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MOLECULAR STRUCTURE AND REGULATION OF VERTEBRATE Na^+/H^+ EXCHANGERS

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Summary

Na^+/H^+ exchangers (NHE), also called antiporters, are vital transmembrane transporters involved in multiple cellular functions including the regulation of intracellular pH, the control of cell volume and transepithelial ion transport. These transporters are highly regulated by a remarkably wide variety of stimuli which can modulate their expression level and activity. Five isoforms of Na^+/H^+ exchangers have been cloned and characterized to date; they define a new gene family of vertebrate transporters. These isoforms share the same overall structure but exhibit differences with respect to amiloride-sensitivity, cellular localization, kinetic variables, regulation by various stimuli and plasma membrane targeting in polarized epithelial cells. Biochemical techniques and molecular genetics tools provide the means of analyzing these transporters at the molecular level. The purpose of this manuscript is to give an overview of the main features of the Na^+/H^+ exchangers with emphasis on recent advances in comprehension of the structure–function relationship and regulation mechanisms of the ubiquitous isoform: NHE-1.

Introduction

Na^+/H^+ antiport (exchange) is one of the primary mechanisms involved in the extrusion of H^+ from vertebrate cells. Originally described by Murer *et al.* (1976) in vesicles from brush-border membranes of kidney tubules, the transporter has since been identified in the plasma membrane of virtually all eukaryotic cells. Biochemical studies and, more recently, molecular cloning have provided increasing evidence about the structure, functional features and regulation of the Na^+/H^+ exchangers, referred to as NHE (see reviews by Counillon and Pouysségur, 1993; Tse *et al.* 1993). Na^+/H^+ exchangers are integral plasma membrane proteins that catalyze the electroneutral exchange of extracellular Na^+ for intracellular H^+ with a stoichiometry of one for one. An essential feature of these exchangers is their allosteric activation by intracellular protons, which are presumed to interact at a 'modifier' site that is separate from the sites involved in Na^+ and H^+ transport.

The activity and expression level of the exchangers can be modulated by a remarkably wide variety of stimuli, including growth factors, tumor promoters, hormones (see review by Grinstein *et al.* 1989) and chronic extracellular acidification (Horie *et al.* 1990), as

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well as by physical factors, such as changes in cell volume (Green *et al.* 1988) or cell spreading (Schwartz *et al.* 1989). The most widely studied NHE isoform, NHE-1, is ubiquitously expressed and is involved in a variety of cellular functions by virtue of its ability to govern intracellular pH. It is inhibited by the diuretic compound amiloride and by the 5-amino-substituted derivatives of amiloride. Besides NHE-1, several other isoforms, initially characterized by their lower sensitivity to amiloride, have been identified, principally in epithelia where they perform more specialized ion transport. Five subtypes of Na^+/H^+ exchangers have been cloned and characterized to date; they share the same structure and define a new gene family of vertebrate transporters.

In this manuscript, we will give an overview of the main features of the Na^+/H^+ antiporters with emphasis on recent progress in understanding the stucture–function relationship and the regulation of the NHE-1 isoform.

Molecular identification of the Na^+/H^+ exchangers

Using a genetic strategy, Sardet *et al*. (1989) were the first to identify a Na^+/H^+ exchanger isoform fully by cloning a human cDNA encoding the amiloride-sensitive growth-factor-activatable Na^+/H^+ antiporter. This cDNA, cloned by complementation of a Na^+/H^+ antiport-deficient cell line, was shown to restore fully the biochemical and physiological features of the transporter, namely pHi regulation, when transfected into antiporter-deficient cells. This human exchanger cDNA, now referred to as NHE-1, is ubiquitously expressed in tissues and cells and, in polarized epithelial cells, generally resides in the basolateral membrane, although exceptions have been reported (D. Roux, J. Noël and J. Pouysségur, in preparation). Pharmacological, kinetic and regulatory properties of Na^+/H^+ exchangers in various cell types, tissues and species had predicted the existence of multiple isoforms of Na^+/H^+ exchangers (see review by Clark and Limbird, 1991). Recent molecular cloning studies have confirmed that Na^+/H^+ exchangers indeed constitute a gene family from which five vertebrate isoforms (NHE-1, βNHE, NHE-2, NHE-3 and NHE-4) have so far been cloned and sequenced (reviewed by Tse *et al.* 1993).

