**Gα13 Stimulates Na\(^+\)-H\(^+\) Exchange through Distinct Cdc42-dependent and RhoA-dependent Pathways**

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Activity of the ubiquitously expressed Na\(^+\)-H\(^+\) exchanger subtype NHE1 is stimulated upon activation of receptor tyrosine kinases and G protein-coupled receptors. The intracellular signaling pathways mediating receptor regulation of the exchanger, however, are poorly understood. Using transient expression of dominant interfering and constitutively active alleles in CCL39 fibroblasts, we determined that the GTPases Ha-Ras and Gα13 stimulate NHE1 through distinct signaling cascades. Exchange activity stimulated by constitutively active RasV12 occurs through a Raf1- and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK)-dependent mechanism. Constitutively active Gα13QL, recently shown to stimulate the Jun kinase cascade, activates NHE1 through a Cdc42- and MEK kinase (MEKK1)-dependent mechanism that is independent of Rac1. Constitutively active Ras attenuates NHE1 through a MEKK1-dependent mechanism, but dominant interfering Rac1N17 does not inhibit Gα13QL-mediated or constitutively active Cdc42V12-mediated stimulation of the exchanger. Conversely, Cdc42N17 does not inhibit RacV12 activation of NHE1, suggesting that Rac1 and Cdc42 independently regulate a MEKK1-dependent activation of the exchanger. Rapid (≤10 min) stimulation of NHE1 with a Gα13/Gα2 chimera also was inhibited by a kinase-inactive MEKK. Gα13QL, but not RasV12, also stimulates NHE1 through a RhoA-dependent pathway that is independent of MEK, and microinjection of mutationally active Gα13 results in a Rho phenotype of increased stress fiber formation. These findings indicate a new target for Rho-like proteins: the regulation of H\(^+\) exchange and intracellular pH. Our findings also suggest that a MEKK cascade diverges to regulate effectors other than transcription factors.

The ubiquitously expressed Na\(^+\)-H\(^+\) exchanger subtype NHE1\(^{1}\) plays a major role in intracellular pH (pHi) homeostasis and in cell volume regulation (1). NHE1 activity is stimulated by hormones, cytokines, and growth factors, resulting in an increase in pHi. Hyperosmotic shock (2) and cell adhesion (3) also activate NHE1. Increases in NHE1 activity are associated with increased cell proliferation (4, 5), differentiation (6, 7), and neoplastic transformation (8–10). Receptor (11, 12), but not osmotic (2), activation of NHE1 is associated with increased phosphorylation of the exchanger on serine residues, suggesting kinase-dependent regulatory mechanisms. Although activation of protein kinase C stimulates NHE1, growth factors and vasoactive agents can stimulate the exchanger independently of this kinase. Mutational activation of three GTPases, Ha-Ras (8, 9), Gα13 (13–15), and Gα13 (13–15), stimulates NHE1 activity. Of these GTPases, only Gα13 activates the exchanger through a protein kinase C-dependent mechanism (14). The downstream signaling events mediating Ha-Ras and Gα13 stimulation of NHE1 have not been identified. Ha-Ras and Gα13 regulate two parallel MAP kinase signaling cascades, and these cascades include serine/threonine protein kinases that could potentially modulate exchange activity.

Stimulation of one of these cascades, the extracellular signal-regulated kinase (ERK) cascade, by activated growth factor receptor tyrosine kinases is mediated by the GTPase Ras. GTP-bound Ras recruits an immediate downstream effector, Raf1, to the plasma membrane, where it is activated by an unidentified mechanism (16, 17). The serine/threonine kinase Raf1 activates the MAP kinase kinases MEK1 and MEK2 (18). These dual-specificity kinases phosphorylate and activate the MAP kinases ERK1 and ERK2 (19). Activated ERKs regulate a wide range of cytosolic and nuclear proteins involved in cell proliferation and neoplastic transformation, including phospholipase A2, p90\(^{rsk}\), c-Myc, and c-Fos (19, 20). Heterotrimeric G proteins, both α (21–23) and βγ (23–25) subunits, also regulate the ERK cascade, although their action on ERK is more cell type-specific than that of Ras. For example, mutational activation of the ubiquitously expressed Gα13 subunit stimulates growth and neoplastic transformation in Rat1 (22) and NIH3T3 (26, 27) fibroblasts; however, it enhances epidermal growth factor-stimulated ERK activity in the former cell type (22), but does not affect this kinase in the latter (26, 27). In already transformed COS-7 cells, mutationally activated Gα13 has been found both to inhibit\(^3\) and to have no effect (24, 28) on ERK activity. In contrast to its cell-specific effects on the ERK cascade, Gα13 consistently stimulates NHE1 activity in a wide range of cell types, suggesting a divergence in its actions on ERK and the exchanger.

