Identification of Sites in the Second Exomembrane Loop and Ninth Transmembrane Helix of the Mammalian Na⁺/H⁺ Exchanger Important for Drug Recognition and Cation Translocation*

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Mammalian Na⁺/H⁺ exchanger (NHE) isoforms are differentially sensitive to inhibition by several distinct classes of pharmacological agents, including amiloride- and benzoyl guanidinium-based derivatives. The determinants of drug sensitivity, however, are only partially understood. Earlier studies of the drug-sensitive NHE1 isoform have shown that residues within the fourth membrane-spanning helix (M4) (Phe165, Phe166, Leu167, and Gly174) and a 66-amino acid segment encompassing M9 contribute significantly to drug recognition. In this report, we have identified two residues within M9, one highly conserved (Glu356) and the other non-conserved (Gly356), that are major determinants of drug sensitivity. In addition, residues in the second exomembrane loop between M3 and M4 (Glu152, Phe157, and Pro158) were also found to modestly influence drug sensitivity. A double substitution of crucial sites within M4 and M9 of NHE1 with the corresponding residues present in the drug-resistant NHE3 isoform (i.e. L167F/G356A) greatly reduced drug sensitivity in a cooperative manner to levels nearing that of wild type NHE3. The above mutations did not appreciably affect Na⁺/H⁺ affinity but did markedly decrease the catalytic turnover of the transporter. These data suggest that specific sites encompassing M4 and M9 are critical determinants of both drug recognition and cation translocation.

Na⁺/H⁺ exchangers (NHE)¹ are present at the cell surface and various organelar compartments of mammalian cells and mediate the electroneutral exchange of Na⁺ for H⁺, a process driven by the relative concentration gradients of the respective cations. To date seven distinct isoforms (NHE1 to NHE7) have been isolated that share ~20–70% amino acid identity (calculated Mf, ranging from ~74,000 to 93,000) and exhibit similar membrane topologies, with 12 predicted N-terminal membrane-spanning (M) α-helices and a large C-terminal cytoplasmic region (1–5). They show considerable differences in their properties, sensitivity to pharmacological antagonists, and responsiveness to various signaling pathways. Consistent with their molecular diversity, the exchangers participate in a broad spectrum of physiological processes, including the regulation of intracellular pH (pHₗ), maintenance of cell volume, and transepithelial transport of electrolytes. In addition, activation of certain exchangers appear to facilitate cellular growth and proliferation in response to numerous growth factors and other mitogens and are associated with events leading to apoptosis (6–8).

The NHE is a known target for inhibition by the diuretic compound amiloride and its analogues (9). Amiloride analogues containing hydrophobic substituents on the 5-amino group of the pyrazine ring, such as 5-(N-ethyl-N-isopropyl) amiloride (EIPA), have higher affinity and specificity for NHE relative to other ion transporters. Comparison of the NHE isoforms in heterologous expression systems show that they have varying affinities for amiloride and its analogues that span more than 2 orders of magnitude, with the following order of sensitivity: NHE1 ≥ NHE2 > NHE5 > NHE3 (10–12). NHE4 also has an apparent low affinity for many of these antagonists, but its activity in transfected fibroblasts can only be detected under specialized experimental conditions (13, 14) that preclude direct comparisons with other ectopically expressed isoforms. Recently, novel benzoyl guanidinium compounds (e.g. HOE694, HOE642 or cariporide, and EMD85131) have been developed that inhibit the NHE isoforms with a similar rank order but over a larger concentration range (3–4 orders of magnitude) (15–18). The more selective binding properties of these compounds for NHE1 have been exploited therapeutically as effective agents in the treatment of cardiac ischemia and reperfusion injuries (17–22) and may prove beneficial in the prevention of diabetes-induced vascular hypertrophy (23). More recently, a preferential antagonist (S3226) of NHE3 has been synthesized that may also facilitate functional studies of this isoform in renal and intestinal epithelia (24, 25).

