The Cation/Ca^{2+} Exchanger Superfamily: Phylogenetic Analysis and Structural Implications

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Cation/Ca^{2+} exchangers are an essential component of Ca^{2+} signaling pathways and function to transport cytosolic Ca^{2+} across membranes against its electrochemical gradient by utilizing the downhill gradients of other cation species such as H^+, Na^+, or K^+. The cation/Ca^{2+} exchanger superfamily is composed of H^+/Ca^{2+} exchangers and Na^+/Ca^{2+} exchangers, which have been investigated extensively in both plant cells and animal cells. Recently, information from completely sequenced genomes of bacteria, archaea, and eukaryotes has revealed the presence of genes that encode homologues of cation/Ca^{2+} exchangers in many organisms in which the role of these exchangers has not been clearly demonstrated. In this study, we report a comprehensive sequence alignment and the first phylogenetic analysis of the cation/Ca^{2+} exchanger superfamily of 147 sequences. The results present a framework for structure-function relationships of cation/Ca^{2+} exchangers, suggesting unique signature motifs of conserved residues that may underlie divergent functional properties. Construction of a phylogenetic tree with inclusion of cation/Ca^{2+} exchangers with known functional properties defines five protein families and the evolutionary relationships between the members. Based on this analysis, the cation/Ca^{2+} exchanger superfamily is classified into the YRBG, CAX, NCX, and NCKX families and a newly recognized family, designated CCX. These findings will provide guides for future studies concerning structures, functions, and evolutionary origins of the cation/Ca^{2+} exchangers.

Introduction

It has been estimated that about one-third of the proteins encoded by genomes are membrane proteins, about one-third of which function as membrane transporters catalyzing substrate movement across the membrane (Paulsen, Sliwinski, and Saier 1998; Paulsen et al. 2000). Ca^{2+} signaling plays an important role in mediating numerous cellular processes in virtually all types of cells in plants (Sanders, Brownlee, and Harper 1999; White and Broadley 2003) and animals (Berridge, Bootman, and Roderick 2003), and, therefore, its cytosolic concentration is tightly regulated by three classes of membrane transporters—Ca^{2+} channels (Berridge, Bootman, and Roderick 2003), Ca^{2+} ATPases (MacLennan, Rice, and Green 1997; Sze et al. 2000), and cation/Ca^{2+} exchangers consisting of Na^+/Ca^{2+} exchangers (NCX; Blaustein and Lederer 1999; Philipson and Nicoll 2000) and H^+/Ca^{2+} exchangers (CAX; Hirschi 2001). To maintain long-term cellular Ca^{2+} homeostasis, cation/Ca^{2+} exchangers, as an essential component of Ca^{2+} efflux systems, remove the cytosolic signaling Ca^{2+} and restore resting cytosolic Ca^{2+} levels, as well as refill the intracellular Ca^{2+} stores. Biochemical identification and functional characterization of cation/Ca^{2+} exchangers in various organisms have advanced our understanding of functional properties and physiological roles of these exchangers in cellular ion homeostasis (Rosen 1987; Norris et al. 1996; Blaustein and Lederer 1999; Hirschi 2001).

More recently, molecular analysis of these exchangers has revealed important structure-function relationships (Philipson and Nicoll 2000; Hirschi 2001). As first noted by Schwarz and Benzer in 1997, CALX, an NCX homolog from *Drosophila melanogaster*, displayed two internally homologous regions. These two regions, one each in the two clusters of hydrophobic domains, are designated the α-1 and α-2 repeats and are highly conserved among known NCX homologs. The α-repeats are believed to have arisen from an ancient gene-duplication event and may form the ion binding sites for translocation (Philipson and Nicoll 2000). Alignment of protein sequences identified by Blast (Altschul et al. 1997) searches of the GenBank database suggested that the α-repeat regions were also evident in two H^+/Ca^{2+} exchangers—one from *Saccharomyces cerevisiae* and another from *Arabidopsis thaliana* (Philipson and Nicoll 2000)—as well as other proteins of unknown functions encoded by plants, bacteria, and archaea (Schwarz and Benzer 1997).

In plant cells, Ca^{2+} signaling is involved in pollen tube elongation, seed germination, hyperosmotic stress, and oxidative stress (White and Broadley 2003). Unlike animals, which use Na^+ as the driving force, plants use H^+ almost exclusively for coupled ion transport (Sze, Li, and Palmgren 1999). Plant H^+/Ca^{2+} exchangers are present in the tonoplast and plasma membranes (Hirschi 2001; White and Broadley 2003). The stoichiometry of the vacuolar CAX has been estimated to be 3 H^+/1 Ca^{2+} (Blackford, Rea, and Sanders 1990). The first plant H^+/Ca^{2+} exchangers to be functionally expressed, AthCAX1 and AthCAX2, were cloned from *A. thaliana* by suppression of yeast mutants defective in vacuolar Ca^{2+} transport (Hirschi et al. 1996). AthCAX1 and AthCAX2 share high amino acid identity in two transmembrane domains, with no obvious similarity in the N-terminus and the central acidic motif. There are several other putative H^+/Ca^{2+} exchangers in *Arabidopsis* (Mäser et al. 2001). H^+/Ca^{2+} exchangers have also been cloned from bacteria (Ivey et al. 1993) and fungi (Cunningham and Fink 1996); however, information on molecular properties of CAX exchangers is primarily limited to *Arabidopsis* CAXs (Mäser et al. 2001; Cheng et al. 2002).

In animal cells, increases in the cytosolic Ca^{2+} concentration can cause muscle contraction, trigger the

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release of neurotransmitters from nerve terminals and of hormones from secretory cells, and regulate gene expression (Berridge, Bootman, and Roderick 2003). Na^+/Ca^{2+} exchangers, present at the plasma membrane of most animal cells, are a fast and high-capacity Ca^{2+} transporting system. Detailed functional and molecular studies have revealed the existence of two families of Na^+/Ca^{2+} exchanger proteins (Blaustein and Lederer 1999; Philipson and Nicoll 2000). One family, K^+-independent Na^+/Ca^{2+} exchangers (NCX), is thought to catalyze the electrogenic exchange of 3 Na^+ for 1 Ca^{2+} (Blaustein and Lederer 1999; but see also Fujioka, Komeda, and Matsuoka 2000; Dong, Dunn, and Lytton 2002). The NCX family is composed of three distinct gene products: NCX1 (Nicoll, Longoni, and Philipson 1990), NCX2 (Li et al. 1994), and NCX3 (Nicoll et al. 1996*b*). All three exchangers share about 70% overall amino acid identity that rises to more than 80% within the predicted transmembrane segments (TMS; Nicoll et al. 1996*b*). The second family, K^+-dependent Na^+/Ca^{2+} exchangers (NCKX), catalyzes the electrogenic countertransport of 4 Na^+ for 1 Ca^{2+} and 1 K^+ (Cervetto et al. 1989; Dong et al. 2001). NCKX exchangers differ from NCX proteins in their absolute requirement for K^+, lower Ca^{2+} transport rates, and primary amino acid sequence outside the α-repeats (Blaustein and Lederer 1999). The NCKX family consists of six distinct exchanger molecules: NCKX1 (Reilander et al. 1992), NCKX2 (Tsoi et al. 1998), NCKX3 (Kraev et al. 2001), NCKX4 (Li, Kraev, and Lytton 2002), and NCKX6 (Cai and Lytton 2004); NCKX5 has been identified but not yet characterized (GenBank accession number AB085629; Schnetkamp 2004). A comparison of the sequences between the three human NCX exchangers and the first four human NCKX molecules shows a high sequence identity restricted exclusively to the α-repeat regions (Lytton et al. 2002). Mutagenesis studies have indicated the α-repeat regions are indeed essential for transport functions of NCX (Nicoll et al. 1996*a*), NCKX (Winkfein et al. 2003), and CAX exchangers (Kamiya and Maeshima 2004).

