceptible site(s). Whole cell lysates from untreated (Cntl) or chymotrypsinized platelets were resolved by SDS-PAGE, transferred to Immobilon, and immunoblotted with anti-NHE1 antibody. Arrows indicate wild-type NHE1 and 2 cleavage products. Blot is representative of 4 similar experiments. *Position of albumin, which produced variable amounts of spurious immunostaining.

Fig. 1. Chymotryptic cleavage of human Na⁺/H⁺ exchanger (NHE1). Isolated human platelets were incubated for indicated times with 100 U/ml chymotrypsin. A: whole cell lysates from untreated (Cntl) or chymotrypsinized platelets were resolved by SDS-PAGE, transferred to Immobilon, and immunoblotted with anti-NHE1 antibody. Arrows indicate wild-type NHE1 and 2 cleavage products. Blot is representative of 4 similar experiments. *Position of albumin, which produced variable amounts of spurious immunostaining. B: cell lysates from parallel samples were resolved by SDS-PAGE, and gel was stained with Coomassie blue. Arrow, actin binding protein (ABP).
lently with ε-amino groups of lysine residues. Intact platelets were reacted with NHS-LC biotin and then incubated for 5 min in the presence or absence of chymotrypsin. As shown in Fig. 2B, biotinylation did not alter the electrophoretic mobility of NHE1 nor the ability of chymotrypsin to cleave the exchanger, as revealed by immunoblotting. The occurrence of biotinylation was verified by overlaying the same blots with peroxidase-coupled avidin (Fig. 2C). Importantly, although the presence of covalently bound biotin is readily detectable in the full-length NHE1, the probe was absent after the initial rapid cleavage catalyzed by chymotrypsin. Because the antibody used for immunoblotting detects the COOH-terminal region of the protein, we conclude that not all of the lysine residues proposed to be exposed to the external medium are accessible for biotinylation. Our data suggest that only the lysine residues contained within ~25 kDa of the NH2-terminal end of the protein are accessible (see Fig. 2A).

Effect of extracellular chymotryptic cleavage on NHE1 function. Earlier structure-function analysis revealed that NHE activity persists in mutants lacking most of the cytosolic COOH-terminal tail (22). It was therefore concluded that the transmembrane, NH2-terminal region is necessary and sufficient for transport. However, the regions of the NH2-terminal domain required for activity were not defined. The finding that the protein can be proteolytically severed at two sites near the NH2 terminus enabled us to test the role of this domain in ion exchange. We measured NHE activity in platelets by recording their ability to recover from an acid load imposed by prepulsing with NH4Cl. As shown in Fig. 3A, control cells recovered very rapidly from the acid load, but only when Na+ was present in the medium. This recovery was sensitive to both amiloride and

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**Fig. 2.** Chymotryptic cleavage and biotinylation of human NHE1. A: diagram of putative topology of human NHE1 depicting residues that are potential chymotrypsin cleavage sites (aromatic amino acids; circles) and biotinylation sites (lysines; triangles). Probable chymotrypsin cleavage sites in first external loop are marked with an arrowhead. Site of N-linked glycosylation and potential O-glycosylation sites are also depicted (squares). B and C: isolated human platelets were treated with (+) or without (−) NHS-LC biotin as described in METHODS and, when specified, were subsequently incubated for 5 min with 100 U/ml chymotrypsin. NHE1 was immunoprecipitated, resolved by SDS-PAGE, and blotted onto Immobilon. Specificity of precipitation was assessed with preimmune serum (pi). B: immunoblot using anti-NHE1 antibody. Slight decrease in mobility in biotinylated sample in rightmost lane was not a reproducible observation. C: blot in B was stripped and reprobed by using horseradish peroxidase-conjugated avidin. B and C are representative of 2 separate experiments.
HOE-694, which is a relatively specific inhibitor of NHE1 when used in the low micromolar range (6). Pretreatment of the platelets with chymotrypsin had no obvious effect on the ability of the cells to recover from the acid load nor on the susceptibility of NHE to amiloride or HOE-694 (Fig. 3B). Even after a 60-min treatment with chymotrypsin, the rate of Na⁺-induced alkalization was decreased only marginally and was not statistically different from the control rate (Fig. 3C). Importantly, the Na⁺-induced extrusion of H⁺ (equivalents) was virtually eliminated by 1 µM HOE-694 in both the control and protease-treated cells (Fig. 3).