Recently, a parallel MAP kinase cascade, the Jun kinase (JNK) or stress-activated protein kinase cascade, has been described (18, 29, 30). Identified substrates of JNK/stress-activated protein kinase are the transcription factors c-Jun and AFT2 (31). The JNK cascade is activated by epidermal growth factor...
factor (32) and UV irradiation (33) through a Ras-independent mechanism by cytokines (30, 32) and UV irradiation (33) through a Ras-dependent mechanism. Recently, Gα13 was found to activate the JNK cascade through a mechanism that is interrupted by the dominant interfering allele RasN17 (28). Extracellular signals activating JNK are mediated through activation of MEK kinase (MEKK1), a mammalian homolog of the STE11 kinase involved in the yeast pheromone mating pathway (35). MEKK1 directly activates the stress-activated protein-kinase kinase SEK1 (JNK kinase), a direct upstream regulator of JNK (36, 37). MEKK1 (MEKK1), a mammalian homolog of the STE11 kinase involved in the yeast pheromone mating pathway (35). MEKK1 directly activates the stress-activated protein-kinase kinase SEK1 (JNK kinase), a direct upstream regulator of JNK (36, 37).

In this study, we used dominant interfering alleles of kinases and GTPases to determine the mechanisms by which Ha-Ras and Gα13 activate NHE1. Although Ha-Ras and Gα13 regulate both the ERK and JNK cascades, our results indicate that Ha-Ras stimulates the exchange through a Raf1- and MEK1-dependent mechanism that is independent of MEKK1. In contrast, Gα13 activates NHE1 through a MEKK1-dependent mechanism that requires Cdc42, but not Rac1. Gα13, but not Ras, also stimulates exchange activity through a Raf-independent mechanism that is dependent of MEKK1.

MATERIALS AND METHODS

Cell Culture and Transfections—Chinese hamster lung CCL39 fibroblasts were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% heat-inactivated fetal bovine serum. 1 h prior to transfections, cells were plated at a density of 0.8 × 10^6 in 60-mm dishes. Cells were transfected using the Lipofectamine method (Life Technologies, Inc.) with 1–3 μg of DNA. pcDNA empty vector was used to maintain transfected DNA constant. 1 h after transfection, cells were resuspended in serum-containing Dulbecco’s modified Eagle’s medium into glass coverslips, allowed to adhere, and then maintained for an additional 18–24 h in serum-free Dulbecco’s modified Eagle’s medium until used for measuring NHE1 activity.

Expression Plasmids—Ha-RasV12, Gα13QL, Gα13αq, and Dα1 dopamine receptor subclone into pcDNA1 were obtained from T. Voyno-Yasenetskaia and H. Bourne and were previously described (13, 22). v-Raf, Naf, and wild-type Raf were provided by A. Madinich and subcloned into pcDNA3 (Invitrogen) at EcoRI/XbaI sites. These Raf1 constructs have a 5′ untranslated sequence from the human Raf1 gene and a KT3 epitope tag at their 3′ends (43). MEK and MEKK constructs were provided by G. Johnson (32, 35). The kinase-inactive form of mouse MEK, referred to as MEK-Km (K343M point mutation), was subcloned into pcDNA3 at BamHI/EcoRI sites with a BamHI-blunted HindIII fragment. Wild-type MEKK1 and its kinase-inactive form, MEKK1-Km (K342A point mutation), were subcloned into pcDNA3 at EcoRI/XhoI sites. The constitutively active MEKK1Δ1 allele contains an N terminus truncation (amino acids 1–352), but retains an intact catalytic domain (amino acids 353–674). The MEKK1Δ1 construct was made by placing a NcoI-Sphi fragment into a Smal site of pCMV. Both MEKK1 and MEKK1-Km were C-terminally tagged with a HA epitope. pEXV-MyRac1V12 and pEXV-MyRac1N17 were previously described (44), as were pEXV-MyRhoAV14 and pEXV-MyRhoAN19 (referred to in this paper as RhoAV14 and RhoAN19, respectively) (45). pCMV-MycCdc42V12 and pCMV-MycCdc42N17 were gifts of M. Hart and A. A. Abo. pEXV-PA encoding protein A was provided by J. Hancock.