In addition, based solely on sequence alignment, a group of bacterial proteins are also found to be related to NCX and NCKX exchangers (Philipson and Nicoll 2000). Like eukaryotic cells, bacteria also maintain a steep Ca^{2+} gradient across the cell membrane. The role of Ca^{2+} signaling in bacteria has not been clearly demonstrated and information on bacterial cation/Ca^{2+} exchangers is quite limited (Norris et al. 1996; Michiels et al. 2002). Current information from completely sequenced genomes of bacteria, archaea, and eukaryotes have revealed the presence of genes that encode homologues of cation/Ca^{2+} exchangers in many organisms, but the majority of them have not been systematically analyzed.

We have recently reported cloning and characterization of a novel K^+-dependent Na^+/Ca^{2+} exchanger, NCKX6 (Cai and Lytton 2004). Although clearly functioning as a K^+-dependent Na^+/Ca^{2+} exchanger in a heterologous expression system, the amino acid sequence of NCKX6 is surprisingly divergent from other known NCX and NCKX exchangers, even in the conserved α-repeat regions (Cai and Lytton 2004), suggesting that NCKX6 may be a distantly related exchanger member.

In this study, starting from bioinformatic analysis of NCKX6 and its homologous proteins, we have undertaken a comprehensive sequence comparison and phylogenetic analysis of related sequences present in the protein databases to describe patterns of conservation and divergence. Five protein families with distinct evolutionary relationship and highly conserved signature motifs are defined, which provide novel insights into the structure and function relationships of the cation/Ca^{2+} exchangers and builds a foundation for future studies.

Methods

Database Searching, Editing, and Sequence Alignments

PSI-Blast (Altschul et al. 1997) searches with iterations to convergence were run at the National Center for Biotechnology Information (NCBI) Web site (www.ncbi.nlm.nih.gov) to screen the nonredundant protein database. Default values for PSI-Blast searches were used, except that the expect value was 1 and the threshold value for inclusion in subsequent rounds was 0.001. Protein alignments were performed using ClustalW (Thompson, Higgins, and Gibson 1994) at the Canadian Bioinformatics Resources Server (www.cbr.nrc.ca) and were subsequently manually edited to improve alignments in Genedoc Multiple Sequence Alignment Editor and Shading Utility (Nicholas, Nicholas, and Deerfield 1997). The evaluation of percentage conservation of amino acid residues in multiple sequence alignments was performed using the Blosum62 Similarity Scoring Table (Henikoff and Henikoff 1992). Some retrieved sequences were discarded on the basis of the following criteria: (1) partial sequences or sequences resulting from frameshifts in the underlying mRNA as a result of cloning artifacts or possibly aberrant alternative splicing; (2) duplicated database submissions of the same sequence; (3) alternatively spliced isoforms; (4) sequences only displaying similarity to regions in the large hydrophilic loop. Tables containing all the remaining 147 sequences with information on species, sizes, and their accession numbers are available as Supplementary Material online.

Next, regions containing each of the two clusters of hydrophobic domains were extracted for further analysis, since the N-terminus including the signal peptides, the C-terminus, and the central hydrophilic loops were very poorly conserved between independent clades. Previous mutagenesis studies in NCX1 and NCKX1 indicated that only the two sets of hydrophobic segments were essential for exchanger function (Philipson and Nicoll 2000; Szerencsei et al. 2000). Therefore, sequences from these two regions were subjected to ClustalW alignments and Genedoc editing again as described above.

Phylogenetic Analysis

Multiple alignment files edited by Genedoc were exported to files in PHYLIP format. Phylogenetic analyses were performed using the PHYLIP package (Felsenstein 1996) version 3.6 alpha3.

Tree diagrams were prepared by using the maximum parsimony method in conjunction with bootstrap resam-

pling to search for the minimum number of amino acid substitutions with the PROTPARS program. Consensus trees were then created using the CONSENSE program. Tree topology was further confirmed by bootstrapped neighbor-joining analysis with the Jones-Taylor-Thornton substitution matrix (PROTDIST program), followed by the neighbor-joining algorithm as implemented in the program Neighbor. The trees of individual protein families defined in the composite tree were further analyzed with one outgroup sequence chosen from one of the other protein families. The trees were displayed with the TreeView program (Page 1996) version 1.6.6.

Topological Analysis

Hydropathy analysis and prediction of putative transmembrane spanning regions for proteins in the prokaryotic family were conducted using the TopPred II program (Claros and von Heijne 1994). The accuracy of TopPred II algorithm prediction for prokaryotic proteins is over 95%, but it is only 83% for eukaryotic proteins (Claros and von Heijne 1994). Topological models for other protein families are based on currently available experimental evidence for CAX (Kamiya and Maeshima 2004), NCX (Philipson and Nicoll 2000; Nicoll, Ottolia, and Philipson 2002), and NCKX (Schnetkamp 2004).

Results and Discussion

The cation/Ca^{2+} exchanger superfamily is defined by the presence of two highly conserved α-repeat regions in two clusters of hydrophobic domains separated by a central hydrophilic loop rich in acidic residues (Schwarz and Benzer 1997; Hirschi 2001). In 1997, Schwarz and Benzer reported a sequence alignment of about 20 sequences related to the *D. melanogaster* CALX exchanger (Schwarz and Benzer 1997), and in 2000, Philipson and Nicoll proposed four different families in the exchanger superfamily, based solely on sequence alignment of 29 sequences identified by Blast searches of the GenBank database (Philipson and Nicoll 2000). In 2002, Kraev and MacLennan presented preliminary genomic analysis of Ca^{2+} transporters limited to *Caenorhabditis elegans*, *D. melanogaster*, and *Homo sapiens* genomes (Kraev and MacLennan 2002). In this study, we extend these early reports by setting out to compile a comprehensive database consisting of bacterial, archaea and eukaryotic sequences related to cation/Ca^{2+} exchangers, and we present the first phylogenetic tree displaying the evolutionary relationships among the members of the superfamily. The current classification may also be applied to new homologs derived from ongoing sequencing projects.

The Cation/Ca^{2+} Exchanger Superfamily Is Diverse and Large

To identify cation/Ca^{2+} exchanger homologous sequences, we used the human full-length NCKX6 sequence (Cai and Lytton 2004) as a query to carry out PSI-Blast (Altschul et al. 1997) searches on the NCBI nonredundant protein databases. When the PSI-Blast search converged, 346 amino acid sequences were initially identified. These 346 sequences were subjected to multiple sequence alignments with ClustalW and sequence screening as described in Methods. One hundred forty-seven sequences (a complete list can be seen in table 1 in the Supplementary Material online) were retained for further analysis. Identical results were obtained by using the human full-length NCX1 (Komuro et al. 1992) and NCKX2 (Prinsen, Szerencsei, and Schnetkamp 2000) sequences as a query.