Biochemical analyses indicate that inhibition by amiloride compounds (26) and HOE694 (15) is reduced by high external Na⁺ (Na⁺ₑ). This competitive inhibition suggests they bind near the Na⁺ₑ transport site and may also share a common site. However, under chloride-free buffer conditions, amiloride and its derivatives also inhibit transport noncompetitively, suggesting that the Na⁺ₑ and amiloride binding sites may not be identical (27, 28). Furthermore, the Na⁺ₑ- and amiloride-binding sites can be altered independently of each other using genetic selection techniques (29). Taken together, these data indicate that amiloride and other antagonists probably interact with multiple sites on the exchanger.

Consistent with the above notion, mutational analyses have identified residues in the predicted fourth membrane-spanning helix (M4) of NHE1 (Phe161, Leu163, and Gly174) of human

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¹ The abbreviations used are: NHE, Na⁺/H⁺ exchanger; EIPA, 5-(N-ethyl-N-isopropyl) amiloride.

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NHE1) that confer sensitivity to amiloride and its analogues but do not seemingly affect Na\textsuperscript{+} affinity (30, 31). By contrast, mutation of a neighboring residue, Phe\textsuperscript{162}, was found to decrease affinities for both Na\textsuperscript{+} and HOE642 (32). In addition, pharmacological analyses of chimeras of rat NHE1 and NHE3 defined a 66-amino acid segment encompassing M9 and its adjacent loops (residues 327–392 of rat NHE1) as a major determinant of the differential drug sensitivity between these two isoforms (16). Homologous substitution of this region between NHE1 and NHE3 caused a reciprocal change in their determinants of the differential drug sensitivity between these adjacent loops (residues 327–392 of rat NHE1) as a major determinant of the differential drug sensitivity.

The objective of the present study was to define specific residues that may contribute to the large difference in drug sensitivity between NHE1 and NHE3. The results show that sites within transmembrane α-helix M9 as well as between helices M3 and M4 are significant determinants of drug recognition. These sites were also found to be important for optimal cation translocation. These data provide additional insight in NHE drug interactions and may aid in the design of more potent isoform-specific drugs with therapeutic potential.

**EXPERIMENTAL PROCEDURES**

**Materials**—Carrier-free \textsuperscript{22}NaCl (radioactivity, 5 mCi/ml) was obtained from PerkinElmer Life Sciences. Amiloride and ouabain were purchased from Sigma, and the amiloride derivative EIPA was obtained from British Drug House Inc. (St. Laurent, Quebec) or Fisher Scientific (Pittsburgh, PA). Potassium perchlorate (K\textsubscript{2}SO\textsubscript{4}) was purchased from PerkinElmer Life Sciences. Amiloride and ouabain were used as 5 mM solutions, and EIPA was used as 2 mM solution.

**Potassium-Selective Drugs**—The more NHE-selective drugs, EIPA and HOE644, were used at 2 mM concentrations containing the K\textsuperscript{+}-K\textsuperscript{+} exchange inhibitor HEPES-K\textsuperscript{+}-ATPase. Influx of \textsuperscript{22}Na\textsuperscript{+} was terminated by rapidly washing the cell monolayers three times with four volumes of ice-cold isotonic saline solution (130 mM Na\textsuperscript{+}, 1 mM Mg\textsuperscript{2+}, 2 mM Ca\textsuperscript{2+}, 20 mM HEPES-NaOH, pH 7.4). The washed cell monolayers were solubilized in 0.25 ml of 0.5 M NaOH, and the wells were washed with 0.25 ml of 0.5 M HCl. Both the solubilized cell extract and wash solutions were added to vials, and radioactivity was assayed using an enzyme-linked immunosorbent assay (ELISA) plate reader. The results are expressed as the difference between the initial rates of H\textsuperscript{+}–exchange specific to the Na\textsuperscript{+}/H\textsuperscript{+} exchanger and the Na\textsuperscript{+}–K\textsuperscript{+}–ATPase.