NHE Activity—For pHi determinations, cells were transferred to a nominally HCO3-free HEPES-buffered medium (46) and loaded with a 1 μM concentration of the acetoxymethyl ester derivative of the pH-sensitive dye 2′,7′-bis(carboxyethyl)-5(6)carboxyfluorescein (Molecular Probes, Inc.) for 15 min at 37°C. 2′,7′-Bis(carboxyethyl)-5(6)-carboxyfluorescein fluorescence was measured using a Shimadzu RF5000 spectrofluorometer by alternately exciting the dye at 500 and 440 nm at a constant emission of 530 nm. Fluorescence ratios were calibrated with 10 μM nigericin in 105 mM KCl (47). Cells were acid-loaded by the application (10 min) and removal of 20 mM NH4Cl (48). Rates of recovery from this acid load (dpHi/dt) were determined by evaluating the derivative of the slope of the pHi tracing at pHi, IP values of 0.05. Data represent the mean ± S.E. of the indicated number of separate cell transfections.

Immunoblotting—Immunoblot analyses were made from cells prepared for pHi determinations to ensure that samples used for Western blots and pHi determinations had similar levels of protein expression. After the glass coverslips were removed for fluorescence measurements, cell lysates were prepared from the remaining adherent cells. Samples were normalized for protein content, and 25 μg of protein was resolved by SDS-polyacrylamide gel electrophoresis. Resolved proteins were transferred to nitrocellulose (Alameda Chemicals & Science, Inc.). After nonspecific binding sites were blocked, the filters were incubated with an anti-Myc antibody at a 1:1000 dilution. Immunosensitive proteins were detected by chemiluminescence (Amersham Biosciences). Microinjection and Immunofluorescence—CCL39 cells were microinjected with plasmids in the nucleus, incubated in serum-free Dulbecco’s modified Eagle’s medium for 5 h, and fixed in 4% formaldehyde in Ca2+/Mg2+-free phosphate-buffered saline. Immunofluorescence procedures using anti-Myc antibodies to visualize injected cells were carried out essentially as described previously (16). Cells were also stained with fluorescent isothiocyanate-labeled phalloidin (Sigma) at 0.5 μg/ml.

RESULTS

Ras and Gα13 Use Distinct Kinase Cascades to Stimulate NHE1—Both Ras and Gα13 regulate ERK and JNK. To determine whether the kinase cascades leading to ERK and JNK activation mediate stimulation of NHE1, we transiently expressed constitutively active kinases and GTPases and dominant negative kinases in CCL39 fibroblasts. NHE1 activity was determined by monitoring the rate of pHi recovery from an acute acid load induced by 20 mM NH4Cl. We first studied the role of Raf1, an immediate downstream effector of Ras (49). Expression of constitutively active v-Raf, RasV12, and α13QL stimulated NHE1 activity (Fig. 1A). The resting pHi of 6.97 ± 0.02 (mean ± S.E.) in vector controls also increased, to 7.18 ± 0.02 with v-Raf, to 7.11 ± 0.02 with RasV12, and to 7.12 ± 0.01 with α13QL (n = four separate transfections). We then cotransfected a kinase-deficient Raf1 (Naf) allele with these GTPases. This dominant interfering kinase specifically blocks activation of endogenous Raf1 activity (43). Naf completely inhibited RasV12-activated exchange activity (Fig. 1A) and decreased RasV12-induced resting pHi to 6.97 ± 0.03 (n = 4). Coexpression of wild-type Raf1 at a 1:1 ratio with Naf rescued the Raf1-induced activation by RasV12 (Fig. 1A). Naf, however, had no effect on α13QL-stimulated exchange activity (Fig. 1A) or α13QL-induced resting pHi (7.11 ± 0.03 in the presence of Naf; n = 4). In three separate transfections, coexpression of Naf also failed to inhibit activation of NHE1 by mutationally active Gα13RC (data not shown). These findings suggested that Ras, but not Gα13 or Gαq, stimulates NHE1 through a Raf1-dependent mechanism.

