Annu. Rev. Pharmacol. Toxicol. 2002. 42:527–52

THE CHANGING FACE OF THE Na^+/H^+ EXCHANGER, NHE1: Structure, Regulation, and Cellular Actions

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Key Words intracellular pH, ion exchanger, signaling, cytoskeleton, cell proliferation

■ **Abstract** The NHE family of ion exchangers includes six isoforms (NHE1–NHE6) that function in an electroneutral exchange of intracellular H^+ for extracellular Na^+. This review focuses on the only ubiquitously expressed isoform, NHE1, which is localized at the plasma membrane where it plays a critical role in intracellular pH (pH_i) and cell volume homeostasis. All NHE isoforms share a similar topology: an N-terminus of 12 transmembrane (TM) α-helices that collectively function in ion exchange, and a C-terminal cytoplasmic regulatory domain that modulates transport activity by the TM domain. Extracellular signals, mediated by diverse classes of cell-surface receptors, regulate NHE1 activity through distinct signaling networks that converge to directly modify the C-terminal regulatory domain. Modifications in the C-terminus, including phosphorylation and the binding of regulatory proteins, control transport activity by altering the affinity of the TM domain for intracellular H^+. Recently, it was determined that NHE1 also functions as a membrane anchor for the actin-based cytoskeleton, independently of its role in ion translocation. Through its effects on pH_i homeostasis, cell volume, and the actin cortical network, NHE1 regulates a number of cell behaviors, including adhesion, shape determination, migration, and proliferation.

INTRODUCTION

Proton fluxes across biological membranes drive a number of physiological processes, including communication within and between cells, cell migration, and the rate at which cells grow, divide, and differentiate. Proton fluxes at the plasma membrane are regulated by several families of ion exchangers, including the Na^+/H^+ exchangers (NHEs) and HCO_3^- transporters, such as the Na^+/HCO_3^- cotransporters (NBCs), Cl^-/HCO_3^- exchangers (AEs), and the Na^+ driven Cl^-/HCO_3^- exchanger (NDAE). The focus of this review is the NHE family, with primary emphasis on

the ubiquitously expressed NHE1 isoform. NHE1 catalyzes an electroneutral exchange of intracellular H^+ for extracellular Na^+, and in so doing regulates pH_i and cell volume. The latter is achieved through Na^+-driven Cl^- and osmotically obliged H_2O fluxes. This review highlights recent findings on NHE1, focusing on its structural topology, regulation, and cellular effects.

To date, six NHE family members have been identified. NHE1 through NHE5 share ~34% to 60% amino acid identity. NHE1 is ubiquitously expressed and plays a central housekeeping role in pH_i and cell-volume homeostasis (see 1, 2 for reviews). In contrast, NHE2 through NHE5 have a more limited tissue distribution and are more specialized in function. NHE2 and NHE4 are expressed predominantly in the kidney and gastrointestinal tract, where apically localized NHE2 functions primarily in Na^+ reabsorption (3), and may act in coordination with basolateral NHE4 (4–7), to promote osmoregulation of renal inner medullary cells. NHE3 is specifically targeted to the apical membrane of renal proximal tubule epithelia (8) and the brush border of mature intestinal epithelia (9, 10). In the kidney, NHE3 is important in Na^+ and HCO_3^- reabsorption, which contributes significantly to maintenance of blood osmolarity and acid-base balance (see 11 for review). NHE5 is expressed predominantly in the brain (12, 13) and is thought to regulate pH_i in neurons (14). The most recently identified NHE family member, NHE6, is the most divergent in sequence, sharing only ~20% identity with the other isoforms. NHE6 is localized exclusively to the mitochondria, with greatest expression in highly metabolic tissues, such as heart, brain, and skeletal muscle (15).

The NHE gene family is evolutionarily conserved. NHEs have been identified in a number of lower organisms, beginning with the bacteria *Streptococcus faecalis* (16) and *Escherichia coli* (NhaA and NhaB) (17), where they are electrogenic and share very little homology with NHEs of higher organisms. Genes coding for NHEs have also been identified in the plant *Arabidopsis thaliana* (18) and the yeast strains *Schizosaccharomyces pombe* [sod2; (19)] and *Saccharomyces cerevisiae* [NHX1 (20); NHA2 (15)]. The protein product of the NHX1 gene, *Nhx1p*, is found predominantly in a prevacuole compartment and is important for endocytic trafficking (21). NHE genes have also been identified in *Caenorhabditis elegans* (22) and *Drosophila melanogaster* (23), although specific isoforms and functions have not been characterized. Finally, in higher organisms, NHEs have been identified in a number of vertebrates, including human (12, 24), rat (4, 25), mouse (26, 27), pig (27a), *Xenopus laevis* (27b), and trout (27c).

STRUCTURAL TOPOLOGY OF NHE1

In mammals, NHE1 is comprised of ~813 to 822 amino acids, with a calculated molecular mass of ~91 kDa. It has a structural topology predicted to be shared by other families of plasma membrane transport proteins critical for pH_i homeostasis, specifically the AEs, NBCs, and NDAE. Based on hydropathy profiles, members of these families are predicted to consist of 10 to 13 transmembrane (TM) segments

(28) with cytoplasmic N- and C-terminal domains (29). NHE1 has a relatively short N-terminal domain and a long C-terminal domain, whereas AE1, NBC1, and NDAE have a long N-terminal domain, and a relatively short C-terminal domain. Hence, NHE1 is a mirror image of AE1 and NBC1. The unifying function of these transporters, to regulate pH_i, is followed by conservation of form or structural topology, which suggests that their distinct structural differences are likely determinants of functional differences, such as ion selectivity and regulation of transport activity.

The 12 α-helices of the TM region of NHE1 (TM-1 to TM-12) (Figure 1) collectively function in the exchange of extracellular Na^+ for intracellular H^+ in an electroneutral 1:1 stoichiometry. The amino acid sequence of the TM domain for NHE isoforms is highly conserved—TM-6 and TM-7 have 95% identity—which suggests that these segments likely participate in the translocation of Na^+ and H^+. Amino acid substitution of Glu-262 to Iso (E262I) in TM-7 of human NHE1 abolishes H^+ ion translocation (30). The N-terminus of NHE1 is thought to be intracellular, although there is a putative signal peptide sequence within the first extracellular loop (EL) of the protein, and evidence for cleavage is controversial (31, 32). The sequence identity of the C-terminal cytoplasmic domain among NHE isoforms is much lower ($\sim$25% to 35%) than that of the N-terminal domain. Functionally, the NHE C-terminal domain regulates ion translocation by the TM region of the molecule. Hence, the topological variation of the C-terminus among NHEs is in keeping with differential regulation by this domain.