Out of 147 sequences, 52 belong to noneukaryotic sequences—10 from archaea and 42 from bacteria. In these 52 sequences, YRBG, an *Escherichia coli* inner membrane protein, is the only protein whose gene has been cloned and expressed (Saaf, Baars, and von Heijne 2001; Ruknudin and Schulze 2002). These noneukaryotic proteins have an average size of about 341 (archaea) ~ 352 (bacteria) residues. For eukaryotic sequences, three are from protozoa (average 491 residues), 13 from fungi (~692 residues), 20 from plants (~501 residues), 24 from invertebrates (~662 residues), four from nonmammalian vertebrates (~807 residues), and 31 from mammals (~846 residues). Most vertebrate proteins as well as some plant proteins have been functionally characterized, but little is known about sequences from protozoa and fungi (table 1 in Supplementary Material online). In addition, compared with bacteria and archaea, which contain only 1~2 sequences within one species, eukaryotes tend to have more exchanger members within a species—for example, five for *Neurospora. crassa*, 11 for *Arabidopsis*, nine for *C. elegans*, and eight for *H. sapiens* (table 1 in Supplementary Material online). This is consistent with the hypothesis that gene families in eukaryotes arise from gene duplication followed by divergence (Lundin 1999). The eukaryotic exchangers are also larger than their noneukaryotic counterparts, having central (or occasionally N-terminal) hydrophilic domains of up to 200~550 residues, which are poorly conserved between independent clades. These regions are not required for ion transport function of exchangers but instead may have evolved to gain different regulatory properties (Philipson and Nicoll 2000; Szerencsei et al. 2000).

Therefore, regions containing the two clusters of hydrophobic domains, corresponding to amino acids 84–249 and 804–955 of human NCX1 (Komuro et al. 1992), were used for further sequence alignment and phylogenetic analysis. The alignment of 147 sequences can be found in Supplementary Material online. Figure 1 shows an alignment of 15 representative sequences from the different families. Several features conserved between prokaryotic and eukaryotic exchangers are noteworthy. The first is two absolutely conserved acidic residues (D/E) at positions 65 and 246, as shown in figure 1, one in each of the two α-repeats. Electrostatic calculations demonstrate that when an ion is transported across the hydrophobic barrier of the membrane, it must overcome an energy barrier that is at its maximum at the membrane center (Parsegian 1969). As demonstrated in the KcsA channel structure (Doyle et al. 1998), ion channels seem to use vestibule charges and dipoles near the selectivity filter to

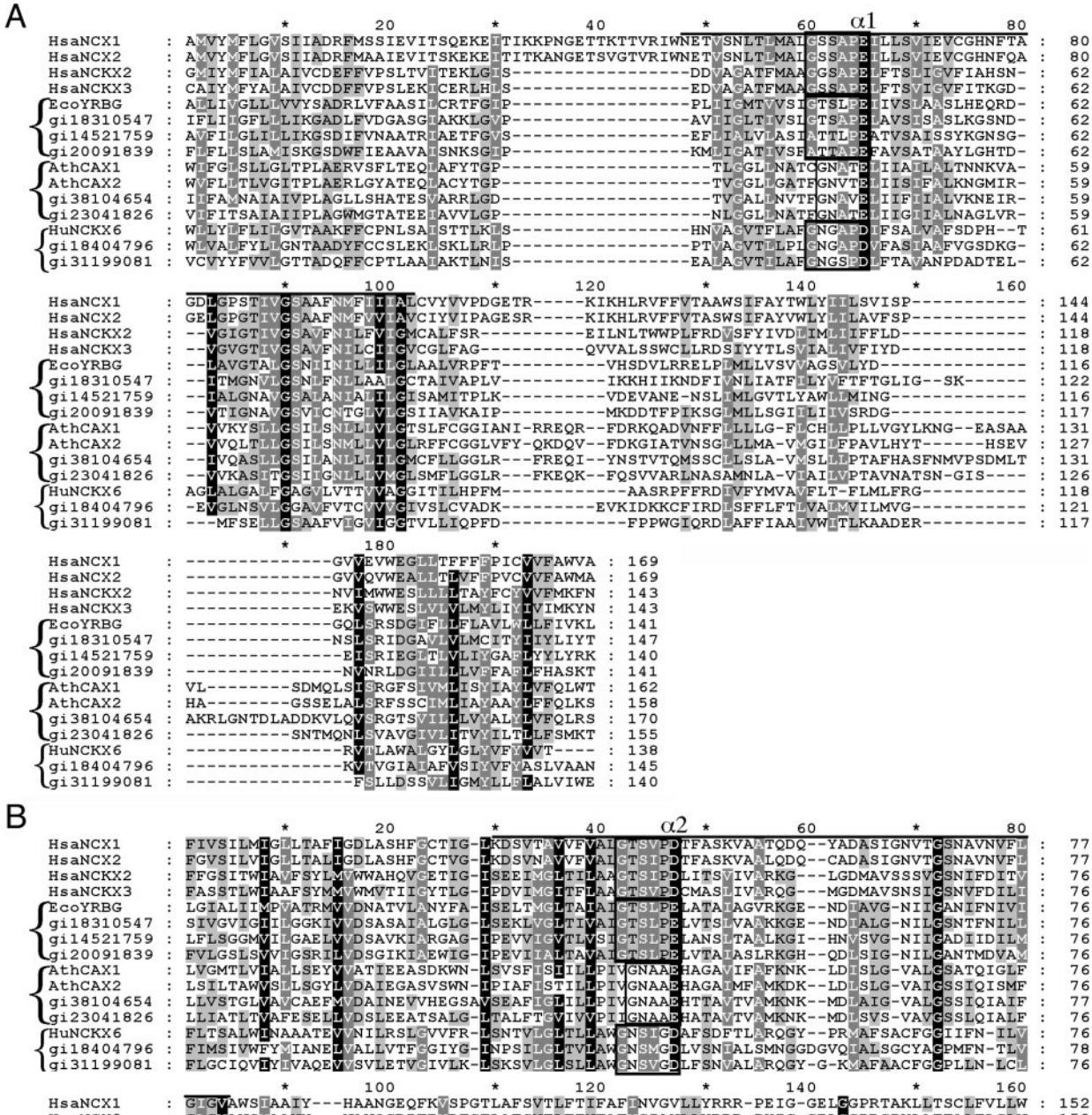

FIG. 1.—Multiple sequence alignment of selected sequences from the cation/Ca^{2+} exchanger superfamily. The amino acid sequences from the two clusters of hydrophobic regions of selected cation/Ca^{2+} exchanger superfamily members were aligned with ClustalW and improved by subsequent manual editing. The regions compared correspond to amino acids 84–249 (*A*) and 804–955 (*B*) of human NCX1 (*HsaNCX1*, gi|10863913). The evaluation of percentage conservation of amino acid residues in multiple sequence alignments was performed using the Blosum62 Similarity Scoring Table (Henikoff and Henikoff 1992). Conserved amino acids are highlighted as follows (white lettering on black background, conserved in 90% or more of the sequences; white lettering on dark gray background, conserved in 70% or more of the sequences; black lettering on light gray background, conserved in 50% or more of the sequences). Dashes indicate gaps introduced to maximize the alignment. The two highly conserved and internally homologous regions are overlined and labeled α1and α2, respectively. The position of the labels α1and α2 also indicates the location of two key conserved acidic residues (E/D). Signature motifs for the α-repeats within each family are highlighted with open squares. Sequences gi|18310547, gi|14521759, and gi|20091839 are from the YRBG family, with gi|38104654 and gi|23041826 from the CAX family and gi|18404796 and gi|31199081 from the CCX family. The names of representatives from each subfamily and the GI numbers of other sequences are shown on the left, grouped by bracket. The complete multiple sequence alignment file of all 147 sequences is available as Supplementary Material online. *Hsa*, *Homo sapiens*; *Eco*, *Escherichia coli*; *Ath*, *Arabidopsis thaliana*.

