Membrane Topology of NHE3

EPITOPES WITHIN THE CARBOXYL-TERMINAL HYDROPOLIC DOMAIN ARE EXOPLASMIC*

Daniel Biemesderfer‡, Brenda DeGray, and Peter A. Aronson
From the Departments of Internal Medicine and of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut 06520-8029

Experimental data indicate that the relatively hydrophilic carboxyl-terminal domains of Na\(^+\)-H\(^+\) exchangers mediate the regulation of transporter activity through interactions with cytoskeletal effectors. It has therefore been assumed that this entire domain lies on the cytoplasmic surface of the plasma membrane. The purpose of the present study was to determine the membrane orientation of the COOH-terminal 131 amino acids of Na\(^+\)-H\(^+\) exchanger isoform NHE3 by use of three monoclonal antibodies that recognize at least two distinct epitopes within this region. Enzyme-linked immunosorbent assay studies demonstrated binding of these monoclonal antibodies (mAbs) to intact right-side-out renal brush border membrane vesicles in the absence of detergent. Moreover, when coupled to an affinity matrix to isolate membrane vesicles, the anti-NHE3 mAbs bound structures that were morphologically identical to intact microvilli. To confirm the identity of the exoplasmic antigen bound by the antibodies, immunoprecipitation studies were performed. Intact right-side-out brush border membrane vesicles were incubated with the mAbs in the absence of detergent. The membranes were pelleted, supernatant with unbound antibody was removed, the pellet was solubilized, and then immunoprecipitation with secondary antibody was performed. Immunoblot analysis indicated that NHE3 was precipitated after binding of the mAbs to intact membranes. Finally, the localization of the mAb epitopes was determined using high resolution immunocytochemistry. Ultrathin cryosections of rat kidney were labeled with the mAbs and bound antibody detected with the colloidal gold technique. Labeling was restricted to the exoplasmic surface of microvilli of the proximal tubule. Taken together, these findings indicate that epitopes within the carboxyl terminus of the Na\(^+\)-H\(^+\) exchanger isoform NHE3 are exposed to the outside of the plasma membrane.

Na\(^+\)-H\(^+\) exchangers mediate the electroneutral, amiloride-sensitive exchange of Na\(^+\) and H\(^+\) across plasma membranes (1). Molecular cloning studies of the Na\(^+\)-H\(^+\) exchanger gene family reveal proteins with similar structure sharing between 45 and 70% overall identity at the amino acid level (2–4). Based upon hydropathy analysis, these proteins contain two structural domains. The amino terminus is very hydrophobic and is predicted to have 10–12 α-helices that are long enough to span the lipid bilayer. In contrast, the carboxyl terminus is relatively hydrophilic and has no predicted membrane-spanning α-helical segments. Functional studies have indicated that ion transport is mediated via the amino-terminal hydrophobic domain and that this activity is regulated through the interaction of cytosolic proteins with the carboxyl-terminal hydrophilic domain (5–10). It has therefore been assumed that the latter domain lies entirely on the cytoplasmic surface of the membrane (3).

To test this assumption directly, we used three monoclonal antibodies (mAbs)† to determine the membrane orientation of the COOH-terminal 131 amino acids of Na\(^+\)-H\(^+\) exchanger isoform NHE3. Our findings indicate that epitopes within this COOH-terminal domain of NHE3 are located on the exoplasmic surface of the plasma membrane. A preliminary account of this work has been published previously as an abstract (11).

† The abbreviations used are: mAb, monoclonal antibody; BBMV, brush border membrane vesicles; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; PLP, paraformaldehyde-lysine-periodate.

