

Evolutionary origins of eukaryotic sodium/proton exchangers

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Brett, Christopher L., Mark Donowitz, and Rajini Rao. Evolutionary origins of eukaryotic sodium/proton exchangers. *Am J Physiol Cell Physiol* 288: C223–C239, 2005; doi:10.1152/ajpcell.00360.2004.—More than 200 genes annotated as Na⁺/H⁺ hydrogen exchangers (NHEs) currently reside in bioinformation databases such as GenBank and Pfam. We performed detailed phylogenetic analyses of these NHEs in an effort to better understand their specific functions and physiological roles. This analysis initially required examining the entire monovalent cation proton antiporter (CPA) superfamily that includes the CPA1, CPA2, and NaT-DC families of transporters, each of which has a unique set of bacterial ancestors. We have concluded that there are nine human NHE (or SLC9A) paralogs as well as two previously unknown human CPA2 genes, which we have named HsNHA1 and HsNHA2. The eukaryotic NHE family is composed of five phylogenetically distinct clades that differ in subcellular location, drug sensitivity, cation selectivity, and sequence length. The major subgroups are plasma membrane (recycling and resident) and intracellular (endosomal/TGN, NHE8-like, and plant vacuolar). HsNHE1, the first cloned eukaryotic NHE gene, belongs to the resident plasma membrane clade. The latter is the most recent to emerge, being found exclusively in vertebrates. In contrast, the intracellular clades are ubiquitously distributed and are likely precursors to the plasma membrane NHE. Yeast endosomal ScNHX1 was the first intracellular NHE to be described and is closely related to HsNHE6, HsNHE7, and HsNHE9 in humans. Our results link the appearance of NHE on the plasma membrane of animal cells to the use of the Na⁺/K⁺-ATPase to generate the membrane potential. These novel observations have allowed us to use comparative biology to predict physiological roles for the nine human NHE paralogs and to propose appropriate model organisms in which to study the unique properties of each NHE subclass.

Na⁺/H⁺ exchanger; NHX; cation proton antiporter; phylogenetic analysis

A BASIC PROPERTY OF LIFE is the ability of an organism to regulate cellular pH, volume, and ion composition. The transmembrane exchange of protons for sodium ions (Na⁺) is ubiquitous in organisms across all phyla and kingdoms, and underlies fundamental homeostatic mechanisms to control these ions. The family of Na⁺/H⁺ exchangers (NHEs) plays an important role in diverse physiological processes, including control of cell cycle and cell proliferation (114, 117), transepithelial Na⁺ movement (174), salt tolerance (93, 130), vesicle trafficking, and biogenesis (5, 22). In mammals, NHE dysfunction is associated with pathophysiological conditions that include hypertension, epilepsy, postischemic myocardial arrhythmia, gastric and kidney disease, diarrhea, and glaucoma (36, 106, 174). Drugs such as S8218 and cariporide, which target specific NHE isoforms, are used to reduce the duration of apnea in animal studies and in clinical trials for the prevention of cardiac ischemia-reperfusion injury, respectively (2, 23, 29). In plants, NHE family members are the principal determinants of salt tolerance and, as such, are of enormous importance to agriculture and biotechnology (93, 130).

In the past few years we have witnessed an explosive growth in the number of sequenced genomes that now await functional analysis. Automated annotation programs have identified

>200 candidate genes for NHEs in databases. The majority of these putative exchangers are bacterial homologs of NhaP antiporters, and the remaining eukaryotic homologs include members of the NHX, NHA/SOD, and SOS genes of yeast, plants, worms, and insects and the NHE (or SLC9A, solute carrier 9A; HUGO nomenclature, <http://www.gene.ucl.ac.uk/nomenclature>; Ref. 154) genes of mammals and fish. Collectively, these prokaryotic and eukaryotic genes encode the monovalent cation proton antiporter (CPA) superfamily of transporters as defined by Saier and colleagues in the Transport Protein Database (<http://tcdb.ucsd.edu/tcdb/>; Ref. 31).

The primary purpose of this study was to explore the evolutionary relations among members of the NHE family, use comparative biology to predict functions of uncharacterized genes, and identify appropriate model organisms for the study of NHE orthologs. Our analysis has confirmed the existence of a total of nine NHE paralogs in the human genome and identified two new human genes belonging to the CPA2 subgroup of antiporters. We also have identified structural features unique to orthologous NHE proteins within a distinct phylogenetic clade and summarized their functions across species spanning all phyla. Together, these studies provide new insights into the evolutionary origins and specific functions of human NHE paralogs.

DATABASE ANNOTATION: DEFINITION OF NHE

Currently, >550 NHE sequence entries have been identified by automated annotation projects on the basis of sequence

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conservation within 10 transmembrane spans that constitute TM3–TM12 within the NH₂-terminal half of proteins that perform Na⁺/H⁺ exchange. Candidate NHE sequences are identified on the basis of sequence similarity to conserved protein motifs, originally established by known sequences and reformed on the basis of newly found sequences. For example, four NHE-specific motifs are listed on the Conserved Domain Database at the National Center for Biotechnology Information (NCBI): 1) the Na₂H₂Exchanger motif (Pfam00999; PSSM-Id 25668), 2) the KOG3826 conserved domain for a subset of eukaryotic Na⁺/H⁺ antiporters (PSSM-Id 21605), 3) the KOG1966 conserved domain for a subset of eukaryotic sodium/hydrogen exchanger proteins (PSSM-Id 19752), and 4) the COG0025 conserved domain for prokaryotic NhaP-type antiporters (PSSM-Id 9901). Other sites use similar signature protein motifs to annotate NHE genes: the PRINT NAHEX-CHNGR protein family fingerprint (no. PR01084), the NaH₂exchanger InterPro sodium/hydrogen exchanger subfamily motif (no. IPR004709), and the a_{cpa1} and b_{cpa1} Tigr protein family motifs (TIGRFAM nos. TIGR00831 and TIGR00840, respectively). At the NCBI, Kef-type CPA2 genes are identified using the COG475 conserved domain for Kef-type K⁺ transporters (PSSM-Id 10348) and the COG4651 conserved domain for proteobacterial Kef-type K⁺ transport systems with a predicted NAD-binding component (PSSM-Id 13795). Initially, all of these motifs were used to identify or confirm identified NHE sequences used in this publication (please refer to Table 1S, published online as Supplementary Material¹ for this article at the *American Journal of Physiology-Cell Physiology* web site). However, postanalysis, we reevaluated the specificity of each conserved domain used. What makes each motif unique is the subset of NHE sequences used to generate the motif itself. Motifs that draw on confirmed NHE sequences are more stringent, e.g., KOG3826, which uses only known mammalian NHE sequences. In contrast, motifs that incorporate unconfirmed, more divergent NHE sequences are less stringent but identify more distantly related candidate sequences, e.g., Pfam00999, which uses nearly all identified NHE sequences. The inherent problem with the latter is that once more distant sequences are identified and incorporated, specificity for the sequences of original interest is lost. Indeed, certain members of both the CPA1 and CPA2 gene families have been identified by and incorporated into Pfam00999, a motif originally used to identify NHE sequences (entries identified by this motif are annotated as “Na⁺/H⁺ exchanger”). Thus Pfam00999 is actually a CPA superfamily conserved domain, and newly identified sequences found using this motif should be annotated as CPA genes, rather than the current use of “NHE,” which refers to a well-defined subset of the CPA1 gene family.

ALIGNMENTS AND SEARCHES

BLAST searches (7) were performed using known protein and mRNA sequences for HsNHE6, ScNHX1, ScNHA1, ScKHA1, and Mm-spermNHE. Predicted and known protein sequences of interest were obtained from (and confirmed using) the GenBank, RefSeq, PDB, SwissProt, PIR, PRF, EMBL,

and DDBJ databases. Gene names, species names, accession numbers, and family assignments of selected genes are listed in Table 1S. Multiple alignments were performed using ClustalW (70) and ClustalX (142). Unrooted trees were prepared according to the neighbor-joining method using Clustal, TreeView-PPC (110), and PAUP 4.0b10 (Sinauer, Sunderland, MA; <http://paup.csit.fsu.edu/index.html>). In all cases, bootstrapping was performed (100 replicates), and most nodes showed high confidence values with three noteworthy exceptions indicated (see Figs. 1, 2, and 4). In a few cases, conflicting gene names have been resolved, and where appropriate, gene names have been assigned in a manner that denotes function on the basis of family assignment and follows existing nomenclature.

MONOVALENT CPA SUPERFAMILY

To better understand the origins and extent of the eukaryotic NHE family, we undertook a phylogenetic analysis of the more diverse CPA superfamily of monovalent cation/proton antiporters that share a common transmembrane organization of 10–12 hydropathic helices with detectable sequence similarity (see above description of conserved domains). As shown in Fig. 1, the annotated CPA superfamily is subdivided into three gene families as defined by the Transport Protein Database established by Milton Saier and colleagues: CPA1 (2.A.36), CPA2 (2.A.37), and NaT-DC (3.B.1; Na⁺-transporting carboxylic acid decarboxylase), each of which has a unique set of ancestral prokaryotic genes (31). The CPA1 family includes bacterial NhaP transporters, and members of the CPA2 family share origins with prokaryotic NhaA and KefB, whereas the NaT-DC family includes bacterial MadA. Although the CPA1 and CPA2 families evolved and diversified in eukaryotes, to date only prokaryotic examples of the NaT-DC family have been identified. The latter mediate the transmembrane export of 1–2 Na⁺ in exchange for an extracellular H⁺ as part of a multiprotein complex catalyzing the decarboxylation of oxaloacetate, malonyl-CoA, or glutaconyl-CoA (26). The CPA1 family includes many well-studied examples of Na⁺/H⁺ exchangers from fungi, plants, and mammals and is discussed in detail below. In contrast, virtually all of the eukaryotic genes associated with the CPA2 family, including a novel cluster in animals that we have identified in the present work, are currently uncharacterized.