lower electrostatic energy for a cation. The two key acidic residues of the cation/Ca^{2+} exchangers may be involved in neutralizing the two positive charges on Ca^{2+}, and thus they may help to overcome the energy barrier required to transport a divalent cation. These two acidic residues in NCX1 (Nicoll et al. 1996*a*) and NCKX2 (Winkfein et al. 2003) are very sensitive to mutation. Second, surrounding the two conserved acidic residues there are other acidic residues and numerous threonine, serine, and asparagine residues. Side chain oxygens from these residues likely contribute to coordinating Ca^{2+} binding or creating a hydrophilic microenvironment lining the Ca^{2+} transport pathway. Third, the two α-repeat regions are rich in glycine and alanine residues flanking both sides of the two conserved acidic residues. Two glycine residues, one in each α-repeat region about 20 residues away following the key acidic residue, are absolutely conserved. The structural flexibility endowed by these residues may be associated with the conformational changes of exchangers during the course of a consecutive reaction cycle with alternatively accessible binding sites, in a way that is analogous to the structural flexibility of a glycine residue implicated in the gating mechanism of K^+ channels (Jiang et al. 2002) or the bending of TMS5 of the sarco/endoplasmic reticulum Ca^{2+} ATPase (Toyoshima and Nomura 2002). Fourth, Several leucine/isoleucine/valine residues are also highly conserved, but the pattern does not fit the common features of leucine-rich repeats or the leucine zipper motif.

The Cation/Ca^{2+} Exchanger Superfamily Is Composed of Five Families

Phylogenetic analysis defined five major branches of the cation/Ca^{2+} exchanger superfamily (fig. 2). The clustering pattern generally reflects the source organism type and known functional properties of some eukaryotic proteins. Therefore, four branches were named based on names of characterized proteins within the branch—YRBG, CAX, NCX, NCKX. The branch containing the protein we originally designated NCKX6 is temporarily named CCX (for cation/Ca^{2+} exchanger) until further information on the substrate specificities on this group of exchangers is available. A complete list of 147 sequences sorted by different families is available as Supplementary Material online (table 2). The topology of the trees was not affected when all positions with more than 20% gaps were removed from the multiple sequence alignments used for phylogenetic analysis.

The tree shows that NCX and NCKX families, almost exclusively composed of sequences with animal origins, branch together. Similarly, YRBG and CAX families, both containing many sequences with archaea and bacterial origins, also branch together, while the CAX family also includes many sequences from fungi and plants. Consistent with the original idea presented in the introduction, NCKX6 and its related proteins indeed form a unique branch, CCX, in the exchanger superfamily. The current topological models for representative members from each group are shown in figure 3 and will be discussed below.

YRBG Family Is a Bacterial and Archaea Group

The YRBG family consists of 34 sequences, 25 from bacteria and nine from archaea. With the completion of more prokaryotic genomes, the YRBG family is expected to grow faster than the other four families. YRBG from *E. coli* contains two homologous N- and C-terminal halves, each having five TMSs with opposite membrane orientations (fig. 3*A*; Saaf, Baars, and von Heijne 2001). This arrangement is suggestive of a gene duplication event, and thus it predicts the existence of a primordial "half" exchanger protein containing only five TMSs. To date, however, there is no evidence of such a molecule. Because two oppositely oriented repeat domains seem to be required for cation/Ca^{2+} exchangers to function, the properties of a half-molecule are difficult to predict. Possibly, the signature motif within the α-repeats of the cation/Ca^{2+} exchanger superfamily would not be sufficiently conserved in such a half-molecule to allow identification by the current database search model.

Most proteins in the YRBG family are modeled to have 10 TMSs like the *E. coli* YRBG protein. Eight bacterial proteins, however, are predicted to have 11 membrane spanning segments (fig. 4). Like other proteins in the YRBG family, these eight proteins still have two oppositely oriented α-repeats, with α-1 spanning TMS 2 and 3 and α-2 spanning TMS 7 and 8, but they also possess an additional hydrophobic segment (TMS11) at the C-terminus (fig. 3*A*). Indeed, when extracting the sequences corresponding to the two hydrophobic domains for phylogenetic analysis, 36–49 residues containing the extra hydrophobic segment were removed as the non-homologous C-terminal sequences from these eight proteins, while only 1–6 residues were deleted from other YRBG family members at the C-termini (data not shown).

Overall, these bacteria and archaea proteins share more sequence similarities within the two α-repeats with NCX and NCKX family members than molecules in CAX and CCX families (fig. 1 and Supplementary Material online), especially in regions surrounding the two key acidic residues. The signature motif of the YRBG family in the α-repeat regions is (G/A)T(S/T)xPE (fig. 1). Functional properties are not known for any of the proteins in this family. Overexpression of YRBG in *Xenopus* oocytes did not result in Na^+/Ca^{2+} exchange activity (Ruknudin and Schulze 2002). Although the Na^+ gradient is utilized in some bacteria for coupled transport, H^+ is the most common coupling ion for bacterial bioenergetic functions (Dimroth 1991). Thus, it is likely that the bacterial and archaea exchangers, for instance, YRBG, utilize the H^+ gradient, instead of Na^+, to extrude cytosolic Ca^{2+}. These ideas will need to be explored experimentally.

Our PSI-Blast searches did not identify the characterized *E. coli* H^+/Ca^{2+} exchanger, chaA (Ivey et al. 1993). Indeed, although chaA was initially proposed to function as an H^+/Ca^{2+} exchanger (Ivey et al. 1993), more recent functional studies have suggested its physiological role may be as a major Na^+ extrusion system in *E. coli* (Ohyama, Igarashi, and Kobayashi 1994; Sakuma et al. 1998; Shijuku et al. 2002). Multiple sequence alignments