An alternative model to the widely accepted topological model of NHE1 predicted by Kyte-Doolittle hydropathy analysis was recently described by Wakabayashi et al. [Figure 1, (31)]. This model was generated by using cysteine mutagenesis of a heterologously expressed human NHE1 molecule, and then determining the accessibility of the cysteines to biotin maleimide in the presence and absence of cell permeabilization. The major differences between the original model and the model presented by Wakabayashi are in the regions from TM-10 through EL-6. The new model predicts TM-10 to be located within the membrane, but not spanning it as previously thought. In addition, the new model predicts IL-5 to be located extracellularly, rather than intracellularly, and predicts EL-6 to span the membrane as TM-11. In support of the recent model proposed by Wakabayashi, work by Shrode et al. (32) suggests that EL-5 and EL-6 of the hydropathy-based model may not be fully exposed extracellularly. Hence, if the latest model is correct, several "new" amino acids may reside within the TM domain, and thus may be potential candidates for participation in ion translocation.

The amino acid sequence of NHEs predicts that several isoforms contain potential glycosylation sites. NHE1 contains sequences for both *N*- and *O*-linked glycosylation, and there is evidence that Asn-75 in EL1 of NHE1 is glycosylated (33). In contrast, NHE2 possesses only *O*-linked glycosylation sites (34), and NHE3 does not appear to be glycosylated (8, 33). Moreover, NHE1 and NHE3 may form functional homodimers by establishing physical contacts within the TM regions (30), possibly through formation of disulfide bonds (35). Recently, Gebreselassie et al. (36) determined the secondary structure of the C-terminal cytoplasmic domain

of NHE1 by using circular dichroism spectroscopy. The C-terminus was found to consist of 35% alpha-helix, 17% beta-turn and 48% random coil, which suggests that this regulatory domain contains distinct secondary structures that may be important for function.

MOLECULAR PHYSIOLOGY OF NHE1

Most NHE isoforms exhibit simple Michaelis-Menten transport kinetics for extracellular Na^+ and H^+, which suggests that each of these ions has a single binding site on the extracellular face of the protein. The dependence of NHE1 activity on extracellular Na^+ is saturable and follows a hyperbolic relationship with apparent affinity constants (K_{Na}) in the range of 3 to 50 mM (39–43). The extracellular Na^+-binding site of NHE1 can also bind and transport H^+ and Li^+, acting to competitively inhibit NHE1 activity (37, 38). In contrast, NHE4 activation exhibits either a hyperbolic (44) or a sigmoidal (45) response to increasing extracellular Na^+ concentrations, which suggests allosteric or cooperative binding kinetics.

NHE1 is highly sensitive to changes in intracellular H^+ (H_i^+), such that reduced pH_i allosterically activates the protein (46). Hence, the kinetics of NHE activity in response to H_i^+ is more complex than that observed for extracellular substrates. NHE1, NHE2, and NHE3 are extremely sensitive to low pH_i. At physiological pH_i, NHE1 and NHE2 are essentially inactive, but they are rapidly activated upon reduction in pH_i (38, 46), whereas NHE3 is active at neutral pH_i because it has a higher affinity for H_i^+. Moreover, NHE1, NHE2, and NHE3 have Hill coefficients of $\sim$2 for the dependence of H_i^+ on NHE activity, which suggests there may be more than one binding site for H^+ on the intracellular face of the protein. In contrast, NHE5 has been shown to exhibit first-order dependence on H_i^+ concentration (39); this suggests that there may be a second type of H^+ binding site, in addition to the transport site, with positive cooperative binding characteristics. In fact, this is not a new concept. Aronson et al. (46) first suggested that the exquisite sensitivity of NHEs to pH_i may be due to a "H^+ modifier site" located in the TM domain at a site separate from the H^+ binding site that acts to set pH_i sensitivity (pH_i setpoint) (46, 47). However, it is not clear whether allosteric activation by H_i^+ is due to direct protonation of ionizable groups in the protein (48), acting to alter NHE conformation and stimulate activity, or is the result of cell-specific regulators that allosterically stimulate NHE activity through pH_i- or hormone-sensitive conformational changes (47, 49).

PHARMACOLOGICAL INHIBITION OF NHE1

Two major classes of pharmacological agents are currently used to inhibit NHE1 activity (Figure 2). One class includes amiloride and its 5′ alkyl-substituted derivatives (40, 50, 51), such as ethylisopropylamiloride (EIPA), dimethylamiloride

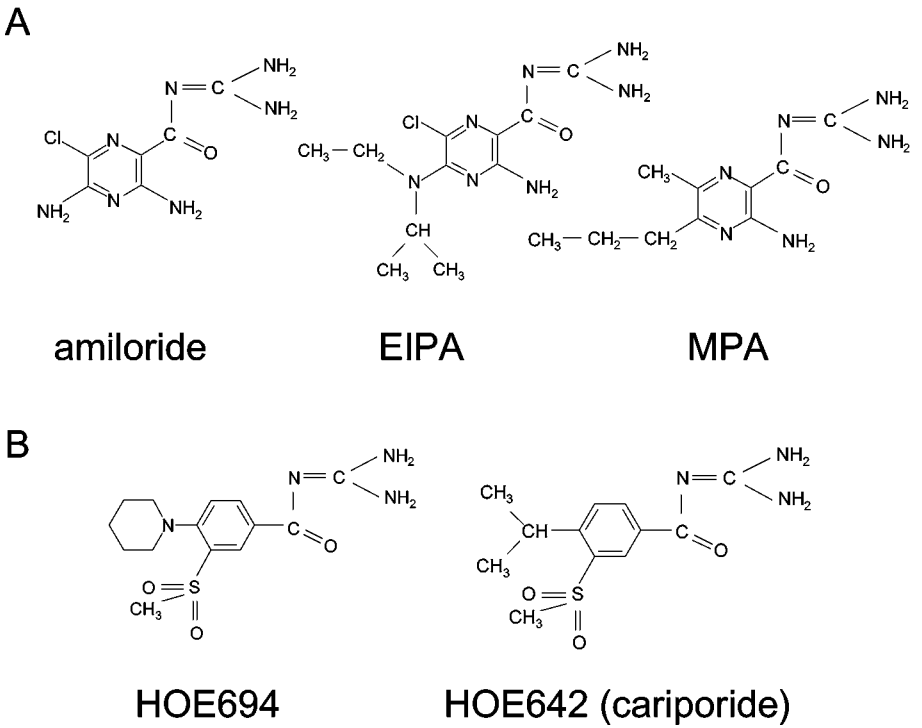


Figure 2 The two major classes of pharmacological agents currently used to inhibit NHE1 activity.

(DMA), and 5-*N*-(methylpropyl)amiloride (MPA). Another class of inhibitors includes the benzoylguanidines and derivatives such as HOE694 (50) and HOE642 (cariporide) (52). Both classes of inhibitors demonstrate higher specificity for NHE1 than NHE3, with the amiloride compounds $\sim 10^2$-fold more specific and the HOE compound $\sim 10^3$- to 10^5-fold more specific. Thus, due in part to their ability to inhibit NHE1 activity, but not renal NHE3 activity, cariporide (53) and other HOE compounds (54) are currently being developed as potential treatments for NHE1-mediated ischemia-reperfusion injury.

NHE1 and NHE2 are both sensitive to inhibition by amiloride and its derivatives, although NHE2 is slightly less sensitive (40, 50). NHE3 and NHE4 are both amiloride insensitive. The sensitivity of NHE5 to inhibition by amiloride and its derivatives is in between that of NHE1 and NHE3, but closer to that of NHE3 (39). Thus, the order of sensitivities to NHE-inhibitory drugs under similar experimental conditions is NHE1 > NHE2 > NHE5 > NHE3. Although the sensitivity of NHE6 to these inhibitors has yet to be determined in a heterologously expressed system, mitochondrial NHE has been shown to be amiloride resistant, but sensitive to various amiloride derivatives (42, 55, 56).