A more detailed comparison of the kinetic properties of NHE in control and chymotrypsin-treated platelets is presented in Fig. 4. The dependence of the exchanger on pH₁, which is typically sigmoidal with half-maximal activity near pH 6.65, was very similar after treatment for 60 min with up to 200 µM chymotrypsin (Fig. 4A). Because the buffering power was similar in the two populations of cells and to facilitate comparison, relative rates of recovery, rather than absolute H⁺ (equivalent) fluxes, are illustrated. Comparable results were obtained in eight control and four chymotrypsin-treated experiments. Finally, the extracellular Na⁺ concentration dependence, measured at pH₁ 6.4, was also indistinguishable before and after chymotryptic treatment (Fig. 4B).

Expression and characterization of epitope-tagged NHE1. Although the predicted topology for all the NHE isoforms suggests that the COOH-terminal tail is located intracellularly, a recent report (3) suggests that the extreme COOH terminus of NHE3 might in fact be extracellular. On the other hand, antibodies to the putative cytosolic domain of NHE1 reacted with the exchanger only after permeabilization of the cell membrane (19), implying that the epitopes are intracellular. However, these experiments were performed using antibodies raised to a fusion protein encompassing the COOH-terminal 157 amino acids, and the precise antigenic determinants are not known. It is therefore conceivable that although the immunoreactive part of the tail is indeed located intracellularly, the extreme COOH terminus is exposed extracellularly, by virtue of an additional transmembrane crossing.

A similar caveat applies to our experiments in platelets, which used a polyclonal antibody to the COOH-terminal 157 residues. An extracellular COOH-terminal fragment could conceivably have been cleaved by chymotrypsin, remaining undetected if it did not contain antigenic determinants or was too small to be resolved by the PAGE system used. To establish more precisely the location of the COOH terminus of NHE1, we constructed a vector to express full-length NHE1 tagged at its COOH terminus with an influenza virus HA peptide (Fig. 5A). This epitope-tagged exchanger was stably transfected into NHE-deficient AP-1 cells, yielding AP-1-NHE1-HA cells. To verify expression of the HA-tagged NHE1, the transfectants were lysed, resolved by SDS-PAGE, and immunoblotted with a monoclonal anti-HA antibody. As illustrated in Fig. 5B, two immunoreactive bands were detected in AP-1-NHE1-HA cells: a major, diffuse band of ~100 kDa, corresponding to mature, tagged NHE1, and a sharper, smaller band of ~80 kDa, which probably represents incompletely glycosylated NHE1 trapped in the secretory pathway. A similar immature form was reported earlier after heterologous expression of NHE1 (6). Importantly, no immunoreactive bands were detected in untransfected AP-1 cells (Fig. 5B).

To use the tagged constructs for assessment of the topology of NHE1, it was important to ascertain that introduction of the epitope did not alter the properties of the exchanger. This was evaluated functionally. AP-1-NHE1-HA cells as well as the parental AP-1 cells were acid loaded as pH₁ was recorded fluorometrically (Fig. 6). The transfectants recovered from the acidosis...
on reintroduction of Na\(^+\) to the medium, whereas the untransfected parental cells remained acidic despite the addition of Na\(^+\) (Fig. 6A). These findings imply that at least a fraction of the tagged NHE1-1 reaches the plasmalemma, in which it effectively catalyzes Na\(^+\)/H\(^+\) exchange. In fact, the rate of exchange was greater in AP-1-NHE1-HA cells than in wild-type Chinese hamster ovary cells (WT5) expressing the endogenous antiporter. As shown in Fig. 6, B and C, the activity observed in the transfectedants bears all the hallmarks of NHE1: the extrusion of H\(^+\) was found to be Na\(^+\) dependent and sensitive to micromolar doses of ethylisopropyl amiloride and HOE-694.

