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The Nck-interacting Kinase (NIK) Phosphorylates the Na^+-H^+ Exchanger NHE1 and Regulates NHE1 Activation by Platelet-derived Growth Factor*

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NIK, a recently identified Nck-interacting kinase, acts upstream of the MEK kinase MEKK1 to activate the c-Jun N-terminal kinase JNK. We now show that NIK binds to and divergently activates the plasma membrane Na^+-H^+ exchanger NHE1. In a genetic screen, NHE1 interacted with NIK at a site N-terminal (amino acids 407–502) to the Nck-binding domain, and this site is critical for its association with NHE1 *in vivo*. NIK also phosphorylates NHE1; however, the phosphorylation sites, which are distal to amino acid 638, are distinct from the NIK-binding site on NHE1 (amino acids 538–638). Expression of wild-type, but not a kinase-inactive, NIK in fibroblasts increased NHE1 phosphorylation and activity. The kinase domain of NIK, however, was not sufficient for this response *in vivo*. Full phosphorylation and activation of NHE1 required both the kinase and the NHE1-binding domains of NIK, suggesting that the NHE1-binding site functions as a targeting signal. The functional significance of an interaction between NIK and NHE1 was confirmed by the ability of a kinase-inactive NIK to selectively inhibit activation of NHE1 by platelet-derived growth factor but not by thrombin. Moreover, although NIK activates JNK through a mechanism dependent on MEKK1, it phosphorylated and activated NHE1 independently of MEKK1. These findings indicate that NIK acts downstream of platelet-derived growth factor receptors to phosphorylate and activate NHE1 divergently of its activation of JNK.

Mitogen-activated protein kinases are components of signaling modules that are evolutionarily conserved. Each of these modules includes a hierarchy of kinases, including mitogen-activated protein kinases, that are activated by mitogen-activated protein kinase kinases (MKKs),[1] which in turn are activated by MKK kinases (MKKKs) (1). Mitogen-activated protein kinase modules are regulated by plasma membrane receptors for growth factors, hormones, and cytokines (1, 2). The activation of mitogen-activated protein kinase modules by receptor tyrosine kinases is probably best understood, and this is mediated by the adaptor proteins Grb2 and Nck (3, 4), which are composed exclusively of SH2 and SH3 domains. Adaptor proteins relay signals to divergent signaling pathways by the binding of their SH2 domains to a phosphotyrosine moiety and the binding of their SH3 domains to proline-rich sequences in effector molecules.

The adaptor protein Nck interacts via its single SH2 domain with activated tyrosine kinase receptors and with insulin receptor substrate-1 (5–9), and it binds to multiple effector proteins via its three SH3 domains. Nck has been reported to bind to the Wiskott-Aldrich syndrome protein (WASP) (10), the serine/threonine kinase NAK (11), and Sos (12), which is a guanine nucleotide exchange factor for Ras. The biological significance of the association of Nck with these effector proteins, however, is not known. Recently, Skolnik and co-workers (13) determined that the SH3 domains of Nck also bind to a novel serine/threonine kinase, NIK (Nck-interacting kinase). Subsequent studies identified Misshapen (Msn) as a *Drosophila* homolog of NIK (14) and determined a direct association between Msn and Dock, the *Drosophila* homolog of Nck (15). NIK belongs to the SPS1 family of Ste20-related serine/threonine kinases, which includes the germinal center kinase (GCK) and hematopoietic progenitor kinase-1 (16, 17). These related kinases have N-terminal kinase domains, C-terminal regulatory domains, and no binding domains for Rac or Cdc42 GTPases. In contrast, members of the PAK family of Ste20-like kinases have N-terminal regulatory domains, C-terminal kinase domains, and a p21 binding domain for Rac and Cdc42 (18–20). Although the upstream regulation of NIK has not been clearly elucidated, NIK and Msn directly interact with the tumor necrosis factor receptor-associated factor TRAF1 and with the adaptor proteins Nck and Dock, respectively (13, 15, 21). Recently NIK kinase activity was shown to be activated by EphB1 and EphB2 receptors (22). Additionally, genetic analysis indicates that Msn acts downstream of Frizzled receptors and Disheveled to mediate Wnt signaling (23). Downstream, NIK couples to the activation of the c-Jun N-terminal kinase (JNK) through a MEKK1- and MKK4-dependent pathway (13). The C-terminal regulatory domain of NIK interacts with MEKK1, and NIK activation of JNK is inhibited by dominant-inactive MEKK1 and MKK4 (13).

We have determined that the Na^+-H^+ exchanger, NHE1, is

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[1] The abbreviations used are: MKK, mitogen-activated protein kinase kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; NIK, Nck-interacting kinase; PAK, p21-activating kinase; JNK, c-Jun N-terminal kinase; PDGF, platelet-derived growth factor; HA, hemagglutinin; GST, glutathione *S*-transferase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HEK cells, human embryonic kidney cells; PAGE, polyacrylamide gel electrophoresis.

also a downstream target of NIK. NHE1 is a ubiquitously expressed plasma membrane ion exchanger that regulates intracellular pH (pH_i) homeostasis and cell volume through an electroneutral exchange of extracellular Na^+ for intracellular H^+. The structural topology of NHE1 includes a series of 12 membrane-spanning α helices that collectively function in ion translocation and a relatively long (300 amino acids) C-terminal cytoplasmic regulatory domain (24). The cytoplasmic domain binds to a number of regulatory proteins (25–27), and it contains a series of distal serine residues that are phosphorylated in response to growth factor activation (28–31). The association between NHE1 and NIK was determined by using a yeast two-hybrid screen to identify proteins interacting with the NHE1 C-terminal regulatory domain, and an *in vivo* association was confirmed in fibroblasts. We found that NHE1 is a substrate for NIK kinase activity; however, an NHE1-binding site C-terminal to the kinase domain of NIK and a NIK-binding site N-terminal to the phosphorylation domain of NHE1 are required for this response, indicating that the kinase activity of NIK is not sufficient, but that a direct binding between these proteins is also required. We also found that NIK kinase activity is required for activation of NHE1 by platelet-derived growth factor (PDGF). Moreover, although we previously reported that NHE1 acts downstream of MEKK1 in a pathway regulated by Rac and Cdc42 GTPases (32), our current findings indicate that NIK activates NHE1 independently of MEKK1.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Myc-tagged wild-type NIK and kinase-inactive NIK containing a D152N substitution in the catalytic domain (NIK-D152N) subcloned into pRK5 were provided by E. Y. Skolnik (New York University Medical Center) and previously characterized (13). For *in vitro* translation, wild-type NIK was subcloned into the *Eco*RV site of pBluescript (pB-NIK). A truncated NIK containing only the N-terminal kinase domain (amino acids 1–357) (NIKΔ357) was obtained by restriction digestion of pB-NIK with *Cla*I and *Pst*I and subcloned into the FLAG-tagged pCMV-Tag2A vector (Stratagene). A truncated NIK containing the N-terminal kinase domain and the NHE1-binding domain (amino acids 1–533) (NIKΔ533) was polymerase chain reaction-amplified, incorporating a *Bam*HI restriction site on the 5′ end and a *Hin*dIII site on the 3′ end, and subsequently cloned in-frame into pCMV-Tag2A. Myc-tagged dominant-activated p160ROCK (ROCKΔ3) and PAK(T423E) were provided by S. Narumiya (Kyoto University) and J. Chernoff (Research Institute of Scripps Clinic), respectively, and were previously characterized (30, 33). HA-tagged wild-type MEKK1 and kinase-inactive MEKK1 (D1369A) were obtained from M. Cobb (University of Texas Southwestern Medical Center), HA-tagged mutationally activated RacL61 was obtained from M. Symons (Picower Institute for Medical Research), and HA-tagged JNK was provided by M. Karin (University of California San Diego). The full-length C-terminal cytoplasmic domain of human NHE1 (amino acids 503–815) and C-terminal fragments containing truncations or deletions were subcloned into pGEX vector, and glutathione S-transferase (GST) fusion proteins with NHE1 were purified by affinity chromatography using glutathione-Sepharose 4B (Amersham Pharmacia Biotech) as previously described (30).