NHE1 inhibitors are thought to act by competitively inhibiting Na^+ binding at the extracellular cation-binding site (38, 50, 57–59). Amiloride and its derivatives, however, have also been shown to inhibit ion translocation noncompetitively, which suggests that external Na^+- and amiloride-binding sites may not be identical (60, 61). Although it is not known whether NHE1 inhibitors and Na^+ compete for the same binding site, several studies have shown that amino acid residues in TM4 (31, 51, 62) and TM9 (31, 48, 63) are important for both ion translocation and amiloride binding.

Recent findings indicate that the actions of pharmacological inhibitors on NHE1 may not be completely understood. As discussed below in the sections on NHE1 regulation and effects, a novel function of NHE1 as a plasma membrane anchoring protein for actin filaments has been described (64). This function, which occurs independently of ion translocation, is an important determinant in cell adhesion and in dynamic reorganization of cortical actin filaments. It is interesting that cells treated with the pharmacological inhibitors EIPA and HOE694 at concentrations selective for NHE1 have shown impaired adhesion and actin filament assembly (65–67). These findings suggest that inhibition of transport activity by existing pharmacological inhibitors might also be associated with conformational changes in NHE1 that impair its association with the cytoskeleton. Although this possibility has not been experimentally confirmed, it indicates caution in interpreting the action and cellular effects of these inhibitors. Moreover, the ability of NHE1 to independently regulate ion translocation and cytoskeletal anchoring dictates the need to develop drugs that selectively target these separate functions.

REGULATION OF NHE1 ACTIVITY

A distinct characteristic of NHE1 is that its activity is regulated by diverse classes of cell-surface receptors, including receptor tyrosine kinases, G protein–coupled receptors, and integrin receptors. Acting through a number of well-characterized signaling networks, receptor-mediated signals converge on a limited number of NHE1-interacting proteins that regulate modifications in the C-terminal cytoplasmic regulatory domain of NHE1. These modifications, including phosphorylation, the binding of regulatory proteins, and conformational changes, regulate transport activity by changing the affinity of the transmembrane internal H^+ transport site. Wakabayashi et al. (49) found that deleting the C-terminal cytoplasmic domain markedly alters the pH_i sensitivity of NHE1. Hence, the C-terminal domain functions as a sensor to discriminate converging signals which, through a mechanism that is currently unclear, ultimately modulates the allosteric activation of NHE1 by H_i^+. In contrast, receptor-dependent activation of the NHE3 isoform is regulated through changes in V_{max} and trafficking between the plasma membrane and recycling endosomes (68, 69). The entire population of NHE1, however, is thought to remain constitutively at the plasma membrane and is not regulated by recruitment of internal stores (32, 68).

Signaling Networks Regulating NHE1

Activation of receptor tyrosine kinases increases NHE1 activity through the classical Ras-mediated ERK cascade. Early work by Hagag et al. (70) and Maly et al. (71) found that NHE1 activity is increased in cells transformed with oncogenic Ras, and this was later confirmed by expressing mutationally active Ras in fibroblast cell lines (72–74). Activation of NHE1 by downstream effectors of Ras, including Raf-1 (72, 75), MEK1/2 (75–77), and ERK or p42/44 MAPK (75, 78), was subsequently determined by using mutationally regulated kinases and pharmacological inhibitors. ERK, however, does not directly activate or phosphorylate NHE1 (75). Takahashi et al. (79) recently determined that p90 ribosomal S6 kinase ($p90^{RSK}$), a serine/threonine kinase acting downstream of ERK, phosphorylates NHE1 and is a likely direct regulator mediating NHE1 activation by growth factors. Moor & Fliegel (77) subsequently confirmed that $p90^{RSK}$ mediates a MEK-ERK-dependent phosphorylation and activation of NHE1.

Activation of NHE1 by receptor tyrosine kinases also occurs by alternative Ras-independent signaling cassettes. Consistent with the recruitment of phospholipase C and phosphatidylinositol 3-kinase (PI3K) to activated receptor tyrosine kinases (80), activation of NHE1 by epidermal growth factor (EGF) (81) and by platelet-derived growth factor (PDGF) (82, 83) is blocked by the inhibition or downregulation of protein kinase C (PKC), through a mechanism that is both dependent (82) and independent (83) of PI3K activity. PDGF activation of NHE1 was also recently found to be mediated by the Nck-interacting kinase NIK (84). Nck is a Grb2-like adaptor protein that binds to the cytoplasmic domain of receptor tyrosine kinases, and, as described below in the section on direct regulation of NHE1, its downstream kinase, NIK, binds to and phosphorylates the C-terminal cytoplasmic domain of NHE1.

Although a number of G protein–coupled receptors regulate NHE1 activity, in most cell types only two G protein α subunits, $G\alpha_q$ and $G\alpha 13$ (74, 85, 86), couple to the activation of NHE1. The expression of mutationally active $G\alpha_s$ and $G\alpha_i$ has no effect on NHE1 activity (74, 87), nor do bacterial toxins that regulate the activity of these α subunits (74, 87–89). Moreover, in most cell types, changes in cAMP have no effect on NHE1 activity (88, 90, 91). Two independent studies using chimeric NHEs confirmed that the cytoplasmic domain of NHE1 is insensitive to cAMP levels. Borgese et al. (90) found that although wild-type NHE1 is not responsive to changes in cAMP, substituting its cytoplasmic domain with that of the trout erythrocyte βNHE1, which contains consensus sites for phosphorylation by protein kinase A, confers activation by cAMP. A similar strategy was used by Cabado et al. (92) to analyze NHE1/NHE3 chimeras. Although increased cAMP inhibits the wild-type NHE3 isoform and a chimeric NHE1/3 containing the transmembrane segments of NHE1 and the cytoplasmic domain of NHE3, it has no effect on an NHE3/1 chimera comprised of the transmembrane and cytoplasmic domains of NHE3 and NHE1, respectively. Given the wide range of cell functions regulated by $G\alpha_s$, $G\alpha_i$, and cAMP, their lack of effect on NHE1 is surprising and clearly indicates selective regulatory mechanisms by G protein–coupled receptors.

Activation of NHE1 by $G\alpha_q$ is mediated through a PKC-dependent mechanism (86), and inhibition of PKC activity impairs the activation of NHE1 by a number of G_q-coupled receptors, including vasopressin (93), bombesin (94), α1-adrenergic (95), and endothelin-1 (96). A PKC-independent activation of NHE1, however, has also been found for a number of Ca^{2+}-mobilizing receptors (97, 98). Moreover, although the C-terminal cytoplasmic domain of NHE1 contains consensus sites for phosphorylation by PKC, there is no indication for direct phosphorylation or regulation by PKC. The link between Ca^{2+}-dependent activation and the direct regulation of NHE1 was first reported by Bertrand and colleagues (99), who found that calmodulin binds directly to NHE1. As described below, a Ca^{2+}-calmodulin-dependent activation of NHE1 likely involves conformational changes in the C-terminus of the exchanger that occur independently of phosphorylation.

