p160ROCK mediates RhoA activation of Na–H exchange

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The ubiquitously expressed Na–H exchanger, NHE1, acts downstream of RhoA in a pathway regulating focal adhesion and actin stress fiber formation. p160ROCK, a serine/threonine protein kinase, is a direct RhoA target mediating RhoA-induced assembly of focal adhesions and stress fibers. Here, stress fiber formation induced by p160ROCK was inhibited by the addition of a specific NHE1 inhibitor, ethylisopropylamiloride, in CCL39 fibroblasts, and was absent in PS120 mutant fibroblasts lacking NHE1. In CCL39 cells, NHE1 activity was stimulated by expression of mutationally active p160ROCK, but not by mutationally active protein kinase N, another RhoA target kinase. Expression of a dominant interfering p160ROCK inhibited RhoA-, but not Cdc42- or Rac-activation of NHE1. In addition, the p160ROCK-specific inhibitor Y-27632 inhibited increases in NHE1 activity in response to RhoA, and to lysophosphatidic acid (LPA), which stimulates RhoA, and it also inhibited LPA-increased phosphorylation of NHE1. A C-terminal truncation of NHE1 abolished both LPA-induced phosphorylation and activation of the exchanger. Furthermore, mutationally active p160ROCK phosphorylated an NHE1 C-terminal fusion protein in vitro, and this was inhibited in the presence of Y-27632. Phosphopeptide maps indicated that identical residues in NHE1 were phosphorylated by p160ROCK in vivo and in vitro. These findings identify p160ROCK as an upstream, possibly direct, activator of NHE1, and suggest that NHE1 activity and phosphorylation are necessary for actin stress fiber assembly induced by p160ROCK.

Keywords: cytoskeleton/lysophosphatidic acid/Na–H exchanger/p160ROCK/RhoA

Introduction

NHE1 is a ubiquitously expressed member of the Na–H exchanger family that catalyzes the extrusion of intracellular proton (H⁺) ions in exchange for extracellular sodium (Na⁺) ions, thereby regulating intracellular pH (pH₇) and cell volume (Noel and Pouyssegur, 1995). This exchanger not only operates under the basal conditions of cells but also is activated by various extracellular stimuli such as hormones, mitogens, oncogenes and extracellular matrix (ECM) proteins. This activation has been shown to be associated with anchorage-dependent growth, cell motility, cell transformation and cell adhesion and spreading (Simchowitz and Cragoe, 1986; Ingber et al., 1990; Krump et al., 1997; Tominaga and Barber, 1998). Quite recently, a defective mutation of the mouse Nhe1 gene was found (Cox et al., 1997). The mutant mice show selective neuronal death, and a unique epilepsy syndrome, suggesting that NHE1 is required for regulation of neuronal excitability and survival. How NHE1 is activated in a particular signaling pathway and how this activation is linked to cell behavior, however, remain largely unknown.

Low molecular weight GTPases of the Rho family function as control points in regulating the organization of the actin cytoskeleton. In cultured cells, RhoA links mitogen activation to the assembly of focal adhesions and stress fibers (Ridley and Hall, 1992), Rac controls the dynamics of lamellipodia (Ridley and Hall, 1992), and Cdc42 regulates the formation of filopodia (Kozma et al., 1995; Nobes and Hall, 1995). RhoA also regulates integrin-induced cell adhesion (Tominaga et al., 1993; Laudanna et al., 1996) and contractility (Chrzanowska-Wodnicka and Burridge, 1996), cell secretion (Price et al., 1995), cytokinesis (Kishi et al., 1993; Mabuchi et al., 1993), proliferation (Yamamoto et al., 1993; Olson et al., 1995) and Ras-induced neoplastic transformation (Qiu et al., 1995b). We previously found that RhoA stimulates NHE1 activity and mediates NHE1 activation by lysophosphatidic acid (LPA) and the GTPase Gα₁₃, and that activation of NHE1 is necessary for RhoA-induced reorganization of the actin cytoskeleton (Hooley et al., 1996; Vexler et al., 1996). RhoA-mediated assembly of stress fibers and focal adhesions is inhibited in NHE1-expressing cells treated with NHE1-selective inhibitors. Furthermore, stress fiber induction by RhoA is absent in NHE1-deficient fibroblasts and is restored by expression of NHE1 (Vexler et al., 1996; Tominaga and Barber, 1998). Interestingly, NHE1 activity is not required for Rac-induced lamellipodia formation (Vexler et al., 1996), although Rac1 also acts upstream of NHE1 to stimulate exchanger activity (Hooley et al., 1996). These findings suggest that NHE1 selectively regulates cytoskeletal events induced by RhoA.