Yeast Two-hybrid Analysis—The C-terminal cytoplasmic domain of NHE1 (nucleotides 1536–2463) was polymerase chain reaction-amplified incorporating an *Nde*I site on the 5′ end and a *Bam*HI site on the 3′ end and subsequently cloned in-frame with the GAL4 DNA binding domain in the yeast expression vector pAS2.1 (CLONTECH, Palo Alto, CA). Using common techniques (34), the yeast strain Y190 was first transformed with the resulting vector, pKWN8, and then with a 9.5-days post-conception murine cDNA fusion library in the VP16 acidic activation domain expression vector pVP16 (courtesy of S. Hollenberg, Howard Hughes Medical Institute, Seattle, WA). 1.8×10^6 transformants were plated on minimal dextrose medium lacking tryptophan, leucine, and histidine and containing 25 mM 3-aminotriazole and allowed to grow at 30 °C for 1 week. Positive clones were streaked for single isolates and tested for β-galactosidase expression. To help eliminate false positives, we routinely re-transformed the isolated plasmid into Y190 pKWN8 to confirm the interaction and swapped the bait and prey between GAL41–147 and VP16 acidic activation domain vectors. The library plasmid was then isolated using cycloheximide counterselection and transferred from yeast into bacteria, and the cDNA insert was sequenced and analyzed using the BLAST algorithm with the NCBI nucleotide data base.

Cell Culture and Transfections—Chinese hamster lung CCL39 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS). CCL39 cells were plated at a density of 5×10^5 cells/60-mm dish, maintained for 18 h, and transfected with the indicated cDNAs (3–6 μg) using the Transfast method (Promega). The cells were maintained in transfection medium for 6 h and then transferred to DMEM containing 0.5% FBS. Cells were used 18 h after transfection. HEK293 cells (human embryonic kidney cells) grown in DMEM containing 10% FBS were seeded at a density of 1.5×10^6 in 100-mm dishes for 18 h and then transfected with 4–6 μg of DNA using the Superfect transfection method (Qiagen). pcDNA3.1 vector was used to balance total transfected DNA. COS-7 cells stably expressing NHE1 tagged at the C terminus with an HA epitope were established by co-transfection of pRSV-neo (1.0 μg) with NHE1 plasmid (10 μg of pCMV-NHE1) using the LipofectAMINE transfection method (35). Cell clones were screened for the localization of NHE1 in the plasma membrane by indirect immunofluorescence and for the abundance of NHE1 by immunoprecipitation and immunoblotting using anti-HA IgG 12CA5 (Roche Molecular Biochemicals).

In Vitro Pull-down Assay—HEK293 cells transfected with Myc-NIK or FLAG-tagged truncated NIK were maintained for 48 h in DMEM supplemented with 5% FBS, washed with phosphate-buffered saline, and lysed in lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotinin). The total cell lysate was collected by centrifugation at 13,000 rpm for 10 min at 4 °C. Protein concentration was measured using the Bio-protein assay. 500 μg of the total cell lysate was incubated with GST-NHE1 fusion proteins coupled to glutathione-agarose beads for 2 h at 4 °C. After incubation, the beads were collected by centrifugation, washed three times with lysis buffer, boiled in SDS-PAGE sample buffer, and resolved on SDS-PAGE. The binding of NIK to GST-NHE1 was detected by immunoblotting with anti-Myc (Santa Cruz Biotechnology) or anti-FLAG (Sigma) antibodies.

Immunoprecipitation—Wild-type COS-7 cells and COS-7 cells stably expressing NHE1-HA were transfected with Myc-NIK or FLAG-tagged truncated NIK using the LipofectAMINE transfection method (35). After transfection, the cells were maintained for 48 h in DMEM supplemented with 5% FBS, washed in phosphate-buffered saline, and lysed as described above. Cell lysates were precleared with protein A-Sepharose or with anti-chicken IgY-agarose, normalized to total protein concentration, and incubated with anti-HA antibodies (12CA5; Roche Molecular Biochemicals) or anti-NHE1 antibodies for 60 min at 4 °C. The NHE1 antibody was developed by our laboratory and generated in chickens using a fusion protein of residues 639–815 (GST-NHE1 639–815, obtained from Larry Fliegel, University of Alberta). The immune complexes were collected after incubation with protein A-Sepharose or anti-IgY-agarose (60 min; 4 °C), washed 3 times with lysis buffer, and resolved on SDS-PAGE. The presence of NIK and the abundance of NHE1 in the immune complexes were determined by immunoblotting with anti-NHE1, anti-NIK (Santa Cruz), anti-Myc (Santa Cruz), anti-FLAG (Sigma), or anti-HA antibodies.

In Vitro Binding Assay—*In vitro*-translated NIK was produced by using the transcription/translation-coupled reticulocyte lysate system (Promega). Briefly, 50 μl of *in vitro* translation mixture (37.5 μl of transcription/translation (TNT) lysate, 2 μl of reaction buffer, 1 μl of TNT RNA polymerase, 1 μl of amino acid mixture without methionine, 2 μl of [^{35}S]methionine, 1 μl of RNase ribonuclease inhibitor, 1 μg of pB-NIK, and nuclease-free water to a final volume of 50 μl) was incubated at 30 °C for 90 min. *In vitro*-translated ^{35}S-labeled NIK was resolved by SDS-PAGE and examined by autoradiography. *In vitro* binding assays were performed by incubating ^{35}S-labeled NIK with GST-NHE1 coupled to glutathione-Sepharose 4B beads in the binding buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA) for 1 h at 4 °C. The beads were pelleted and washed three times with binding buffer. The bound complexes were resolved by SDS-PAGE and visualized by autoradiography.