Activation of NHE1 by Gα13 is mediated by a signaling pathway involving the low molecular weight GTPase RhoA (72). Expression of a mutationally active RhoA in fibroblasts constitutively increases NHE1 activity, and expression of a mutationally inactive RhoA inhibits activation of NHE1 by Gα13 and by G protein–coupled receptors for lysophosphatidic acid (LPA) and thrombin. The G13-RhoA activation of NHE1 acts through the Rho-kinase ROCK, which, as described below, directly phosphorylates C-terminal serine residues on the cytoplasmic domain of NHE1 (67). Regulation of NHE1 by the Gα13-related family member Gα12 is controversial. Depending on the cell type, expression of mutationally activated Gα12 either stimulates (86) or inhibits (87) exchange activity. Activation of several G protein–coupled receptors, including the somatostatin SSTR1 subtype (88, 100) and the dopamine D_2R subtype (91), inhibits NHE1 activity. Although the GTPase mediating G protein–coupled receptor inhibition of NHE1 is unknown, Gα12 is a likely candidate because it is the only α subunit shown to attenuate exchange activity.

Integrin receptors were first shown to couple to the activation of NHE1 by Ingber & Schwartz (101–103). Integrin activation, either by plating cells on a fibronectin matrix (103) or by treating cells with insoluble fibronectin (102), and integrin aggregation by cell spreading on an extracellular matrix (101, 104) stimulate NHE1 activity and increase pH_i in the presence of HCO_3^-. Signals from integrin receptors converge with those from heptahelical receptors coupled to Gα13 to activate NHE1 through a RhoA-ROCK cascade (66, 67). This is in agreement with recent reports that integrins activate RhoA (105) and that inhibition of ROCK activity blocks integrin-induced focal adhesion assembly (106). Moreover, activation of NHE1 provides an "inside-out" signal to regulate the membrane clustering of integrin receptors and their downstream effects, including the assembly of focal adhesions and actin filaments (64, 66). As described below in the section on downstream effects of NHE1, NHE1 regulates the cytoskeletal actions of integrins by functioning as a tether for actin-associated proteins, anchoring the cortical cytoskeleton to the plasma membrane.

Receptor-independent regulation of NHE1 has also been described. Probably the best characterized of these regulatory mechanisms is the activation of NHE1 by

hyperosmotic stress. Increased transport activity by hyperosmolarity likely functions as a physiological response to induce volume restoration in response to cell shrinkage. Consistent with this possibility, Krump et al. (107) found that hyperosmotic activation of NHE1 in neutrophils is a response to decreased cell size, and not to osmolarity or ionic strength. How does NHE1 sense changes in cell size? Activation of NHE1 by hyperosmotic stress occurs independently of NHE1 phosphorylation (108), changes in intracellular [Ca^{2+}] (109–111), or MAPK signaling (77). One possible mechanism, however, involves activation through dynamic changes in the actin-based cytoskeleton. Denker et al. (64) recently determined that NHE1 associates with actin filaments by a direct association with actin-binding proteins of the ERM (ezrin, radixin, moesin) family. As described below, this cytoskeletal association regulates the membrane distribution of NHE1 and is a determinant in NHE1-regulated organization of cortical actin filaments. However, it may also be important in modulating transport activity. Consistent with cytoskeleton-mediated activation of NHE1, Watson et al. (112) found that serum stimulates NHE activity in Caco-2 cells by a mechanism dependent on F-actin, and Shrode et al. (111) found that inhibition of myosin light chain kinase blocks shrinkage-induced activation of NHE1. An actin-mediated activation in response to cell shrinkage could also be a mechanism shared with receptor-regulated NHE1 activity, as indicated by the shrinkage-dependent regulation of the exchanger in response to muscarinic agonists (113) and LPA (114). LPA regulates a Rho-dependent activation of ROCK, which, in addition to directly phosphorylating NHE1, also stimulates myosin-based contractility by increasing phosphorylation of myosin light chain (115). Together, these findings support an emerging model that places NHE1 both upstream and downstream of cytoskeletal reorganization.

Direct Regulation of NHE1

Regulation of NHE1 activity in response to growth factors (49), hormones (116) and osmotic stress (108) is mediated by the relatively long (∼300 amino acids) C-terminal cytoplasmic domain that acts to alter the affinity of a transmembrane H^+ binding site (49). The C-terminus is comprised of a number of distinct subdomains modified by phosphorylation and by the binding of regulatory proteins (Figure 3). It is currently unclear how C-terminal modifications convey changes in H^+ affinity within the transmembrane region. In AE1, however–which shares a similar topology with NHE1–the juxtamembrane region of the cytoplasmic domain is thought to act as a flexible hinge region that may facilitate conformational changes and an interaction between cytoplasmic and transmembrane segments (117).

PHOSPHORYLATION Sardet et al. (24) first determined that NHE1 is a phosphorylated glycoprotein. A phosphorylation domain at the distal C-terminus (amino acids 656–815 of human NHE1) contains a number of serine residues constitutively phosphorylated in quiescent cells that have increased phosphorylation levels in response to activation of the exchanger by growth factors (118). Three

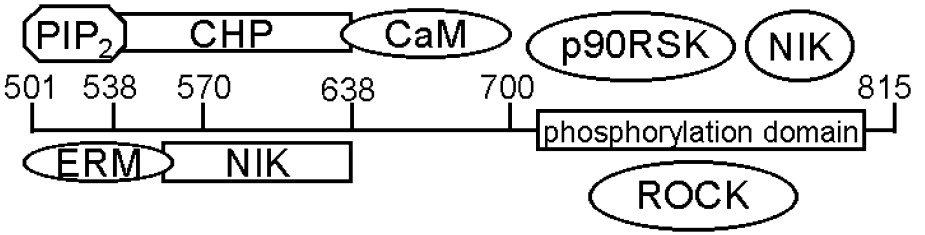


Figure 3 Binding and interaction sites for structural and regulatory proteins on the C-terminal cytoplasmic domain of NHE1. PIP_2 (phosphatidylinositol 4,5-bisphosphate), ERM (ezrin, radixin, and moesin), NIK (Nck-interacting kinase), CHP (calcineurin B homologous protein), and CaM (calmodulin) directly bind to NHE1. ROCK (Rho kinase), p90RSK (ribosomal S6 kinase), and NIK are serine/threonine kinases that phosphorylate the distal C-terminus of the cytoplasmic domain. Of these kinases, only NIK directly binds to NHE1.

serine/threonine kinases, ROCK (67), $p90^{RSK}$ (79), and NIK (84), directly phosphorylate NHE1. As described above, ROCK acts directly downstream of Rho to mediate signals from integrins and receptors coupled to G13. $p90^{RSK}$ phosphorylates Ser-703 and plays a key role in NHE1 activation by serum. The link between NIK and NHE1 was determined by using a yeast-two-hybrid screen, which indicated that NIK directly associates with the C-terminus of NHE1. NIK is the only kinase shown to bind directly to NHE1, and the NIK-binding site (amino acids 538–638; Figure 3) is distinct from and upstream of phosphorylation sites (serine residues distal to 638). Although the kinase domain of NIK is sufficient to phosphorylate NHE1 in vitro, phosphorylation in vivo requires the NHE1-binding site on NIK. These findings suggest that NHE1 acts as a targeting signal to sequester cytoplasmic NIK to the plasma membrane. Moreover, overexpression of a kinase-inactive NIK blocks activation of NHE1 by two upstream pathways: one mediated by PDGF signaling (84), and another mediated by dishevelled signaling (W. Yan & D.L. Barber, unpublished observations). The ability of dishevelled to activate NHE1 suggests that its upstream regulators, frizzled receptors and Wnt ligands, also regulate the exchanger, although this remains to be determined.