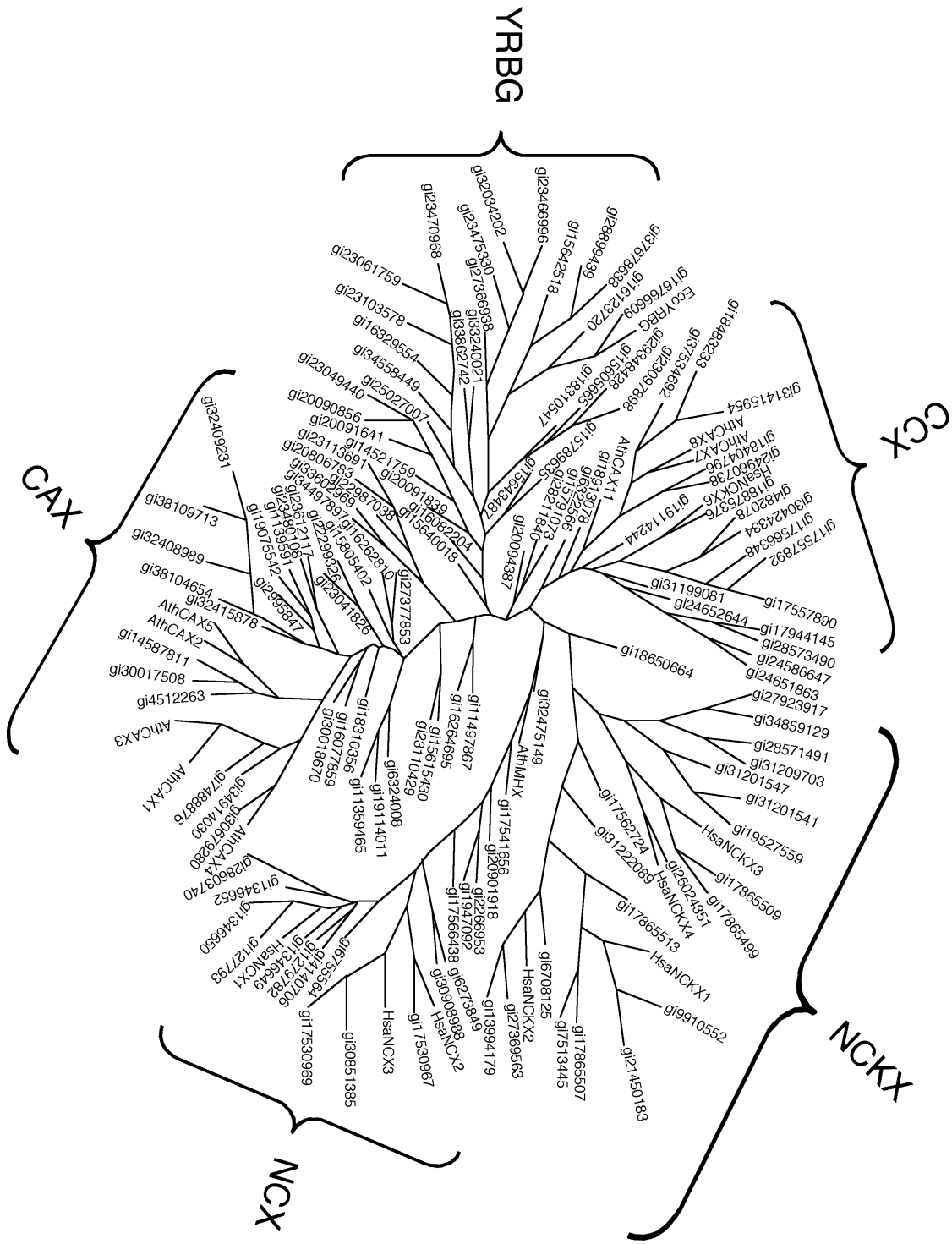

FIG. 2.—A phylogenetic tree based on sequences from two clusters of hydrophobic domains showing the evolutionary relationship between the cation/Ca^{2+} exchangers from bacteria, archaea, and eukaryotes. The phylogenetic tree, constructed by using maximal parsimony method and confirmed by the neighbor-joining analysis, reflects the structural and functional properties of 147 proteins in the cation/Ca^{2+} exchanger superfamily. Five major groups are defined and named after the previously characterized representative members from each group (*YRBG*, *CAX*, *NCX*, and *NCKX*) or temporarily assigned "*CCX*" for the group containing *HsaNCKX6*. Each branch of the tree is labeled with the GI numbers in the NCBI protein database for most organisms and assigned with names in the GenBank for *Arabidopsis thaliana* (*Ath*) and *Homo sapiens* (*Hsa*) exchangers.

show that chaA shares only very limited sequence similarity with plant and yeast CAX molecules (Hirschi et al. 1996) or with YRBG, NCX, NCKX, or CCX exchangers (data not shown) within the α-repeat regions as well as TMSs. Of note, the two key acidic residues of the α-repeats are conserved in chaA. In addition, no obviously conserved repeated motif is present between the two halves of chaA (data not shown). Thus, it is possible that, in

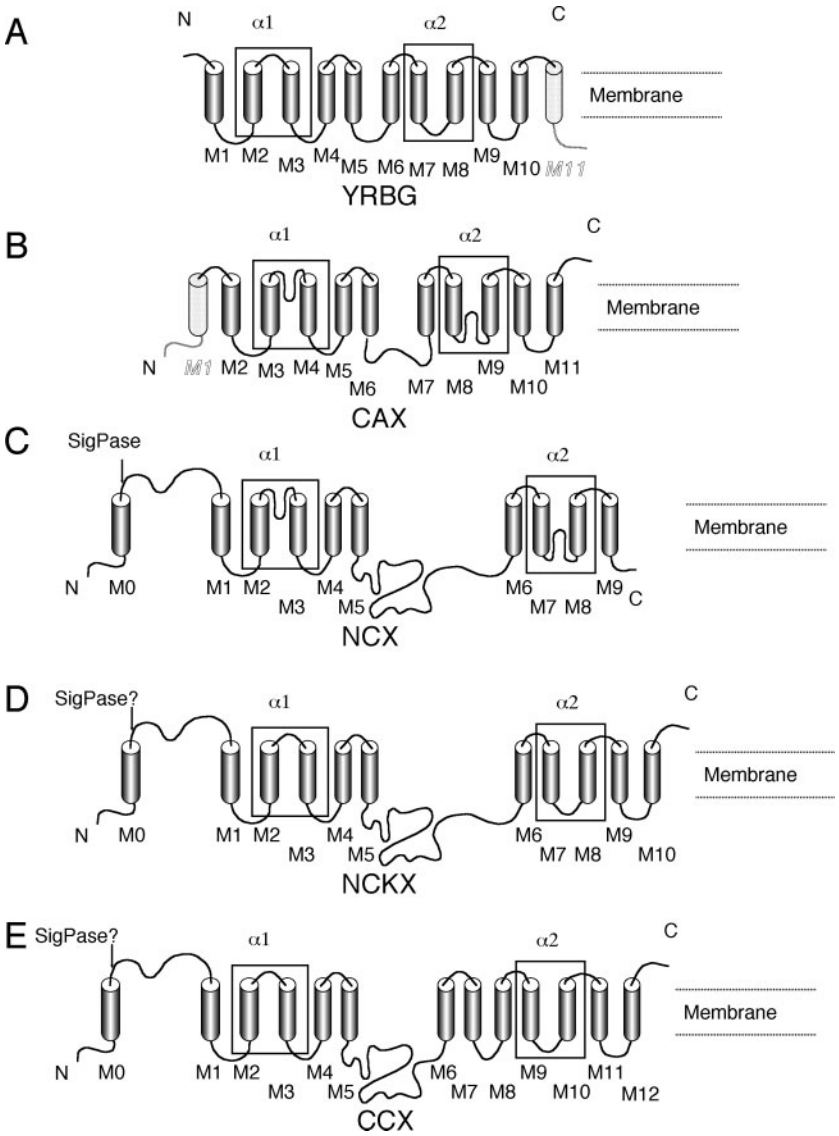

FIG. 3.—Topological models for five cation/Ca^{2+} exchanger families. Putative transmembrane spanning regions for all proteins in the YRBG family and noneukaryotic proteins in the CAX family are predicted using the TopPred II program (42) and illustrated with cylinders indicating the TMSs. Topological models for the NCX (Philipson and Nicoll 2000; Nicoll, Ottolia, and Philipson 2002) and NCKX (Schnetkamp 2004) families and eukaryotic proteins in the CAX (Kamiya and Maeshima 2004) family are based on current experimental information. *M11* in the YRBG family and *M1* in the CAX family, which are absent in some bacterial and archaea members, are indicated by gray cylinders. The loops in α-1 and α-2 of CAX (Kamiya and Maeshima 2004) and NCX (Philipson and Nicoll 2000) have been shown to form membrane reentrant loops. *SigPase*, signal peptidase cleavage site.