Although Raf1 may act on several substrates, the MAP kinase protein MEK1 and MEK2 are its preferred targets (18). We used a catalytically inactive MEK-Km allele (35) to investigate MEK-dependent activation of NHE1. Coexpression of MEK-Km completely inhibited RasV12 activation of NHE1.
Distinct kinases mediate RasV12 and α13QL stimulation of NHE1. The rate of pH recovery (dpH/dt $\times 10^{-4}$ pH/s) from an acute acid load was determined in CCL39 cells transiently expressing empty vector (pcDNA) or the indicated kinases and GTPases. Data are expressed as the recovery rate at pH 6.75. A, Raf1 regulation of NHE1 activity was determined by measuring recovery rates in cells expressing pcDNA or constitutively active v-Raf, RasV12, and α13QL alone (control) or coexpressed with a dominant interfering Raf1 (Naf). Recovery rates also were determined in cells coexpressing RasV12, Naf, and wild-type Raf (+ Naf + wtRaf). B, MEK-regulated NHE1 activity was determined in cells expressing pcDNA, RasV12, and α13QL in the absence (control) or presence (+ MEK-Km) of a dominant interfering MEK. C, the role of MEKK1 in stimulating NHE1 was determined in cells expressing pcDNA, constitutively active MEKK1, RasV12, and α13QL alone (control) or coexpressed with a dominant interfering MEKK1 (+ MEKK-Km). Data represent the mean ± S.E. of recovery rates in four to five separate transfections.

(Fig. 1B) and lowered RasV12-induced resting pH, from 7.13 ± 0.02 to 6.97 ± 0.03 (n = 4). Ras therefore activates NHE1 by the same pathway as it activates ERK. MEK-Km, however, had no effect on α13QL-induced NHE1 activity (Fig. 1B) or on resting pH (data not shown).

Ras (29, 30) and Ga13 (28) also activate the JNK cascade, which suggests that another signaling pathway may mediate the effect of these GTPases on NHE1. The lack of suitable dominant interfering or constitutively active alleles of JNK and its upstream regulator JN kinase limited our study to the role of MEKK1, an upstream regulator of JNK (36, 37), in stimulating the exchanger. Expression of a constitutively active carboxyl-terminal truncated MEKK1 (MEKK1Δ) increased NHE1 activity (Fig. 1C) and increased the resting pH, from 7.00 ± 0.02 in vector controls to 7.16 ± 0.04 (n = 3). Coexpression of a kinase-inactive MEKK1-Km with RasV12 had no effect on Ras-stimulated exchange activity (Fig. 1C) or on Ras-induced increases in pH, (7.12 ± 0.02 in the absence and 7.11 ± 0.01 in the presence of MEKK1-Km; n = 4). Coexpression of MEKK1-Km with α13QL, however, completely inhibited G13-stimulated exchange activity (Fig. 1C) and reduced the G13-induced resting pH, from 7.12 ± 0.02 to 7.00 ± 0.03 (n = 5). Ga13, but not Ras, therefore uses a MEKK1-dependent pathway to stimulate NHE1.

Members of the Rho Family of GTPases Couple to the Activation of NHE1—Recently, two members of the Rho subfamily of GTPases, Rac1 and Cdc42, were determined to activate the JNK cascade (38, 39). A third member of this family, Rho, does not couple to this signaling pathway (39, 50). To examine whether these GTPases can regulate NHE1 activity, we transiently expressed the constitutively active alleles Rac1V12, Cdc42V12, and RhoAV14 in CCL39 fibroblasts. In three separate transfections, expression of each constitutively active GTPase resulted in an increase in exchange activity (Fig. 2). Additionally, the resting pH of 6.98 ± 0.01 in vector controls increased to 7.14 ± 0.02 with Rac1V12, to 7.18 ± 0.04 with Cdc42V12, and to 7.09 ± 0.02 with RhoAV14. Coexpression of MEK-Km had no effect on Rac1V12 or Cdc42V12 stimulation of NHE1 (Fig. 2). Coexpression of MEKK1-Km with these alleles, however, completely inhibited their activation of the exchanger (Fig. 2) and reduced resting pH, to 7.01 ± 0.02 with Rac1V12 and to 7.02 ± 0.03 with Cdc42V12. In contrast, MEKK1-Km failed to reduce increases in either NHE1 activity (Fig. 2) or resting pH, induced by RhoAV14, confirming the specificity of its inhibitory effects on Rac1V12 and Cdc42V12. Hence, the mechanism whereby these small G proteins activate the ex-
Fig. 3. α13QL stimulation of NHE1 activity is inhibited by Cdc42N17 and RhoAN19, but not Rac1N17. The rates of pH$_i$ recovery at the indicated pH$_j$ values were determined in CCL39 cells transiently expressing empty vector (pcDNA) or α13QL or coexpressing α13QL and the indicated dominant interfering Rho family proteins. Data represent the mean ± S.E. of recovery rates in four to six separate transfections. A, coexpression of Rac1N17 had no effect on α13QL stimulation of NHE1 activity. B, coexpression of Cdc42N17 blocked α13QL-stimulated exchange. C, coexpression of RhoAN19 also blocked α13QL-stimulated exchange. D, expression of Myc-tagged Rac1N17 and Cdc42N17 was determined by immunoblotting with an anti-Myc antibody.