The significance of phosphorylation as a factor regulating NHE1 activity, however, is controversial. Deletion of the phosphorylation domain by a C-terminal truncation at amino acid 636, or functional blockade of the domain with site-specific antibodies, partially (47) or completely (116) inhibits activation of the exchanger. Full activation of transport activity by receptor-mediated signaling mechanisms, including the response to thrombin (47, 79, 116), endothelin (116), LPA (67), and activation of integrins (66) requires NHE1 phosphorylation. In contrast, activation of NHE1 by osmotic stress (108, 110) or intracellular acidification (79) and inhibition of NHE1 by ATP depletion (119) occur without detectable changes in phosphorylation of the exchanger. Together, these findings suggest

that phosphorylation may be a major determinant of NHE1 activity in response to receptor-dependent, but not receptor-independent, regulation. They also indicate that although the exchanger can be regulated by phosphorylation of its distal C-terminal domain, additional phosphorylation-independent regulatory sites are also important.

CALMODULIN Bertrand et al. (99) first identified two calmodulin-binding sites on the cytoplasmic domain of NHE1 at amino acids 636–656 and 664–684, with high ($K_d \sim 20$ nM) and low ($K_d \sim 350$ nM) affinities, respectively. The high-affinity calmodulin-binding domain regulates NHE1 activity in response to Ca^{2+}-mediated signaling mechanisms (47, 120). In quiescent cells, this site may function as an autoinhibitory domain by interacting with the transmembrane domain and inhibiting ion translocation. The model presented by Wakabayashi and colleagues (47, 120) suggests that upon activation, NHE1 undergoes a conformational change that releases the interaction of this autoinhibitory domain with the transmembrane segment and allows the Ca^{2+}-dependent binding of calmodulin. This model is supported by the observation that deletion of the calmodulin-binding domain results in constitutive activation of NHE1 (120).

ADDITIONAL REGULATORY SITES A critical regulatory domain (amino acids 567–635) is required for growth factor–stimulated NHE1 activity. Deletion of this domain, in the presence of an intact phosphorylation domain, completely abolishes activation by thrombin and EGF (49). Pouysségur and colleagues (121) suggested that a phosphoprotein binds to this domain, although the identity of this putative regulatory protein remains undetermined. One possible candidate is the Ca^{2+}-binding phosphoprotein, CHP (calcineurin B homologous protein). CHP, identified in a genetic screen for NHE1-interacting proteins, binds to the critical regulatory domain of the exchanger (122). Constitutive binding of CHP to NHE1, however, inhibits ion translocation. Further, Goss et al. (123) found an unknown phosphopeptide of ~24 kD, similar to the mobility of CHP, to be constitutively associated with the C-terminus of NHE1. Hence, a negative regulatory protein such as CHP could constitutively bind to NHE1 in the resting state to maintain reduced transport activity. Release of CHP could then allow either a conformational change in the C-terminus of NHE1 or the binding of a positive regulator to this site, which would promote increased transport activity. Interestingly, the binding site for NIK, a positive regulator, overlaps with that for CHP, and a speculative model would include competitive binding of NIK to displace CHP and allow phosphorylation and activation of NHE1.

Additional binding sites for regulatory proteins have recently been identified in the juxtamembrane region of the C-terminal cytoplasmic domain, which contains two distinct clusters of positively charged residues. These two charged clusters are invariant in NHE1 across species, but are less well defined in other plasma membrane NHE isoforms. An analogous charged region located in the juxtamembrane domain of the N-terminus of the AE family of anion exchangers (124) functions as a binding site for the cytoskeletal-associated proteins ankyrin and band 4.1.

Denker et al. (64) recently found that the band 4.1 family members, ezrin, radixin, and moesin, bind directly to the distal cluster of positively charged residues in the juxtamembrane region of NHE1. In fibroblasts, NHE1 has a predominant distribution at the leading edge of lamellipodia (125, 126), where it colocalizes with ERM proteins [Figure 4; (64)]. As described below in the section on downstream effects of NHE1, ERM binding is critical for localizing NHE1 to lamellipodia and for NHE1-dependent organization of cortical actin filaments. The direct association of ERM proteins with NHE1, however, is distinct from the indirect association of ERM proteins with NHE3. The C-terminal domain of NHE3 binds directly to the homologous PDZ domain-binding adaptor proteins NHE-RF (also termed EPB50) and E3KARP, which in turn bind directly to ezrin (127, 128). Ezrin also functions as an anchoring protein for protein kinase A (129), and by binding to an NHE-RF/ezrin/PKA complex, NHE3 might be regulated by cAMP. This is not the case for NHE1 because NHE1 neither directly binds NHE-RF in vitro (X Lin, S Denker & D Barber, unpublished observations) nor is regulated by cAMP (88, 90). Hence, the direct binding of ERM proteins to NHE1 and their indirect association with NHE3 likely have distinctly different functional consequences.

Positively charged residues in the juxtamembrane domain of NHE1 also function as a binding site for phosphatidylinositol 4,5-bisphosphate [PIP_2; (130)]. Mutant forms of NHE1 lacking the PIP_2-binding site have attenuated transport activity. PIP_2 binding could induce conformational changes in NHE1 to maintain the cytoplasmic domain in an unfolded state to allow the association of additional regulatory proteins. Alternatively, because the PIP_2 binding site overlaps with that for ERM proteins, mutations in this site could disrupt the association of NHE1 with the actin cytoskeleton, which, as described above, might be an important determinant in regulating transport activity.

The ability of NHE1 to interact with multiple regulatory proteins suggests that it may function as a scaffold for signaling complexes. In support of this possibility, the C-terminal domain of NHE1 contains proline-rich motifs predicted to modulate a phosphorylation-dependent assembly of proteins. Whether C-terminal modifications regulate the assembly of signaling complexes or the localization of NHE1 is an interesting question for future investigation.

CELLULAR ACTIONS OF NHE1

The well-established function of NHE1 is an ion translocation-dependent regulation of pH_i and cell volume. Changes in pH_i and cell volume have pleiotropic effects that can in turn alter a number of cell functions. A role for NHE1, either obligatory or permissive, in the specific cell functions of proliferation, survival, and migration has received the most attention and is reviewed in this section.