Structural features

On the basis of their hydropathy profiles, all Na^+/H^+ exchangers exhibit similar topologies: the molecule has two separate functional domains: an N-terminal hydrophobic domain (made up of 10–12 membrane-spanning domains) with approximately 500 amino acids and a C-terminal hydrophilic domain with 300 amino acids (Fig. 1). The N-terminal transmembrane part of NHE-1 is necessary and sufficient to catalyze the ion exchange, whereas the cytoplasmic C-terminal domain determines the pH set point value of the exchanger and is crucial for mediating the activation of the exchanger by growth factors, hormones and hyperosmotic stress (Wakabayashi *et al.* 1992).

The isoforms of Na^+/H^+ exchangers cloned so far are homologous (reviewed by Counillon and Pouysségur, 1993; Tse *et al.* 1993). NHE-1, NHE-2, NHE-3 and NHE-4

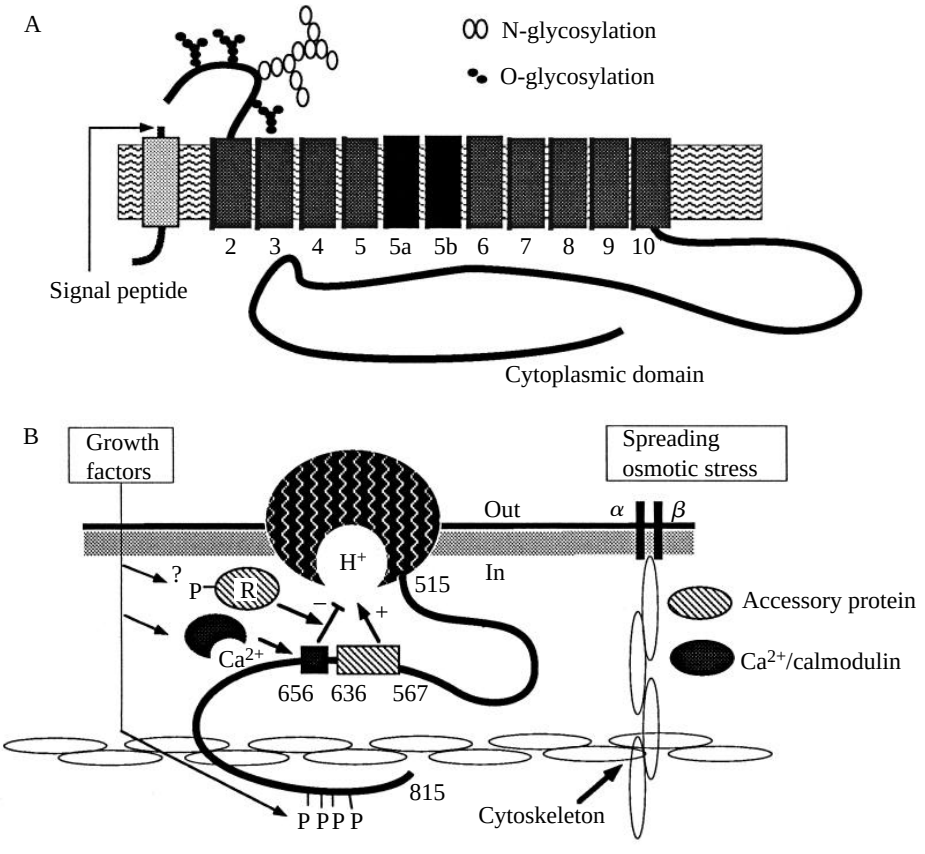

Fig. 1. (A) Membrane topology of NHE-1. We have highlighted 5a and 5b, the most conserved transmembrane segments within the NHE gene family, the O- and N-glycosylation sites in the first extracellular loop and the potential signal peptide. (B) Model for growth factor activation of NHE-1. Deletion 515 (removal of the whole cytoplasmic domain) preserves ion translocation; internal deletion 567–636 abolishes growth factor activation. Different pathways of NHE-1 regulation are represented: growth factors are believed to interact with NHE-1 through an accessory protein (R), as yet unidentified and potentially phosphorylated; they could also interact *via* the binding of Ca^{2+}/calmodulin to the high-affinity calmodulin-binding domain (636–656); finally, they could interact through phosphorylation (P represents the putative phosphorylation sites located in the cytoplasmic tail of NHE-1). Activation of NHE-1 by other stimuli, such as cell spreading and osmotic stress, could be mediated through association of NHE-1 with the cytoskeleton. α and β represent integrins.