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‡ To whom correspondence should be addressed: Section of Nephrology, Dept. of Internal Medicine, Yale University School of Medicine, 333 Cedar St., LMP 2073, P. O. Box 208029, New Haven, CT 06520-8029. Tel.: 203-785-4186; Fax: 203-785-7068; E-mail: daniel.biemesderfer@yale.edu.
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In some experiments (Fig. 1), polyvinylidene difluoride membranes were transferred to polyvinylidene difluoride membranes (Millipore Immobilon-P) at 300 mA for 6–10 h at 4 °C with a Transflector transfer electrophoresis unit (Hoefer Scientific Instruments, San Francisco) and stained with Ponceau S at 0.5% trichloroacetic acid. Immunoblotting was performed as follows. Strips of polyvinylidene difluoride were incubated first in Blotto (5% nonfat dry milk and 0.1% Tween 20 in PBS, pH 7.4) for 1–3 h to block nonspecific binding of antibody, followed by overnight incubation in primary antibody diluted 1:2,000 in Blotto. The strips were then washed in Blotto and incubated for 1 h with an appropriate horseradish peroxidase-conjugated secondary antibody (goat anti-mouse IgG, gamma chain-specific or rabbit anti-guinea pig IgG; Zymed, San Francisco). Bound antibody was detected with the ECL chemiluminescence system (Amersham Corp.) according to manufacturer’s protocols. In some experiments (Fig. 1), polyvinylidene difluoride membranes were transferred according to the manufacturer’s protocols. Beads that had been previously coated with rabbit anti-mouse IgG were in turn incubated with primary antibody after stripping away the first antibody by incubation in 2% SDS, 100 mM β-mercaptoethanol, 50 mM Tris (pH 6.9) for 60 min at 70 °C. ELISA—ELISAs were performed in a manner similar to that described previously (12). BBMV (protein concentration, 30 mg/ml) were diluted 1:1600 in PBS (150 mM NaCl, 10 mM phosphate, pH 7.4); then, 100-μl aliquots of this antigen were applied to each well of a 96-well microtitre plate (Costar), and the plates were incubated overnight at 4 °C. The following day, the plates were washed four times in PBS (pH 7.4) and once in PBS with 1% BSA (PBS/BSA) to block nonspecific antibody binding. Primary antibodies were diluted either in PBS/BSA or in PBS/BSA with 0.1% Triton X-100, and then 100-μl aliquots were applied to wells of the 96-well plate and incubated for 1 h at room temperature. Plates were washed four times in PBS with 0.1% Triton X-100 (PBS/TX100) and once in PBS/BSA/TX100. Bound antibody was detected by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (gamma chain-specific, Zymed, San Francisco). Bound antibody was detected with the ECL chemiluminescence system (Amersham Corp.) according to manufacturer’s protocols. Beads that had been previously coated with rabbit anti-mouse IgG were in turn incubated with primary antibody after stripping away the first antibody by incubation in 2% SDS, 100 mM β-mercaptoethanol, 50 mM Tris (pH 6.9) for 60 min at 70 °C.

RESULTS

We had previously generated three mAbs against a fusion protein representing the carboxyl-terminal 131 amino acids (amino acids 702–832) of NHE3 (12). We first examined whether these antibodies were directed at identical or different epitopes within this segment of NHE3. Immunoblotting experiments were carried out using rabbit renal brush border membranes expressing native (full-length) NHE3 or cells transfected with rabbit NHE3 truncated at amino acid 756 (NHE3Δ). As shown in Fig. 1, both mAb 2B9 and mAb 4F5 labeled the native 80-kDa NHE3 polypeptide expressed in rabbit kidney. However, only mAb 2B9 stained NHE3Δ in transfected fibroblasts. Results with mAb 19F5 were identical to those observed for 4F5 (not shown). We conclude that mAb 2B9 is directed at an epitope that lies between amino acids 702 and 756 of NHE3, whereas the epitope(s) for 4F5 and 19F5 must be COOH-terminal to amino acid 756.