In Vitro Kinase Assay—Total cell lysates from HEK293 cells transiently expressing Myc-tagged NIK, PAK(T423E), ROCKΔ3, or NIK-D152N were precleared with protein A-Sepharose (Zymed Laboratories Inc.) and immunoprecipitated with 5 μl of anti-Myc polyclonal antibody (Santa Cruz). For determining JNK kinase activity, coexpressed HA-tagged JNK was immunoprecipitated with anti-HA IgG 12CA5 (Roche Molecular Biochemicals). The immune complexes were collected after centrifugation and washed three times with lysis buffer. An aliquot of

the immune complex was used for immunoblotting to determine the expression of transfected kinases. For kinase reactions, the immune complex was washed once with wash buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 0.1% Nonidet P-40, 10% glycerol, 1 mM Na_3VO_4, 5 mM β-mercaptoethanol) and incubated with 5 μg of myelin basic protein, GST or GST-NHE1 (638–815), GST-NHE1 (501–815), or c-Jun as indicated. The kinase reaction was initiated by the addition of 20 μl of pre-mixed kinase reaction buffer (25 mM Hepes, pH 7.5, 1 mM dithiothreitol, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 1 μM Na_3VO_4, 10 μM ATP, 0.5 μCi of [λ-^{32}P]ATP) and maintained at 30 °C for 20 min. SDS-PAGE sample buffer was added to stop the reaction. Substrates were separated by SDS-PAGE, and incorporation of ^{32}P was determined by autoradiography.

In Vivo Phosphorylation of NHE1—COS-7 cells stably expressing HA-tagged NHE1 were transfected with NIK, NIK-D152N, NIKΔ357, or NIKΔ533, serum-starved for 18 h, and then incubated with phosphate- and serum-free medium for 2 h. Cells were labeled by adding [^{32}P]orthophosphate (100 μCi/ml) for an additional 3 h. After the labeling period, the cells were washed twice with ice-cold phosphate-buffered saline and lysed with lysis buffer (50 mM Hepes-NaOH, pH 7.4, 150 mM NaCl, 3 mM KCl, 12.5 mM sodium pyrophosphate, 1 mM ATP, 1% Nonidet P-40, 5 mM EDTA supplemented with protease inhibitors). The lysate was centrifuged for 10 min at 13,000 rpm. After centrifugation, the supernatant was collected, precleared with protein A-Sepharose, normalized to total protein concentration, and then incubated with anti-HA IgG (12CA5; Roche Molecular Biochemicals). The immunoprecipitated proteins were boiled in Laemmli sample buffer and separated by SDS-PAGE. One-third of the immunoprecipitated proteins was used for immunoblotting to detect NHE1 expression.

Intracellular pH Measurements—NHE1 activity and pH_i were determined in populations of CCL39 fibroblasts and HEK293 cells as previously described (35, 36) and by imaging single CCL39 cells. For cell populations, cells plated on glass coverslips were maintained in DMEM supplemented with 0.5% serum for 16–18 h, transferred to a nominally HCO_3^--free Hepes buffer, and loaded with 1 μM acetoxymethyl ester derivative of the pH-sensitive dye 2,7-biscarboxylethyl-5(6)-carboxyfluorescein (Molecular Probes) for 15 min at 37 °C without CO_2. The Hepes buffer contained 145 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM Mg_2Cl, 1.8 mM $CaCl_2$, 1 mM KH_2PO_4, and 30 mM Hepes titrated to pH 7.4. The coverslips were placed in a thermostatically controlled (37 °C) cuvette holder within a Shimadzu RF5000 spectrofluorometer and perfused at a continuous flow rate of 2 ml/min using a 4-channel peristaltic pump. 2,7-Biscarboxylethyl-5(6)-carboxyfluorescein fluorescence was measured by alternately exciting the dye at 500 and 440 nm at a constant emission of 530 nm. Fluorescence ratios were converted to pH_i by calibrating each experiment with 10 μM nigericin (Molecular Probes) in 105 mM KCl, as previously described (37). Receptor-mediated activation of NHE1 was determined by including thrombin (30 nM; Enzyme Research Labs) or PDGF-BB (25 ng/ml; Roche Molecular Biochemicals) during the prepulse and acid recovery periods. NHE1 activity was determined by measuring the pH_i recovery from an acid load induced by prepulsing cells for 10 min with 30 mM NH_4Cl (38). The pH_i-dependent rate of pH_i recovery (dpH_i/dt) was calculated by evaluating the derivative of the slope of the time-dependent pH_i recovery at pH_i intervals of 0.05.

To determine single-cell NHE1 activity, cells were plated onto 10-mm glass coverslips at 35% confluency in 35-mm wells, then transfected 24 h before pH analysis with 1.6 μg of pRK5-NIKD152N and 0.4 μg of pIH3-CD8, a plasmid expressing the CD8+ antigen (courtesy of Dr. Brian Seed, Harvard University), using a 5:1 ratio of Superfect reagent (Quiagen, Valencia CA). Immediately after transfection, the cells were transferred into DMEM supplemented with 0.1% FBS overnight. The following day, cells were loaded with 2,7-biscarboxylethyl-5(6)-carboxyfluorescein as described above, and coverslips were placed in a superfusion chamber mounted on a Nikon Diaphot 200 microscope interfaced with an imaging workbench (Axon Instruments, Foster City, CA). Cells were washed briefly with anti-CD8+ antigen-coated Dynabeads M-450 (Dynal, Oslo, Norway) in solution 1 (135 mM NaCl, 5.4 mM KCl, 0.4 mM KH_2PO_4, 0.33 mM NaH_2PO_4, 0.8 mM $MgSO_4$, 1.2 mM $CaCl_2$, 10 mM glucose, 20 mM Hepes, pH 7.4, with Tris base). Bead-coated cells were identified visually. To induce an intracellular acid load, cells were perfused first with solution 2, in which 30 mM NaCl was replaced with NH_4Cl, followed by solution 3, in which NaCl was replaced by *N*-methyl-D-glucamine. Cells were excited at 490 and 440 nm and emitted fluorescence measured at 530 nm. Measurements were taken every 10 s over a 5-min period after the onset of Na^+-dependent recovery. Intracellular pH was estimated by *in situ* calibration of the F_{490}/F_{440} fluorescence ratio using the nigericin high K^+ method (37). On each coverslip, the recovery of from 4 to 8 transfected cells was monitored, averaged, and compared with the recovery of from 4 to 8 nontransfected cells examined in parallel. Four coverslips were assayed, creating 4 sets of averaged data, for a total of 16–32 cells in each control and experimental group. For these four sets of averaged data, the rate of pH change was compared with the internal pH using a scatter plot, and the best linear fit of the data was determined.