The expression of NHE1-HA was further evaluated by immunofluorescence. In accordance with the immunoblotting data, AP-1 cells failed to react with the HA-specific antibody (Fig. 7, left). In contrast, those cells stably transfected with NHE1-HA demonstrated a diffuse, membrane-associated labeling (Fig. 7, middle). In some cells, perinuclear intracellular staining was observed, consistent with accumulation of some of the immature NHE1 in the endoplasmic reticulum, likely due to overexpression (see inset, Fig. 7, middle). Importantly, immunolabeling was observed only after the AP-1-NHE1-HA cells had been permeabilized with Triton-X-100 (Fig. 7, middle) but not in fixed, unpermeabilized cells (Fig. 7, right). This indicates that the HA epitope is intracellular and therefore implies that the COOH-terminal end of NHE1 is located intracellularly as well.

Chymotryptic cleavage of rat NHE1. Treatment of the platelet exchanger with chymotrypsin and use of the polyclonal antibody indicated that cleavage occurred near the NH\(_2\)-terminal domain of NHE1 but could not rule out the occurrence of COOH-terminal cleavage. This possibility was considered experimentally using the heterologously expressed rat NHE1, which was epitope tagged at its COOH terminus. AP-1-NHE1-HA cells were exposed to chymotrypsin for various times, and whole cell lysates were resolved by SDS-PAGE, followed by immunoblotting with a monoconal antibody to HA. Within 5 min, chymotrypsin cleaved NHE1-HA, yielding an immunoreactive fragment of apparent molecular mass of 64 kDa (Fig. 8). The immunoreactivity appeared to increase in the 80-kDa region, suggesting an alternate cleavage site. However, the appearance of this product was obscured by the presence of the immature form of NHE1 in the transfectants. No further cleavage products were noted for up to 20 min (not illustrated). These results imply that chymotrypsin cleaves only at sites located in the NH\(_2\)-terminal half of the protein and are consistent with the notion that the COOH terminus is localized entirely in the cytosolic environment.

Localization of glycosylation sites in rat NHE1. The human NHE1 was shown earlier to exhibit both N- and O-linked carbohydrates (6). The sequence of the rat homologue (Fig. 5A) similarly predicts several potential O-linked glycosylation sites and a potential N-linked glycosylation site. As shown in Fig. 8, incubation of lysates, obtained from AP-1-NHE1-HA cells, with N-glycosidase F increased the mobility of the mature rat NHE1 from an apparent molecular mass of ~100 to ~83 kDa. The mobility was similarly increased by a combined treatment with neuraminidase and O-glycosidase. These observations suggest that, like the human NHE1, the rat homologue is glycosylated on both asparagine and serine residues. It is noteworthy that the minor band of ~75 kDa was virtually unaffected by the glycosidases, consistent with the notion that it is a poorly glycosylated immature form of NHE1.

Having established that proteolysis occurs only near the NH\(_2\)-terminus, we proceeded to define the position of the cleavage sites with respect to the sites of glycosylation. Cells were initially treated with chymotrypsin, then lysed, and subjected to deglycosylation. As shown...
in Fig. 8, the mobility of the chymotryptic products was similar, ~65 kDa, whether the samples had been treated with or without N-glycosidase F or with the combination of neuraminidase and O-glycosidase. These findings imply that the carbohydrate moieties had been removed by the protease chymotrypsin. The increased sharpness of the bands obtained after proteolysis is consistent with this conclusion.

**DISCUSSION**

The experiments described above provide evidence to complement our understanding of the structure of NHE1 predicted by analysis of hydropathy.

COOH terminus. Earlier biochemical studies suggested that most of the COOH-terminal domain of NHE1 (~300 residues) is located intracellularly. The evidence supporting this notion includes the following: 1) the finding that antibodies raised to the terminal 157 amino acids react only after cells are permeabilized (19); 2) the observation that all the known phosphorylation sites are within the COOH-terminal domain (22); and 3) the report that calmodulin can bind to two sites within this domain (2). On the other hand, a more recent result indicates that at least part of the COOH-terminal region of NHE3 appears to be exposed extracellularly (3).