share 40–60 % overall amino acid identity: the membrane-spanning domains constitute the most highly conserved portions of the molecules and exhibit 50–60 % amino acid identity. It is noteworthy that, among them, the two segments M5a and M5b appear to be almost identical in all the cloned isoforms, suggesting that this part of the exchanger should play a crucial role in the structure of the molecule and/or in the ion transport mechanism. In addition, all the isoforms possess a first putative transmembrane segment which displays the features of a cleavable signal peptide (Counillon *et al.* 1994). The

cytoplasmic domains share only 30–40 % homology; all of them contain variable putative protein kinase consensus sequences. The βNHE isoform, cloned from trout red blood cells, exhibits a large degree of homology with NHE-1: 74 % homology for the transmembrane domain and 48 % for the cytoplasmic domain (Borgese *et al.* 1992).

NHE-1 is glycosylated. The NHE-1 sequence possesses three consensus sites for N-linked glycosylation at asparagine residues 75, 370 and 410. Indeed, treatment of NHE-1 with endoglycosidase F was found to reduce the apparent size of NHE-1 from 110 to 90 kDa, indicating that NHE-1 is a glycoprotein (Sardet *et al.* 1990). To analyze the potential functional consequences of deglycosylation, site-directed mutagenesis at the individual N-glycosylation consensus sites (Asn to Asp) was performed and the cDNAs containing combinations of these three mutations were generated (Counillon *et al.* 1994). Expression of the mutated cDNAs in antiporter-deficient PS120 fibroblasts showed that all constructs produced functional transporters that had transport rates and pharmacological profiles similar to those of the wild-type molecule, indicating that the glycosylation of NHE-1 is unnecessary for its expression and function. In addition, this study demonstrated that only the first glycosylation site (Asn-75) is used in NHE-1.

NHE-1 and NHE-3 form stable homodimers in intact cells. A recent study (Fafournoux *et al.* 1994) demonstrated that both NHE-1 and NHE-3 exist as stable dimers in the membrane, suggesting that oligomerization might be a common feature of Na^+/H^+ exchange transporters. Importantly for membrane sorting, co-expression of NHE-1 and NHE-3 in the same cells did not lead to the formation of heterodimers, demonstrating an isoform specificity for the subunit interaction. In addition, this study showed that, despite the formation of oligomers as the basic structural unit, the monomer seems to represent the minimal functional unit for NHE-1. However, further work is required before a definitive conclusion can be attained.

The NHE-1 isoform

Amiloride-sensitivity and identification of the amiloride binding site

Amiloride and its 5-amino-substituted derivatives are reported to inhibit the transporter by competing with Na^+ for its external binding site (see review by Benos, 1988). The amiloride binding site appears to be located in the N-terminal domain of the exchanger since NHE-1, after removal of the entire cytoplasmic domain, remains sensitive to amiloride (Wakabayashi *et al.* 1992). NHE-1 is the isoform that is most sensitive to amiloride and its analogues (L'Allemain *et al.* 1984; Tse *et al.* 1993). Identification of the amiloride binding site of NHE-1 was pursued by our group. A one-point mutation was identified by selecting mutant cells expressing an exchanger resistant to amiloride or its analogues (Franchi *et al.* 1986), and their exchanger cDNAs were subsequently cloned and sequenced. A leucine to phenylalanine substitution at position 167 (hamster amino acid sequence), which is responsible for the decreased affinity for 5-amino substitutives of amiloride, was observed in the selected cells (Counillon *et al.* 1993*a*). This residue is located within a highly conserved part of the fourth putative transmembrane domain of the antiporter. In addition, mutation of a close residue in this fourth transmembrane domain, Phe-165 to Tyr, was shown to generate mutants exhibiting a three- to fourfold decrease in

transport rate for Na^+ and a 40-fold increased $K_{0.5}$ (the concentration of amiloride that inhibits 50 % of the initial rate of Na^+ influx) for amiloride (Counillon *et al.* 1993*a*). Interestingly, the Leu-167 to Phe substitution was found to occur naturally in the sequence of NHE-3 that displays an amiloride-resistant phenotype. These results strongly suggest that the fourth transmembrane segment is part of the amiloride binding domain.