Recently, it was determined that NHE1 has an additional function as a plasma membrane anchor for cortical actin filaments, independent of its role in ion translocation (64). Through a direct association of its C-terminal domain with the ERM

family of actin-binding proteins, NHE1 tethers the cortical actin cytoskeleton to the leading edge of lamellipodia in fibroblasts. The actin anchoring function of NHE1, but not its function in ion translocation, is critical for dynamic reorganization of the cortical cytoskeleton in response to extracellular signals. NHE1 acts downstream of G protein–coupled receptors and integrin receptors in a RhoA pathway regulating cytoskeletal organization, the assembly of focal adhesions, the formation of actin stress fibers, and cell shape (65–67). NHE-deficient cells have impaired cytoskeletal organization; however, this is completely rescued by the expression of wild-type NHE1 or a translocation-defective NHE1-E266I but not by an NHE1 with C-terminal mutations that prevent ERM binding, NHE1-KR/A. Moreover, in fibroblasts expressing NHE1-KR/A, NHE1 is redistributed from the leading edge of lamellipodia to a more uniform distribution, indicating that ERM binding also promotes clustering of the exchanger. It is interesting that pharmacological inhibitors of NHE1, including EIPA and HOE694, also impair cell adhesion and actin filament organization, which suggests that in addition to blocking ion translocation, they might induce conformational changes in NHE1 that disrupt its association with the cytoskeleton.

The ability of transport proteins and ion channels to act as membrane anchors for the cortical actin cytoskeleton is a recurring theme that may have a number of functional consequences. One function involves the regulation of membrane integrity and cell shape, as noted for the well-established association of AE1 with ankyrin in erythrocytes (131). Additionally, cytoskeletal associations restrict transmembrane proteins to localized microdomains, as evidenced by the importance of ankyrin binding for the clustering of voltage-gated sodium channels at axonal initial segments (132). The association of NHE1 with the cytoskeleton is clearly important for the organization of cortical actin filaments and cell shape; however, it might also be a determinant in mediating an actin-dependent regulation of NHE1 activity (112) or, as discussed below in the section on migration, in restricting the distribution of NHE1 to a particular microdomain of the membrane to maintain a localized H^+ efflux.

Proliferation

A role for NHE1 in cell proliferation was initially inferred from the growth-promoting effect thought to be associated with mitogen-induced increases in pH_i. Pouysségur and colleagues (133) first reported that the proliferative rate of mutant fibroblasts lacking NHE activity was markedly impaired compared to parental NHE-competent cells at neutral and acidic pH_i. Subsequent studies using pharmacological inhibitors of NHE1, such as EIPA or HOE694, indicated that NHE1 has a permissive, but not an obligatory, role in promoting proliferation in response to mitogens (134–136) and oncogene expression (137, 138).

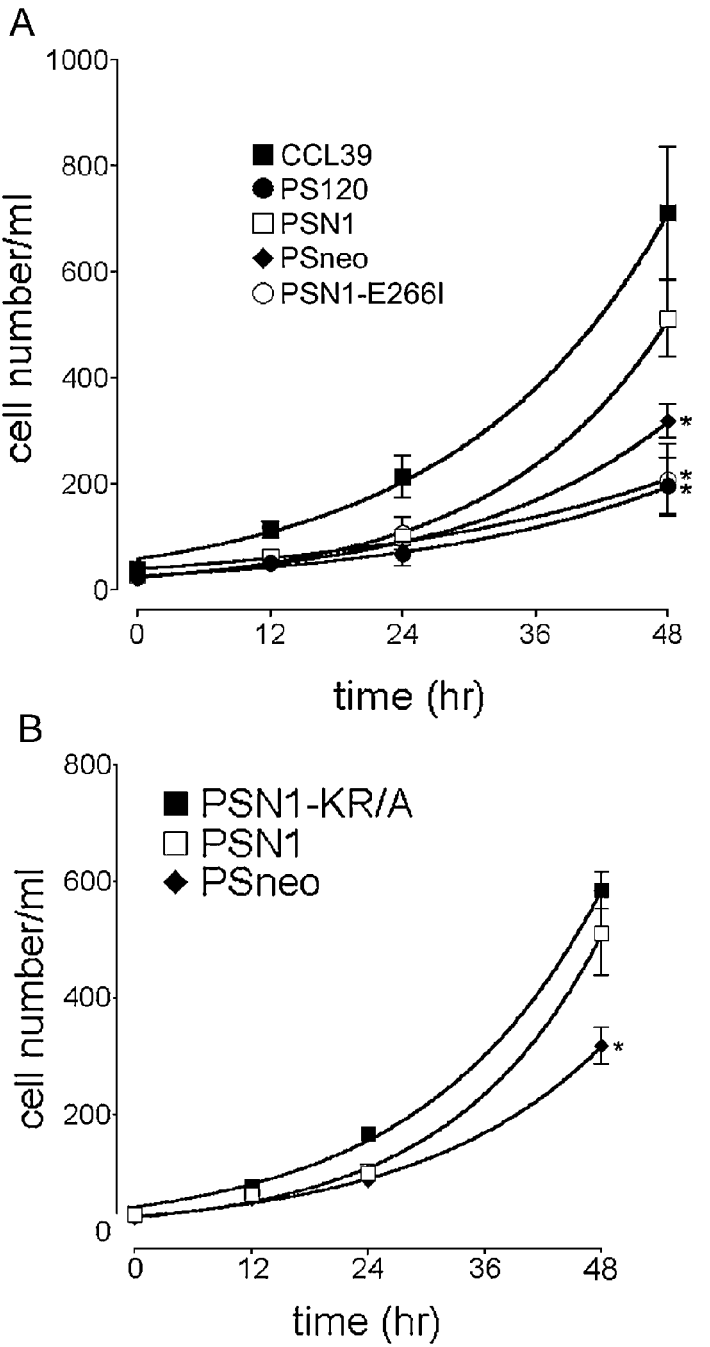
The permissive effect of NHEs on cell proliferation was clearly established by Kapus and colleagues (139), who stably expressed NHE1 in an NHE-deficient cell line derived from a parental CHO clone. Although NHE-deficient cells proliferated in the continuous presence of serum, the rate of proliferation markedly increased

with NHE1 expression. It is interesting that expression of NHE2 and NHE3 also conferred an increased proliferative response. More recently, ion translocation by NHE1, but not its action as a cytoskeletal anchor, was found to be critical for conferring enhanced proliferation (64). Proliferation of NHE-deficient PS120 cells, derived from parental CCL39 fibroblasts, was enhanced four-fold after 48 h in culture by expression of wild-type NHE1 or NHE1-KR/A, which has impaired binding to ERM proteins and actin filaments (Figure 5). In contrast, the proliferative rate of PS120 cells was not rescued by expression of an ion-translocation-deficient NHE1-E266I. These studies were performed in the continuous presence of HCO_3^-, which indicates a selective role for NHE1 in cell proliferation that cannot be compensated by anion exchangers. Precisely how NHE1 promotes proliferation, however, is unclear. The increased H^+ efflux by NHE1 in activated cells reduces metabolic acid generated by glycolysis, glucose utilization, and lactic acid production. This "housekeeping" mechanism, however, is probably too simplistic to explain why proliferation is reduced in the absence of NHE1, particularly in the presence of HCO_3^-, when the NDAE-acid extruder is still functional. Moreover, although ion translocation is required, the relative contribution of changes in pH_i versus cell volume has not been established. Transit through the cell cycle is accompanied by an increase in cell volume, and achieving a critical size, which could be regulated by NHE1, is thought to regulate entry into mitosis (140). Reshkin and colleagues (137) recently found that enhanced proliferation by increasing pH_i occurs independently of the E2F-mediated transcriptional activation of cell-cycle regulator genes. However, under physiological conditions, cells expressing a translocation-defective NHE1-E266I have a delayed transit through G2-M, coincident with altered expression of critical regulatory genes of the G2-M checkpoint, including wee-1 kinase and 14-3-3 tau/theta (L Putney & D Barber, manuscript in preparation).