Our data using both native and epitope-tagged NHE1 indicate that the extreme COOH terminus of this protein lies within the cell, since it was not accessible to external antibodies or to an impermeant biotin derivative. Moreover, none of the hydrophobic residues that are substrates for chymotryptic cleavage in the COOH-terminal tail are exposed to the protease when it is added to intact cells. Thus our findings are most easily reconciled with the original model, reproduced in Fig. 5A, in which the COOH-terminal hydrophilic domain lies entirely within the cytosol. The discrepancy between our findings and those of Biemesderfer et al. (3) may reflect differences between isoforms. However, preliminary experiments in our laboratory indicate that, as in the case of NHE1, an epitope tag placed at the COOH terminus of NHE3 is accessible only after permeabilization of the cells and is resistant to proteolytic treatment in intact but not in permeabilized cells (unpublished observations).

NH₂ terminus. As reported for the human NHE1 (6), we found that the rat homologue of the exchanger contains both N- and O-linked carbohydrates (Fig. 8). In the human NHE1, mutagenesis studies found that N75 is the only potential N-glycosylation site utilized, implying that the first putative loop is indeed extracellular. These observations could not be confirmed by epitope tagging of the extreme NH₂ terminus, possibly because this portion of the protein is cleaved cotranslationally as part of a signal sequence.

As an alternative approach, we used chymotryptic treatment to verify the transmembrane disposition of NHE1. In platelets, the protease cleaved the exchanger quantitatively under conditions in which intracellular proteins remained unaffected. These findings indicate that the entire population of NHE1 is present on the surface membrane of unstimulated platelets. This rules out recruitment of intracellular exchangers as a viable mechanism of stimulation, as found to be the case for H⁺ pumps and glucose transporters in other tissues (e.g., Ref. 12).

As reported earlier (6), two immunoreactive bands can be observed in transfected cells. The smaller of these polypeptides was proposed to be an intermediate form in the biosynthesis of NHE1 (6). Our observations in the AP-1-NHE1-HA cell line, which are stably transfected with HA-tagged NHE1 (AP-1-NHE1-HA) and their untransfected counterparts (AP-1) were lysed, resolved by SDS-PAGE, transferred to Immobilon, and immunoblotted with an anti-HA antibody. Representative of 3 similar experiments.
second site may exist in rat NHE1, but the product of its cleavage is obscured by the presence of the ~80-kDa immature species in the transfected. The precise location of the cleavage sites is difficult to establish based solely on the molecular mass of the products, because the contribution of the carbohydrate to the electrophoretic mobility of the protein is uncertain and because the sites of O-linked glycosylation are not defined. We therefore proceeded to deglycosylate NHE1 after proteolysis. As shown in Fig. 8, the ~65-kDa fragment yielded by chymotrypsin treatment did not contain detectable N- or O-linked carbohydrate. On the basis of the amino acid composition of the rat NHE1, we can estimate that cleavage occurred at most ~150 residues from the NH₂ terminus, i.e., in the first or second putative extracellular loops (see arrowheads in Fig. 5A). The portion cleaved off includes the sites of attachment of both the N- and O-linked sugars: these probably include N76 and one or more of the serine residues between residues 35 and 66 in the first external loop.

Biotinylation experiments using an impermeant succinimidyl derivative indicated that at least one lysine residue in human NHE1 is readily exposed extracellularly. These sites were found to be near the NH₂ terminus, inasmuch as they were cleaved by short incubation with chymotrypsin (Fig. 2C). There are 10 lysine residues in the predicted extracellular loops of NHE1-HA expression in AP-1 cells. A: AP-1 or AP-1-NHE1-HA cells were loaded with BCECF and acidified by an NH₄Cl prepulse. Tail end of prepulse is illustrated. pH was measured fluorometrically in Na⁺-free [N-methyl-D-glucammonium ion (NMDG) medium] or Na⁺-rich medium, as indicated by bars. B: AP-1-NHE1-HA cells were BCECF loaded and acidified as in A. Recovery from acidification was monitored in Na⁺-rich medium in presence or absence of 1 µM ethylisopropyl amiloride (EIPA), as indicated. C: accumulated data of rates of Na⁺-induced recovery from an acidification in wild-type Chinese hamster ovary cells (WT), AP-1 cells, or AP-1-NHE1-HA cells. Presence or absence of Na⁺, HOE-694 (HOE), and EIPA is as indicated. Data are means ± SE of no. of experiments indicated. Last 3 columns were compared with respective control (AP-1-NHE1 cells with Na⁺) and significance of difference is noted. **P < 0.01; ***P < 0.001.