Several direct targets of RhoA have been identified recently, including protein kinase N (PKN) (Amano et al., 1996b; Watanabe et al., 1996), rhophilin (Watanabe et al., 1996), rhoetkin (Reid et al., 1996), p140mDia (Watanabe et al., 1997) and a family of Rho-associated kinases, p160ROCK (ROCK-I) (Ishizaki et al., 1996) and ROKα/ Rho-kinase/ROCK-II (Leung et al., 1995; Matsui et al., 1996; Nakagawa et al., 1996). Although the functions of most RhoA targets remain unknown, p140mDia and Rho-associated kinase isozymes mediate RhoA effects on the actin cytoskeleton. p140mDia, a mammalian homolog of
Drosophila diaphanous, controls actin polymerization by binding and accumulating the actin-binding protein profilin (Watanabe et al., 1997). The Rho-associated kinase iso-zymes, p160ROCK (Ishizaki et al., 1997) and ROKα/Rho-kinase/ROCK-II (Leung et al., 1996; Amano et al., 1997), mediate RhoA-induced assembly of focal adhesions and actin stress fibers. p160ROCK and ROKα/Rho-kinase/ROCK-II are coiled-coil-forming serine/threonine kinases sharing ~90% identity within the kinase domain. These kinases are suggested to regulate cell contractility by indirectly increasing phosphorylation of myosin light chain through the inhibition of myosin phosphatase activity (Kimura et al., 1996) or by directly phosphorylating myosin light chain independently of myosin light chain kinase (Amano et al., 1996a). The objective of the current study was to determine whether p160ROCK, which is required for RhoA-induced stress fiber and focal adhesion formation, regulates NHE1 activity. We found that p160ROCK specifically mediates activation of NHE1 by LPA, Gα13 and RhoA, and not by Cdc42 and Rac1. p160ROCK mediates LPA-induced phosphorylation of NHE1 in vivo and directly phosphorylates NHE1 in vitro. Furthermore, we have found that NHE1 phosphorylation was necessary for activation of NHE1 in the LPA–RhoA–p160ROCK pathway. These findings indicate that NHE1 is a downstream, possibly direct, target of p160ROCK and suggest that activation of NHE1 collaborates with increased contractility via myosin to induce p160ROCK-mediated changes in actin cytoskeleton reorganization.

Results

NHE1 activity is required for p160ROCK-induced stress fiber formation

Focal adhesions and stress fibers are clustered structures of, respectively, integrin complexes bound to ECM proteins and actin filaments ligated to the complexes. Such clustering is generated by the force of myosin-based contractility (Chrzanowska-Wodnicka and Burridge, 1996). Because the RhoA-associated kinases such as p160ROCK and ROKα/Rho-kinase have been suggested to enhance phosphorylation of myosin light chain in cells and to work downstream of Rho to induce focal adhesions and stress fibers, it is generally accepted that they induce cytoskeletal effects presumably by regulating myosin contractility through phosphorylation of its light chain. We previously determined that NHE1 activity regulates a RhoA pathway leading to stress fiber formation (Vexler et al., 1996). The relationship between Rho-associated kinases and NHE1, however, remains unknown. To determine whether NHE1 activation is also prerequisite for stress fiber induction by RhoA-associated kinases, we transiently expressed a mutationally activated p160ROCKΔ3 in CCL39 fibroblasts. p160ROCKΔ3 is a mutant of p160ROCK truncated at amino acid residue 727, which results in deletion of the Rho-binding domain, the pleckstrin homology domain and the cysteine-rich zinc finger domain. The constitutive activity of the Δ3 mutant was demonstrated previously (Ishizaki et al., 1997) and confirmed by its ability to phosphorylate histone in vitro (see Figure 6B). As it does in cultured HeLa cells (Ishizaki et al., 1997), this mutant induced stress fibers and focal adhesions in CCL39 cells (Figure 1A and B). This induction was abolished by the addition of ethylisopropylamiloride (EIPA; 25 μM), an NHE1 inhibitor, suggesting that NHE1 activity was required for p160ROCK-induced stress fiber formation (Figure 1C and D). This suggestion...
was supported in experiments using PS120 cells, which are derived from parental CCL39 cells but lack Na–H exchangers (Pouyssegur et al., 1984). No stress fibers were induced by the expression of p160ROCKΔ3 in these NHE1-deficient cells (Figure 1E and F). We previously determined that inhibition of NHE1, either in EIPA-treated CCL39 cells or in PS120 cells, selectively inhibits RhoA-mediated cytoskeletal remodeling, as it has no effect on Rac-induced lamellipodia or membrane ruffling, or on Cdc42-induced filopodia formation (Vexler et al., 1996; Tominaga and Barber, 1998). To confirm that the effect of NHE1 on stress fiber formation was not specific to CCL39 cells, we demonstrated that induction of stress fibers in HeLa cells expressing p160ROCKΔ3 was also inhibited by EIPA (Figure 1G–J).

**p160ROCK stimulates NHE1 activity**

The above results demonstrate that NHE1 activity is required for p160ROCK-induced stress fiber formation. They do not, however, necessarily indicate that p160ROCK lies upstream of NHE1 and activates it. To determine whether p160ROCK couples to the regulation of NHE1 activity, we determined the activity of the exchanger in CCL39 cells expressing the Δ3 mutant. The steady-state intracellular pH (pH i) in a HEPES buffer increased from 7.18 ± 0.03 (mean ± SEM; n = 4 transfections) in vector controls to 7.36 ± 0.01 (n = 4) in the Δ3-expressing cells. Additionally, NHE1 activity, determined as the rate of pH i recovery (dpH i/dt) from an NH 4Cl-induced acid load, was increased in cells transfected with Δ3, compared with vector controls (Figure 2A and B). In two separate cell transfections, expression of p160ROCKΔ1, which is constitutively active due to a truncation at amino acid residue 1080 (Ishizaki et al., 1997), also stimulated NHE1 activity (Figure 2B, inset). To determine the specificity of p160ROCK-induced activation of NHE1, we examined the activity of the exchanger in CCL39 cells transfected with a constitutively active PKN. Although PKN also acts directly downstream of RhoA (Amano et al., 1996; Watanabe et al., 1996) and recently was determined to bind α-actinin (Mukai et al., 1997), its functional importance in RhoA-mediated signaling events remains unknown. Myc-PKN, which is constitutively active due to the Myc epitope at the N-terminus (G.Martin, personal communication), was able to phosphorylate histone in vitro (see Figure 6B) but had no effect on pH i (data not shown) or NHE1 activity (Figure 2A and C) compared with vector controls (Figure 2A and C). We also determined that another constitutively active PKN allele, containing only the kinase domain, had no effect on NHE1 activity when transfected into CCL39 cells (data not shown).