confirm the independent actions of the Rho family of GTPases on NHE1, we examined the ability of their dominant interfering alleles to inhibit stimulation by their constitutively active alleles. Rac1N17 had no effect on Cdc42V12; Cdc42N17 had no effect on Rac1V12 or RhoAV14 stimulation; and RhoAN19 had no effect on Cdc42V12 stimulation (Fig. 4).

Members of the Rho family have distinct effects on the actin cytoskeleton. Rac1 regulates membrane ruffling and lamellipodia (40, 41); Cdc42 controls the formation of filopodia (41, 42); and RhoA induces stress fiber formation (40). We next examined the effect of α13QL on the actin cytoskeleton by plasmid microinjection followed by immunofluorescence microscopy. CCL39 cells expressing α13QL were contracted and showed a marked increase in stress fiber formation (Fig. 5, A and B), which was very similar to the phenotype induced by expression of RhoAV14 (Fig. 5, C and D). In contrast, expression of Rac1V12 caused cell spreading and stimulated lamellipodia formation (Fig. 5, E and F), whereas Cdc42V12-expressing cells showed enhanced formation of filopodia and, to a much lesser extent, of lamellipodia as well (Fig. 5, G and H). These results suggest that of the Rho family members, RhoA may be the primary target of Go13 activation.

Acute Activation of NHE1 by a Go13/α13 Chimera Is Mediated by MEKK1—We have primarily studied NHE1 stimulation by the mutationally activated α13QL, which allows us to determine effects ascribed to a single GTPase. The effects of α13QL, however, could reflect an indirect response because this constitutively active Go subunit was expressed for 48 h before NHE1 activity was measured. To study rapid effects of Go13, we used an α13/α13 chimera, chimeric protein that allows specific receptor-mediated activation of Go13. This chimera was constructed by substituting the five carboxy-terminal residues of α13, which are thought to specify receptor recognition, with cognate residues of Go13 (13). Because the D2-dopamine receptor (D2R) activates Go13, but not Go13, we previously used this chimeric allele in HEK293 cells to demonstrate rapid stimulation of exchange is different, as Rac1 and Cdc42, but not RhoA, act through a MEKK1-dependent pathway. The MEKK1-independent effects of both Ras and Rho on NHE1 suggested that a Rho-mediated pathway may be an additional mechanism linking Ras to the exchanger. In three separate transfections, however, coexpression of a dominant interfering RhoAN19 had no effect on Ras-induced increases in exchange activity or resting pH$_i$ (data not shown).