**Influx Measurements**—The cells were grown to confluence in 24-well plates. NHE activity was determined by the calcium phosphate-DNA coprecipitation technique of Chen and Okayama (37). After 48 h after transfection, the AP-1 cells were selected for survival in response to repeated (5–6 times over a 2-week period) acute NH\textsubscript{4}Cl-induced acid loads (i.e., H\textsuperscript{+}–killing technique) (10, 38) to discriminate between Na\textsuperscript{+}/H\textsuperscript{+}–exchanger positive and negative transfectants. The positive clones for each transfectant were pooled and used for subsequent analyses.

**Immunoblotting**—Stably transfected cells were grown to confluence in 60-mm dishes and lysed with 1% Triton X-100. Total cellular protein extracts (30 μg) were resolved by 6% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech). The blots were briefly rinsed with phosphate-buffered saline (PBS), blocked with 5% nonfat skim milk in PBST (phosphate-buffered saline with 0.1% Tween 20), and then incubated with a rabbit polyclonal anti-NHE1 antibody (dilution 1:5,000). After extensive washes with PBST, the blots were incubated with anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (Amersham Pharmacia Biotech) recorded as enhanced chemiluminescence (Amersham Pharmacia Biotech) recorded on x-ray film. For quantitation of protein levels, the amount of protein was titrated in preliminary experiments, and the film exposure times were selected to ensure that the chemiluminescent signals were within the linear range of the x-ray film.

**RESULTS**

**Determinants of Drug Sensitivity in M9**—Earlier studies have identified sites within M4 (Pha\textsuperscript{165}Pha\textsuperscript{166}Leu\textsuperscript{167}Gly\textsuperscript{178}) numbered according to the rat NHE1 sequence) and a 66-amino acid segment encompassing M9 (residues 327–392) as important elements of NHE drug recognition. The location of these sites are depicted in a secondary structural model of rat NHE1 (Fig. 1), as defined by recent topological mapping studies (41). The region surrounding M9 is of particular interest because it appears to account for large differences (2–3 orders of magnitude) in the differential drug sensitivity of NHE1 (high affinity) and NHE3 (low affinity) (16) and is proposed to form part of the pore-lining region with M4 (41). These two isoforms share ~50% amino acid identity in this region, but the precise...
Determination of non-conserved sites encompassing transmembrane helix M9 that confer differences in drug sensitivity between NHE1 and NHE3. A, comparison of amino acid sequences of rat NHE1 and NHE3 in the region containing transmembrane helix M9. The shaded boxes indicate sites in NHE1 that were mutated to the equivalent residues in NHE3. The gray and black shading indicates those sites that, when mutated, had either no effect or had significantly altered drug sensitivity, respectively. B–D, AP-1 cells stably expressing either wild type NHE1, wild type NHE3, or mutant NHE1/G356A were grown to confluence in 24-well plates. Before Na\(^{+}\) influx measurements, the cells were washed with Na\(^{-}\)-free isotonic choline chloride solution and then incubated in assay medium containing carrier-free NaCl (1 μCi/ml) and increasing concentrations of amiloride (B), EIPA (C), or HOE694 (D) (for details, see “Experimental Procedures”). Data were normalized as a percentage of the maximal rate of Na\(^{+}\) influx. Values represent the average of two or three experiments, each performed in triplicate.
Drug Binding Determinants of the Na\(^+\)/H\(^+\) Exchanger