addition to the generally accepted gene duplication hypothesis (Schwarz and Benzer 1997; Philipson and Nicoll 2000), internally homologous regions in H^+/Ca^{2+} exchangers may have evolved from other classes of transporters such as chaA by convergent mutations in hydrophobic regions surrounding the key acidic residues.

CAX Family Consists of H^+/Ca^{2+} Exchangers

The CAX family comprises one sequence from archaea, 16 from bacteria, and 24 from eukaryotes—two from protozoa, 11 from fungi, and 11 from plants (fig. 5). Six members from plants have been characterized to catalyze H^+/Ca^{2+} exchange across vacuolar membranes, driven by the pH gradient generated by vacuolar H^+-ATPases and H^+-pyrophosphatase (Hirschi et al. 1996; Hirschi 2001; Cheng et al. 2002). The archaea and nine of the bacterial proteins in the CAX family are predicted to have a 10-TMS topology, typical for the YRBG family. Analysis of the remaining sequences suggests a putative model with 11 TMSs (fig. 3*B*). In contrast to what was found in the YRBG family, the molecules in the CAX family with 11 TMSs have an additional hydrophobic segment at the N-terminal end, not at the C-terminal end. Indeed, about 27–70 residues from bacterial proteins with 11 TMSs were excluded as the N-terminal nonhomologous region from phylogenetic analysis, but only 0–7 residues in the C-terminus. The cause of differences in localization of the extra hydrophobic segment between bacterial

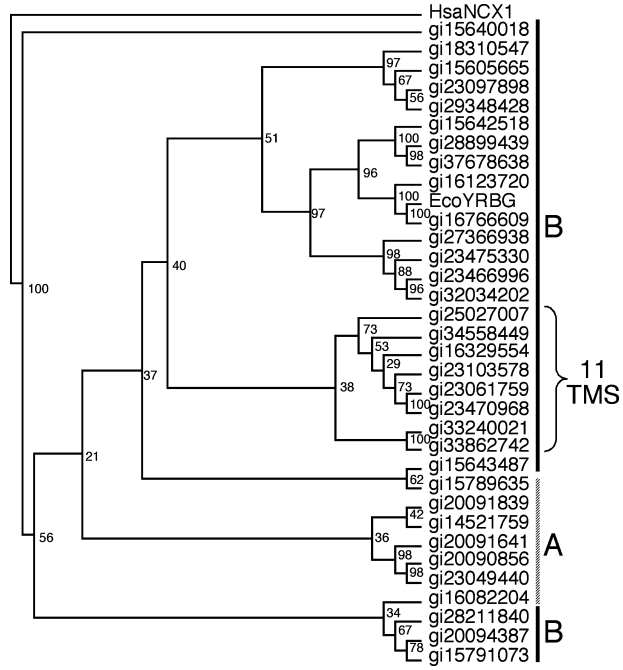

FIG. 4.—Phylogenetic tree of the YRBG family. The tree shown was further refined with maximal parsimony analysis by using sequences of the two hydrophobic clusters from the YRBG branch defined in figure 2 and the *Homo sapiens* NCX1 as an outgroup, and it was confirmed by the neighbor-joining method. Bootstrap values (out of 100 trials) obtained with CONSENSE are indicated at the nodes. Bacterial proteins containing 11 TMSs, predicted by the TopPred II program, are indicated on the right. *B*, bacteria; *A*, archaea.

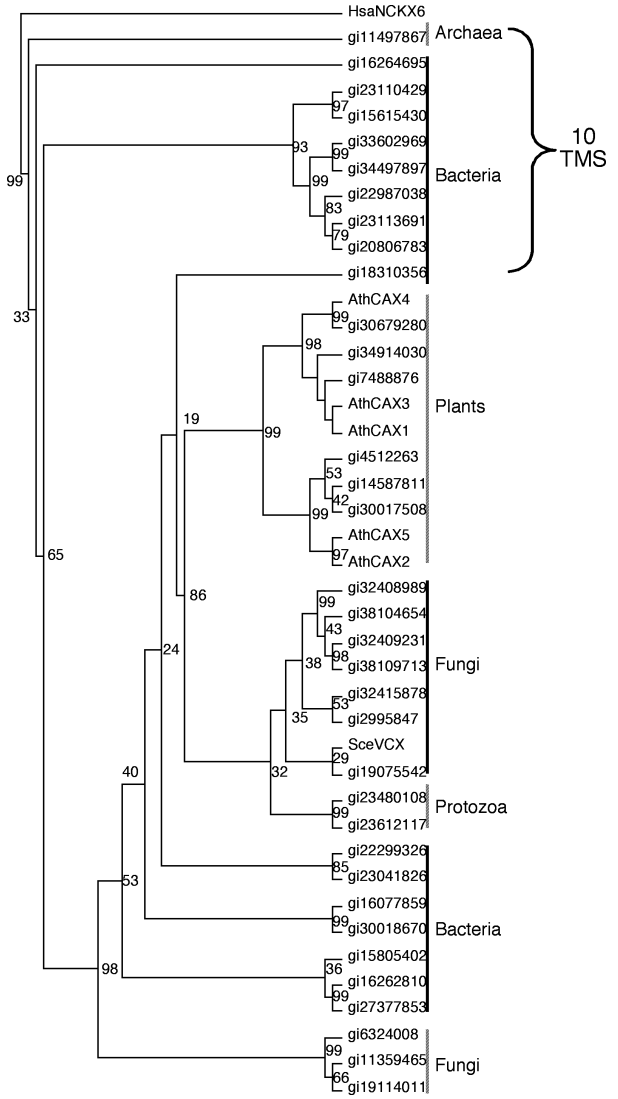

FIG. 5.—Phylogenetic tree of the CAX family. The tree shown was further refined using *Homo sapiens* NCKX6 as an outgroup, as described in the figure 4 legend. The sequences containing 10 TMSs, as predicted by the TopPred II program, are bracketed on the right.

sequences of the YRBG and CAX families is not clear. Possibly, they may have evolved as different signal sequences for protein targeting to discrete sites (Martoglio and Dobberstein 1998).

The signature motif of CAX family members in the α-repeat regions is different from all other members of the cation/Ca^{2+} exchanger superfamily. Both repeats share a highly conserved GNxxE motif where the two Glu residues are the key acidic residues essential for ion transport (Kamiya and Maeshima 2004). His residue is also highly conserved immediately following the second key acidic residue as in "GNAAEH" (fig. 1), and this essential histidine cannot be replaced by Arg or Ala (Kamiya and Maeshima 2004).