**p160ROCK selectively mediates activation of NHE1 by RhoA**

NHE1 activity is stimulated by activation of RhoA, Cdc42 and Rac1 (Hooley et al., 1996). To determine whether p160ROCK selectively mediates NHE1 activity by a specific Rho family GTPase, we co-transfected CCL39 cells with KD-IA, a kinase-inactive p160ROCK, KD-IA, which contains a K105→A substitution in the kinase domain and an I1009→A substitution in the Rho-binding domain, functions as a dominant-interfering allele to block RhoA-dependent assembly of actin stress fibers (Ishizaki et al., 1997). In CCL39 cells transfected with mutationally active RhoAV14, steady-state pH i increased from 7.23 ± 0.02 in vector controls to 7.44 ± 0.01, and this increase in pH i was reduced to 7.29 ± 0.03 by co-transfection with KD-IA (n = 7 separate matched transfections). Additionally, RhoA-induced increases in NHE1 activity were completely inhibited by co-transfection with KD-IA (Figure 3A). KD-IA had a small inhibitory effect on...
NHE1 activity in vector control cells (Figure 3A, inset), suggesting that an upstream regulator of p160ROCK might be constitutively active in serum-starved CCL39 cells. Co-transfection of KD-IA, however, had only a minor inhibitory effect on NHE1 activity stimulated by mutationally active Cdc42V12 (Figure 3B) or mutationally active Rac1V12 (Figure 3C). The magnitude of this inhibition was similar to that observed in vector control cells (Figure 3A, inset). Immunoblot analysis indicated that KD-IA expression was similar in cells transfected with RhoAV14, Cdc42V12 or Rac1V12, and that co-transfection of KD-IA had no effect on the expression of mutationally active GTPases (Figure 3D). The ability of p160ROCK to mediate specifically RhoA activation of NHE1 was confirmed further by treating cells with the p160ROCK-selective inhibitor Y-27632. The compound Y-27632 is a novel pyridine derivative that suppresses RhoA- and p160ROCK-induced stress fiber assembly but has no effect on Rac-induced membrane ruffling or Cdc42-induced filopodia formation (Uehata et al., 1997). In four separate cell preparations, Y-27632 significantly inhibited activation of NHE1 in cells transfected with RhoAV14 ($P = 0.01$), but not in those with Cdc42V12 (Figure 3E). These findings indicate that although three members of the Rho family of GTPases couple to the stimulation of NHE1, p160ROCK selectively mediates only RhoA activation of the exchanger.

p160ROCK mediates activation of NHE1 by Gα13

The α subunit of the heterotrimeric GTPase G13 couples to the stimulation of NHE1 (Dhanasekaran et al., 1994; Voyno-Yasenetskaya et al., 1994; Kitamura et al., 1995). Although previous findings indicated that Gα13 acts downstream of the LPA receptor to activate NHE1 through both Cdc42- and RhoA-dependent signaling pathways (Hooley et al., 1996), expression of mutationally active Gα13Q19 in fibroblasts induces a RhoA-like phenotype of increased stress fiber formation, but not a Cdc42-like phenotype of filopodia extension (Buhl et al., 1995; Hooley et al., 1996). Hence, RhoA, and not Cdc42, may be the preferred downstream effector of Gα13 in vivo. To determine whether p160ROCK mediates Gα13 activation of NHE1, we used co-transfections in CCL39 cells. Transfection of mutationally active Gα13Q19 alone increased the steady-state pH$_i$ from 7.16 ± 0.04 in vector controls to 7.37 ± 0.03, but co-transfection with KD-IA completely inhibited Gα13Q19-induced increases in pH$_i$, resulting in a value of 7.14 ± 0.03 ($n = 3$ separate
matched transfections). Co-transfection of KD-IA also completely inhibited the stimulation of NHE1 activity by Gα13QL (Figure 4). Together with our previous findings (Voyno-Yasenetskaya et al., 1994; Hooley et al., 1996; Vexler et al., 1996), these results suggest that NHE1 activity is stimulated by a signaling cascade involving LPA→Gα13QL→RhoA→p160ROCK.