Table I

| Transfectants | Rates of Transport | n |
|---------------|-------------------|---|
| Untransfected AP-1 cells | 49 ± 14 | 4 |
| NHE1, wild type | 12,482 ± 3,665 | 12 |
| G152A | 12,108 ± 3,984 | 2 |
| P157S/P158F | 13,383 ± 3,355 | 12 |
| L167F | 20,596 ± 7,010 | 11 |
| H329A | 18,457 ± 1,405 | 4 |
| L336G | 7,848 ± 739 | 4 |
| Y341I | 4,617 ± 350 | 4 |
| Y342A | 15,815 ± 1,764 | 4 |
| Y343N | 9,372 ± 771 | 4 |
| Y346N | 7,484 ± 275 | 4 |
| Y346D | 6,672 ± 328 | 4 |
| Y346D | 3,750 ± 456 | 4 |
| E350N | 3,204 ± 574 | 4 |
| E350D | 6,678 ± 515 | 4 |
| E350Q | 2,035 ± 590 | 12 |
| F352L | 2,794 ± 170 | 4 |
| S355A | 2,204 ± 128 | 4 |
| G356A | 9,103 ± 1,685 | 12 |
| G356S | 5,628 ± 299 | 4 |
| G356L | 490 ± 20 | 2 |
| G356D | 4,299 ± 829 | 4 |
| G356K | 350 ± 24 | 2 |
| L167F/G356A | 11,605 ± 4,191 | 11 |
| I361T | 1,906 ± 744 | 2 |
| A362F | 5,870 ± 589 | 2 |
| V366C/M367C | 13,807 ± 1,286 | 4 |
| P669K | 8,603 ± 612 | 4 |
| Y370A | 14,571 ± 1,140 | 4 |
| E372K | 12,404 ± 1,094 | 4 |
| N374A | 7,985 ± 450 | 4 |
| S376A | 16,928 ± 1,217 | 4 |
| H377E | 7,546 ± 886 | 3 |
| S379A | 14,190 ± 1,140 | 4 |
| H380A | 8,587 ± 376 | 4 |
| T381A/T382A | 3,660 ± 445 | 4 |
| Y385A | 1,786 ± 272 | 4 |
| F386T | 7,989 ± 716 | 2 |
| W390L | 10,433 ± 540 | 4 |
| V393G | 11,631 ± 1,183 | 4 |
| NHE3, wild type | 13,970 ± 2,923 | 6 |
| A305G | 12,589 ± 2,946 | 4 |

Values represent raw data for rates of carrier-free \(^{22}\text{Na}\) influx of untransfected or stably transfected AP-1 cells expressing wild type or mutant NHEs after intracellular acidification with a prepulse of 50 mM NH\(_4\)Cl (mean ± S.D. of n determinations).

Critical Sites in M3-M4 Region

Table I illustrates the rates of transport for wild type and mutant exchangers. As illustrated in Fig. 8, the rates of drug-resistant mutants are modestly reduced (−50%) in their rates of transport in the presence of low concentrations of EIPA. It was also noted that the activities of the E350Q/D/N mutants could not be completely blocked by amiloride or EIPA, achieving only 80–85% inhibition at the highest concentrations tested, whereas wild type NHE1 and NHE3 were rendered inactive.

To test this possibility, these sites were mutated in NHE1 to the corresponding residues present in NHE3 (i.e. single G152A and double P157S/P158F substitutions). For comparison, a previously characterized mutation within M4 (L167F) that is known to reduce drug sensitivity was also constructed (30). As shown in Fig. 6B and summarized in Table II, both the single G152A and dual P157S/P158F substitutions modestly reduced sensitivity to EIPA by 3- and 7-fold, respectively. Consistent with previous studies, the L167F mutation significantly reduced (−30-fold) the effectiveness of EIPA to block transport. These data directly affect internal H\(^+\) affinity, i.e. (E350Q; G356A) were also measured as a function of the Na\(^+\) concentration for wild type and mutant exchangers, as illustrated in Fig. 8, the rates of \(^{22}\text{Na}\) influx gradually approached saturation, with increasing Na\(^+\) concentrations for wild type and mutant exchangers, consistent with simple Michaelis-Menten kinetics. Analysis of the data using a hyperbolic fit function yielded apparent Na\(^+\) affinity constants (K\(_{\text{Na}}\)) for the mutants that were not appreciably different from wild type (Table III) and corroborated earlier findings for L167F. Thus, the new drug-sensitive sites identified herein do not contribute to Na\(^+\) binding but, rather, are more likely involved in non-competitive drug interactions.