Figure 1 highlights the phylogenetic relations among members of the CPA superfamily, including representative members from *Escherichia coli* (EcKefB, EcNhaA, and EcYjcE) and *Arabidopsis* (AtKEA1, AtCHX1, AtSOS1, and AtNHX1) and all homologs from yeast (ScKHA1, ScNHA1, and ScNHX1) and humans (HsNHA1 and HsNHA2, HsNHE1–HsNHE9, Hs-spermNHE). The complete phylogenetic tree and accession numbers of individual genes can be found in the Supplemental Material available online (Fig. 1S and Table 1S). It is noteworthy that the recently discovered sperm-specific Na⁺/H⁺ exchangers found in mouse (156), rat, macaque, and human do not cluster with other mammalian NHE genes and only weakly associate with the NaT-DC clade. Because they have no distinct orthologs in nonmammalian genomes sequenced thus far, their evolutionary origins remain obscure. Bacterial antiporters of the NhaB, NhaC, and NhaD subgroups share a common origin within the IT (ion transporter) superfamily that includes diverse transporters for cations and anions

¹ Supplemental material for this article may be found at <http://ajpcell.physiology.org/cgi/content/full/00360.2004/DC1>.

and for organic and inorganic substrates (115) and are not represented here. However, we have found that bacterial NhaA shares common ancestry with the fungal NHA exchangers and falls within the CPA2 family.

The CPA2 Family

The CPA2 family consists of several clades, each consisting of prokaryotic members that share origins with newly identified plant and animal homologs, as shown in Fig. 2. The KefB and KefC genes of *E. coli* encode glutathione-gated K^+ efflux systems and are the closest bacterial homologs of an uncharacterized cluster of plant genes, including *Arabidopsis* KEA1–3. The plant CHX transporters, represented by 28 genes in *A. thaliana*, were recently reported to be preferentially expressed in the male gametophyte and sporophytic tissues and developmentally regulated during gametogenesis (88, 140). AtCHX17 is also present in the epidermal and cortical cells of mature root zones, and knockout mutant plants accumulate less K^+ in the root when stressed with salt or K^+ starvation, consistent with K^+ transport (28). Another clue to their function may come from their homology with the fungal KHA members: *Saccharomyces cerevisiae* KHA1 has been implicated in K^+/H^+ exchange (51, 120). The yeast NHA1/SOD2 family of $Na^+, K^+/H^+$ exchangers appear to be distantly related to the well-characterized bacterial NhaA antiporters and are also clustered with another subset of plant KEA, represented by KEA4–KEA6 in *Arabidopsis*. The fungal NHA clade includes ScNHA1, SpSOD2, and CtNHA1 (as well as ZrSOD2, PsNHA1, and PsNHA2; not shown). These transporters range in length from 698 to 1,085 amino acids, are found on the plasma membrane, and have been shown to be important for cytoplasmic Na^+ , K^+ , and pH homeostasis (14, 15, 75, 116, 137). Deletion of CaCNH1 in the pathogenic fungus *Candida albicans* resulted in unusual elongated cell morphology and retarded growth, even in the absence of salt stress (135). More recently, two regions within the cytoplasmic COOH terminus of ScNHA1 were shown to be important for cell cycle-dependent regulation of function (133). It is noteworthy that there is no evidence that fungal NHAs perform electrogenic Na^+/H^+ antiport as do the related bacterial NhaA genes, which are also major contributors to NaCl tolerance in many bacterial species and perform electrogenic $(n+1)H^+/nNa^+$ exchange (109). Electron cryomicroscopy studies of EcNhaA have revealed a 7×14 -Å structure with 12 tilted, bilayer-spanning helices in a dimer (58, 121, 163). Having now established that NhaA is a member of the CPA superfamily, we can apply this structural model to other CPA genes (including human NHE paralogs) to provide insight into how transporter structure relates to function.

We have identified, for the first time, a new family of related genes in animals that we have termed NHA on the basis of their

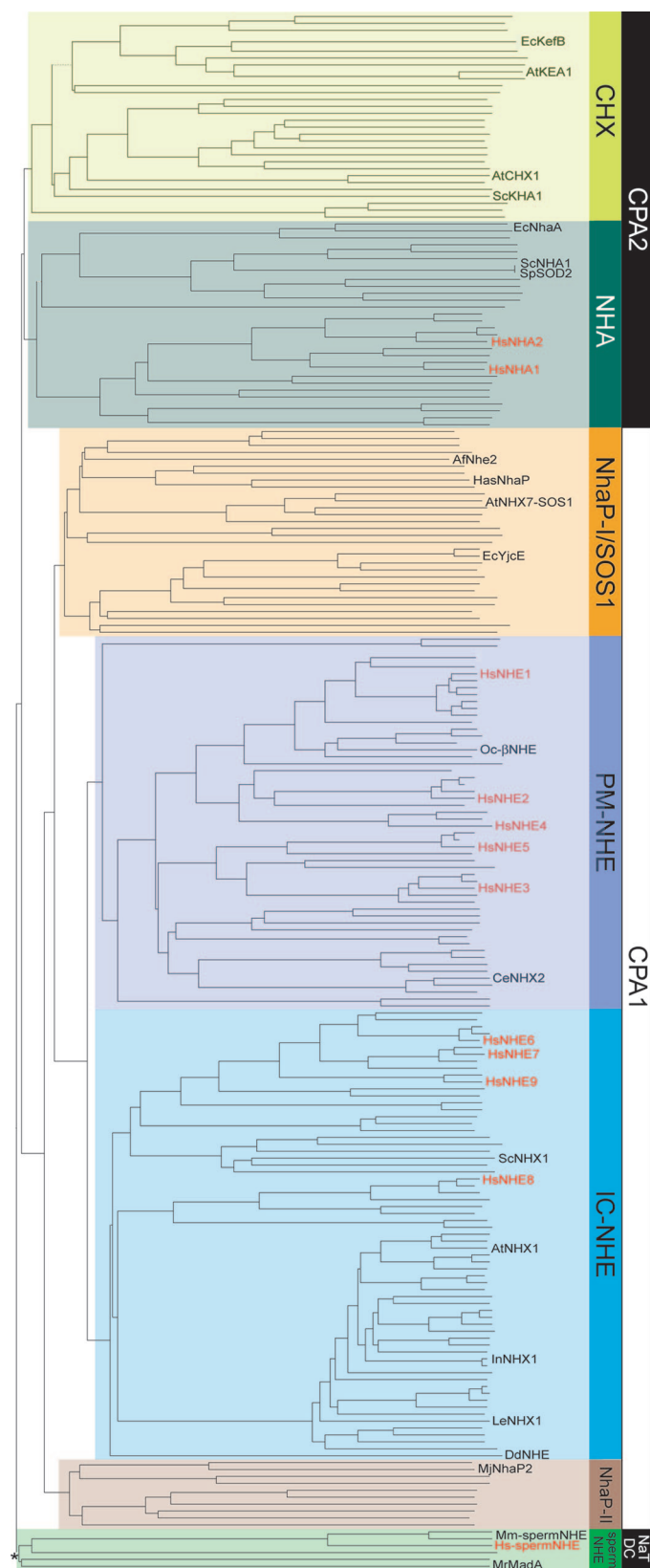
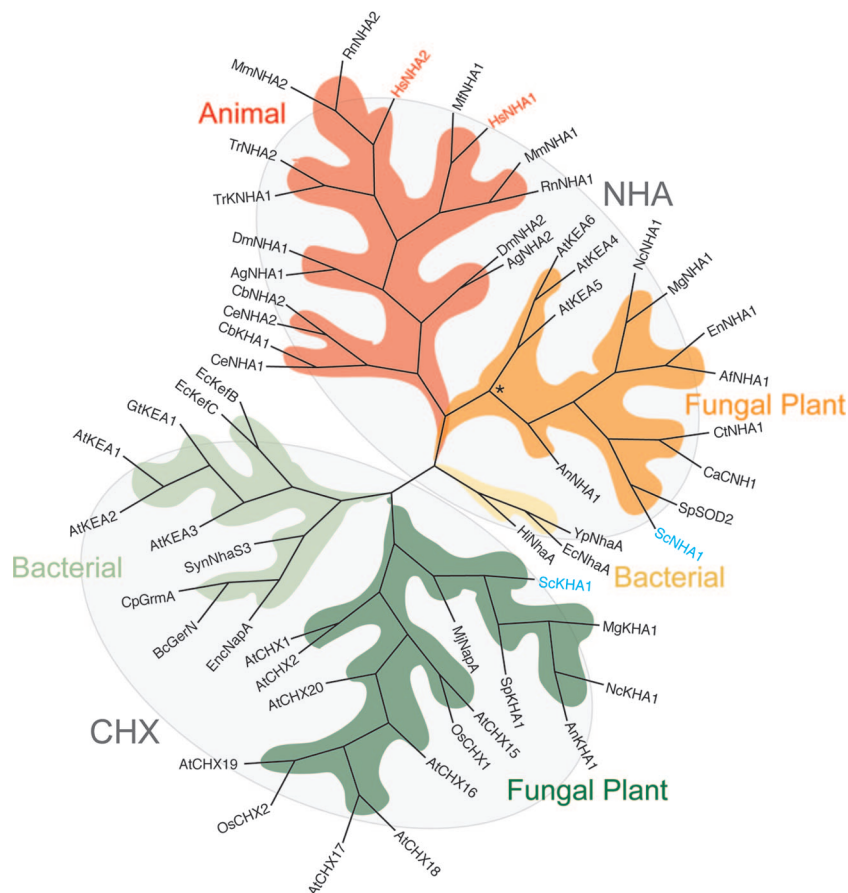