**p160ROCK mediates LPA-induced phosphorylation of NHE1 in vivo**

Growth factor activation of NHE1 is associated with increased phosphorylation of the cytoplasmic domain of the exchanger on serine residues (Sardet et al., 1991). To determine whether p160ROCK regulates the phosphorylation of NHE1, we stably expressed full-length human NHE1, tagged at the C-terminus with an EE epitope, in NHE-deficient PS120 cells (PS120N cells). The transient transfection efficiency of PS120 cells is <10%, which prevented us from studying the effects of mutationally active or dominant-interfering p160ROCK on NHE1 phosphorylation. We therefore determined whether an LPA pathway involving p160ROCK phosphorylated NHE1. LPA, which activates RhoA (Ridley and Hall, 1992) and stimulates NHE1 activity (Vexler et al., 1996), increased the phosphorylation of NHE1 (Figure 5A, lane 3), a result similar to the previously described effect of growth factors (Sardet et al., 1991). In PS120N cells pre-treated with the specific p160ROCK inhibitor Y-27632 (30 μM), however, LPA-induced phosphorylation of NHE1 was reduced to basal levels (Figure 5A, lane 4). Y-27632 had no effect on basal NHE1 phosphorylation (Figure 5, lane 2). Immunoblot analysis indicated that the amount of immunoprecipitated NHE1 in the absence and presence of LPA was similar (Figure 5B). These findings suggest that p160ROCK mediates LPA-induced phosphorylation of NHE1 and, as shown below (Figure 8C), LPA-stimulated NHE1 activity.

**p160ROCK directly phosphorylates NHE1 in vitro**

We next investigated whether p160ROCK directly phosphorylates NHE1 in vitro. Mutationally activated Myc-tagged p160ROCKΔ3 and PKN were transiently expressed in CCL39 cells and immunoprecipitated with anti-Myc mAb. Immunoprecipitates were then used for in vitro kinase assays with either histone or GST–NHE1 as a substrate. The GST–NHE1 fusion protein included amino acid residues 638–815 of the C-terminal cytoplasmic domain and contained all serine residues that are phosphorylated in vivo (Sardet et al., 1991; Wakabayashi et al., 1992; see Figure 8B). The abundance of kinases in the immunoprecipitates was determined by immunoblotting with anti-Myc antibodies (Figure 6A). As previously reported, both Δ3 (Ishizaki et al., 1997) and PKN (Mukai et al., 1997) phosphorylated histone (Figure 6B). In contrast, only Δ3 phosphorylated GST–NHE1 (Figure 6B). This finding correlates with the ability of p160ROCK, but not PKN, to stimulate NHE1 activity (Figure 2). GST alone was not phosphorylated by either kinase (data not shown). In the presence of Y-27632 (100 μM) added to the kinase reaction buffer, Δ3-induced phosphorylation of GST–NHE1 was completely inhibited (Figure 6B, lane 3), suggesting that p160ROCK acts directly on the exchanger. We also confirmed that the NHE inhibitor EIPA had no effect on p160ROCK activity. Phosphorylation of histone by immunoprecipitated Δ3 was decreased in the presence of Y-27632 in a dose-dependent manner, but was unchanged in the presence of EIPA (3–300 μM; Figure 6C).

To assess further whether p160ROCK directly phosphorylates NHE1, two-dimensional phosphopeptide mapping was performed to analyze the sites of NHE1 phosphorylated in vivo and in vitro. EE-tagged NHE1 phosphorylated in vivo was isolated by SDS–PAGE, transferred to nitrocellulose membranes and digested with trypsin. Incorporation of 32P into several phosphopeptides increased in response to LPA (Figure 7A and B). Two phosphopeptides from in vivo-phosphorylated NHE1 (spots a and b in Figure 7B) appeared also to be generated in GST–NHE1 phosphorylated by p160ROCKΔ3 (Figure 7C). This was confirmed by mixing the two reactions (Figure 7D). These findings suggest that identical residues...
Increased phosphorylation of NHE1 is necessary for LPA-stimulated exchange activity

Our findings suggest that p160ROCK increases the phosphorylation and activity of NHE1. Previous findings indicate that NHE1 activity is regulated by both phosphorylation-dependent and -independent mechanisms (Grinstein et al., 1992; Wakabayashi et al., 1992, 1994a; Winkel et al., 1993; Goss et al., 1994). To determine the functional importance of phosphorylation in p160ROCK-mediated NHE1 activity, we stably expressed NHEΔ635-EE in NHE-deficient PS120 cells (PS120Δ635). All C-terminal serine residues are deleted in this truncated exchanger. The expression of NHEΔ635-EE was confirmed and compared with the expression of full-length NHE1 in PS120N cells (Figure 8A). Lysates from PS120 cells were used to confirm the specific immunoprecipitation of NHE1 (Figure 8A, lane 1 and Figure 8B, lane 1). The phosphorylation of full-length NHE1 in quiescent PS120N cells increased with serum (10% for 10 min; Figure 8B, lanes 1 and 2) or with LPA, as shown in Figure 5, lane 3. In Figure 8B, the relatively high basal phosphorylation of NHE1 is probably due to constitutive integrin activation of NHE1 in adherent cells (Tominaga and Barber, 1998). We were unable to detect basal or stimulated phosphorylation of NHE1 in PS120Δ635 cells (Figure 8B, lanes 3 and 4), confirming the loss of phosphorylation of NHEΔ635.