Molecular mechanism of activation

Most mitogens activate the NHE-1 isoform, leading to an intracellular alkalization (see review by Grinstein *et al.* 1989), most easily detectable in the absence of bicarbonate (L'Allemain *et al.* 1985; Ganz *et al.* 1989). At least three groups have reported that mitogenic agents activate the antiporter by shifting the pH-dependence of the modifier site, adjusting the set point upwards by 0.15–0.30 pH units (Aronson *et al.* 1982; Moolenaar *et al.* 1983; Paris and Pouysségur, 1984; Grinstein *et al.* 1985). As a result, the exchanger is activated, but only temporarily, returning to near quiescence when intracellular pH (pHi) attains the new set point value. It had been repeatedly suggested that the alteration in the set point was mediated by phosphorylation of the antiporter itself or of an ancillary protein. Indeed, immunoprecipitation of NHE-1 in ^{32}P-labeled fibroblasts demonstrated that 'resting' NHE-1 is phosphorylated and that mitogenic stimulation is accompanied by an increase in phosphorylation of NHE-1 with a time course similar to that of the rise in intracellular pH (Sardet *et al.* 1990). Moreover, okadaic acid, a serine/threonine protein phosphatase inhibitor, can by itself trigger activation of NHE-1 in correlation with stimulation of its phosphorylation (Bianchini *et al.* 1991; Sardet *et al.* 1991). It has been hypothesized that phosphorylation of NHE-1, which has been demonstrated to occur exclusively on serine residues whatever the stimulus used (Sardet *et al.* 1990), directly triggers the activation of the antiporter. Recent evidence, however, shows that the situation is more complex. Wakabayashi *et al.* (1994) pursued their investigations on the role of phosphorylation in the activation of NHE-1 by growth factors. Creating an internal deletion within the cytoplasmic region between amino acids 567 and 635 (Fig. 1) abolished growth factor activation, lowered the affinity of the H^+ sensor but did not change the growth-factor-induced phosphorylation profile. By contrast, deletion of the cytoplasmic tail from residue 635, which removes all the major phosphorylation sites, was shown to reduce by only 50 % the growth factor response, supporting the existence of an additional mechanism not requiring direct phosphorylation of the antiporter for mediating the growth factor signal. The authors therefore postulate the existence of a regulatory protein which would control NHE-1 activation and might itself be regulated by phosphorylation (Wakabayashi *et al.* 1994). In cultured fibroblasts, NHE-1 preferentially accumulates at focal adhesions, where it is likely to interact with cytoskeletal proteins (Grinstein *et al.* 1993). This interaction might be involved in the activation of NHE-1 by osmotic shrinking or during cell spreading. Several cDNA clones encoding proteins putatively interacting with the cytoplasmic domain of NHE-1 have already been isolated in our group by P. Fafournoux using the double hybrid technique (P. Fafournoux, unpublished results).

Very recently, a study by Wakabayashi and colleagues provided more insight into the mechanism of NHE-1 activation. By studying the direct interaction of calmodulin with

NHE-1, they identified and characterized two Ca^{2+}/calmodulin-binding sites located in the middle of the cytoplasmic regulatory domain of NHE-1 (Bertrand *et al.* 1994). They demonstrated that deletion of the high-affinity calmodulin-binding region (residues 636–656) and point mutations of positively charged residues within this region reduced the cytoplasmic alkalization in response to growth factors and hyperosmotic stress, with a concomitant loss of the calmodulin-binding ability, suggesting that binding of Ca^{2+}/calmodulin to the high-affinity calmodulin-binding region is involved in the activation of NHE-1 in response to extracellular signals (Bertrand *et al.* 1994). In addition, the authors showed that mutations that prevented calmodulin binding to this high-affinity region rendered NHE-1 constitutively active by inducing an alkaline shift in the pHi-dependence of NHE-1 (Wakabayashi *et al.* 1994). These results suggest a model in which calmodulin-binding region 636–656 functions as an autoinhibitory domain for NHE-1 and Ca^{2+}/calmodulin activates NHE-1 by binding to this region and thus abolishing its inhibitory effect.

The other NHE isoforms

Pharmacological profiles

Although all Na^+/H^+ exchangers are inhibited by amiloride and its 5-amino-substituted analogues, they exhibit a wide range of sensitivities to these drugs depending upon the cell type. Moreover, within the plasma membrane domains of the same cell in polarized epithelia, the response differs in the apical and basolateral regions. Pharmacological studies on expressed cDNAs of the recently cloned isoforms NHE-2 and NHE-3 have shown that NHE-3 is highly resistant to amiloride and its derivatives whereas NHE-2 exhibits the same sensitivity to amiloride as NHE-1 but has a decreased (25-fold) affinity for ethyl isopropyl amiloride (EIPA) (Orlowski, 1993; Counillon *et al.* 1993*b*; Tse *et al.* 1993).