Cell Survival and Apoptosis

An additional effect of NHE1 on cell proliferation may be mediated through enhanced cell survival or inhibition of apoptosis (141). Intracellular acidification and decreased cell volume are hallmarks of apoptosis, and the intracellular alkalinization and regulatory volume increases by NHE1 could provide an antiapoptotic signal. Consistent with this possibility is the inhibition of NHE1 activity that parallels apoptosis (142). Additionally, activation of Fas receptors inhibits NHE1

Figure 5 Effect of NHE1 activity on cell proliferation (cell number). (A) The proliferation rate of NHE1 competent, CCL39 (*closed square*) and PSN1 cells (*open square*), is greater compared to NHE1 deficient, PS120 (*closed circle*) and PSneo cells (closed diamond), or ion-translocation defective, PSN1-E266I cells (*open circle*). The growth rate of cells expressing a mutant NHE1 unable to bind ERM proteins, PSN1-KR/A, however, was similar to that of PSN1 (B). The asterisk indicates different from PSN1 cells ($p < 0.05$) by Student's t test. Reproduced with permission from *Molecular Cell* (64).


A
1000
800
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400
200
0
cell number/ml
CCL39
PS120
PSN1
PSneo
PSN1-E266I
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400
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cell number/ml
PSN1-KR/A
PSN1
PSneo
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12
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time (hr)

activity, and pharmacological inhibition of NHE1 accelerates Fas-induced DNA fragmentation (141). A role for NHE1 in cell survival is also supported by the observation that fibroblasts expressing an ion-translocation defective NHE1-266I are less able to tolerate serum deprivation than fibroblasts expressing a wild-type form (S Denker & D Barber, unpublished observations). Further evidence that NHE1 activity promotes cell survival comes from the analysis of tissues derived from mice expressing various truncated or deleted isoforms of NHEs. A spontaneous mutation that resulted in truncation of the NHE1 protein after TM11 caused severe neurological defects in mice, including ataxia and slow-wave epilepsy (143). *NHE1-null* animals showed selective neuronal death in tissues with high metabolic activity, including the cerebellum and brainstem (143). Moreover, targeted disruption of the *NHE2* gene in mice increased necrosis in parietal cells of the gut that predominantly express *NHE2* (143a).

Cytoskeletal Organization and Migration

Emerging new areas of investigation focus on the role of NHE1 in regulating organization of the actin-based cytoskeleton and in promoting cell migration. As discussed in previous sections, through its anchoring function, but independently of ion translocation, NHE1 acts downstream of integrin receptors and Rho GTPases to regulate a number of cytoskeletal events, including cell adhesion, cell shape, and the assembly of actin filaments (Figure 6). These findings raise the question of whether there is a functional basis for the use of NHE1 specifically as an actin-anchoring protein. A corollary of the anchoring function of NHE1 might be to restrict the exchanger within dynamic regions of the cell membrane to create localized pH_i or osmotic changes important for signaling or regulatory events.

A likely significance of the localization of NHE1 in lamellipodia is that it functions in regulating migratory behavior (see 144 for review). Early work by Simchowitz & Cragoe (145), which was later confirmed by Ritter and colleagues (146), found that pharmacological inhibition of NHE1 inhibits neutrophil chemotaxis and chemokinesis in response to the chemoattractant *N*-formyl-Met-Leu-Phe. Inhibition of NHE1 activity impairs directed, but not random, motility, which indicates that cells are motile but inefficient in their motility. Pharmacological inhibition of NHE1 has also been reported to impair the migration of endothelial cells (147) and Madin-Darby canine kidney (MDCK) cells (148, 149). Furthermore, fibroblasts expressing a translocation-defective NHE1-E266I, which is associated with the actin cytoskeleton and localizes to the leading edge of lamellipodia (64), are unable to migrate in a polarized manner in a standard wound-healing assay (S. Denker & D. Barber, manuscript in preparation). The striking defects observed include a loss of forward motion and an inability to disassemble the trailing edge.

If ion translocation by NHE1 at the leading edge of lamellipodia is necessary for membrane protrusion, the molecular mechanisms mediating this action remain to be determined. Current models implicate actin polymerization as the driving

force for membrane protrusion (150). If this is the case, NHE1 could regulate actin nucleation by altering the activity of pH-sensitive proteins, such as cofilin (151). An alternative model for the driving force of membrane protrusion incorporates localized increases in osmotic pressure in conjunction with actin polymerization. This hydrostatic pressure model (152) involves a swelling of the actin network in response to changes in membrane-associated osmotically active particles, which could be a direct consequence of NHE1 activity.

Recent findings on NHE1 will lead to exciting new areas for future investigation. Still unanswered is how C-terminal modifications confer changes in the pH_i sensitivity of the TM domain. Also of interest is whether the binding of phospholipids, such as PIP_2, at a juxtamembrane flexible hinge region might regulate conformational changes that allow interactions between transport and regulatory domains. Studies on the newly identified function of NHE1 as a cytoskeleton-anchoring protein are at an early state, and it remains to be determined whether ERM binding is regulated by extracellular signals and, importantly, by pharmacological inhibitors recognized to block ion translocation by NHE1. Moreover, how this anchoring function and its associated effects on the cortical actin network regulate other cell behaviors is unknown. Finally, findings that NHE1 activity contributes to migratory responses indicates the need for reevaluation of how membrane protrusion, and perhaps actin polymerization, might be regulated by pH_i or by changes in osmotic pressure.

ACKNOWLEDGMENTS

We thank S. Wakabayashi for contributing the NHE1 topology models shown in Figure 1 and P.M. Cala for helpful communication on membrane transporters. Moreover, we apologize to all those investigators who have significantly contributed to the field of NHE research, but whose work we were unable to include in this review. We thank E. Leash for editorial review.