We next determined whether phosphorylation of NHE1 is important for an increase in NHE1 activity induced by an LPA-p160ROCK pathway. As explained above, the low (<10%) transfection efficiency of PS120 cells prevented us from studying NHE1 activity in response to transient expression of mutually active or dominant-interfering RhoA or p160ROCK. We therefore used LPA to stimulate the activity of full-length NHE1 expressed in PS120 cells, as previously described (Vexler et al., 1996), and found that this increase in activity was blocked by the p160ROCK inhibitor Y-27632 (Figure 8C). In PS120Δ635 cells, quiescent NHE1 activity was greater than in PS120N cells (Figure 8C), possibly due to the loss of an internal calmodulin-binding site at residues 636–656, which has been suggested to function as an autoinhibitory domain in quiescent cells (Wakabayashi et al., 1994b). The LPA-induced increase in exchanger activity observed in PS120N cells was reduced by 80% in PS120Δ635 cells (Figure 8C), although the absolute activity of both exchangers in the presence of LPA was similar. In contrast, the magnitude of serum-induced increases in NHE1 activity in PS120Δ635 cells, relative to PS120N cells, was reduced by only 45% (Figure 8C). Hence, NHE1Δ635 almost completely lost the ability to be stimulated by LPA, but only partially lost the ability to be stimulated by serum.

Discussion

In this study, NHE1 activity was stimulated by the dominant active forms of p160ROCK, a RhoA-associated kinase, and RhoA-induced stimulation of NHE1 activity was blocked by the dominant-negative form of this kinase. These findings suggest that p160ROCK acts downstream of RhoA and upstream of NHE1 to mediate RhoA activation of the exchanger. Furthermore, Y-27632, a specific inhibitor of p160ROCK, not only inhibited NHE1 activation by LPA but also suppressed phosphorylation of NHE1 induced by this stimulus. Consistently, no phosphorylation and greatly reduced activation by LPA were found with NHE1Δ635, in which all serine residues in the cytoplasmic domain are deleted. These findings suggest that increased phosphorylation of NHE1 may be necessary for a p160ROCK-mediated pathway to stimulate exchange activity. The importance of NHE1 phosphorylation in regulating exchange activity has been controversial. Deletion of the C-terminal phosphorylation sites in NHE1 inhibits growth factor-induced increases in pH, by only 50% (Wakabayashi et al., 1992, 1994a). However, microinjection of antibodies directed against the C-terminal phosphorylation domain completely inhibits activation of NHE1 by thrombin and endothelin (Winkel et al., 1993). On the other hand, activation of NHE1 by osmotic stress (Grinstein et al. 1992) and inhibition of NHE1 by ATP

Fig. 6. p160ROCK directly phosphorylates NHE1 in vitro. (A) Myc-tagged p160ROCKΔ3 (lane 1) and PKN (lane 2), transiently expressed in CCL39 cells, were immunoprecipitated and subjected to immunoblotting with anti-Myc antibodies. (B) These same immunoprecipitates were used for in vitro kinase assays with histone or GST–NHE1638–815 as a substrate. Lanes 1 and 4, vector control; lane 2, p160ROCKΔ3; lane 3, p160ROCKΔ3 in the presence of Y-27632; lane 5, PKN. Exposure time of lanes 1 and 2 is 30 min and that of lanes 3–5 is 4 h. (C) Effects of Y-27632 and EIPA on p160ROCKΔ3 kinase activity were determined by phosphorylation of histone in the presence of the indicated concentrations of these inhibitors. The results shown are representative of 2–3 determinations.
depletion (Goss et al., 1994) occur without detectable changes in phosphorylation of the exchanger. Our current finding that deletion of the C-terminal domain abolished most of the LPA stimulation of NHE1 but suppressed only 50% of serum stimulation indicates that, depending on the extracellular stimulus or intracellular signaling pathway, NHE1 activity is regulated by phosphorylation-dependent and -independent mechanisms, and that the LPA–RhoA pathway utilizes only the phosphorylation-dependent mechanism to stimulate the exchanger. Our findings that the in vitro phosphorylation of a GST–NHE1 fusion protein by p160ROCK is inhibited by Y-27632, and that identical sites on NHE1 are phosphorylated in vivo and in vitro further suggest that p160ROCK may regulate NHE1 by direct phosphorylation.

We previously determined that NHE1 acts downstream of RhoA in a pathway regulating the actin cytoskeleton (Hooley et al., 1996; Vexler et al., 1996). Activation of NHE1 is necessary for RhoA-induced stress fiber formation but is not sufficient. Increased NHE1 activity in the absence of a RhoA-mediated signal has no effect on stress fiber formation (Vexler et al., 1996). A direct RhoA target molecule, p160ROCK, was shown previously to induce stress fiber formation (Ishizaki et al., 1996). Our current results show that p160ROCK itself can activate NHE1 and selectively mediates activation of NHE1 by RhoA, but not by Rac1 or Cdc42. We also found that activation of NHE1 is necessary for p160ROCK-induced stress fiber formation. p160ROCK (Ucheta et al., 1997) and ROKα/Rho-kinase (Kimura et al., 1996) previously were shown to increase myosin light chain phosphorylation and to induce cell contractility, and this action on contractility has been suggested as a mechanism for RhoA-induced stress fiber formation (Chrzanoswska-Wodnicka and Burridge, 1996). Our current study indicates that activated NHE1 acts cooperatively with this myosin-based mechanism to mediate the RhoA action. The NHE1 action may precede the action on myosin, because without activation of the exchanger, p160ROCK cannot induce the assembly of stress fibers (Figure 1). What is the NHE1 action in this event? Our recent findings suggest that activation of NHE1 is required for cell spreading on a fibronectin-