Kinetic Properties of Drug-resistant Mutant Na\(^+\)/H\(^+\) Exchangers—To evaluate whether the above mutations had additional functional consequences other than affecting drug sensitivity, we performed a detailed comparison of their intrinsic kinetic properties (cation affinities and catalytic turnover). Earlier kinetic measurements indicate that NHE antagonists can function as either simple competitors (26) or, under Cl\(^−\)-free conditions, mixed competitors (27, 28) of external Na\(^+\) binding. Consistent with these analyses, mutation of certain sites that decreased drug sensitivity (Leu\(^{167}\), Gly\(^{178}\), and His\(^{353}\)) were found to have no demonstrable effect on Na\(^+\) affinity (30, 31, 33), whereas mutation of a neighboring residue, F166S, influenced both Na\(^+\) and drug affinities (32). Hence, it was of interest to examine whether the single (E350Q; G356A) or double (P157S/P158F; L167F/G356A) substitutions generated in this study also influenced Na\(^+\) affinity. For comparative purposes, the previously characterized L167F mutation was included in the analysis. NHE activity, defined as the initial rates of EIPA-inhibitable H\(^+\)-activated \(^{22}\text{Na}\) influx, was measured as a function of the Na\(^+\) concentration in the wild type and mutant exchangers. As illustrated in Fig. 8, the rates of \(^{22}\text{Na}\) influx gradually approached saturation, with increasing Na\(^+\) concentrations for wild type and mutant exchangers, consistent with simple Michaelis-Menten kinetics. Analysis of the data using a hyperbolic fit function yielded apparent Na\(^+\) affinity constants (K\(_{\text{Na}}\)) for the mutants that were not appreciably different from wild type (Table III) and corroborated earlier findings for L167F. Thus, the new drug-sensitive sites identified herein do not contribute to Na\(^+\) binding but, rather, are more likely involved in non-competitive drug interactions.

The transport activities of the M9 drug-resistant mutants (i.e. E350Q and G356A) were also measured as a function of pH to assess whether H\(^+\) affinity was affected. The residues in the exomembranous loop between transmembrane helices M3 and M4 were not examined because they are less likely to directly affect internal H\(^+\) sensitivity. As shown in Fig. 9, both mutations had no appreciable effect on the H\(^+\) dependence of the exchanger over the range of pH, 5.4–7.4. Similarly, other
Comparison of drug inhibition constants of wild type and mutant rat Na⁺/H⁺ exchangers

Values for half-maximal inhibition (\(K_{50}\)) were determined from the logit transformation of the sigmoidal inhibition data presented in Figs. 2–7. The transformation involved plotting the In[P/(100 − P)] (where P represents the percentage of inhibition) as a function of the log[inhiber]. The \(K_{50}\) is the concentration when logit = 0. Values represent the mean ± S.D. of two to three separate experiments, each performed in quadruplicate. ND, not determined.

| Exchanger  | Amiloride  | EIPA         | HOE694       |
|------------|------------|--------------|--------------|
| NHE1       | 2.5 ± 0.1 \times 10^{-6} | 1.1 ± 0.2 \times 10^{-8} | 1.2 ± 0.3 \times 10^{-7} |
| NHE1/G152A | ND         | 3.2 ± 0.4 \times 10^{-8} | ND           |
| NHE1/P157R/P158F | ND          | 7.9 ± 0.8 \times 10^{-8} | ND           |
| NHE1/L167F | 7.5 ± 1.6 \times 10^{-6} | 3.2 ± 0.8 \times 10^{-7} | 4.5 ± 0.9 \times 10^{-6} |
| NHE1/E350Q | 5.0 ± 0.7 \times 10^{-5} | 1.4 ± 0.4 \times 10^{-6} | ND           |
| NHE1/E350D | ND         | 5.0 ± 3.5 \times 10^{-6} | ND           |
| NHE1/E350N | ND         | 6.3 ± 1.2 \times 10^{-7} | ND           |
| NHE1/G356A | 1.2 ± 0.1 \times 10^{-5} | 3.6 ± 0.3 \times 10^{-7} | 4.1 ± 2.6 \times 10^{-5} |
| NHE1/G356S | ND         | 7.9 ± 2.6 \times 10^{-7} | ND           |
| NHE1/G356D | ND         | 3.6 ± 0.4 \times 10^{-7} | ND           |
| NHE1/L167F/G356D | ND     | 1.8 ± 0.3 \times 10^{-6} | ND           |
| NHE3/A305G | ND         | 7.9 ± 0.8 \times 10^{-7} | ND           |
| NHE3      | 1.1 ± 0.2 \times 10^{-4} | 3.2 ± 0.3 \times 10^{-6} | 6.3 ± 1.5 \times 10^{-4} |