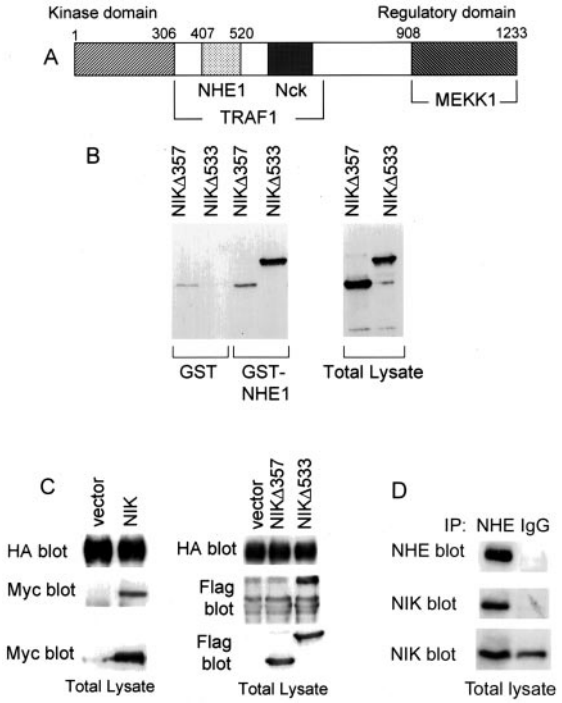

FIG. 1. **NHE1-binding site on NIK.** *A*, schematic diagram of NIK indicating the N-terminal kinase domain, the C-terminal regulatory domain, and domains for binding NHE1, Nck (13), TRAF1 (21), and MEKK1 (13). *B*, lysates prepared from COS-7 cells transiently expressing FLAG-tagged NIKΔ533 or NIKΔ357 were incubated with GST alone or GST-NHE1 (503–815). Fusion proteins were precipitated with glutathione-Sepharose beads and separated by SDS-PAGE, and bound NIK was visualized by immunoblotting with anti-FLAG antibodies. Also indicated is the relative expression of NIKΔ533 and NIKΔ357 in total cell lysates. *C*, NHE1-HA was immunoprecipitated from lysates prepared from cells transiently expressing Myc-tagged wild-type NIK or FLAG-tagged NIKΔ533 or NIKΔ357, and the presence of NIK in immune complexes was determined by immunoblotting (Myc and FLAG blots). The abundance of NHE1 in immune complexes was determined by immunoblotting with anti-HA antibodies. *D*, to confirm the association of NIK with endogenous NHE1, lysates from cells transiently expressing wild-type NIK were incubated with anti-NHE1 antibodies or IgG, and the presence of NIK in immune complexes and total cell lysates was determined by immunoblotting with anti-NIK antibodies.

RESULTS

NIK Binds to NHE1—The C-terminal cytoplasmic domain of NHE1 functions as a regulatory domain to confer changes in ion translocation by the transmembrane domain. We previously used a genetic screen to identify a novel Ca^{2+}-binding protein, CHP, which binds directly to the C terminus of NHE1 to regulate ion translocation (27). To identify additional NHE1-interacting proteins, we used the entire C-terminal cytoplasmic domain of rat NHE1 (amino acids 512–820) as bait to screen a 9.5–10.5 days post-conception embryonic library. Positive clones detected by a combination of HIS3 and β-galactosidase expression were selected and analyzed. Two out of 30 selected clones revealed a partial sequence (amino acids 407–520) of the serine/threonine kinase NIK. The yeast two-hybrid analysis indicated that a NIK fragment between amino acid residues 407 and 520 was sufficient for binding to NHE1. As shown in Fig. 1, adjacent to the NHE1-binding site are N-terminal proline-rich motifs that bind to Nck, and overlapping the Nck- and NHE1-binding sites is a binding site for TRAF1 (21). Addition-

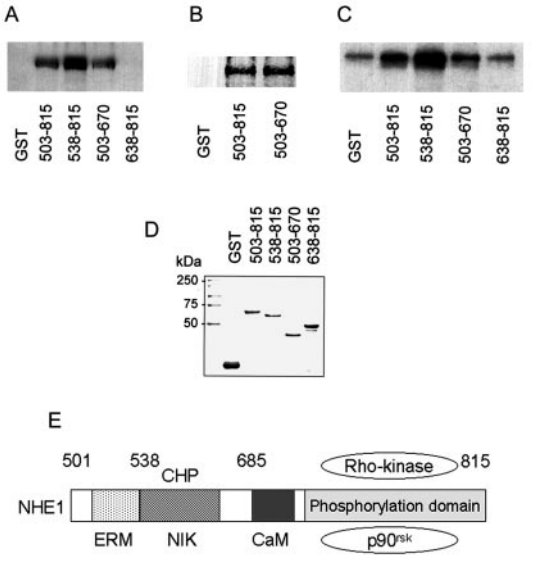

FIG. 2. **NIK-binding site on NHE1.** *A* and *B*, GST-NHE1 fusion proteins were incubated with lysates prepared from HEK293 cells transiently expressing full-length Myc-NIK (*A*) or kinase inactive Myc-NIK-D152N (*B*). Bound NIK was precipitated and visualized by immunoblotting as described in Fig. 1*B*. The NHE1 fusion proteins included C-terminal amino acid residues 503–815, 538–815, 503–670, and 638–815. *C*, ^{35}S-labeled *in vitro*-translated NIK was incubated with GST-NHE1 fusion proteins containing C-terminal residues as indicated in *A*. NIK co-precipitating with fusion proteins was separated by SDS-PAGE and examined by autoradiography. *D*, Coomassie Blue staining of GST-NHE1 fusion proteins separated by SDS-PAGE. *E*, schematic diagram of the C-terminal cytoplasmic domain of NHE1, indicating binding sites for the regulatory proteins ERM (ezrin, radixin, and moesin) (39), NIK, CHP (calcineurin homologous protein) (27), and CaM (calmodulin) (26), and a phosphorylation domain containing serine residues that are regulated by the kinases Rho kinase (30) and p90^{rsk} (31).

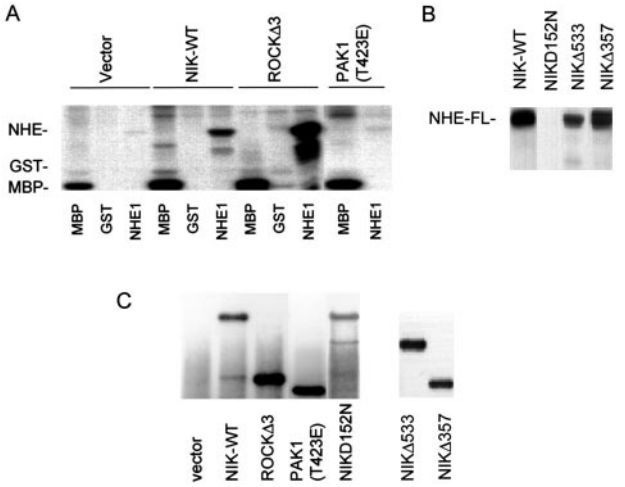

FIG. 3. ***In vitro* phosphorylation of NHE1.** *(A)* Myc-tagged wild-type (*WT*) NIK, mutationally activated p160ROCKΔ3, mutationally activated PAK1-T423E, and pcDNA3.1 vector were transiently expressed into HEK293 cells, immunoprecipitated and used for *in vitro* kinase assays with myelin basic protein (*MBP*), GST, or GST fusion proteins containing the C terminus of NHE1 (amino acids 638–815; NHE1) as the substrate. *(B)* Myc-tagged wild-type NIK and kinase-inactive NIK-D152N, and Flag-tagged NIKΔ533 (*NHE-FL*) and NIKΔ357 were transiently expressed into HEK293 cells, immunoprecipitated and used for *in vitro* kinase assays with a full-length C terminus GST-NHE1 (501–815) *(C)* Immunoblot of the indicated kinases in the immune precipitate.