Cdc42 and RhoA, but Not Rac1, Mediate Go13 Activation of NHE1—Because all three members of the Rho family stimulate NHE1 activity, we examined their role in mediating Go13 actions. Expression of a dominant interfering Rac1N17 had no effect on the actions of α13QL on exchange activity (Fig. 3A) or on resting pH$_i$ (7.12 ± 0.01 in the absence and 7.13 ± 0.02 in the presence of Rac1N17; n = 6). Dominant interfering Cdc42N17 (Fig. 3B) and RhoAN19 (Fig. 3C), however, completely blocked NHE1 stimulation by α13QL. The resting pH$_i$ also decreased, from 7.12 ± 0.01 with α13QL alone to 6.97 ± 0.02 (n = 4) with coexpression of Cdc42N17 and to 6.96 ± 0.03 (n = 4) with coexpression of RhoAN19. The inhibitory actions of Cdc42N17 and RhoAN19 were specific, as neither dominant interfering allele blocked RasV12 activation of the exchanger (data not shown). Rac1N17 was expressed at levels comparable to Cdc42N17 (Fig. 3D), suggesting that its inability to block α13QL activation of NHE1 was not attributed to the lack of protein expression. Rac1N17 also failed to inhibit NHE1 activity stimulated by RasV12 (data not shown). Although we found no condition that could serve as a positive control to confirm the dominant interfering function of Rac1N17, this plasmid construct was previously used to block the transforming action of RasV12 (44). Hence, the inhibition of α13QL-stimulated NHE1 activity by Cdc42N17, but not by Rac1N17, suggests that Rac1 may not act downstream of Cdc42, as was previously suggested by morphological studies (41, 42). Recent reports on Cdc42 and Rac1 activation of JNK also failed to establish a clear hierarchical action of these Rho-related GTPases (38, 39). To further
NHE1 by activation of the D2R (13). In CCL39 cells, activation of an α13/α1z chimera also resulted in a rapid stimulation of the exchanger (Fig. 6). Quinpirole, a D2R agonist, had no effect on NHE1 activity in cells expressing empty vector (pcDNA) or the D2R. In cells coexpressing the D2R and the α13/α1z chimera, however, quinpirole stimulated exchange activity (Fig. 6A), inducing an increase in the rate of pH<sub>i</sub> recovery from an acid load (Fig. 6B). Stimulation of NHE1 by quinpirole activation of α13/α1z was completely inhibited by MEKK1-Km, whereas MEK-Km had no effect (Fig. 6C). These findings indicate that rapid as well as constitutive activation of NHE1 by Gα13 can be mediated by MEKK1. This mechanism of acute stimulation of the exchanger suggests that nuclear transcription factors, the previously described targets of a MEKK1-dependent signaling cascade, are probably not involved in a Gα13-MEKK1-NHE1 pathway. Coupling to NHE1 may therefore represent a divergence in MEKK1 signaling.

**DISCUSSION**

The ubiquitously expressed Na<sup>+</sup>-H<sup>+</sup> exchanger NHE1 is one of several ion exchangers involved in cytoplasmic pH homeostasis. Activation of NHE1 increases the rate of H<sup>+</sup> efflux from the cell, resulting in a rise in pH<sub>i</sub>. Although the kinetics of NHE1 activation by growth factors, hormones, and cytokines has been extensively studied (1), post-receptor signaling mechanisms regulating the exchanger remain largely unknown. This study focuses on the signaling pathways mediating activation of NHE1 by Ha-Ras and Gα13. Although these GTPases regulate similar kinase cascades, our findings indicate they use distinct mechanisms to stimulate NHE1 activity (Fig. 7). It is not surprising that Raf1 and MEK, which act downstream of Ras, mediate RasV12 activation of the exchanger. ERK, a selective substrate for MEK (19), is also a likely component in Ras activation of NHE1, although there is currently no direct evidence for an ERK-dependent regulation of the exchanger. Gα13 also regulates ERK (22); however, it stimulated NHE1 activity independently of the Ras/Raf pathway. It is unknown how Gα13 regulates ERK; if the Gα13 signal acts directly on ERK, then the dominant interfering alleles of Raf1 and MEK used in this study would not block an ERK-mediated activation of NHE1.