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**Fig. 7.** Phosphopeptide mapping of NHE1 phosphorylated by LPA *in vivo* and by p160ROCKΔ3 *in vitro*. EE-tagged NHE1 immunoprecipitated from 32P-labeled quiescent (A) and LPA-stimulated (B) PS120N cells, and GST–NHE1 phosphorylated *in vitro* by p160ROCKΔ3 (C). Phosphorylated NHE1 was isolated by SDS–PAGE, digested with trypsin and samples were loaded onto thin-layer cellulose plates. Phosphopeptides were separated by electrophoresis (horizontal dimension) and chromatography (vertical dimension), and visualized by Image Analyzer. Phosphopeptides derived *in vivo* (B) and *in vitro* (C) were mixed and analyzed (D). Identical radioactive spots observed with *in vivo* and *in vitro* reactions are indicated by arrows (a) and (b).
Na–H exchange and p160ROCK

Fig. 8. Increased phosphorylation of NHE1 is necessary for LPA-stimulated exchange activity. (A) PS120 cells (lane 1) and PS120 cells stably expressing the EE epitope-tagged full-length NHE1 (PS120N) (lane 2) or the EE epitope-tagged C-terminally truncated NHE1Δ635 (PS120Δ635) (lane 3) were labeled with [35S]methionine. Each exchanger variant was then immunoprecipitated with anti-EE antibodies and analyzed on a 7.5% acrylamide SDS–PAGE gel. (B) Serum-starved and 32P-labeled PS120 (lane 1), PS120N (lanes 2 and 3) and PS120Δ635 cells (lanes 4 and 5) were stimulated for 10 min with 10% serum. Phosphorylated NHE1 was immunoprecipitated and analyzed. Black arrows indicate the position of wild-type NHE1, and white arrows indicate the position of truncated NHE1. (C) The rate of pH\textsubscript{i} recovery from an acid load, determined at pH\textsubscript{i} 6.6 in PS120N and PS120Δ635 cells. Data are expressed as the mean ± SEM of 4–6 separate cell preparations.

coated dish (Tominaga and Barber, 1998). Activation of NHE1 was found previously to be associated with cell spreading, and this activation required integrin ligation of ECM proteins (Ingber et al., 1990; Schwartz et al., 1991). Probably the initial attachment of integrins to ECM proteins triggers activation and recruitment of p160ROCK and NHE1 to a particular site of the membrane, and the subsequent activation of NHE1 further strengthens the integrin binding to facilitate cell spreading. Consistent with this idea is the report that both LPA activation and attachment to integrins are required for cell spreading and stress fiber formation (Hotchin and Hall, 1995). It is interesting in this respect that p160ROCK is recruited to the cytoskeleton complexes in a manner dependent on integrin ligation of ECM proteins (Fujita et al., 1998) and that NHE1 molecules accumulate focally with cytoskeletal proteins such as vinculin, talin and F-actin along the border of lamellipodia of spreading cells (Grinstein et al., 1993; Plopper et al., 1995). A proposed sequence of events is depicted in Figure 9.

The specific signal that NHE1 is contributing to regulate Rho-mediated cytoskeletal remodeling remains to be determined. The effects of NHE1 are likely to be mediated by changes in intracellular concentrations of H\textsuperscript{+} or Na\textsuperscript{+}, or by changes in cell volume. The predominant localization of NHE1 at sites of focal contact (Grinstein et al., 1993; Plopper et al., 1995) suggests that if H\textsuperscript{+} is an important signal, then perhaps localized pH\textsubscript{i} gradients might be critical for the assembly of focal adhesions and the focal attachment of actin stress fibers. If localized pH\textsubscript{i} gradients are an important signal, our findings suggest that these are generated primarily by NHE1, as HCO\textsubscript{3}–-dependent exchangers are unable to compensate for the loss of NHE1 activity. An alternative possibility is that NHE1 is structurally linked to the actin cytoskeleton, analogously to the role of the erythrocyte Cl–HCO\textsubscript{3} exchanger, AE1. AE1 and NHE1 share a similar structural topology of 12 transmembrane domains and a long cytoplasmic domain, and they share a similar function in regulating pH\textsubscript{i}. AE1, however, also functions to tether actin to the plasma membrane through the binding of its cytoplasmic domain to the actin-associated proteins ankyrin (Ding et al., 1996) and protein 4.1 (An et al., 1996).