Our first method to examine the topology of the mAb epitopes (inside or outside) was to test the ability of the antibodies to bind to intact BBMV. We took advantage of the fact that BBMV isolated from renal cortex by divalent cation precipitation are predominantly sealed and right-side-out (19, 27). Fig. 2 shows the results of ELISA in which anti-NHE3 mAbs were allowed to bind to BBMV in the presence or absence of 0.01% Triton X-100 as a permeabilizing agent. For all three anti-NHE3 monoclonal antibodies, binding to BBMV was nearly as great in the absence of detergent as in the presence of detergent. In contrast, binding of an mAb specific for the microvillar core luminal membrane marker γ-glutamyl transpeptidase (18).

Immunoprecipitation of NHE3—Rabbit brush border membrane vesicles (250 μg) were suspended in 1 ml of PBS (pH 7.4). To expose the mAbs to only the extracellular domain of microvilli, antibodies were added to the suspended BBMV in the absence of detergent and allowed to incubate on a rotator for 1 h at 4 °C. The mixture was then diluted with 20 ml of PBS containing 1% BSA, and the membranes were collected by centrifugation at 100,000 × g for 1 h in a Beckman ultracentrifuge. The supernatant containing unbound antibody was removed, and the membrane pellet was solubilized in 1% Triton X-100 in PBS. Samples were cleared of insoluble material by centrifugation for 10 min at top speed in a table top centrifuge. The immune complexes were then collected using protein G-Sepharose and analyzed by SDS-PAGE and immunoblotting. Parallel immunoprecipitations were carried out in which mAbs were exposed to all microvillar epitopes by first solubilizing BBMV in PBS containing 1% Triton X-100 for 30 min before addition of antibodies.

Although the brush border membrane vesicles that were used in these antibody binding experiments (Fig. 2) were en-
riched 10–15-fold in microvillar membrane markers, some contaminating membranes were also present. To verify that the antibody binding described in Fig. 2 was actually to microvillar rather than to contaminating membranes, we tested the ability of the antibodies to affinity isolate microvillar vesicles. For these studies, the mAbs were coupled to the surface of magnetic beads. After mixing the antibody-coated beads with BBMV, we examined the morphology of the specifically bound membranes by electron microscopy. As seen in Fig. 3A, very little membrane bound to beads coated with a control (anti-villin) mAb. In contrast, shown in Fig. 3B, beads coated with the anti-NHE3 mAb 4F5 isolated large numbers of membranous structures, the majority of which had microvillar architecture (Fig. 3B, inset). This experiment confirms that the anti-NHE3 mAbs are capable of binding to epitopes on the outside of the microvillar membrane.

Another set of experiments was designed to directly localize bound mAb in brush borders using immunoelectron microscopy. Ultrathin (50–100 nm) cryosections of PLP-fixed rat kidney were incubated with the mAbs, and then bound antibody was visualized in the electron microscope by use of a colloidal gold-conjugated secondary antibody (Fig. 4). This approach has two distinct advantages for mapping epitopes of membrane proteins. First, the ultrathin cryosections allow equal antibody access to all regions of the cell including both surfaces of the lipid bilayer. Second, the colloidal gold particles appear as discrete electron-dense spheres of uniform size (in these studies 10 nm) and permit the localization of epitopes to within several nanometers.

To test the method and to ensure that we could reliably distinguish between cytoplasmic and exoplasmic labeling of membrane proteins, we used control antibodies to other known renal proteins. We used an mAb to the α-subunit of the Na,K-ATPase as a control for labeling of a cytoplasmic epitope of a transport protein (Fig. 4A). We used an anti-villin mAb to label a cytosolic epitope in microvilli (Fig. 4B). Finally, as a control for labeling an exoplasmic epitope of microvilli, we used a mAb to dipeptidyl peptidase IV (Fig. 4C).