Fig. 1. Phylogenetic tree of the monovalent cation proton antiporter (CPA) superfamily. The unrooted dendrogram shows phylogenetic relationships between 222 CPA genes. The bars at right indicate classification into the 3 CPA families (from top to bottom; number of genes used in analysis): CPA2 (59), CPA1 (147), and NaT-DC (6). These can be further subclassified into 7 subfamilies (as indicated by the colored boxes): CPA2 is divided into the CHX and NHA clades, CPA1 is represented by the NhaP-I/SOS1, plasma membrane (PM)-NHE, intracellular (IC)-NHE, and NhaP-II subfamilies; and the NaT-DC family also includes the distantly related mammalian sperm NHE genes. There are 21 representative, well-characterized CPA genes shown for each subfamily (black text). The 12 human CPA genes are shown in red and include 9 NHE paralogs, a sperm-NHE, and 2 newly identified NHA genes. Corresponding GenBank accession nos. listed in Table 1S; all branches of this dendrogram are identified in Fig. 1S (see Supplemental Material for this article online). Bootstrap analysis showed a confidence value <50 at the node indicated by the asterisk.



similarity to fungal NHA1, likely to be $\text{Na}^+, \text{K}^+/\text{H}^+$ exchangers. There are two paralogs, NHA1 and NHA2, in all completely sequenced metazoan genomes that we examined, including *Caenorhabditis elegans*, fly, puffer fish, mouse, and human (Fig. 2). In a BLAST search against the human genome, the two novel human NHA orthologs are most similar to the SLC9A (NHE) genes of the CPA1 family and are distant from SLC8A ($\text{Na}^+/\text{Ca}^{2+}$ exchangers), SLC10A (polypeptide or bile acid/ Na^+ transporters), and SLC7A (positively charged AA/ H^+ exchangers) genes (see HUGO, <http://www.gene.ucl.ac.uk/nomenclature>; Ref. 154). The cellular distribution and physiological roles of this new family remain to be explored. An electrogenic $2\text{Na}^+/\text{H}^+$ activity has been reported in gill epithelial cells of the euryhaline green shore crab (*Carcinus maenas*) and apical membranes of single hepatopancreatic epithelial cell suspensions of the Atlantic lobster (*Homarus americanus*); however, the molecular identity of this transport mechanism is unresolved (48, 87, 128, 144). Of note, it has been suggested that the CmNHE1 transporter, cloned from the gills of green crab, may be responsible for this reported electrogenic activity (128, 144). However, the ion exchange properties of this antiporter have not been characterized, and in our analysis CmNHE1 was found in the recycling plasma membrane clade of the NHE family (CPA1), which includes NHE orthologs known to exhibit electroneutral exchange activity (see below). We suggest that a CPA2 homolog may be responsible, because the activity of the well-characterized bacterial CPA2 gene EcNhaA is known to be electrogenic and CPA2 orthologs exist in every animal species examined.

The CPA1 family arose from ancestral NhaP genes in prokaryotes. NhaP is known to transport Na^+ or Li^+ in exchange for H^+ in an electroneutral and pH-dependent manner (66, 69, 79, 149). Exogenously expressed ApNhaP from a halotolerant cyanobacterium conferred salt tolerance in a fresh water cyanobacterium (*Synechococcus* sp. PCC 7942), allowing it to grow in seawater (153). Many bacterial species have multiple NhaP paralogs (e.g., MjNhaP1 and MjNhaP2), each with distinguishing transport characteristics, suggesting unique physiological roles (50, 69, 72). As shown in Fig. 1, the bacterial NhaP members fall into distinct clusters, of which one branch shares ancestry with plasma membrane Na^+/H^+ exchangers from plants, first identified in *Arabidopsis* as SOS1 (salt overly sensitive; Ref. 129). The latter is also somewhat confusingly named NHX7 and is closely related to AtNHX8, although both are distinct from other NHX genes in *Arabidopsis* that are members of the NHE family (see EVOLUTION OF THE EUKARYOTIC NHE GENE FAMILY; Ref. 88). Other members of this cluster have been identified in rice (*Oryza*), moss (*Physcomitrella*; Ref. 18), sea grass (*Cymodocea*), and protozoans (*Plasmodium*, *Cryptosporidium*). The SOS1 gene was identified in a screen for salt sensitivity in *Arabidopsis* and encodes a plasma membrane electroneutral $\text{Na}^+(\text{Li}^+)/\text{H}^+$ antiporter. Other components of the SOS pathway include the calcium-binding protein SOS3 that regulates a protein kinase SOS2, which in turn activates SOS1 (93, 130). A detailed view of the phylogenetic relations between the plant SOS1 and bacterial NhaP

families is shown in Fig. 3, with the more abundant NHE members omitted for the sake of clarity.

EVOLUTION OF THE EUKARYOTIC NHE GENE FAMILY

The eukaryotic NHE clade is the best characterized of the CPA families and contains the first eukaryotic NHE sequence identified, HsNHE1, for which this subfamily is named (123). According to the dendrogram shown in Fig. 4, the NHE gene family can be divided into two well-defined clades that we have termed intracellular and plasma membrane, based on the subcellular locations of the best-characterized members in each of these two groups: yeast NHX1 and human NHE1. These two main clades can be further divided into five distinct subgroups on the basis of sequence similarity: three within the intracellular clade that we have termed “endosomal/TGN,” “plant vacuolar,” and “NHE8-like” clusters, and two plasma membrane subgroups, “recycling” and “resident.”

This comprehensive phylogenetic analysis has led to the novel conclusion that the eukaryotic NHE gene family originated as intracellular exchangers first seen in yeast, slime mold, and plant species. These include endosomal/TGN NHE genes of yeast (ScNHX1) and the plant vacuolar NHE genes (e.g., AtNHX1), as well as the NHE8-like clade with the earliest representation in slime mold (DdNHE). Further up the evolutionary ladder, new NHE genes emerged in nematodes and insects (e.g., DmNHE2) that are found on the plasma membrane in addition to the endosome. Members of this group have

been shown to recycle between these two membranes. Most recently in evolutionary history, the resident plasma membrane NHE genes emerged first in fish (e.g., rainbow trout β -NHE) and are exclusive to vertebrates. Four of these NHE clusters are represented by at least one human NHE isoform, as discussed in detail below.

The Endosome/TGN NHE Clade

From Fig. 4, it is clear that there are representative members of the intracellular subgroups in all phyla. One of the oldest members is in fungi and was first identified as NHX1 (YDR456W) in *S. cerevisiae* (94). It is important to clarify that this is identical to ScNHA2 that was initially suggested to reside in mitochondria (103, 106) (examples of current incorrect database entries include NCBI protein accession no. Q04121, OMIM entry no. 300231; ProDom family no. PD000631). However, numerous independent studies have since confirmed that ScNHX1 is found in the endosomal compartment of yeast, where it contributes to salt sequestration and halotolerance, osmoregulation, vacuolar pH regulation, and vesicle trafficking (5, 22, 38, 94–96). To avoid confusion with the phylogenetically distinct NHA antiporters of fungi and persistent incorrect citations of mitochondrial localization in the literature, we propose that the term ScNHA2 be discontinued in favor of the original gene name of ScNHX1 that is now widely adopted in the nomenclature of plant and invertebrate NHE homologs. ScNHX1 has orthologs in all eukaryotes whose genomes are completely sequenced, including two in *A. thaliana* (AtNHX5 and AtNHX6), at least one in tomato (*Lycopersicon esculentum* Mill. cv. MoneyMaker, LeNHX2; Ref. 150), one in *C. elegans* (CeNHX5), one in *Drosophila melanogaster* (DmNHE3), three in the puffer fish (*Takifugu rubripes*, TrNHE6, TrNHE7, and TrNHE9), and three in mammals (HsNHE6, HsNHE7, and the most recently identified HsNHE9).

The endosomal (and secretory granule) location of HsNHE6 has been confirmed in cell culture models (25, 91), and HsNHE7 has been shown to be present in the *trans*-Golgi network (TGN; 102). The subcellular distribution and function of HsNHE9 remains to be determined. Interestingly, all three human paralogs are highly expressed in brain tissue (39, 102, 103; also see the HUGE database, <http://www.kazusa.or.jp/huge/>, accession no. D97743 for HsNHE6), and CeNHX5 is predominantly found in granular structures of neuronal cell bodies (98). A potential disruption of HsNHE9 has been linked to adult attention deficit hyperactivity disorder (39). Unlike plasma membrane NHE transporters, ScNHX1, as well as orthologous genes in plants and humans, e.g., LeNHX2 and HsNHE7 (102, 150), catalyzes K^+/H^+ exchange in preference to Na^+/H^+ exchange.