Fig. 7. Detection of NHE1-HA in intact and permeabilized AP-1 cells by immunofluorescence. AP-1 (left) or AP-1-NHE1-HA cells (middle and right) were fixed with 4% paraformaldehyde and treated with (left and middle) or without (right) 0.1% Triton X-100 in PBS for 20 min. Samples were then probed with a monoclonal anti-HA antibody, followed by a Cy3-labeled goat anti-mouse secondary antibody. Inset to middle illustrates perinuclear, probably endoplasmic reticular staining.

Fig. 8. Chymotryptic cleavage of HA-tagged rat NHE1: effect on glycosylation sites. AP-1-NHE1-HA cells were incubated with (+) or without (−) 100 U/ml chymotrypsin for 10 min. Cell lysates were then prepared and treated with indicated glycosidases for 5 h, as described in METHODS. Samples were then resolved by SDS-PAGE, transferred to Immobilon, and immunoblotted with anti-HA antibody. Treatment with chymotrypsin, N-glycosidase F (N-Gly), neuraminidase (Neura), or O-glycosidase (O-Gly) is indicated. Representative of 3 experiments.
the human NHE1, 2 on the first loop and the remainder on the fifth and sixth loops (Fig. 2A). We favor the interpretation that only one or both of the lysine residues in the first loop are accessible from the outside. This would imply that the putative extracellular loops between transmembrane domains 9–10 and 11–12, despite their size and hydrophobicity, are not fully exposed to the outside medium. In accordance with this interpretation, chymotrypsin failed to cleave in this region, despite the presence of multiple tyrosine and phenylalanine residues that are theoretical substrate sites for the protease (see Figs. 2C and 5A). Hence the regions between transmembrane domains 9–10 and 11–12 may lie along the membrane surface or be folded within the protein, perhaps contributing to the aqueous path for ion translocation. In addition, it is possible that these two loops are involved in dimerization of NHE1 (8), which may involve intermolecular disulfide linkage (9).

Functional consequences of cleavage. Extensive chymotryptic cleavage of human NHE1, which reduced the size of the polypeptide containing the antigenic determinants to ~70 kDa, had little effect on ion exchange activity. Neither the rate nor the apparent affinity of motryptic cleavage of human NHE1, which reduced the possible that these two loops are involved in dimerization of NHE1 (8), which may involve intermolecular disulfide linkage (9).

Functional consequences of cleavage. Extensive chymotryptic cleavage of human NHE1, which reduced the size of the polypeptide containing the antigenic determinants to ~70 kDa, had little effect on ion exchange activity. Neither the rate nor the apparent affinity of the exchanger for external Na\(^+\) or internal H\(^+\) were measurably affected. Therefore structural integrity is not essential for NHE1 activity. It is noteworthy, however, that because our antibodies could not detect the NH\(_2\)-terminal region of the protein, the cleaved fragments may have remained associated with the membrane and the COOH-terminal portion of the protein after proteolysis, possibly continuing to contribute to the functional activity. Resolution of these alternatives will require development of antibodies to the first external loop or epitope tagging of this region.

We thank Dr. G. Goss for his contribution to preliminary experiments that facilitated this work.

This work was supported by grants from the Medical Research Council (MRC) of Canada (to S. Grinstein and J. Orlowski). L. D. Shrode is the recipient of a postdoctoral fellowship from the Arthritis Society of Canada. B. S. Gan is supported by grants from the Plastic Surgery Educational Foundation, Plastic Surgery Research Fund, and Toronto Hospital Dept. of Surgery. S. J. A. D’Souza is the recipient of a postdoctoral fellowship from the MRC. S. Grinstein is cross-appointed to the Dept. of Biochemistry, University of Toronto, and is an International Scholar of the Howard Hughes Medical Institute, an MRC Distinguished Scientist, and current holder of the Piblado Chair in Cell Biology.

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Received 4 November 1997; accepted in final form 15 April 1998.

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