Shown in Fig. 4, the topology of the epitope relative to the plasma membrane was easily seen for each of these control antibodies. The epitope of the Na,K-ATPase recognized by mAb C62.4 was localized to the cytoplasmic surface of the basolateral plasma membrane (Fig. 4A). Staining for villin, as predicted, showed the protein to be located exclusively in the microvillar core (Fig. 4B). In contrast, staining for dipeptidyl peptidase IV was clearly on the outside or exoplasmic surface of the microvillar membrane (Fig. 4C). These data confirm that immunoelectron microscopy using the colloidal gold/ultrathin cryosectioning technique can distinguish cytoplasmic and exoplasmic epitopes of membrane proteins.

When the anti-NHE3 mAb 4F5 was used in these studies, gold label was observed almost exclusively on the exoplasmic surface of the microvillar membrane (Fig. 4D), a pattern similar to that seen for the exoplasmic control dipeptidyl peptidase IV. The same staining pattern was found with all three anti-NHE3 mAbs (not shown). These findings clearly demonstrate...
that the epitopes recognized by the anti-NHE3 mAbs are exposed to the exoplasmic surface of the plasma membrane.

Having shown by three different methods that the anti-NHE3 mAbs bind to the external surface of the microvillus membrane, we conducted a final set of studies to confirm that the exoplasmic binding of the mAbs was actually to NHE3. In these experiments the anti-NHE3 mAbs were incubated with intact BBMV. The membranes were then washed to remove unbound antibody, and the membranes were solubilized. Immune complexes were then collected using protein G-Sepharose and analyzed by SDS-PAGE and immunoblotting. An anti-NHE3 guinea pig antiserum was used to detect NHE3 in the immunoprecipitates. For comparison, and to detect total NHE3, parallel samples were run in which immunoprecipitation was performed directly from Triton X-100-solubilized brush border membrane vesicles.

Shown in Fig. 5, each of the three anti-NHE3 mAbs immunoprecipitated almost as much NHE3 when incubated with intact BBMV as when incubated with solubilized protein. As a control for BBMV integrity in these studies, a similar immunoprecipitation experiment was conducted with an anti-villin mAb. As also shown in Fig. 5, immunoprecipitation of villin was greatly reduced when the anti-villin mAb was incubated with intact BBMV as compared with solubilized membranes, confirming that the cytoplasmic space of intact BBMV is not accessible to antibodies in the absence of detergent. The finding

Fig. 4. Immunoelectron microscopic localization of anti-NHE3 mAb binding sites. Anti-NHE3 mAb 4F5 (D) or mAbs to known epitopes were used to immunolabel ultrathin cryosections of PLP-fixed rat kidney. Bound antibody was detected with rabbit anti-mouse IgG-coated 10-nm colloidal gold. As controls for intracellular epitopes, mAbs to the α-subunit of the Na,K-ATPase (A) and to villin (B) were used. As a control for an extracellular epitope, an mAb to dipeptidyl peptidase IV was used (C). A, staining for the Na,K-ATPase along the basolateral membrane of a principal cell in a cortical collecting duct. B and C, staining for villin and dipeptidyl peptidase IV, respectively, in cross-sections of microvilli of proximal tubules. Panel D, representing the apical region of a proximal tubule, shows staining for an epitope within the COOH terminus of NHE3. Magnification: A, 46,400×; B, 54,300×; C, 60,000×; and D, 54,300×.
that anti-NHE3 mAbs can immunoprecipitate NHE3 after exposure to intact BBMV confirms that a COOH-terminal region of NHE3 must be exposed on the exoplasmic surface of microvilli.

**DISCUSSION**

Using mAbs to at least two distinct epitopes of the Na\(^+\)-H\(^+\) exchanger isoform NHE3, we have demonstrated that regions of the carboxyl-terminal hydrophilic domain are exposed on the exoplasmic surface of the plasma membrane. The topology of the mAb epitopes was shown by four independent methods: 1) antibody binding to intact brush border membrane vesicles as detected by ELISA, 2) affinity isolation of right-side-out microvillar vesicles by immobilized antibody, 3) localization of antibody binding by immunogold labeling of ultrathin cryosections of PLP-fixed kidney examined by electron microscopy, and 4) immunoprecipitation of NHE3 after antibody exposure to intact brush border vesicles with removal of unbound antibody prior to solubilization. These findings indicate that the antibody epitopes, one between amino acids 702 and 756 and the other(s) between amino acid 756 and the COOH terminus, must be exposed on the external surface of the plasma membrane.