ally, at the distal C terminus is a MEKK1-binding site that is critical for NIK activation of JNK (13). Further confirmation of the NHE1-binding site was obtained by using a pull-down assay. Lysates prepared from HEK293 cells transiently expressing either a C-terminal-truncated NIKΔ533, which contains both the N-terminal kinase domain and the NHE1-binding domain, or a C-terminal-truncated NIKΔ357, which contains the kinase domain but not the NHE1-binding domain, were incubated with GST alone or with GST-NHE1 (503–815). Although the expression of both constructs in total cell lysates was comparable, the binding of NIKΔ533 was more than 10-fold greater than that of NIKΔ357 (Fig. 1*B*). As shown in Fig. 2*A*, full-length NIK also bound to NHE1 in a similar assay.

To confirm that full-length NHE1 associates with NIK *in vivo*, we used a co-immunoprecipitation approach. COS-7 cells stably expressing wild-type NHE1 tagged at the C terminus with an HA epitope were transfected with Myc-tagged wild-type NIK or FLAG-tagged truncated NIK. After 48 h, NHE1 was immunoprecipitated by using anti-HA antibodies. Western analysis revealed that wild-type NIK (Fig. 1*C*; *Myc blot*) and NIKΔ533, but not NIKΔ357 (Fig. 1C; *FLAG blot*), co-precipitated with NHE1. The abundance of NHE1 in immunoprecipitates, determined by immunoblotting, was similar in all conditions (Fig. 1*C*; *HA blot*), and immunoblotting confirmed the expression of wild-type and truncated NIK (Fig. 1*C*; *Total Lysate*). Because the abundance of NHE1 in these cells was 2-fold greater than endogenous exchanger (data not shown), we also confirmed that NIK specifically associates with endogenous NHE1 (Fig. 1*D*). These findings confirm that NIK associates with full-length NHE1 *in vivo* and, moreover, that this association is mediated by the NHE1-binding site on NIK (amino acids 407–520) that was identified *in vitro*.

We used two approaches to identify the NIK-binding site in NHE1. First, lysates prepared from HEK293 cells transiently expressing full-length Myc-NIK were incubated with GST fusion proteins containing the full or partial C-terminal domain of NHE1. NIK binding was determined by immunoblotting with anti-Myc antibodies. NIK specifically bound to the full-length cytoplasmic domain of NHE1 (503–815) and to an N-terminal fragment of this domain (503–670), but a C-terminal fragment, 538–815, had the highest binding affinity (Fig. 2*A*). In contrast, NIK did not interact with a distal C-terminal fragment (638–815), which contains serine residues that are phosphorylated by growth factor activation (28), or with GST alone. The more abundant binding of NIK to NHE1 (538–815) compared with NHE1 (503–815) suggests that the region between 503 and 538 might have an inhibitory effect on NIK binding. A similar pull-down assay was used to determine that kinase-inactive NIK-D152N also associated with GST-NHE1 full length and with GST-NHE1 503–670 but not with GST alone (Fig. 2*B*). To further confirm the site of the NIK-binding domain on NHE1, we used an *in vitro* binding assay. *In vitro*-translated NIK metabolically labeled with ^{35}S was incubated with GST-NHE1 fusion proteins, and protein complexes were precipitated with glutathione beads. The binding pattern of *in vitro*-translated NIK to full-length GST-NHE1 and GST-NHE1 fragments was similar to what we observed using the pull-down assay (Fig. 2*C*), with amino acid residues 538–815 having the highest binding affinity. Together, these findings indicate that NIK binds directly to NHE1 at a site located between amino acids 538 and 638 in the C-terminal domain. This site overlaps with the binding site for CHP, a calcineurin B homologous protein that inhibits NHE1 activity (27), but is distinct from binding sites for calmodulin (26) and the ERM proteins ezrin, radixin, and moesin (39) (Fig. 2*E*).

NHE1 Is a Substrate for NIK Kinase Activity—Serine residues in the distal C terminus of NHE1 (amino acids 638–815) are phosphorylated by growth factor activation (28). We previously determined that the RhoA kinase p160ROCK (40) directly phosphorylates the C terminus of NHE1 and that this

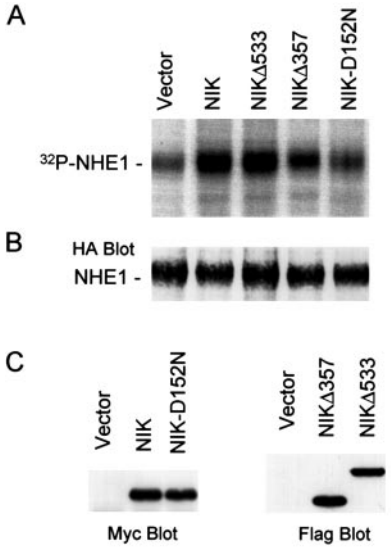

FIG. 4. ***In vivo* phosphorylation of NHE1.** *A*, NHE1-HA stably expressed in COS-7 cells, either alone (vector) or co-expressed with the indicated NIK constructs, was immunoprecipitated with anti-HA IgG 12CA5 after cells were labeled with [^{32}P]orthophosphate. Proteins were separated by SDS-PAGE, and phosphorylation was determined by autoradiography. *B*, abundance of NHE1 in the immunoprecipitates, as determined by immunoblotting with anti-HA antibodies. *C*, abundance of NIK in total cell lysates, as determined by immunoblotting.

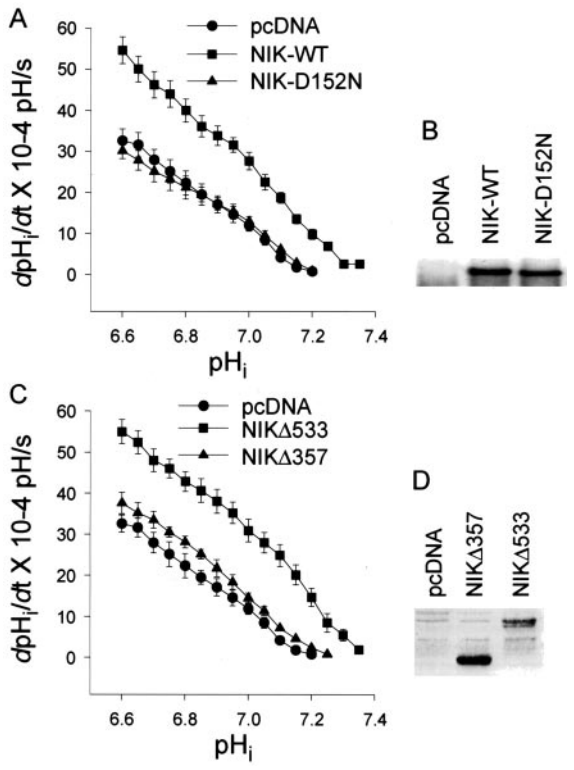

FIG. 5. **NIK stimulates NHE1 activity.** Intracellular pH was determined in CCL39 cells transfected with pcDNA3.1 vector alone, NIK, NIK-D152N, NIKΔ357, or NIKΔ533 and serum-starved for 16 h. *A* and *C*, the rates of pH_i recovery from an acid load at the indicated pH_i values were determined in a nominally HCO_3^--free HEPES buffer and used as an index of NHE1 activity. Data represent the means ± S.E. of 3–8 separate transfections. *B*, immunoblot of Myc-tagged NIK and NIK-D152N expressed in CCL39 cells. *D*, immunoblot of FLAG-tagged NIKΔ357 or NIKΔ533 expressed in CCL39 cells. *WT*, wild type.