NCX and NCKX Families Are Primarily Animal Groups

The NCX (fig. 6) and NCKX (fig. 7) families are composed of 22 and 26 proteins, respectively. Most of them are functionally characterized exchangers from animal species, with two exceptions (table 2 in Supplementary Material online)—in the NCX family, one sequence, AthMHX, (GenBank gi|6492237) from *A. thaliana* has been shown to function as a Mg^{2+} transporter (Shaul et al. 1999), and one sequence (GenBank gi|32475149) in the NCKX family is from a bacterium (*Pirellula sp. 1*). Two mammalian sequences (GenBank gi|27923917 and gi|34859129), as far as can be ascertained from the current NCBI databases, code for the fifth, possibly the last, member of the mammalian NCKX family remaining to be characterized—NCKX5.

The signature motifs for NCX and NCKX family members are G(S/G)SAPE within the α-1 repeat and GTS(I/V)PD within the α-2 repeat (fig. 1). The two key acidic residues in other families are either two Glu residues (YRBG and CAX) or two Asp residues (CCX), but in the NCX and NCKX families, they are a Glu in α-1 and an Asp in α-2. It will be interesting to know whether the asymmetric structure of the transport pathway in the key positions is essential for Na^+/Ca^{2+} exchange activity. The asymmetric nature of key residues in the pore loop region of the voltage-gated ion channels has been shown to be important for different ions selectivity. For example, Asp, Glu, Lys, and Ala in Na^+ channels compared to Glu, Glu, Glu, and Glu in Ca^{2+} channels (Heinemann et al. 1992). In addition, examination of the AthMHX sequence reveals a Gln residue

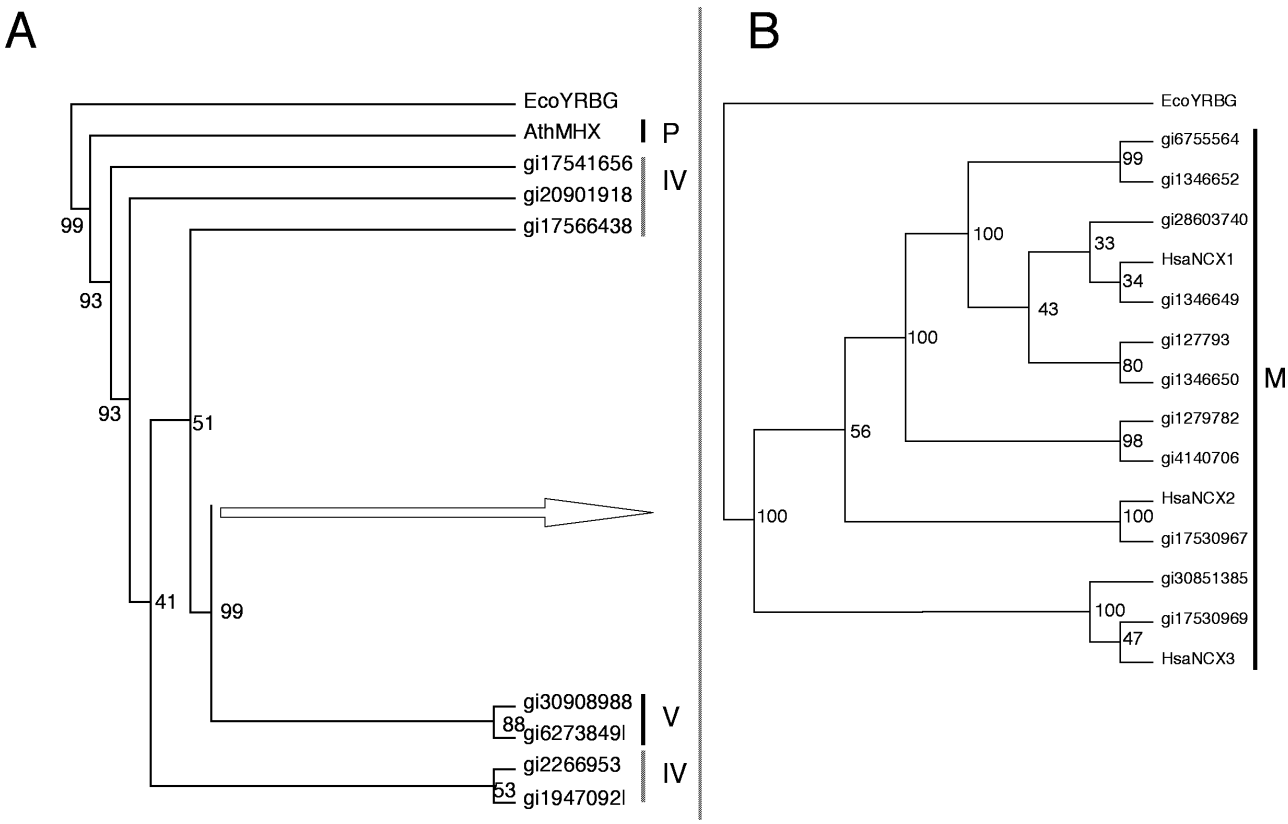

FIG. 6.—Phylogenetic tree of the NCX family. (*A*) The tree shown was further refined using *Escherichia coli* YRBG as an outgroup, as described in the figure 4 legend. (*B*) Expanded view of the mammalian NCX sequences compared using the full-length proteins and the same methods in (*A*). *P*, plant; *IV*, invertebrates; *V*, nonmammalian vertebrates; *M*, mammals.

replacing the key glutamate residue in the α-1 repeat (see alignments in the Supplementary Material online). This change, providing a nitrogen atom in the side chain, might underlie the selectivity of Mg^{2+} over Ca^{2+}.

Hydropathy analysis suggested 12 hydrophobic segments for these animal exchangers (Nicoll, Longoni, and Philipson 1990; Tsoi et al. 1998). Experimental evidence, however, indicated that the first hydrophobic segment served as a cleavable signal sequence and the seventh segment was likely a part of the large central hydrophilic loop (Cai, Zhang, and Lytton 2002; Nicoll, Ottolia, and Philipson 2002). The current models for NCX and NCKX molecules are presented in figure 3. The overall structure and orientations of the two α-repeats in NCKX are reminiscent of the 11-TMS model for exchangers in the CAX family or the 10-TMS model for most YRBG family members with the addition of a signal sequence segment. The C-terminal half of NCX is different, containing only four TMSs. It remains as an intriguing possibility that such structural differences between members of the NCKX and NCX families may underlie their distinctive ion stoichiometry (Cai, Zhang, and Lytton 2002).

Phylogeny of the NCKX family (fig. 7) reveals that branches corresponding to mammalian paralogs also contain invertebrate and nonmammalian vertebrate exchangers. It appears that two distant and sequential gene replication events gave rise to the branches of the NCKX family: one branch containing NCKX1 and NCKX2 and another branch consisting of NCKX3, NCKX4, and putative NCKX5. Thus, the initial gene replication event seems to predate the divergence of vertebrates and invertebrates. In contrast, as shown in figure 6*A*, in the NCX family, invertebrate and nonmammalian vertebrate exchangers diverged before mammalian NCX exchangers split into three branches. These data suggest that mammalian NCX and NCKX exchangers underwent gene replication events at different evolutionary stages.