Tissue specificity

Whereas NHE-1 is ubiquitously distributed, the other NHE isoforms appear to have a more specialized distribution in epithelia (see reviews by Counillon and Pouysségur, 1993; Tse *et al.* 1993). Pharmacological studies had initially identified amiloride-'insensitive' NHE isoforms on the apical membranes of polarized intestinal and renal epithelial cells (reviewed by Clark and Limbird, 1991). To analyze further the targeting specificity of the different isoforms, D. Roux, J. Noël and J. Pouysségur, in our group, expressed in the renal epithelial cell lines OK and MDCK either NHE-1 or an epitope-tagged NHE-3. The use of two approaches, immunodetection by confocal microscopy and pharmacological analysis of amiloride-sensitive ^{22}Na uptake, allowed us to demonstrate that, in these two cell systems, the NHE-1 isoform is expressed both apically and basolaterally whereas the NHE-3 isoform is specifically targeted to the apical membrane (D. Roux, J. Noël and J. Pouysségur, unpublished data). It is presently impossible to determine whether the lack of specificity of NHE-1 expression observed in OK and MDCK cells is physiological or corresponds to a loss of function of the protein trafficking machinery of these cell lines. Nevertheless, these results clearly show that the

NHE-3 isoform possesses all the information to be expressed specifically at the apical face of epithelial cells.

Hormonal regulation

The analysis of the hormonal regulation of the different exchangers is particularly complex because different isoforms are co-expressed in a variety of tissues and exhibit different pathways of regulation (for a review, see Clark and Limbird, 1991). A strategy to overcome these difficulties consists of studying the regulation of the cloned exchangers by expressing single isoforms in NHE-deficient PS120 cells or in well-characterized established cell lines (Tse *et al.* 1993). The results of such studies are interesting, but are limited by their failure to take into account the effects on regulation of the exchangers inherent to the cell type and to the polarity of epithelial cells. Indeed, the effects of protein kinases on the regulation of Na^+/H^+ exchangers differ depending on the cell type and also differ within the same cell depending on the membrane domain: i.e. apical *versus* basolateral domain (Tse *et al.* 1993).

The βNHE isoform isolated from trout red cells provides an interesting example of the activation of NHE by increased levels of cyclic AMP. It has two clustered cyclic-AMP-dependent protein kinase consensus sites (Borgese *et al.* 1992) located in the cytoplasmic domain. When expressed in PS120 fibroblasts, βNHE displayed cyclic-AMP-sensitivity (Borgese *et al.* 1992), whereas there was no effect of cyclic AMP on the activity of the isoforms NHE-1, NHE-2 or NHE-3 transfected into the same cells (Borgese *et al.* 1992). The deletion or point mutation of the two protein kinase A consensus sites of βNHE strongly reduced its response to cyclic AMP (Borgese *et al.* 1992). Replacing the cytoplasmic tail of human NHE-1 with that of βNHE conferred cyclic-AMP-sensitivity to the chimeric NHE-1 isoform (Borgese *et al*. 1994). These results reinforce the hypothesis that the cytoplasmic domain mediates and dictates the nature of the hormonal response. However, another level of regulation can be demonstrated; NHE-1, which is insensitive to variations in cyclic AMP concentration when expressed in fibroblasts, becomes inhibited by agents that raise cyclic AMP levels when expressed in the epithelial cell line OK (Helmle-Kolb *et al.* 1993). This inhibition by cyclic AMP is the typical response of the endogenous apically expressed ‘amiloride-resistant’ isoform in these cells (Helmle-Kolb *et al.* 1993). These findings illustrate that the nature of the hormonal response relies not only on the sequence of the cytoplasmic domain but also on the nature of the cell expressing the NHE isoform.

Concluding remarks

Identification of the primary structure of the Na^+/H^+ exchangers by cloning their cDNAs represented a major advance in the biology of these widespread transporters. Now, with the generation of specific molecular tools and the advance of molecular genetics, future work will describe the complete ‘anatomy’ of the NHE isoforms in the nephron and other organs. Experiments exploiting the combination of site-directed mutagenesis and random mutagenesis with powerful genetic screens will help considerably in the identification of the key residues of the NHE molecules involved in

ion catalysis, H^+-sensing and apical targeting in epithelial cells. Finally, alteration of NHE gene expression in transgenic animals or disruption of the genes encoding each isoform in mice is another area of considerable interest in investigating the physiological role of these exchanger isoforms and their possible implication in human diseases.

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