phosphorylation is critical for ROCK-mediated activation of the exchanger (30). Recently, NHE1 was found also to be a direct substrate for p90^{rsk} (41), which may be an important determinant in NHE1 activation by growth factors. Currently, ROCK and p90^{rsk} are the only kinases recognized to directly phosphorylate NHE1. Because of its association with NIK, we next determined whether NHE1 is a substrate for NIK kinase activity. Myc-tagged wild-type NIK and mutationally activated p160ROCKΔ3 and PAK-T423E transiently expressed in HEK293 cells were immunoprecipitated, and their kinase activity was determined *in vitro* by using myelin basic protein, GST, or GST-NHE1 (638–815) as substrates. Similar to previous findings, wild-type NIK (13), p160ROCKΔ3 (30), and PAK-T423E (42) phosphorylated myelin basic protein (*MBP*) (Fig. 3*A*). NHE1 was phosphorylated by wild-type NIK and, as previously determined, by p160ROCKΔ3 (30). The more abundant phosphosphorylation by p160ROCKΔ3 was likely due to the use of a constitutively active kinase, as compared with phosphorylation by wild-type NIK. The specificity of NHE1 phosphorylation was confirmed, because NHE1 was not a substrate for PAK. GST alone was not phosphorylated by any of the indicated kinases. We also confirmed that phosphorylation of full-length GST-NHE1 (501–815) by wild-type NIK was similar to that obtained using GST-NHE1 (638–815), which does not contain the NIK-binding site (Fig. 3*B*). Additionally, full-length NHE1 was phosphorylated by NIKΔ533 and NIKΔ357 but not by kinase-inactive NIK-D152N (Fig. 3*B*). Hence, the phosphorylation of NHE1 by NIK *in vitro* is not regulated by NIK binding. Immunoblotting confirmed that differences in NHE1 phosphorylation were not due to differences in the amount of immunoprecipitated kinases (Fig. 3*C*).

We also determined that NIK increases NHE1 phosphorylation *in vivo*. In quiescent COS-7 cells stably expressing NHE1-HA, the exchanger is constitutively phosphorylated, as previously described (43). Transient expression of wild-type NIK, but not kinase-inactive NIK-D152N, increased basal NHE1 phosphorylation (Fig. 4*A*). We next determined the importance of NIK binding in NHE1 phosphorylation. The C-terminal regulatory domain and the N-terminal catalytic domain of NIK are required to activate JNK (13). The emerging consensus is that the function of NIK and related mitogen-activated protein kinases depends not only on their kinase activity but also on their ability to act as a scaffolding protein to regulate the localization of signaling complexes (1). We also found that the kinase domain of NIK is not sufficient to fully phosphorylate NHE1. In COS-7 cells transiently expressing the C-terminal-truncated NIKΔ533, which contains both the N-terminal kinase domain and the NHE1-binding domain, we observed an increase in NHE1 phosphorylation (Fig. 4*A*) that was similar to phosphorylation by full-length NIK. In contrast, NHE1 phosphorylation by expression of a C-terminal-truncated NIKΔ357, which contains the kinase domain but not the NHE1-binding domain, was markedly less that than that observed with full-length NIK or NIKΔ533 and only slightly more than quiescent levels (Fig. 4*A*). Immunoblotting confirmed that equivalent amounts of NHE1 were present in the immunoprecipitate for all conditions (Fig. 4*B*) and that the expression of NIK constructs in total cell lysates was comparable (Fig. 4*C*). These findings indicate that the N-terminal kinase domain of NIK is not sufficient to achieve full phosphorylation of NHE1, and they suggest that the NIK-binding site on NHE1 might function as a targeting signal to sequester NIK to the plasma membrane.

NIK Increases NHE1 Activity and Steady-state pH_i—To investigate the functional significance of an interaction between NIK and NHE1, we determined whether NIK regulates NHE1 activity and pH_i. In CCL39 fibroblasts, NHE1 activity, determined as the rate of pH_i recovery (dpH_i/dt) from an NH_4Cl-induced acid load, increased in cells expressing wild-type Myc-NIK compared with vector controls (Fig. 5*A*). Moreover, the increase in NHE1 activity was associated with a significant