The evolutionary history of mammalian NCKX and NCX exchangers is also evident in the exon structures (Li, Kraev, and Lytton 2002). For instance, the exon boundary sites of the *NCKX1* gene within the encoded protein are identical to those of the *NCKX2* gene. Similarly, the *NCKX3* and *NCKX4* genes share nearly identical exon boundaries, while the two pairs have distinct exon arrangements. On the other hand, the exon boundaries of the three *NCX* genes are identical, with the exception that the long coding exon 2 present in *NCX1* and *NCX3* is split into three exons in *NCX2*.

NCX1 sequences from nine mammalian species were identified, compared with only two for NCX2 and three for NCX3. The tree topology in figure 6*A* was not affected by using only *H. sapiens* and *Mus musculus* as representative mammalian species for phylogenetic analysis of the whole NCX family (data not shown). However, relationships of some individual mammalian sequences within the well supported mammalian subgroup (bootstrap value 99; fig.

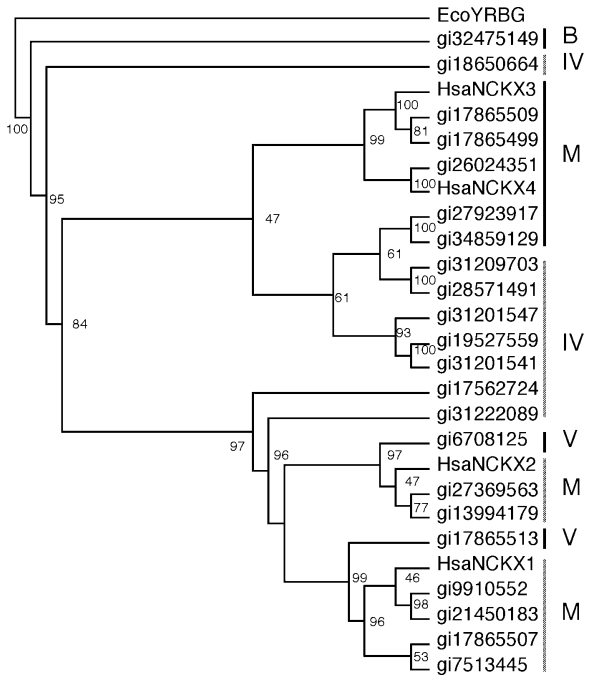

FIG. 7.—Phylogenetic tree of the NCKX family. The tree shown was further refined using *Escherichia coli* YRBG as an outgroup, as described in the figure 4 legend. *B*, bacterium; *IV*, invertebrates; *V*, nonmammalian vertebrates; *M*, mammals.

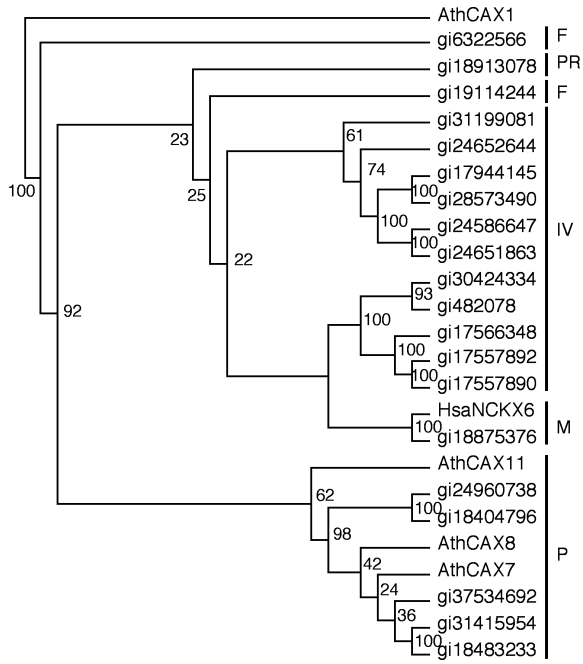

FIG. 8.—Phylogenetic tree of the CCX family. The tree shown was further refined using *Arabidopsis thaliana* CAX1 as an outgroup, as described in the figure 4 legend. *F*, fungi; *PR*, protozoa; *P*, plants; *IV*, invertebrates; *M*, mammals.

6*A*) could not be strongly resolved, possibly due to the very high similarity of the two hydrophobic domains among closely related mammalian NCX sequences. Therefore, the mammalian NCX subgroup was further analyzed using the full-length sequences that might contain additional evolutionary information within the subgroup (fig. 6*B*). This analysis revealed that NCX1 and NCX2 seem to be more closely related to each other compared with NCX3 (fig. 6*B*).

Northern blot and in situ hybridization studies have demonstrated that NCX1, NCX2, and NCX3 and NCKX2, NCKX3, and NCKX4 are highly expressed in brain, and the unique role of each different exchanger in neuronal Ca^{2+} homeostasis and neuronal function awaits further investigation (Lytton et al. 2002). Surprisingly, phylogenetic analysis reveals that the invertebrates—mosquito, nematode, and fruit fly—have also evolved more NCX and NCKX exchangers to regulate cytosolic [Ca^{2+}] than previously appreciated (figs. 6 and 7, online Supplementary Material; Schwarz and Benzer 1997). These model organisms may provide a more tractable way to understand the intriguing question about why nature has evolved so many different Na^+/Ca^{2+} exchangers in neuronal development and function.

CCX Family Is a Separate Group

Phylogenetic analysis and multiple sequence alignments indicate that NCKX6 and its related proteins form a unique group, designated cation/Ca^{2+} exchanger or "CCX," distinct from other members of the exchanger superfamily (figs. 1 and 2). Although NCKX6 is capable of functioning as a K^+-dependent Na^+/Ca^{2+} exchanger in a heterologous system, it may also operate with other cations (Cai and Lytton 2004), just as some CAX exchangers catalyze transport of other cation species in addition to Ca^{2+} (Hirschi 2001). Twenty-four sequences were identified—one from protozoa, two from fungi, eight from plants, 11 from invertebrates, and two from mammals (fig. 8, table 2 in online Supplementary Material). CCX family members share the unique signature motifs GNG(A/S)PD in α-1 and (G/S)(N/D)SxGD in α-2 (fig. 1, online Supplementary Material). The signature sequence is similar to the GNxxE motif in the CAX family, suggesting that the asparagine may also be a key coordinating residue for ion transport.

C. elegans and *D. melanogaster* have five copies of exchanger molecules in the CCX family, more than in the NCX and NCKX families, suggesting CCX proteins may play a particularly important role in those organisms. One interesting finding about the mammalian CCX exchanger, NCKX6, is its ubiquitous expression in all tissues examined (Cai and Lytton 2004). Further characterization of this class of exchangers will no doubt advance our understanding of Ca^{2+} homeostasis in mammalian cells and tissues.

In summary, this study has presented the first comprehensive bioinformatic analysis of the cation/Ca^{2+} exchanger superfamily, integrating genomic, functional, structural, and evolutionary information. The phylogenetic analysis, defining five distinct families, provides a useful guide for future molecular, biophysical, and biochemical analyses of function and mechanism for the cation/Ca^{2+} exchanger superfamily members.

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