What are the functional consequences of the p160ROCK-induced activation of NHE1? NHE1 has been proposed to be involved in anchorage-dependent cell proliferation and, paradoxically, cell transformation (Grinstein et al., 1989; Maly et al., 1989; Ingber et al., 1990; Kaplan and Boron, 1994). Similarly, RhoA has been suggested to be involved in the G\textsubscript{1}–S progression and cell transformation. In the former process, RhoA is presumed, but has not been proved, to induce focal adhesions and to
transmit the growth signal from these complexes (Yamamoto et al., 1993; Schwartz, 1997). Quite recently, RhoA has been shown to induce p27Kip1 degradation, which is essential in G1–S progression (Hirai et al., 1997). The p27 degradation is also dependent on cell adhesion, at least in some instances. On the other hand, RhoA in collaboration with Raf induces cell transformation manifested as serum independence and anchorag-indepen-dent growth. The transformed cells showed no sign of focal adhesions and stress fibers, although activated RhoA is present (Qiu et al., 1995b), indicating that generation of a growth signal is somehow dissociated from cell adhesion. It would be interesting to test whether these processes are also sensitive to an NHE1 inhibitor such as EIPA. NHE1 and RhoA may also be involved in cell motility (Simchowitz and Cragoe, 1986; Stasia et al., 1991). Cell migration has been proposed to occur by cycling the extension and adhesion in the front and the de-adhesion and retraction in the rear (Mitchison and Cramer, 1996). RhoA, if involved, is supposed to work in the former process. It would be interesting to know whether NHE1 inhibition and RhoA inactivation induce a similar inhibitory phenotype of migrating cells.

In summary, we determined that NHE1 is a downstream, and possibly direct, target of p160ROCK, and that NHE1 activity and/or phosphorylation is necessary for actin stress fiber assembly induced by p160ROCK. Together with our previous studies (Hooley et al., 1996; Vexler et al., 1996; Tominaga and Barber, 1998), these findings suggest that NHE1 activity is a critical component of normal cytoskeletal functions regulated by a RhoA-p160ROCK-mediated pathway, including cell adhesion and contractility. Additionally, a functional link between p160ROCK and NHE1 may be an important determinant in pathophysiological conditions associated with abnormal cytoskeletal organization. Recent experiments using Y-27632 implicate a role for RhoA-p160ROCK in augmenting blood pressure in hypertensive rats (Uehata et al., 1997). Although the inhibitory effect of the Y-compound on hypertension has been attributed solely to its inhibition of smooth muscle contraction, it is quite likely that Y-27632 corrects high blood pressure also by inhibiting enhanced NHE1 activity. NHE1 activity is increased in blood and vascular smooth muscle cells of hypertensive patients and in animal models of genetic hypertension such as the spontaneously hypertensive rat (Aviv, 1996). Additionally, transgenic mice overexpressing recombinant NHE1 have salt-sensitive hypertension (Kuro-o et al., 1995). We expect that similar pathophysiological links between NHE1 and the RhoA-p160ROCK pathway will be found in other disease states.

**Materials and methods**

**Expression plasmids**

pCAG-myc-p160ROCK and related mutants (Ishizaki et al., 1997), pcDNA1-Gnt3Q (Yonoy-Nosenetska et al., 1994), pEXV-myc-RhoAV14 (Qiu et al., 1995b), pEXV-myc-Rac1V12 (Qiu et al., 1995a) and pCMV-myc-Cdc42V12 (Hooley et al., 1996) constructs were produced as previously described. pcAN-myc-PKN was providedinto Dr G.Martin (ONXY Pharmaceuticals). Human NHE1 cDNA was provided by Dr J.Pouyssegur (University of Nice, France) and subcloned into pcDNA1 (Invitrogen) with a Glu–Glu epitope (EE) tag at the C-terminus (NHE1-EE). Using this plasmid as a template, a PCR product was generated that contained residues 1545–1905 of the NHE1 cDNA along with an in-frame sequence encoding an EE epitope tag (Grussmennery et al., 1985) and a BamHI site at the 3′ end. The HindIII-BamHI fragment from this PCR product was subcloned into pCDNA3 (Invitrogen) and sequenced. A HindIII–BglII fragment from the full-length NHE1 cDNA was then subcloned between the HindIII site in this plasmid and the BglII site contained within the NHE1 coding region to restore the 5′ end of the NHE1 coding region (NHE1A635-EE). A GST fusion protein comprising the 178 C-terminal amino acids of rabbit NHE1 was obtained from Dr L.Fliegel (University of Alberta, Edmonton) (Silva et al., 1995) and purified by glutathione–Sepharose 4B beads (Pharmacia) according to the manufacturer’s instructions. EIPA was obtained from Molecular Probes (Eugene, OR).

**Cells**

CCL39 cells, a Chinese hamster lung fibroblast line; PS120 cells, an NHE-deficient clone derived from parental CCL39 cells (Pouyssegur et al., 1984); and PS120 cells stably expressing NHE1 (EIPA) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS). HeLa cells were maintained in DMEM supplemented with 10% heat-inactivated FBS. Full-length NHE1 and NHE1Δ635 were stably expressed in PS120 cells (PS120N and PS120Δ635, respectively) by a calcium phosphate method (Speciality Media Inc., NJ) with 20 μg of cDNA. Clones were selected by geneticin and by sequential proton suicide incubations as previously described (Pouyssegur et al., 1984). This latter selection method eliminates cells not expressing NHE1 because in a HEPES buffer they are unable to extrude protons after an NH₄Cl-induced acid load. Expression of NHE1 was confirmed by measuring the rate of pH recovery from an acid load in a HEPES buffer (Yonoy-Nosenetska et al., 1994) and by immunoprecipitating NHE1 from 35S-labeled cells using mouse anti-EE monoclonal antibody (Grussmennery et al., 1985). For transient expressions, cells were plated at a density of 5×10⁵ cells per 6 cm dish. After 24 h, the cells were transfected using lipofectamine (Gibco Life Technologies, Inc.) for 3–4 h with 2–3 μg of DNA in Opti-MEM. pcDNA3 empty vector was used to maintain total transfected DNA constant. Cells were maintained in growth medium for 3–4 h and then in serum-free DMEM for 16–20 h prior to experiments. Transfection efficiencies in CCL39 cells were routinely 30–35%, as determined by staining with β-galactosidase expression. Phalloidin staining and myc immunostaining were performed as previously described (Vexler et al., 1996).