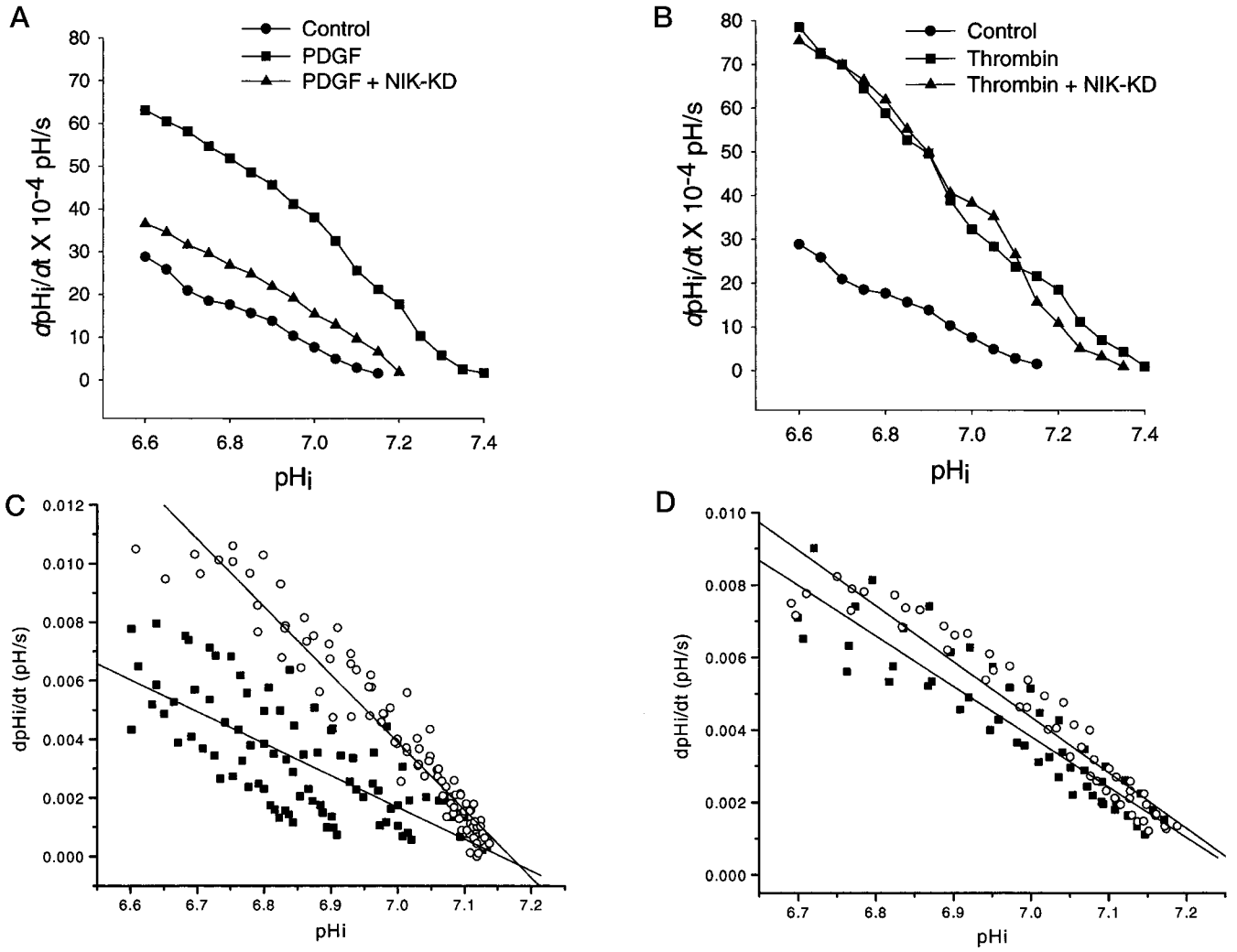

FIG. 6. **NIK mediates activation of NHE1 by PDGF but not by thrombin.** *A* and *B*, NHE1 activity, determined as described in Fig. 5, was analyzed in quiescent CCL39 cells and in CCL39 cells treated with PDGF (25 ng/ml) or thrombin (30 nM) in the absence and presence of transiently expressed NIK-D152N. *C* and *D*, NHE1 activity, determined in individual cells, treated with PDGF (*C*) or thrombin (*D*) in the absence (○, *upper line*) or presence (■, *lower line*) of NIK-D152N.

increase in pH_i from 7.12 ± 0.02 (S.E.) in vector controls to 7.33 ± 0.02 in cells expressing NIK (Student's *t* test; $p < 0.01$; $n = 8$). In contrast, expression of the kinase-inactive Myc-NIK-D152N had no effect on NHE1 activity (Fig. 5*A*) or pH_i (7.15 ± 0.02; $p > 0.2$; $n = 3$). Similar NIK-induced increases in NHE1 activity and pH_i were observed using HEK293 cells (data not shown). Immunoblotting of CCL39 cell lysates confirmed that similar levels of wild-type NIK and NIK-D152N were expressed in CCL39 cells (Fig. 5*B*). These findings are consistent with previous reports that PAK family kinases are constitutively active when expressed as wild-type alleles (13).

As we found with NHE1 phosphorylation *in vivo*, the kinase domain of NIK was not sufficient to activate NHE1. In CCL39 cells transiently expressing NIKΔ533, increases in NHE1 activity (Fig. 5*C*) and pH_i (7.34 ± 0.03) were similar to those in cells expressing full-length wild-type NIK. In contrast, expression of NIKΔ357 had no effect on steady-state NHE1 activity (Fig. 5*C*) or pH_i (7.13 ± 0.02). Immunoblot analysis indicated a comparable expression of NIKΔ357 and NIKΔ533 in cell lysates (Fig. 5*D*).

NIK Mediates Activation of NHE1 by PDGF—To determine the physiological significance of NIK-activated NHE1, we investigated its importance in receptor-mediated regulation of the exchanger. We first determined that kinase-inactive NIK-D152N could act as a dominant interfering allele by confirming that NHE1 activation by wild-type NIK was blocked by co-expression of NIK-D152N at a 1:4 ratio (data not shown). We then found that expression of NIK-D152N in CCL39 cells inhibited activation of NHE1 by PDGF (Fig. 6*A*). To confirm the specificity of this response, we found that activation of NHE1 by thrombin, which is likely mediated by a Rho-ROCK-dependent mechanism (30), is not impaired by NIK-D152N (Fig. 6*B*). In these experiments, however, NIK-D152N was transiently expressed in a subpopulation of cells, and we reasoned that although this might be sufficient to block PDGF activation, it might not be able to block a thrombin response. To confirm these findings, therefore, we determined NHE1 activity in individual cells expressing NIK-D152N, identified by co-expression of a CD8+ antigen. Similar to our results with cell populations, we found that kinase-inactive NIK effectively blocked activation of NHE1 by PDGF but not by thrombin (Fig. 6, *C* and *D*).

MEKK1 Kinase Activity Does Not Regulate Phosphorylation or Activation of NHE1 by NIK—Several previous findings suggest that there could be a functional interaction between NIK and MEKK1 in regulating NHE1. Su *et al.* (13) found that NIK acts upstream of MEKK1 in a pathway activating JNK. Activation of JNK by NIK is blocked by a dominant-negative MEKK1, and it requires a direct association of MEKK1 with the C terminus of NIK (see Fig. 1). We previously reported that MEKK1 acts downstream of Rac and Cdc42 GTPases in a

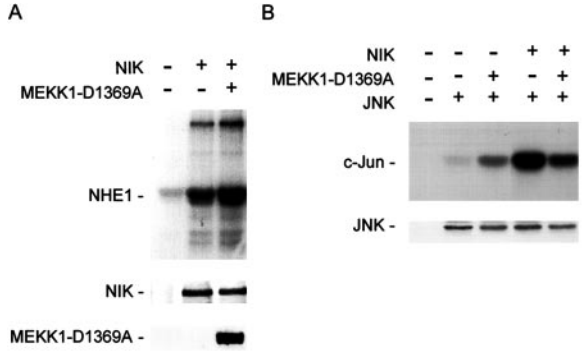

FIG. 7. **Phosphorylation of NHE1 by NIK is independent of MEKK1 kinase activity.** *A*, *upper panel*, Myc-tagged NIK, immunoprecipitated from HEK293 cells transiently expressing NIK alone or co-expressing MEKK1-D1369A, was used for *in vitro* kinase assays with GST-NHE1 as a substrate as described for Fig. 3. *Middle panel*, immunoblot for NIK in the immune complex. *Lower panel*, immunoblot for MEKK1-D/A in total cell lysates. *B*, *upper panel*, HA-JNK immunoprecipitated from HEK293 cells transfected with either JNK alone or co-transfected with NIK or MEKK1-D1369A was used for *in vitro* kinase assays with GST-c-Jun as a substrate. *Lower panel*, immunoblot for JNK in the immune complex.