The Intracellular NHE8-Like Clade

In addition to the early ScNHX1-containing endosomal/TGN subgroup, two additional NHE clades emerged: the NHE8-like and plant vacuolar clades. The NHE8-like clade has its origin in *Dictyostelium discoideum* (slime mold), and single orthologs are present in the genomes of worm (CeNHX8), fruit fly (DmNHE1), puffer fish (TrNHE8), and mammals, including MmNHE8 in mouse and HsNHE8 (also known as KIAA0939) in human. Interestingly, there are no NHE8 mem-

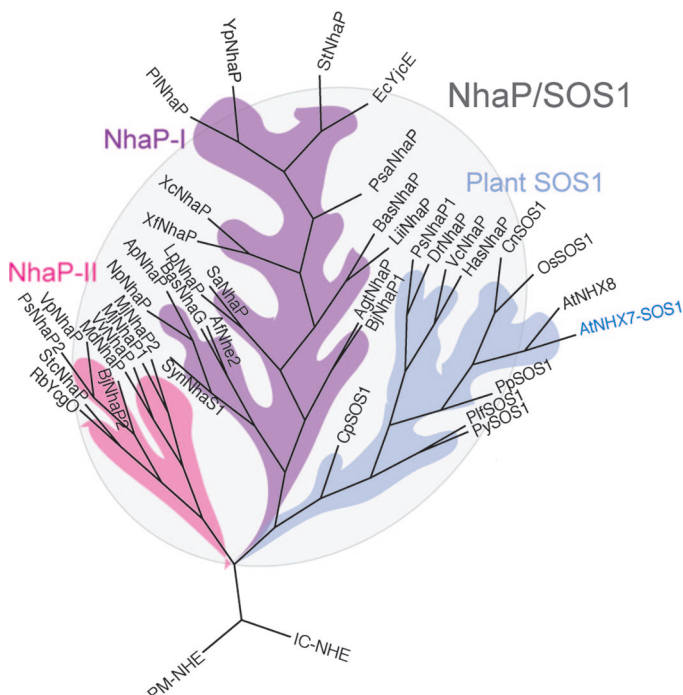
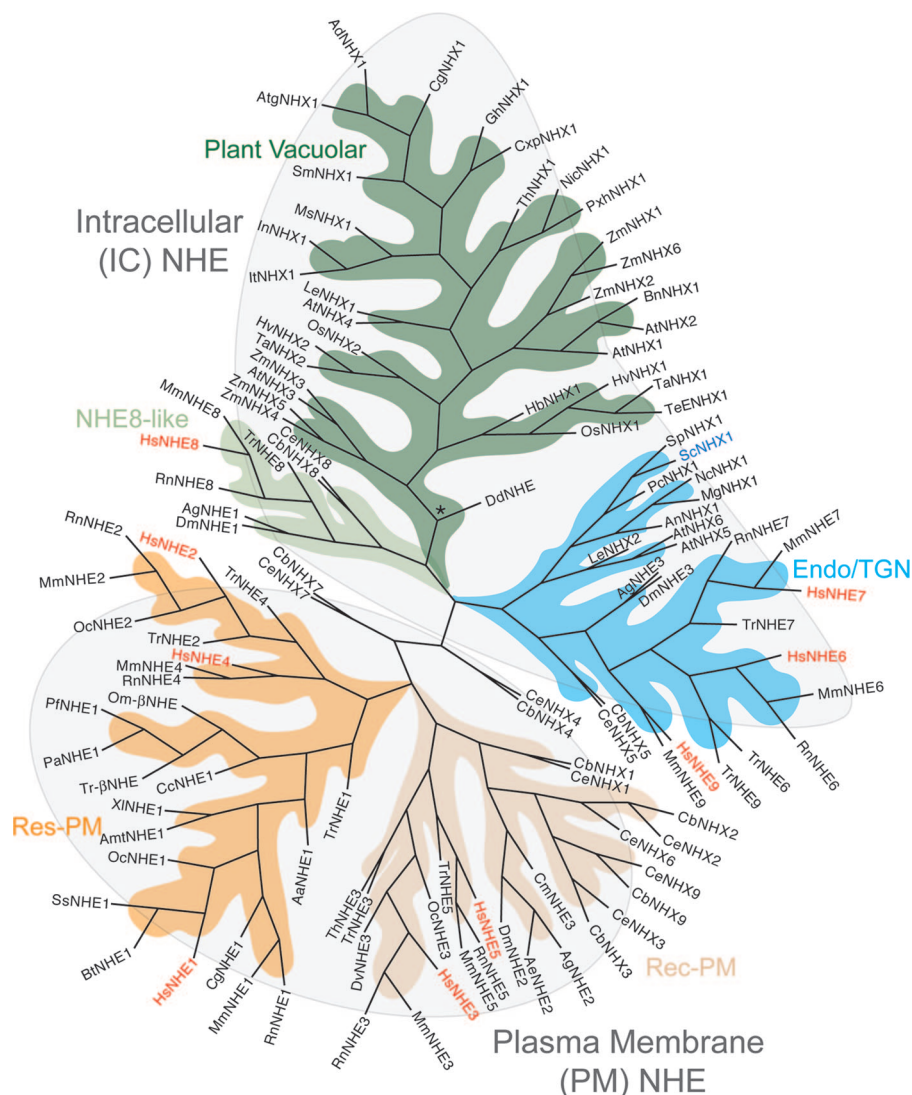


Fig. 3. Phylogenetic tree of CPA1 transporters. Phylogenetic analysis was performed with 39 bacterial NhaP and eukaryotic SOS genes. The NhaP/SOS1 clade (shaded oval) is a CPA1 subfamily restricted to bacteria, protozoa, and plants and shows similarity to both PM-NHE and IC-NHE CPA1 subfamilies (indicated by lower 2 branches). It can be further divided into 3 clades: NhaP-I (shaded violet) and NhaP-II (shaded pink) gene clusters contain ancestral bacterial NHE genes, e.g., ApNhaP; and the plant SOS1 clade (shaded light purple) contains 8 plant and protozoan SOS1 genes, e.g., AtNHX7-SOS1 (blue text), as well as 4 related bacterial NhaP genes. Corresponding GenBank accession nos. are listed in Table 1S.

Fig. 4. Phylogenetic tree of the NHE family. Phylogenetic analysis was performed using 118 eukaryotic CPA1 NHE genes, resulting in the unrooted phylogram shown. The eukaryotic NHE gene family can be divided into 2 major clades on the basis of cellular location, ion selectivity, inhibitor specificity, and protein sequence similarity: IC (top shaded region) and PM (bottom shaded oval). The IC-NHE subfamily can be further divided into 3 clades: the endosomal/TGN cluster (shaded blue), which includes ScNHX1 (blue text), one of the oldest eukaryotic NHE genes, as well as HsNHE6, HsNHE7, and HsNHE9; the NHE8-like clade (shaded light green), which includes 8 animal NHE genes (1 for each species shown) and shows closest similarity to DdNHE; and the plant vacuolar clade (shaded dark green), which includes 32 plant NHE genes (e.g., AtNHX) and the DdNHE gene of slime mold. The PM-NHE subfamily can be divided into 2 clades: the recycling cluster (Rec-PM; shaded beige), which includes only animal NHE genes (24 shown), e.g., HsNHE3 and HsNHE5; and the resident clade (Res-PM; shaded orange), which is restricted to vertebrate NHE genes (25 shown) such as Om-βNHE and HsNHE1, HsNHE2, and HsNHE4. The 9 human NHE paralogs are shown in red text. Corresponding GenBank accession nos. are listed in Table 1S. Bootstrap analysis showed a confidence value of 58 at the node indicated by the asterisk.



bers in plants, although the vacuolar clade may be functionally equivalent. Currently, this subgroup has not been well characterized. Limited studies describe the organellar, as well as apical plasma membrane, distribution of the mouse ortholog MmNHE8 in kidney proximal tubule epithelial cells, suggesting the possibility that MmNHE8 recycles to and from the plasma membrane (63). However, in worm, CeNHX8 is found predominantly in hypodermal seam cell intracellular membranes, supporting an intracellular location (98). RT-PCR and ELISA of the KIAA0939 cDNA clone (containing a 595-amino acid portion of HsNHE8) show moderate expression in brain, liver, and kidney tissues (see the HUGE database, <http://www.kazusa.or.jp/huge/>, accession no. AB023156).

The Plant Vacuolar NHE Clade

The third intracellular NHE clade is abundantly and exclusively represented in plants and includes four genes in *A. thaliana* (thale cress; AtNHX1–AtNHX4; Ref. 88), six in *Zea mays* (corn; ZmNHX1–ZmNHX6), at least two in *Oryza sativa* (rice; OsNHX1 and OsNHX2; Ref. 54), one in tomato (LeNHX1; Ref. 150), one in *Brassica napus* (157), one in *Beta vulgaris* (BvNHX1; Ref. 168), one in *Mesembryanthemum*

crystallinum (common ice plant; McNHX1; Ref. 32), two in *Hordeum vulgare* (barley; HvNHX1 and HvNHX2; Ref. 52), one in *Atriplex gmelini* (AgNHX1; Ref. 67), and one Japanese morning glory ortholog (*Ipomoea nil*; InNHX1; Ref. 55). These orthologous transporters are located in the tonoplasts/vacuolar membranes of cells, particularly in the root, where they function to sequester salt and confer halotolerance (93, 104, 127). Consistent with this important role, gene expression is induced by salt and osmotic stress, and transgenic plants that overexpress NHX1 are highly tolerant to salt stress (8, 53). Ectopic expression of many of the plant NHX genes in yeast *nhx1Δ* mutants partially complements the salt- and hygromycin-sensitive phenotypes of the latter and is routinely used as a first step in functional analysis. Biochemical characterization of representative members has shown that they transport both K^+ and Na^+ (14, 32, 52, 131, 151, 157, 168, 171, 172). The InNHX1 ortholog from the Japanese morning glory also has been shown to regulate vacuolar pH, because a transposon insertion in the upstream region of the gene changes petal coloration from blue to purple, indicating an acidic shift in vacuolar pH (55). More recently, a T-DNA insertional mutant of AtNHX1 was reported to have an altered leaf development,

suggesting that some plant NHX orthologs may have additional physiological roles besides defense against ion toxicity (9).

The Recycling Plasma Membrane NHE Clade

We propose that the plasma membrane NHE genes emerged from these ancient, ubiquitous intracellular NHE. It appears that the plasma membrane NHE genes are more recent, because orthologs are not found in yeasts or plants. The plasma membrane NHE genes can be further classified into two subgroups: recycling and resident. The recycling plasma membrane NHE group appears to be older, with orthologs present in nematodes (CeNHX1, CeNHX2, CeNHX3, CeNHX6, and CeNHX9; Ref. 98), fruit fly (DmNHE2; Ref. 62), mosquito (*Aedes aegypti*; AaNHE2; Ref. 68), puffer fish (TrNHE3 and TrNHE5), and mammals, including two human paralogs (HsNHE3 and HsNHE5; Refs. 24, 76, 146).