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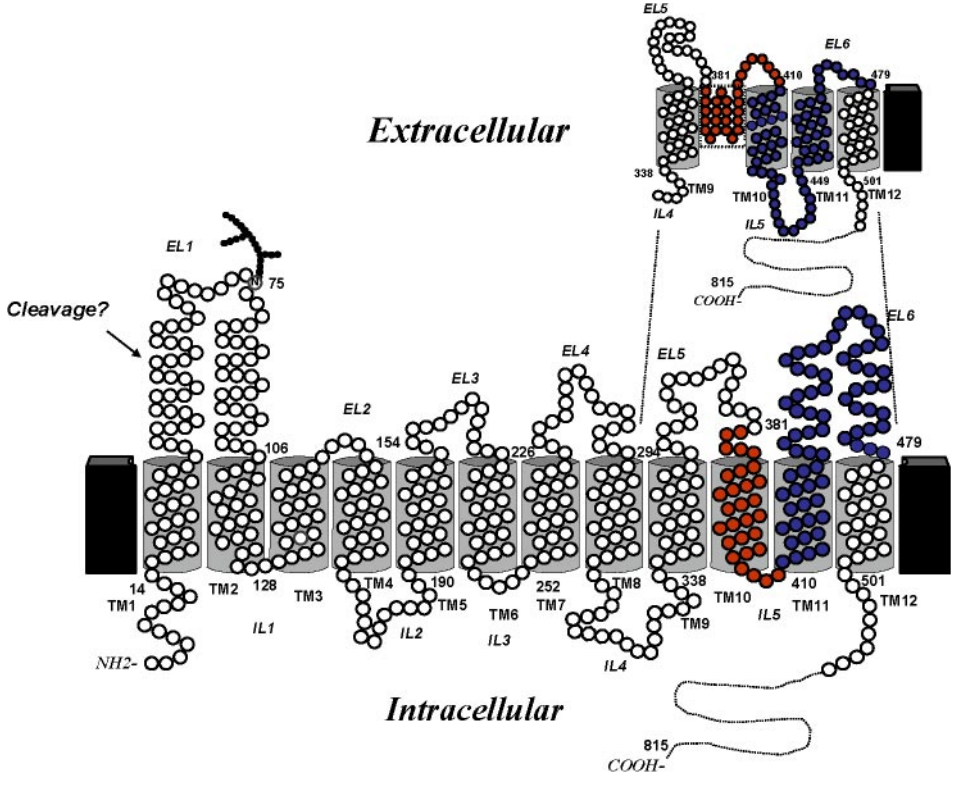

Figure 1 Predicted topological model of NHE1 based on hydropathy analysis (*middle*) and cysteine mutagenesis studies (*top inset*). The distinct differences between the two models are in TM-10 (*red*) and TM-11 through EL-6 (*blue*). Reproduced with permission from S. Wakabayashi (31).

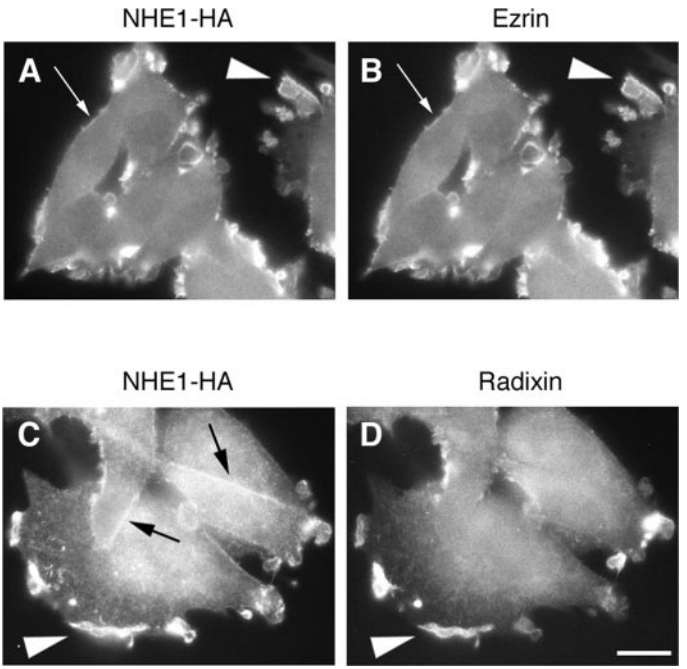

Figure 4 Localization of NHE1, ezrin, and radixin in fibroblasts stably expressing NHE1 tagged with heme agglutinin (HA). HA-tagged NHE1 (*A* and *C*) colocalize with both ezrin (*B*) and radixin (*D*) in lamellipodia (*arrowheads*). NHE1 (*A*, *arrows*) and ezrin (*B*, *arrows*) have an almost identical labeling pattern along the membrane, whereas NHE1 (*C*, *arrows*) appears to have a broader distribution than radixin (*D*) along the membrane. Scale bar, 5 μm. Reproduced with permission from *Molecular Cell* (64).

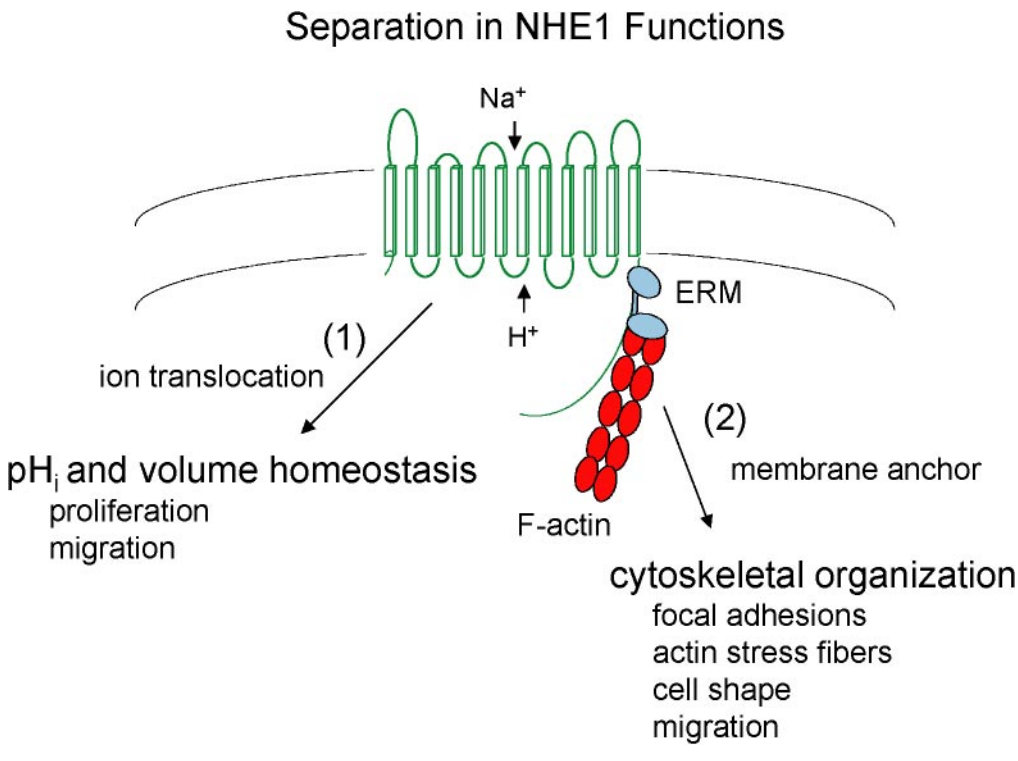

Figure 6 Separation in NHE1 functions. Ion translocation by NHE1 changes pH_i and cell volume, which in turn regulates cell proliferation and migration. Independently of ion translocation, NHE1 binds directly to ERM proteins and acts as a membrane anchor for the actin cortical network. ERM binding to NHE1 is critical for the organized assembly of focal adhesions and actin stress fibers, and for cell shape determination.