Our findings identify NHE1 as a previously undescribed effector of a MEKK1 signaling pathway. A role for MEKK1 in activating the exchanger was determined by several experiments. First, a constitutively activated amino-terminal truncated MEKK1 increased NHE1 activity and pH<sub>i</sub>. Second, a kinase-inactive MEKK1-Km inhibited NHE1 activation by constitutively activated RacV12, Cdc42V12, and α13QL. This inhibition was specific, as MEKK1-Km had no effect on NHE1 activity stimulated by RasV12 or RhoAV14. Third, acute stimulation of the exchanger by D2R activation of an α13/α1z chimeric protein was also inhibited by MEKK1-Km. This latter finding argues against a role for transcriptional regulation in a Gα13-MEKK1-NHE1 pathway, suggesting a divergence in the...
actions of MEKK1 on nuclear factors and NHE1, an integral plasma membrane protein. JNK, a downstream effector of MEKK (37), is stimulated by Ras through a Rac-dependent mechanism (38, 39). Ras activation of NHE1, however, occurs independently of Rac and MEKK1. A likely explanation for this is that perhaps a critical threshold of MEKK1 activation must be attained to signal downstream to the exchanger. Ras stimulates only a modest increase in JNK activity (29, 38, 39) that is much less potent than its activation of ERK. Additionally, we consistently find that JNK activation by α13QL is 3-4 times greater than that by RasV12. The distinct mechanisms whereby Ras and Ga13 stimulate NHE1 are not consistent with recent findings that Ga13 stimulates JNK through a Ras-dependent mechanism (28). A Ras-mediated activation of JNK by Ga13, however, could function in regulating effectors other than NHE1.

Our findings also identify NHE1 as a previously undescribed downstream effector of the Rho family of GTPases. Although three members of this family, Rac1, Cdc42, and RhoA, stimulate NHE1 activity, their upstream regulation and coupling to the exchanger are distinct. Rac1 and Cdc42 activate NHE1 through a MEKK1-dependent mechanism, whereas RhoA couples to the exchanger independently of MEKK1. Ga13 activation of NHE1 is mediated by Cdc42 and RhoA, but not by Rac. The downstream signaling pathways used by MEK, MEKK1, and Rho to stimulate NHE1 are undetermined.

Our morphological studies suggest that RhoA is the primary target of Ga13, as expression of constitutively activated α13QL increased stress fiber formation, similar to a RhoAV14-induced phenotype. How acute activation of Ga13 regulates the actin cytoskeleton is currently unknown, and we cannot exclude the possibility that transient activation of Ga13 might also induce a Cdc42 phenotype. Concertina, a Drosophila homolog of Ga13, also functions in cytoskeletal reorganization (58). Concertina is critical for early gastrulation, as it coordinates cell shape changes during ventral furrow formation. Hence, regulation of

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*C.-Y. Yu and D. L. Barber, unpublished observations.*
the actin cytoskeleton may be a phylogenetically conserved function of Ga13.

It is currently unknown whether the Rho-like GTPases divergently regulate NHE1 and the actin cytoskeleton or whether these effectors lie within a single signaling pathway. If the latter is true, then a critical question is whether activation of NHE1 occurs upstream or downstream of cytoskeletal reorganization. Cytoplasmic pH may be a potent modulator of cytoskeletal reorganization, as the bundling and cross-linking of actin filaments are pH-dependent processes (54). In Dictostelium, changes in pH over the physiological range seen in our study (6.9–7.2) produce dramatic reorganization of the actin cytoskeleton (54). Additionally, pH-influenced cytoskeletal remodelling has been suggested to modulate cell motility (55), differentiation (56), and protein synthesis (57). NHE1 regulation could also occur downstream of cytoskeletal reorganization by the Rho-like GTPases, as osmotically induced changes in cell shape regulate Na\(^+\)-H\(^+\) exchange (2).

At this point, it is not clear how MEK-, MEKK-, and Rho-mediated pathways couple to NHE1. Ultimately, these pathways must converge at the exchanger, and at an upstream regulator of the exchanger. Using interaction cloning, we recently identified a novel protein, NIP1, that coprecipitates with NHE1 in vivo and regulates its activity (5). Overexpression of NIP1 interferes with Ras and Ga13 activation of the exchanger, suggesting that the signaling pathways identified in this study may converge at NIP1 to regulate NHE1. The functional consequence of increased NHE1 activity remains unresolved. Constitutive activation of the GTPases and kinase cascades we studied induces neoplastic transformation, and transformed cells have a higher exchange activity and resting pH than nontransformed cells (8–10). The Rho and JNK pathways may also participate in cell volume regulation, and NHE1 is activated by osmotic challenge (2). Whether activation of NHE1 plays either an obligatory or a permissive role in the cellular actions regulated by these three distinct signaling pathways remains to be determined.

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Note Added in Proof—Buhl et al. recently reported that α130L stimulates a Rho-dependent increase in stress fiber formation (Buhl, A. M., Johnson, N. L., Dhanasekaran, N. and Johnson, G. (1995) J. Biol. Chem. 270, 24631–24634).

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