Mammalian NHE3 orthologs have been extensively studied and are functionally well characterized. These were first observed to cycle between the endosomes and the plasma membrane, hence the classification of this NHE clade as the recycling plasma membrane group. The *C. elegans* clade members (CeNHX1, CeNHX2, CeNHX3, CeNHX6, and CeNHX9) show a similar dual endosomal/plasma membrane distribution (98). CeNHX2 is predominantly found on the apical surface of the worm gut epithelium (97, 98), and AaNHE2 is found in the Malpighian tubule of the mosquito as well as the mid- and hindgut (68). NHE3-like immunoreactivity has been reported in the branchial epithelial cells of the gills of two fish species (rainbow trout and the blue-throated wrasse, *Pseudolabrus tetrius*; Ref. 49), and mammalian NHE3 orthologs are predominantly found on the apical plasma membrane and recycling endomembrane of epithelial cells of the small intestine, colon, gallbladder, kidney proximal tubule, and epididymis, where they are highly selective for Na⁺, in preference to K⁺ (106, 174). These orthologous transporters are important contributors to organismal Na⁺ and osmotic homeostasis by acting to absorb Na⁺ from the lumen of the gut and nephron (in mammals, where they also contribute to HCO₃⁻ reabsorption and H⁺ secretion; Refs. 57, 122) in a highly regulated manner (40, 43, 84, 89, 148). Study of the MmNHE3 knockout mouse confirms these findings: although the homozygous NHE3^{-/-} animal survives, it presents with diarrhea, is mildly acidotic, shows a reduction in blood pressure, and dies on a low-sodium diet (16, 59, 125, 166, 167). More recent findings indicated that NHE3 is found within large protein complexes on the apical surface that include functional regulators and cytoskeletal binding proteins (4, 74, 85, 86, 152, 158). Limited studies also have shown that changes in NHE3 activity regulates the endocytosis and exocytosis of albumin and the albumin receptor megalin in cell culture (42, 44, 45, 60, 83) and in the mouse kidney (61), suggesting the possibility that NHE3 may also play a role in the regulation of membrane recycling between the apical endosome and the plasma membrane.

Although less well characterized, limited studies have shown endomembrane and plasma membrane location of NHE5 in neuronal and fibroblast cultures (138, 139). Expression is believed to be ubiquitous in the 11-week-old fetus, whereas in the adult, mammalian NHE5 is predominantly found in brain tissue, with the highest expression reported in the cerebellum (11, 13). A single study has linked possible

mutation in the regulatory and intronic regions of the HsNHE5 gene to familial paroxysmal kinesigenic dyskinesia (136).

The Resident Plasma Membrane NHE Clade

The resident plasma membrane NHE are the most recent of the NHE to emerge and are exclusive to vertebrates. They have their earliest representation in species of fish, including the rainbow trout (*Onchorynchus mykiss*, Om-βNHE; Ref. 21), puffer fish (TrNHE1, TrNHE2, TrNHE4, and β-NHE), winter flounder (*Pseudopleuronectes americanus*, PaNHE1; Ref. 111), American eel (*Anguilla rostrata*, ArNHE1; not shown), marine long-horned sculpin (*Myoxocephalus octodecimspinosus*, MoNHE1 and MoNHE2; not shown, Ref. 34), and euryhaline killifish (*Fundulus heteroclitus*, FhNHE1 and FhNHE2; not shown, Ref. 34), and in amphibians such as *Xenopus laevis* (XINHE1; Ref. 27). Mammals have three paralogs: NHE1, NHE2, and NHE4 (108, 123, 147). Consistent with a relatively recent gene duplication, NHE2 and NHE4 are found adjacent to each other in many mammalian genomes, e.g., on human chromosome 2 on q11-12. Extensive studies performed on mammalian orthologs of NHE1, NHE2, and, to a much lesser extent, NHE4, suggest that these NHE genes are exclusive residents of the plasma membrane. Of note, the avian species are not represented in this analysis, but a study in chicken colon suggested the existence of NHE3 and NHE2, confirming the presence of resident plasma membrane NHE genes (e.g., NHE2) in all vertebrates (41).

It is worth mentioning that CeNHX4 and CeNHX7 do not clearly fall into any NHE clade (see Fig. 4). It has been suggested that these exchangers are found on the plasma membrane of all cells and the basolateral membrane of intestinal epithelial cells, respectively, resembling the cellular distribution of mammalian NHE1 orthologs (98). Thus these two worm NHE genes may represent distant precursors to vertebrate resident plasma membrane NHE genes. However, CeNHX4 and CeNHX7 show low sequence similarity to identified resident plasma membrane NHE, and we were unable to identify any other invertebrate NHE that had greater homology with resident plasma membrane NHE orthologs of vertebrates.

One of the earliest evolved members of this NHE clade is the Om-βNHE, first identified as the molecular mechanism for driving Na⁺/H⁺ exchange in the red blood cells of the rainbow trout (as well as other fish species; Refs. 78, 80, 99, 100). Similar Na⁺/H⁺ exchange activity has been reported in amphibians (*Rana temporaria*; Ref. 3). Fish orthologs of NHE1 are also found in the accessory cell types on the brachial epithelium (164, 165) and hepatocytes (56), where Na⁺/H⁺ exchange activity has been reported in rainbow trout, black bullhead (*Ameiurus melas*), and American eel (56). Human NHE1 has been extensively studied for 15 years and has been reported to be ubiquitously distributed in all cells of the body, where it contributes to cellular volume and pH homeostasis (106, 118). The NHE1 knockout mouse has been made and is also found as a spontaneous mutant that manifests neurological disorders including epilepsy, ataxia, and motor defects due, in part, to the hyperexcitability of hippocampal CA1 neurons (17, 37, 65, 169, 170). Mammalian NHE2 transporters are known to function on the apical surface of mammalian proximal tubule, small intestine, colon, gallbladder, and epididymal epithelial cells to absorb luminal Na⁺ (1, 33). Although they share

Table 1. *The nine human NHE paralogous genes and three pseudogenes*

Gene Name (in order of evolution)	Human Chromosomal Location	NCBI GeneID No.	Gene Length, bp	Exons	Protein Sequence Length, aa	Subcellular Location	Examples of Shared Neighboring Genes	Ortholog in Oldest Species	Original Species
SLC9A6 (NHE6)	Xq26.3	10479	61,666	16	669–701	Endosome	GAPDH (LOC392549)	ScNHX1	Fungi
SLC9A7 (NHE7)	Xp11.3-p11.23	84679	152,100	17	725	TGN	GAPDH pseudogene (LOC389849); CHST7		
SLC9A9* (NHE9)	3q23-24	285195	583,226	16	645	Organellar, apical PM?	CHST2		
SLC9A8 (NHE8)	20q13.13	23315	81,077	16	666	Organellar?	None	DdNHE	Slime mold
SLC9A3 (NHE3)	5p15.3	6550	51,023	17	834	Recycling endosome/PM	p25 α	DmNHE2	Insect/worm
SLC9A5 (NHE5)	16q22.1	6553	23,242	16	896	Secretory granule/PM	CG1-38 (p25 isoform)		
SLC9A1 (NHE1)	1p36.1-p35	6548	56,097	12	815	PM	GPR3	Om β NHE	Fish
SLC9A2 $\ddagger$ (NHE2)	2q11.2	6549	91,644	12	812	Apical PM	GPR45		
SLC9A4 (NHE4)	2q11-q12	6552	~60,000	12	888	PM	GPR45 $\dagger$		
SLC9A7P1 (NHE7P1)	12q23.1	Pseudogene			NA	NA		NA	
SLC9A3P1 $\S$ (NHE3P1)	10q21.1	Pseudogene			NA	NA		NA	
SLC9A3P2 (NHE3P2)	22q11.21	Pseudogene			NA	NA		NA	

NHE, Na⁺/H⁺ exchanger; aa, amino acids; bp, base pairs; TGN, *trans*-Golgi network; PM, plasma membrane. *Predicted to be the oldest human NHE paralog. $\dagger$ Gene sequence from LOC389015, LOC400991, and two predicted upstream coding exons. $\ddagger$ SLC9A2 and SLC9A4 are neighboring genes on chromosome 2, thus sharing other neighboring genes. Each separate grouping of expressed NHE paralogs indicates a different NHE clade. $\S$ See Ref. 168.

similar tissue distributions, NHE2 differs from NHE3 in that it does not recycle (81, 82). The NHE2 knockout mouse has decreased gastric acid secretion due to parietal cell degeneration apparently caused by failure to protect these cells from acid damage (12, 20, 124). NHE4 is not as well characterized as NHE1 and NHE2, but it is known to be present in the basolateral membrane of parietal cells, in epithelial cells of the thick ascending limb of the nephron, and in the macula densa (112). NHE4 is activated at unusually low pH values (pK_a 6.21; Refs. 30, 113).

In summary, from these analyses we conclude that the NHE gene family originated as intracellular/organellar Na⁺,K⁺/H⁺ exchangers that are common to all eukaryotic cells. Some of the earliest members are found in fungi (ScNHX1) and localize to the endosomes, where they are critical for pH homeostasis and vesicle trafficking. Additional members appeared in higher eukaryotes, presumably for specialized functions in other organelles such as the TGN (HsNHE7). An offshoot of this early clade is the vacuolar NHE, exclusive to plants (AtNHX1), and the NHE8 group, exclusive to animals. The appearance of recycling NHE, found in both endosomes and plasma membrane in metazoan organisms (HsNHE3), occurred next. Most recently in evolutionary history, the resident plasma membrane NHE genes emerged, first in fish (rainbow trout β -NHE), and include HsNHE1, HsNHE2, and HsNHE4; these are found in all vertebrates.