**NHE1 activity and intracellular pH**

To determine NHE1 activity and pH, cells plated on glass coverslips were serum-starved for 16–20 h, transferred to a nominally HCO₃-free HEPES buffer, and loaded for 10 min at 37°C with 1 μM of the acetoxymethyl ester of the pH-sensitive fluorescent dye BCECF. Cells were placed in thermocellula controled (37°C) cuvette holder in a Shimadzu RF5000 spectrofluorometer, and BCECF fluorescence was measured at 530 nm by alternately exciting the dye at 500 and 440 nm. The emission ratio was calibrated to pH for each determination using 10 μM nigericin as previously described (Thomas et al., 1979). To determine NHE1 activity, cells were pulsed for 10 min with 30 mM NH₄Cl and then transferred to a HEPES buffer (Hirai and Weeks, 1976). The rate of pH₁ recovery (dpH₁/dt) from an acid load induced by the rapid removal of NH₄Cl was calculated at pH₁ intervals of 0.05 units and used as an index of NHE1 activity. To study the effects of LPA and serum, these agents were added in the NH₄Cl pulse and in the HEPES recovery buffer. The values for pH₁ and dpH₁/dt were expressed as the mean ± SEM of the indicated number of matched separate transfections or of separate cell preparations for LPA and serum treatment.

**Phosphorylation of NHE1**

Cells grown to sub-confluence in 100 mm dishes were serum deprived for 22 h, then pre-incubated in a nominally phosphate-free, serum-free medium for 2 h and labeled for an additional 5 h at 37°C with [γ³²P]orthophosphate (100–200 μCi/ml). For LPA stimulation, labeled cells were first dissociated by dissociation buffer (Gibco), plated on poly-L-lysine-coated dishes and incubated in serum-free medium for 45 min with or without Y-27632 (Uehata et al., 1997). LPA (10 μM) was then added for an additional 10 min. For serum stimulation, serum (10% final) was added directly to adherent labeled cells for 10 min at 37°C. After stimulation, the cells were washed with ice-cold phosphate-buffered saline (PBS), frozen in liquid N₂ and resuspended in buffer A [50 mM HEPES–NaOH (pH 7.4), 150 mM NaCl, 3 mM KCl, 12.5 mM sodium pyrophosphate, 1 mM ATP, 5 mM EDTA supplemented with protease inhibitors]. Samples were centrifuged for 15 min at 100 000 g. The pellets were resuspended in 500 μl of ice-cold buffer B [buffer A
containing 1% Brij 96 (Sigma), sonicated for 40 s, and centrifugated for 30 min at 100 000 g. The supernatants were pre-cleared with anti-
mouse IgG-agarose (Sigma) and then incubated overnight at 4°C with 5 
µg of mouse anti-EE monoclonal antibodies. Anti-mouse IgG-agarose was added for 1 h at 4°C and then washed five times with buffer B.

Immunoprecipitated proteins were solubilized by boiling in Laemmli
sample buffer and separated by SDS–PAGE (8% polyacrylamide). The gel was dried and subjected to analysis using an Image Analyzer (Molecular Dynamics).

**Kinase assays**

Cells transiently expressing 2 µg of pCAG-myc-p160ROCKΔ3, 3 µg of pCAN-myc-PKN or 2 µg of pcDNA3 were lysed with lysis buffer [10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 µg/ml leupeptin and 2 µg/ml aprotinin], and Myc-tagged kinases were
immunoprecipitated with anti-Myc (9E10) antibody as described above.

After immunoprecipitation, the beads were suspended with 400 µl of
kinase wash buffer (20 mM Tris–HCl, pH 7.4, 1 mM EDTA, 0.1% NP-
40, 10% glycerol, 1 mM Na3VO4, 5 mM β-mercaptoethanol). A 100 µl fraction of the sample was sedimentated and resuspended in 20 µl of
kinase buffer containing 25 mM HEPES–NaOH (pH 7.5), 1 mM dithiothreitol, 10 mM MgCl2, 3 mM MnCl2, 1 mM Na3VO4, 10 mM ATP and 0.3 µCi of [32P]ATP. GST–NHE1 (3 µg) or histone H1 (3 µg) was added as a substrate for kinase activity and the kinase mixture was
incubated at 30°C for 20 min. The reaction was stopped by adding 5 µl of 5 × Laemmli’s sample buffer. Samples were resolved by SDS–PAGE (12% acrylamide) and incorporation of radioactivity was determined by autoradiography (Figure 6B) or scintillation counting of excised
deradioactive bands (Figure 6C). Equal loading of immunoprecipitated
kinases was confirmed by immunoblotting with anti-Myc polyclonal antibody (A-14, Santa Cruz Biotechnology) as described previously
(Tominaga and Barber, 1998). The effects of EIPA and Y-27632 on