![Image](93x391 to 253x511)

**Fig. 3.** Effect of different substitutions of Gly356 on the drug sensitivity of NHE1. AP-1 cells separately expressing wild type (NHE1, NHE3) and mutant (NHE1/G356S, NHE1/G356D) exchangers were grown to confluence in 24-well plates, and their inhibition was assessed by varying the concentrations of EIPA. Transport activity was measured as described in the legend of Fig. 2. Values represent the average of two or three experiments, each performed in quadruplicate.

![Image](98x199 to 248x317)

**Fig. 4.** Effect of mutation of A305 on the drug sensitivity of NHE3. AP-1 cells separately expressing wild type (NHE1, NHE3), and mutant (NHE3/A305G) exchangers were grown to confluence in 24-well plates, and their inhibition was assessed by varying the concentrations of EIPA. Transport activity was measured as described in the legend of Fig. 2. Values represent the average of three experiments, each performed in quadruplicate.

substitutions at these sites (i.e. E350D, E350N, G356S, and G356D) did not alter H⁺ affinity (data not shown).

Last, we assessed whether the catalytic turnover of the mutated transporters was affected. To measure this parameter, it was necessary to estimate the quantity of mature, fully glycosylated NHE1 transporter at the cell surface (separate from the immature, core-glycosylated transporter that resides within intracellular compartments) of the different stable transfectants and express these values in relation to the cellular rates of H⁺-activated ²²Na⁺ influx under near maximal acid-load conditions. This was accomplished immunologically by Western blot analysis using an affinity-purified rabbit anti-NHE1 antibody directed to the cytosolic domain of the exchanger followed by densitometry of the chemiluminescent signals recorded on x-ray film. To obtain a reasonably accurate measure of cellular NHE1 levels, the amount of total cellular protein analyzed by Western blotting was titrated in preliminary experiments, and film exposure times were varied to ensure that the chemiluminescent signals were within the linear range of the x-ray film. Representative immunoblots are shown in Fig. 10A. The anti-NHE1 antibody recognized two major bands, a slower migrating, fully glycosylated form with an apparent molecular mass of ~100 kDa that has been demonstrated by biochemical and immunological means to reside at the plasma-membrane (42, 43) and an immature core-glycosylated form of the protein of ~75 kDa that resides intracellularly, presumably within the endoplasmic reticulum. A faint, nonspecific band of ~62 kDa was consistently observed in all cell extracts and served as a convenient indicator of equal protein loading on the gels. Among the various mutants, it was noted that the upper protein band for NHE1/G356A migrated more diffusely, suggestive of incomplete glycosylation, albeit minor. A more drastic effect was observed for the NHE1/G356L mutant that showed a marked reduction in the level of the fully glycosylated, plasmalemman form and a corresponding increase in the core glycosylated form. It is likely that this substitution destabilized the protein, resulting in incomplete processing and retention in endomembrane compartments. Densitometric analysis of the fully glycosylated band and normalization of the data to that of the wild type exchanger is shown in Fig. 10B. In general, the majority of the mutants proteins were expressed at equivalent or higher levels relative to the wild type transporter in stably transfected cells but generally displayed equivalent or lower rates of H⁺-activated ²²Na⁺ influx on a per-cell basis (Fig. 10C). To estimate the relative activity or turnover of the transporters, the cellular rates of ²²Na⁺ influx were expressed as a function of their respective plasmalemman protein levels (Fig. 10D). Of the various NHE1 constructs, only the NHE1/L167F mutant had a level of activity that was comparable with wild type. All the others showed a marked reduction in activity, suggesting that although these sites do not affect Na⁺ or H⁺ affinity, they do influence the velocity of the transporter and, hence, may be important in conformational changes underlying cation translocation.
DISCUSSION