GENE LENGTHS AND CHROMOSOMAL LOCATIONS OF HUMAN NHE PARALOGS

It has been proposed that within a gene family that has been conserved through evolution, the oldest paralog to be retained in the genome is also the longest (intron length inversely correlates to probability of recombination). An examination of the human genome reveals that although their coding regions are significantly shorter (see below), genes belonging to the intracellular NHE clade are significantly longer (61,666–583,226 base pairs) than the genes of

the plasma membrane clade (23,242–91,644 base pairs; Table 1). The same trend was confirmed for the mouse and rat NHE genes (data not shown), consistent with our hypothesis that the intracellular NHE evolved before their plasma membrane homologs. Furthermore, we note that the largest NHE paralog in the human genome, HsNHE9 (583,226 base pairs), is also the most similar in sequence to yeast ScNHX1. This would indicate that HsNHE9 is the oldest NHE in the human genome, although, ironically, it is the most recent to have been identified.

To further support our phylogenetically based classification scheme, we examined the chromosomal location of human NHE paralogs to identify common neighboring genes. Because gene duplication encompasses surrounding chromosomal segments, we expect that duplicated genes share common neighbors (see Ref. 71). For example, CHST2 and CHST7 genes, encoding paralogous carbohydrate (*N*-acetylglucosamine 6-*O*) sulfotransferases, are close to HsNHE9 and HsNHE7 on chromosomes 3q23-24 and Xp11.3, respectively. Similarly, HsNHE7 and HsNHE6 share proximity with GAPDH pseudogenes, potentially encoding glyceraldehyde 3-phosphate dehydrogenases, on chromosome X. CHST and GAPDH homologs are not found near other human NHE paralogs and thus are only found near the three human paralogs of the endosomal/TGN clade. These findings, taken together with the gene length hypothesis of gene age (see Table 1), suggest that the order of evolution of the early NHE paralogs is HsNHE9 $\gg$ HsNHE7 > HsNHE6.

Similar relationships with chromosomal neighbors are also observed among members of the plasma membrane clade. For example, HsNHE3 and HsNHE5 share ZDHHC and brain-specific protein 25 homologs (both zinc finger DHHC domain-containing proteins) as neighbors on chromosomes 5 and 16, respectively. Note that HsNHE2 and HsNHE4 are neighboring genes, and HsNHE1, HsNHE2, and HsNHE4 share G protein-coupled receptor homologs (GPR3 and GPR45).

DISTINGUISHING CHARACTERISTICS OF INTRACELLULAR AND PLASMA MEMBRANE NHE

There are emerging structural and functional differences between the phylogenetically distinct intracellular and plasma membrane clades of NHE. Cation selectivity in the intracellular group appears to extend to K^+ as well as Na^+ and has been documented for members of the plant vacuolar clade and the endosomal/TGN clade (53, 102, 151). Sensitivity to inhibition by amiloride and its derivatives is a hallmark of the plasma membrane NHE, although there are clear differences in the affinity toward these inhibitors among NHE1–NHE5 (118). However, it is remarkable that members of the intracellular clades are relatively insensitive to inhibition by amiloride, consistent with the divergence of amino acid sequence in the regions implicated in drug binding (see below; Refs. 38, 102). We also note that polypeptide length differs between the two major clades, with the intracellular NHE having shorter chain length (722 ± 46 amino acids) compared with the longer plasma membrane NHE (867 ± 67 amino acids; Table 2). This difference is most apparent between human paralogs, although there are two exceptions from homologs in other species, DdNHE8 and CeNHX2. In general, protein length and isoelectric point (pI) of the conserved NH_2 -terminal transmembrane region (TM3 to TM12) are well conserved across the NHE family (388–451 amino acids; $pI = 6.97 \pm 0.26$; see Table 2; also see Figs. 2S and 3S online). Rather, it is the cytoplasmic regulatory COOH terminus that is more variable in sequence length, pI, and identity between paralogs of each subgroup. Thus plasma membrane orthologs have long (365 ± 52 amino acid), net basic ($pI = 8.12 \pm 0.50$) COOH termini, whereas

intracellular orthologs have shorter (199 ± 46 amino acid), net acidic ($pI = 5.77 \pm 0.79$) COOH termini ($P < 0.05$). These differences correlate with evolutionary age, because plasma membrane homologs with longer COOH termini evolved later, and cellular location, because organellar NHE homologs have more acidic COOH termini. Net charge and sequence lengths of NH_2 termini are highly variable between homologs. For example, plant NHX genes are missing sequences equivalent to TM1, and the pI of NH_2 termini among the human paralogs varies from highly acidic (4.64 and 4.98 for NHE7 and NHE4, respectively) to extremely basic (10.98 and 11.50 for NHE6 and NHE8, respectively; see Table 2 and Fig. 3S). These variations are likely to correlate with specific roles of individual NH_2 termini in membrane targeting and thus are unique to each homolog (91, 159, 175).

Further examination of NHE protein sequence alignment data showed many structural features both shared between and unique to each NHE clade (see Fig. 4S). As an example, we focused on the TM3–TM4 region of selected NHE sequences from all NHE clades, a region known to be important for ion selectivity and drug sensitivity (106) (also shown for NhaA, a CPA2 member; Ref. 58). As shown in Fig. 5, this region contains five residues conserved among all eukaryotic NHE sequences. These residues are presumed or known to be important for function of all NHE homologs (e.g., P167, position refers to the HsNHE1 sequence; Refs. 134, 143). In EL2, mammalian endosomal/TGN NHE orthologs have a 46- to 47-amino acid insert. HsNHE6 is believed to have two splice variants sequenced from brain (669 amino acids) and liver (701 amino acids; Ref. 91). The 32-amino acid insert is in the EL2

Table 2. Domain sequence length and isoelectric point values of selected NHE genes

	Sequence Length, aa				pI			
	Full	N _t	TM3–TM12	C _t	Full	N _t	TM3–TM12	C _t
<i>IC NHE genes</i>								
ScNHX1	633	78	410	145	4.82	4.02	7.12	4.33
HsNHE6	669	88	418	163	6.48	10.98	7.8	4.77
HsNHE7	725	88	449	188	6.41	4.64	9.07	4.72
HsNHE9	645	38	451	156	6.21	4.98	7.54	4.79
DdNHE8	948	136	388	424	4.95	4.98	5.08	4.89
CeNHX8	681	126	396	159	7.66	4.87	7.38	9.64
HsNHE8	666	161	400	105	9.18	11.5	7.63	5.8
AtNHX1	538	35	409	94	7.21	5.69	7.28	7.56
OsNHX1	544	38	409	97	7.59	4.14	8.62	6.81
InNHX1	542	37	409	96	7.4	4.47	7.28	9.53
Mean	659	83	414	163	6.79	6.03	7.48	6.28
SE	38	15	7	31	0.42	0.88	0.33	0.64
<i>PM NHE genes</i>								
CeNHX2	644	65	394	185	5.42	4.89	5	6.23
DmNHE2	1291	212	394	685	6.85	8.85	6.44	6.67
HsNHE3	834	71	394	369	7.68	5.69	6.11	9.18
HsNHE5	896	63	395	438	7.56	4.8	8.65	7.3
Om-βNHE	759	86	389	284	7.55	8.55	6.51	8.22
HsNHE1	815	118	390	307	7.21	9.6	6.25	7.61
HsNHE2	812	97	391	324	9.09	5.88	6.77	9.83
HsNHE4	888	128	427	333	8.77	4.96	6.53	9.95
Mean	867	105	397	366	7.52	6.65	6.53	8.12
SE	67	17	4	52	0.40	0.71	0.36	0.50
P	0.011*	0.335	0.057	0.003*	0.234	0.601	0.071	0.044*

* $P < 0.05$, significant difference between intracellular (IC) and plasma membrane (PM) NHE sequences. Human genes are in bold type. N_t, NH_2 -terminal sequence; C_t, COOH-terminal sequence; TM, transmembrane.

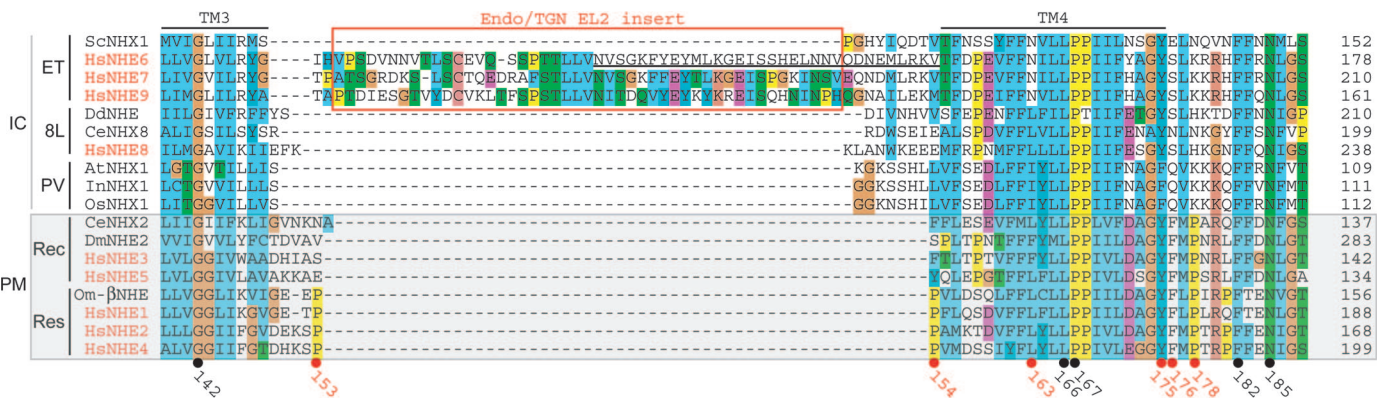


Fig. 5. Protein sequence alignment showing the transmembrane (TM)3–TM4 region of selected NHE homologs. Protein sequences of 10 IC and 8 PM eukaryotic NHE orthologs (shaded gray), representing 5 clades (ET, endosomal/TGN; 8L, NHE8-like; PV, plant vacuolar; Rec, recycling; Res, resident) were aligned using ClustalX software. The TM3–TM4 region (indicated by black lines) of the protein sequence alignment is shown (the full sequence alignment is shown in Fig. 4S); this is represented by A139 to T188 of HsNHE1 (see Ref. 155). Residues discussed are marked by filled circles below. 100% conserved residues are shown in black, and residues that show NHE ortholog-specific conservation are shown in red (e.g., a proline at position 178 is conserved in PM-NHE orthologs but not in IC-NHE sequences). Residue position numbers refer to the HsNHE1 sequence. Note the 46- to 47-amino acid-long insert in extracellular loop 2 (EL2) unique to mammalian endosomal/TGN NHE orthologs (indicated by the red box). The underlined portion of the HsNHE6 sequence indicates a 32-amino acid addition identified as a splice variant in liver tissue (91). Human NHE paralogs are labeled in red text. The conventional ClustalX pseudocolor scheme, based on amino acid chemical properties, is applied to better visualize patterns of conserved residues between protein sequences. Numbers at right indicate the position of the last amino acid shown in each protein sequence. Corresponding GenBank accession nos. are listed in Table 1S.