Algorithms that predict hydropathy have been applied to cloned Na\(^+\)-H\(^+\) exchangers (NHE1–4) and have provided the same general model (2–4). In brief, the amino-terminal 60% of the protein is very hydrophobic and is predicted to have multiple (10–12) membrane-spanning \(\alpha\)-helical segments. The remaining carboxyl-terminal portion is relatively hydrophilic and has no hydrophobic stretches that are of sufficient length (15–20 residues) to constitute membrane-spanning \(\alpha\)-helices. The COOH-terminal hydrophilic domains of NHE proteins contain sites for phosphorylation by protein kinases (6, 8) and for binding to intracellular proteins such as calmodulin (7, 10, 28). Moreover, antibody studies indicate that at least a portion of the COOH-terminal hydrophilic domain of NHE1 is cytoplasmic (3). Given the absence of predicted membrane-spanning \(\alpha\)-helical segments in this region, it has been assumed that the entire COOH-terminal hydrophilic domain of NHE proteins must be cytoplasmic (3). Our findings clearly indicate that this assumption is not correct, at least for the case of NHE3.

Studies of NHE proteins have indicated that the portion of the COOH-terminal hydrophilic domain just after the hydrophobic domain (approximately from amino acids 550–690) is most critical for regulation of NHE activity in response to intracellular signaling mechanisms. For example, amino acids 636–656 represent the high affinity calmodulin binding region of NHE1 (10, 28). A critical region including amino acids 559–661 is essential for protein kinase A activation of a NHE1 homologue in trout red cells (5). Likewise, a region encompassing amino acids 579–684 is essential for mediating protein kinase A inhibition of NHE3 activity (6). These data predict that there must be at least one membrane-spanning segment between the protein kinase A regulatory site and the more distal exoplasmic epitopes identified by our mAbs. It should also be noted that Levine and co-workers (9) have shown that truncating NHE3 at amino acid 756 eliminates the effects of calmodulin on this isoform. However, it is unclear if the calmodulin binding site is located in the COOH terminus (amino acids 756–832) or if the truncation results in allosteric inhibition of binding in other more proximal regions of the protein. If the calmodulin binding site of NHE3 is located between amino acids 756–832, then the data predict a second membrane-spanning segment between the exoplasmic epitope of mAb 2B9 and the presumably internal calmodulin binding site.

Although membrane-spanning \(\alpha\)-helical structure in the C-terminal hydrophilic region of the NHEs is not predicted by hydropathy analysis (29), it is important to note that \(\beta\)-structures, which are not readily identified by such methods, can also span lipid bilayers. In bacteria, some pore-forming proteins such as maltoporin have membrane-spanning domains that consist largely of anti-parallel \(\beta\)-barrels (30). \(\beta\)-Structures may also be important in forming membrane-spanning domains in eukaryotic proteins. For example, the nicotinic acetylcholine receptor in the electroplaque membranes of *Toxopha*rd is the best characterized member of a large family of neurotransmitter-gated ion channels. Hydropathy analysis predicts each of the five subunits to consist of four membrane-spanning \(\alpha\)-helices (31). However, circular dichroism studies predict insufficient \(\alpha\)-helical structure to account for the predicted number of membrane-spanning domains (32). In addition, structural analysis by electron crystallography has suggested that the membrane-spanning regions of the acetylcholine receptor consist of both \(\alpha\)-helices and \(\beta\)-sheets (33). Accordingly, it has been suggested that the NHE proteins may contain membrane-spanning \(\beta\)-sheet structures (34), which might include one or more membrane-spanning segments in the distal regions of the COOH-terminal hydrophilic domain of NHE3.

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