The aim of this study was to further define the structural elements involved in drug recognition by mammalian Na+/H+ exchangers. Prior analyses of chimeras of the drug-sensitive NHE1 and drug-resistant NHE3 isoforms revealed a 66-amino acid sequence containing transmembrane helix M9 as a major segment responsible for drug recognition (16). The present study further demarcates this region by showing that Gly356 of NHE1, which is thought to reside within M9, is a crucial determinant of NHE isoform-specific drug sensitivity. Mutation of this amino acid to the corresponding residue present in NHE3 (i.e. Ala305) substantially reduced drug recognition. Significantly, the effects were greatest for the more NHE-selective drugs, EIPA and HOE694, rather than amiloride, which is consistent with previous analyses of the NHE1/3 chimeras (16). The more substantial changes for EIPA compared with amiloride suggest that Gly356 either directly or indirectly influences the interaction of the 5-amino-substituted moiety of EIPA with this region. This may equally explain the enhanced sensitivity to HOE694, which is structurally similar to EIPA and also has a large substituent group at an equivalent position of its benzoic ring. Conversely, the reciprocal mutation in NHE3 (A305G) caused a modest increase in drug sensitivity. Although the magnitude of this increase for NHE3/A305G is less than the reduction of drug sensitivity for NHE1/G356A, the result is nevertheless significant since a gain-of-function mutation is more indicative of a site that directly contributes to drug recognition than a loss-of-function mutation, which could cause nonspecific alterations in protein structure. The relevance of this site in conferring isoform-specific drug sensitivity is further indicated by the absence of an effect of mutations at neighboring amino acids that differ between NHE1 and NHE3. It is also noteworthy that NHE5, which shows drug affinities that are intermediate to NHE1 and NHE3 (12), also has an alanine residue at the equivalent position (i.e. Ala301). Taken together, these data support the critical importance of this site in drug recognition and possibly binding.
and Asn) were used that would be expected to minimally per-

...ering given that isosteric or conservative replacements (Gln, Asp,

... reduction in drug sensitivity. This effect was particularly strik-

... performed in quadruplicate. The apparent affin-

| NHE1           | $K_{Na}$ (nmol/min/mg of total cellular protein) |
|----------------|-----------------------------------------------|
| Wild type      | 13.2 ± 1.7                                   |
| P157S,P158F    | 12.4 ± 1.6                                   |
| L167F         | 12.4 ± 2.5                                   |
| E350Q        | 15.0 ± 1.7                                   |
| G356A        | 16.3 ± 0.9                                   |
| L167F,G356A  | 14.2 ± 1.3                                   |

In addition to Gly$^{356}$, residues that are divergent between NHE1 and NHE3 in the exomembrane loop between M3 and M4 were also found to influence drug sensitivity. Substitutions of Gly$^{152}$, Pro$^{157}$, and Pro$^{158}$ in NHE1 with the corresponding residues present in NHE3, Ala, Ser, and Phe, respectively, moderately reduced drug sensitivity. We also corroborated earlier findings that replacement of Leu$^{147}$ with Phe (present at the equivalent position of NHE3) in M4 also significantly reduced drug sensitivity (30). Indeed, this site seems to be generally important for drug recognition by the NHEs, since mutagenesis of the equivalent residue in rabbit NHE2 (L143F) also reduced its sensitivity to amiloride compounds (44). To further confirm the involvement of transmembrane helices M4 and M9 in drug recognition, we combined the L167F and G356A mutations. Significantly, this dual substitution caused a synergistic reduction in drug sensitivity of NHE1 to levels closely approaching those observed for wild type NHE3. Thus, these two sites are sufficient to account for much of the difference in drug sensitivity between NHE1 and NHE3 and possibly other isoforms as well. Taken together, these data now extend the elements involved in isoform-specific drug recognition to include not only those sites within M4 but also those that reside on the exofacial surface between M3-M4 and within M9.