region shared by all mammalian endosomal/TGN NHE orthologs, suggesting that the longer HsNHE6 is likely the functional variant. This unique region contains many charged residues, suggesting a potential role in ion selectivity and translocation (see Ref. 160). Also in this region of EL2, two proline residues are found back to back exclusively in resident NHE orthologs, a structural feature that may be involved in extracellular ion (Na^+) or drug (amiloride) binding exclusive to this clade of NHE genes. In IL2, F176 and P178 are only conserved in plasma membrane NHE homologs, whereas F175 of TM4 is exclusively conserved in plant vacuolar NHE orthologs. Residue L163, known to be important for amiloride sensitivity (35, 73, 173), is an asparagine (polar) at the same position in endosomal/TGN NHE orthologs. This may be responsible, at least in part, for the relative insensitivity of HsNHE7 (102) and ScNHX1 (38) to amiloride and ethylisopropyl amiloride (EIPA). Also, plant vacuolar NHE orthologs shown to be relatively sensitive to amiloride and EIPA have an isoleucine at this position (119, 151). Examples of such conserved and unique residues and protein regions can be found throughout the length of the NHE sequences (see Figs. 2S–4S) and offer clues into the structural characteristics that underlie specific and conserved functional properties of NHE paralogs.

EVOLUTION OF FUNCTION IN THE EUKARYOTIC Na^+/H^+ EXCHANGER FAMILIES

Although plasma membrane Na^+/H^+ exchange is ubiquitous to all cells, we find that this function is performed by members of the NHE family in the animal kingdom, whereas in plants and fungi, other members of the CPA superfamily, NHA and SOS, perform an equivalent role. As shown in Fig. 6, this difference correlates with the source of the electrochemical driving force. In plants and fungi, a proton motive force generated by the plasma membrane H^+ -ATPase PMA1 is used to expel Na^+ (and K^+) from the cytosol, thereby acting to acidify the cytoplasm. However, in the animal kingdom, plasma membrane NHE act to alkalize the cytoplasm by

coupling to the Na^+ electrochemical gradient maintained by the Na^+/K^+ -ATPase. There was simultaneous appearance of the Na^+/K^+ -ATPase and the plasma membrane NHE genes in nematodes (105), insects, fish, and mammals. Interestingly, the intracellular NHE found in the endosome, TGN, or vacuole, common to all eukaryotes, couple to the H^+ electrochemical gradient maintained by the V-type H^+ -ATPase (Fig. 6). Thus these intracellular NHE function as a H^+ -leak pathway to alkalize the compartmental lumen (see Ref. 64). In remarkable contrast, NHE3 remains coupled to the Na^+ gradient when present in early endosomes, where it has been shown to acidify the lumen (4, 46). Further correlations in evolution should also be considered, including the coemergence of the plasma membrane NHE and Na^+/K^+ -ATPase with animal-specific mechanically integrated tissues that require rapid ion fluxes for function.

The absence of ATP-powered plasma membrane sodium pumps in plants may also explain the development of the specialized clade of vacuolar NHE in this kingdom, which act to store high concentrations of salt and water in the vacuole. These NHE are critical determinants of salt tolerance and osmoregulation in plants. It is noteworthy that in fungi, the absence of members of the plant vacuolar clade is consistent with the presence of P-type sodium pumps of the ENA family at the plasma membrane, which are distantly related to mammalian Na^+/K^+ -ATPase. The latter play a principal role in salt tolerance so that the role of the endosomal NHX1 in yeast (and presumably in animal cells) is primarily in vesicular pH homeostasis and vesicle trafficking. Because the lysosomes of animal cells do not serve as a major organellar sink for cations, it is unlikely that their NHE would serve an equivalent function to the plant vacuolar clade.

The relocation of NHE from endosomal compartments to the plasma membrane that occurred during metazoan evolution also coincided with a preference for Na^+/H^+ exchange. This was necessary to avoid shunting the gradient set up by the Na^+/K^+ -ATPase. Furthermore, sodium selectivity in the

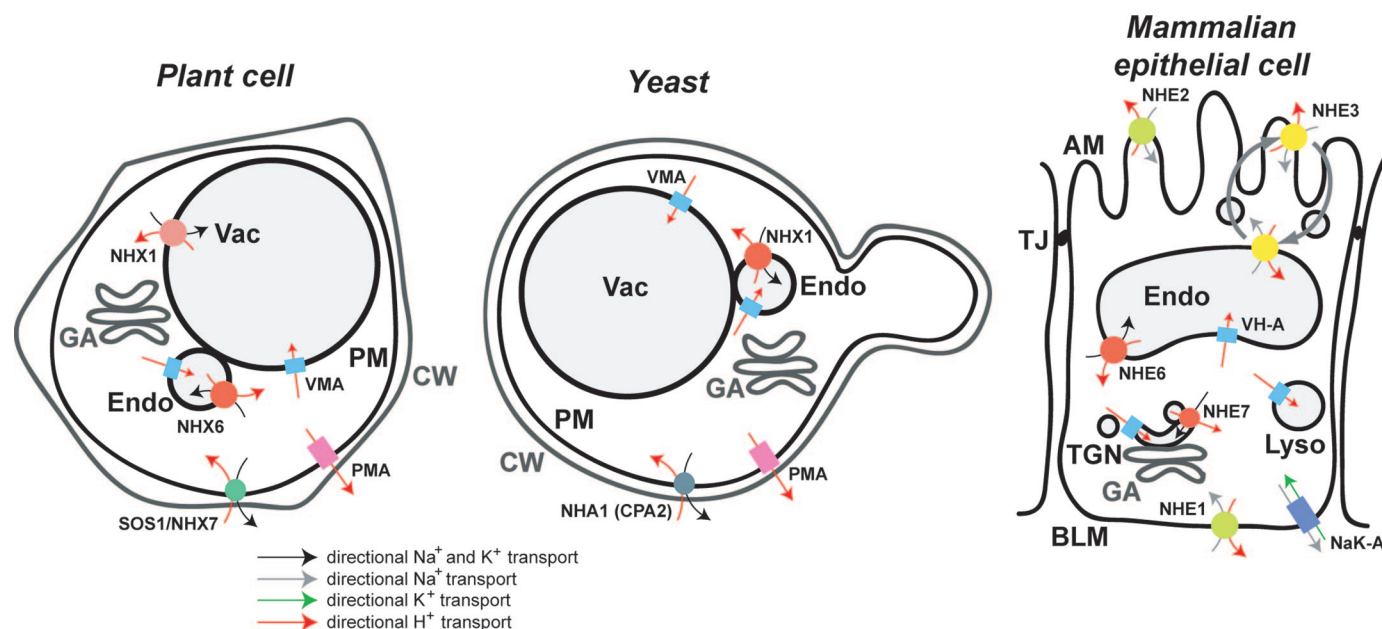


Fig. 6. Cellular location of CPA1 transporters in yeast, plant, and mammalian epithelial cells. Where applicable, the vacuole (Vac), Golgi apparatus (GA), endosome (Endo), *trans*-Golgi network (TGN), lysosome (Lyso), cell wall (CW), tight junction (TJ), plasma membrane (PM), apical plasma membrane (AM), and basolateral plasma membrane (BLM) are shown on representations of (from right to left) plant, yeast, and mammalian epithelial cells. Solid circles represent CPA1 homologs, and orthologs of the same clade share color: endosomal/TGN NHE (red), SOS1 (dark green), plant vacuolar NHE (pink), recycling PM-NHE (yellow), and resident PM-NHE (light green). ScNHA1 (a CPA2 gene; gray circle) is shown on the plasma membrane of the yeast cell. Solid squares represent ion gradient-providing ATPases, and orthologs of the same type share color: organellar V-type H^+ -ATPase (VMA or VH-A; light blue), plasma membrane H^+ -ATPase (PMA; pink), and the plasma membrane Na^+/K^+ ATPase (NaK-A; dark blue). Small arrows indicate the direction and monovalent cation specificity of transport by the corresponding gene. Large shaded arrows represent endo- and exocytosis of the mammalian recycling NHE ortholog NHE3. Function of intracellular CPA1 orthologs is coupled to V-type H^+ -ATPase activity in all cells. Plasma membrane CPA function is coupled to PMA activity in yeast and plants; however, PM-NHE function in mammalian cells is coupled to NaK-A activity.

plasma membrane NHE clade correlates with an overall sensitivity to inhibition by the amiloride class of compounds. Emerging evidence indicates that the intracellular NHE are capable of transporting K^+ , consistent with the cytoplasmic abundance of this cation, and are relatively tolerant to amiloride inhibition. It is interesting that one group has reported that the intracellular LeNHX2 transports K^+ but not Na^+ , whereas RnNHE1 has been reported not only to not transport K^+ but also to be inhibited by K^+ at high concentrations (107), although this observation is controversial (101).

Thus the NHE contribute to ion homeostasis as well as regulation of pH of either an organellar lumen or the cytosol depending on subcellular location, a function that they share with the larger superfamily of CPA genes. However, what is more intriguing and perhaps unique to the NHE family is the emerging role of regulating membrane movement through direct associations of the COOH-terminal NHE tail with proteins of the trafficking and cytoskeletal machinery. Documented examples include the association of ScNHX1 with a Rab-GAP involved in vesicle trafficking (5), the ability of NHE3 to control the rate of surface recycling of the albumin receptor megalin and albumin endocytosis, and numerous proteins that mediate NHE1-cytoskeletal interactions implicated in plasma membrane remodeling and cell migration (174). An increase in the length of the COOH-terminal tail occurred during the evolution of the NHE, with the longest tails occurring in the resident plasma membrane clade. This may correlate with increased complexity of interactions with the cytoskeleton and other cell components, characteristic of higher eukaryotes.

Another interesting observation is that human paralogs found in each subgroup have similar regulatory COOH-terminal tail protein sequences. Variability of sequence within this cytoplasmic region is most apparent between NHE paralogs of different subgroups (e.g., the COOH termini of NHE1 vs. NHE6 are very different), rather than between each paralog (e.g., the COOH-terminal sequences of NHE6 and NHE7 are similar; see Figs. 2S and 4S).

DISTRIBUTION OF NHE PARALOGS IN HUMANS

The late evolution of the HsNHE1 gene, whose orthologs are only present in vertebrates, brings up the question of whether this paralog is truly ubiquitously expressed. Indeed, cell types have already been identified that do not stain for an antibody raised against HsNHE1, such as the epithelial cells of the renal thin descending limb, connecting and collecting tubules, and glomeruli, as well as the intercalated and nonepithelial cells (19). In fact, most early experiments supporting the ubiquitous distribution of HsNHE1 were performed before all NHE paralogs had been identified (e.g., the HsNHE1 protein sequence is very similar to that of HsNHE2 and HsNHE4, all of which are found in the resident plasma membrane NHE clade), and subsequently, both mRNA and protein probes may not have been specific to NHE1. In addition, although highly impaired, NHE1 null mice survive to adulthood, leading to further questioning of the ubiquitous role of NHE1 as a housekeeping gene (17, 37). Ultimately, the distribution of NHE may need to be reassessed with paralog-specific probes.

With a new phylogenetic outlook on the NHE family, we can use comparative biology to better understand the function of specific human NHE paralogs, beginning with the endosomal/TGN subgroup of NHE genes, including human NHE6, NHE7, and NHE9, conserved in all eukaryotes. These NHE transporters must perform a basic fundamental cellular function important for eukaryotic life such as vesicle biogenesis, maintenance, and trafficking by direct regulation of luminal pH and volume. On the basis of this hypothesis, we would predict the combined cellular distribution of the three human paralogs to be ubiquitous.

UTILITY OF MODEL ORGANISMS IN THE STUDY OF NHEs

Insights provided by this phylogenetic analysis should allow researchers to identify appropriate model systems for the study of individual NHEs. The fungal NHX1 is one of the oldest members of the NHE gene family, making it an appropriate model in which to study molecular structure in the context of cation binding and selectivity, drug binding, pH sensitivity, and ion translocation. Furthermore, because ScNHX1 is a founding member of the endosomal/TGN subgroup, yeast is an acceptable model in which to study the cellular physiology of this subgroup of NHE genes. Indeed, much of what we know about the cellular role of this subgroup has been drawn from studies performed in yeast. For example, we would predict that like ScNHX1, orthologous NHE genes play an important role in the regulation of vesicular pH and trafficking. This could be mediated by interaction with components of the trafficking pathway, similar to the role of a Rab-GAP in *S. cerevisiae* (5). Interestingly, yeast contains a single ortholog of each of the three CPA families represented in eukaryotes: NHX1, NHA1, and KHA1 (Fig. 1), making it an appropriate model organism in which to study the contribution of these gene families to cell physiology.

The NHE8-like subgroup of genes has its origins in the slime mold DdNHE. Slime mold is used as a model organism in which to study cell migration and cell-cell interaction, and it is likely that it has an NHE specialized for such a physiological event. Although the phylogenetic clustering with the vacuolar clade would predict a lysosomal distribution in animal cells, NHE8 may be recruited to the plasma membrane, as is the V-type H^+ -ATPase in some cell types (126). It will be of interest to determine whether these NHE are functionally coupled to the H^+ gradient, as suggested by the phylogenetic clustering with the organellar NHE, or to the Na^+ gradient, typical of the plasma membrane NHE.

The plasma membrane recycling subgroup of NHE genes, including human NHE3 and NHE5, has origins in the worm NHX2 gene, the first evolved plasma membrane NHE. *C. elegans* is one of the earliest species to have evolved neurons and a gut, lined by epithelial cells. Preliminary studies in the worm show that CeNHX2 (the ortholog of mammalian NHE3) is recycled on and off the apical membrane of gut epithelial cells and functions to maintain the H^+ gradient required for nutrient absorption, e.g., peptide uptake by OPT-2 (97). Furthermore, in daf-2 mutants, CeNHX2 and pep-2 (an intestinal H^+ /dipeptide symporter homologous to HsPEPT1) are downregulated, suggesting a mechanism of dietary restriction associated with observed longevity (90). CeNHX1, CeNHX2, and CeNHX7 also show nearly exclusive intestinal expression in the worm,

and CeNHX9 is expressed in the H-shaped excretory cell, the nematode equivalent of the kidney (98). Although the presence of mammalian NHE3 on apical membranes of epithelial cells of the intestine and kidney proximal tubule (and in amiloride studies in mammals) initially prompted investigators to conclude that NHE3 is primarily involved in Na^+ absorption, it is likely that NHE3 has an equally important role in mediating the pH-sensitive regulation of endo- and exocytosis of other receptors and transporters between the recycling endosome and the apical plasma membrane. This emerging role for NHE3 in epithelial cells is supported by studies showing that NHE3 function alters the recycling of albumin receptors and albumin in epithelial cell culture models (44, 45, 60, 83) and in mouse kidney (61), and NHE3 is found in large protein complexes containing endocytotic machinery on the surface of epithelial cells (86, 174). Also, if the primary function of the recycling plasma membrane subgroup of NHE genes is exclusively Na^+ absorption, it is unusual that NHE3 (as well as NHE5, the other recycled mammalian NHE) has an additional neuronal distribution in which extensive Na^+ absorption does not occur but regulation of receptor and transporter recycling is very important (29, 161, 162, 170). Similarly, intracellular NHE predecessors to NHE3 (e.g., ScNHX1) are known to be important for vesicle trafficking or membrane remodeling, a function that is likely retained among all NHE genes in some form. It is also noteworthy that insects also evolved a recycling plasma membrane NHE but do not have a resident NHE. Thus, because *Drosophila melanogaster* has only three NHE genes, it would be an excellent model organism in which to study functional interplay among the NHE genes of three different subgroups: DmNHE2 (recycling plasma membrane), DmNHE1 (NHE8-like), and DmNHE3 (endosomal/TGN).

Similarly, we also can look to model organisms to help us understand the unique regulation of the NHE transporters in each subgroup. For example, NHERF-like PDZ-binding proteins known to bind to mammalian NHE3 orthologs appear in the worm (not in yeast), correlating with the emergence of the NHE3 plasma membrane recycling subgroup. In addition, the predicted NHERF2 binding region within the NHE3 sequence is also conserved in mammalian NHE5 sequences, CeNHX2, and DmNHE2 (data not shown).

The resident plasma membrane NHE subgroup, including human NHE1, NHE2, and NHE4, originated in early vertebrates. Fish were the first vertebrates; thus these exchangers may be important for bone formation or resorption. Preliminary studies in osteoclasts showed a role for Na^+/H^+ exchange in H^+ extrusion linked to bone resorption (92, 132) and in pH regulation of chondrocytes (141, 145). Fish were the first species to have a cardiovascular system responsible for respiratory exchange (unlike invertebrate hearts that just circulated nutrients), and although some gastropods have gills, fish species were the first to have evolved advanced gills for respiratory exchange (which evolved into lungs in amphibians and more complex species). This finding further highlights the importance of NHE1 function in the mammalian heart and circulatory system (6). Similarly, unlike NHE3, NHE1 expression has been detected in all regions of the human airway (47). Also, the pronephric kidney was first seen in these early vertebrates. NHE2 and NHE4 can be found in the more advanced metanephric kidneys of mammals (10).

SUMMARY

This analysis has added several types of insights to one of the most extensively studied classes of mammalian transport proteins, the NHE/SLC9A gene family. Although extensive cloning efforts had already identified all nine mammalian members of this gene family, a comprehensive analysis of genome databases has independently confirmed this conclusion. Importantly, this phylogenetic analysis proposes a new system of classification based on evolutionary development that provides an effective framework within which to analyze NHE location, ion transport and specificity, structure/function correlates, and regulation. This classification also serves to identify appropriate model organisms for the study of individual NHE paralogs. The phylogenetic classification prompts a switch in emphasis from the study of individual NHEs to the study of defined NHE classes. Such an approach is likely to produce a more thorough understanding of the range of functions for each NHE subtype and allow extrapolations between studies performed in different model systems. Studies of NHE1 and NHE3 function have been emphasized, whereas the functions of NHE2 and NHE4–NHE9 remain relatively obscure. In particular, the dearth of ion transport data on the novel intracellular subgroup, including HsNHE6–HsNHE9, has seriously hindered efforts to understand their physiological roles. We suggest that investigators performing physiological studies with these NHE orthologs consider experimental approaches based on this classification scheme, in addition to the organ and cellular distribution of the NHE gene of interest.

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