Aside from these sites, we also identified a highly conserved residue (Glu$^{350}$) in M9 that when mutated caused a profound reduction in drug sensitivity. This effect was particularly striking given that isosteric or conservative replacements (Gln, Asp, and Asn) were used that would be expected to minimally per-

... turb protein structure. Substitutions of other conserved residues flanking this site had no effect on drug sensitivity, confirming an important role for this particular site in drug recognition and one that may be shared among all mammalian NHEs, including plasmalemmal (NHE1–5) as well as the more distantly related organellar (NHE6–7) isoforms.

To determine whether these sites were involved in other aspects of exchanger function, we performed a detailed kinetic analysis of the various mutants. Previous studies show that the amiloride-based compounds display either simple-competitive (26, 45, 46) or mixed-competitive (27, 28) inhibition at the external Na$^+$ transport site, suggesting that the external Na$^+$ and amiloride binding sites may not be identical. Furthermore, certain guanidinium derivatives that block Na$^+/H^+$ exchange by competing with Na$^+_o$ were not effective competitors of [3H]ethylpropyl amiloride binding to the exchanger (47), again implicating the involvement of at least two discrete sites in drug binding. In this regard, kinetic analyses of point mutations of NHE1 at positions Leu$^{167}$ (30) and Gly$^{356}$ (31) showed no change in Na$^+$ affinity, although a combined mutation did produce a modest 2-fold reduction in Na$^+$ affinity ($K_{Na}$ 14 to 28 mM) (31). More significantly, mutation of a neighboring residue, F166S (equivalent to F162S in human NHE1), was found to substantially decrease both Na$^+_o$ affinity (−11-fold) and sensitivity to HOE642 (−1500-fold), although affinities for other transportable cations such as Li$^+$ and H$^+$ or the inhibitor guanidinium were unaffected (32). In the present report, mutations that affected drug sensitivity (P157S/P158F; L167F; E350Q; G356A; and L167F/G356A) did not affect Na$^+_o$ affinity. In the cases of E350Q/D/N and G356A/S/D, intracellular H$^+$ concentrations over the range of pH 7.4–5.4. The pH$_i$ was adjusted by the potassium nigericin method, as described in “Experimental Procedures.” To facilitate comparison of the effects of mutating these sites, data were normalized to the maximal uptake rates. Values represent the average of two experiments, each performed in quadruplicate.

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In summary, we have defined several distinct sites between helices M3-M4 (Gly152-Pro157 Pro158) and within M9 (Gly356) that partly account for the differential drug sensitivities between NHE1 and NHE3 and which may apply to other isoforms as well. Significantly, mutations of Gly356 appear to have a greater effect on binding of the more NHE-selective antagonists such as EIPA when compared with amiloride. In addition, we have identified a highly conserved glutamate residue (Glu350) in the ninth transmembrane helix that is also a critical determinant of drug recognition by mammalian NHEs. Mechanistically, these sites are not involved in the competitive interaction between Na\(^{+}\) and drug binding but, rather, appear to contribute significantly to efficient cation translocation. Further molecular dissection of the drug binding region should facilitate the rational design of more potent and isoform-specific drugs that may be therapeutically beneficial in the treatment of certain human diseases.

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Identification of Sites in the Second Exomembrane Loop and Ninth Transmembrane Helix of the Mammalian Na$^+/H^+$ Exchanger Important for Drug Recognition and Cation Translocation

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