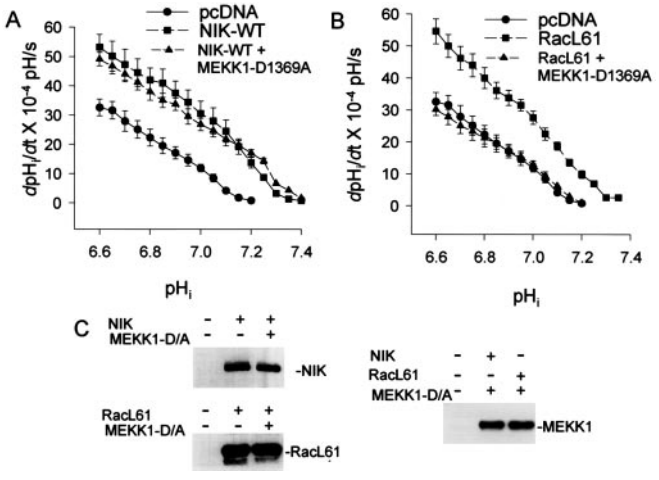

FIG. 8. **Activation of NHE1 by NIK is independent of MEKK1 kinase activity.** *A* and *B*, NHE1 activity was determined in CCL39 cells transfected with pcDNA3.1 vector, wild-type NIK (*NIK-WT*), and mutationally activated RacL61 alone or co-transfected with kinase-inactive MEKK1-D1369A. The rates of pH_i recovery from an NH_4Cl-induced acid load were determined at the indicated pH_i values in a Hepes buffer. *C*, expression of RacL61, NIK, and MEKK1-D1369A in total cell lysates was determined by immunoblotting.

pathway stimulating NHE1 (32). Expression of a mutationally activated MEKK1 constitutively stimulates NHE1 activity, and activation of NHE1 by GTPase-deficient Rac and Cdc42 is blocked by the co-expression of a kinase-inactive MEKK1. Taken together, these findings suggested that MEKK1 might also mediate activation of NHE1 by NIK. As described above, however, a truncated NIKΔ533, which does not include the MEKK1-binding site, phosphorylates and activates NHE1. This indicates that the binding of MEKK1 to NIK is not required to regulate NHE1. It does not establish, however, whether MEKK1 kinase activity is involved in this response. We therefore determined the effects of a catalytically inactive MEKK1-D1369A on the regulation of NHE1 by NIK. Using an *in vitro* kinase assay similar to that described in Fig. 3*A*, we found that phosphorylation of NHE1 by NIK was similar in the absence and presence of co-expressed MEKK1-D1369A (Fig. 7*A*; *MEKK1-D1369A*). In contrast, as previously described (13), co-expression of kinase-inactive MEKK1 markedly reduced NIK activation of JNK (Fig. 7*B*).

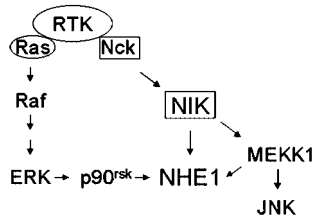

FIG. 9. **Schematic diagram indicating that receptor tyrosine kinases (*RTK*) can activate NHE1 via two pathways mediated by $p90^{rsk}$ and NIK and that NIK divergently regulates NHE1 and JNK.** *ERK*, extracellular signal-regulated kinase.

We also determined that MEKK1 activity is not required for the activation of NHE1 by NIK. Co-expression of catalytically inactive MEKK1-D1369A had no effect on activation of NHE1 by NIK (Fig. 8*A*). In contrast, co-expression of MEKK1-D1369A completely inhibited the activation of NHE1 by mutationally activated RacL61 (Fig. 8*B*). Co-transfection with MEKK1-D1369A had no effect on the expression of NIK or RacL61, as indicated by immunoblotting (Fig. 8*C*). In three separate cell preparations, co-expression of kinase-inactive NIK-D152N did not affect activation of NHE1 by RacL61 or wild-type MEKK1 (data not shown). These results indicate that whereas MEKK1 mediates NIK activation of JNK, as previously described (13), and Rac activation of NHE1, as we previously reported (32), its binding to NIK is not required for the association of NIK and NHE1, nor does its kinase activity regulate phosphorylation and activation of NHE1 by NIK.

DISCUSSION

Our findings identify NHE1 as a previously unrecognized effector of NIK. We determined that NHE1 and NIK interact through identified sites on each protein and that NHE1 is a substrate for NIK kinase activity *in vitro* and *in vivo*. Moreover, transient overexpression of wild-type, but not kinase-inactive, NIK increases NHE1 activity and steady-state pH_i.

An emerging consensus is that the function of NIK and MKK kinases (MKKKs) depends not only on their kinase activity but also on their ability to act as scaffolding proteins to regulate the localization of signaling complexes and impart specificity (1). The ability of NIK to activate JNK is mediated in part by its ability to associate with MEKK1 either by acting as a scaffolding protein to juxtapose NIK with MEKK1 and MKK4 or by imparting specificity of MEKK1 coupling to JNK (13). Additionally, MEKK1 functions as a scaffolding protein by interacting with its downstream substrate MKK4 and the upstream NIK-related kinase hematopoietic progenitor kinase-1 (HPK-1) (1). Our findings confirm that the kinase activity of NIK is not sufficient to phosphorylate or activate NHE1 *in vivo* but, rather, that a direct binding to NHE1 is also required. Unlike the cytoplasmic protein JNK, however, NHE1 is an integral plasma membrane protein, which suggests that the NHE1-binding site in NIK might function as a targeting signal to sequester NIK to the membrane.

The NIK-binding site in NHE1 has been reported to function as a critical domain for regulating NHE1 activity. Deletion of this site completely abolishes activation of NHE1 by growth factors (43), and a putative regulatory protein is postulated to bind to this site to confer regulated NHE1 activity. Although we recently identified a novel Ca^{2+}-binding protein, CHP, that directly binds to this critical NHE1 regulatory domain, binding of CHP, in contrast to NIK, inhibits NHE1 activity (27). Together, these findings suggest that multiple regulatory proteins that confer regulation of NHE1 activity might share the NIK/CHP-binding site. Additionally, it remains to be determined whether there is competitive binding between CHP and NIK at this domain.

NHE1 was previously shown to be a substrate for the Rho kinase p160ROCK (30) and for $p90^{rsk}$ (31). ROCK mediates activation of NHE1 by integrin receptors (35) and by the G protein-coupled receptor for lysophosphatidic acid (30). $p90^{rsk}$ mediates activation of NHE1 by growth factors, most likely by acting downstream of the mitogen-activated protein kinase ERK (extracellular signal-regulated kinase), which has been shown to indirectly activate NHE1 (44). Unlike NIK, however, neither ROCK nor $p90^{rsk}$ has been shown to bind to NHE1, and a truncated ROCK containing only the kinase domain is sufficient to activate and phosphorylate NHE1 (30), which suggests that an NHE1-targeting domain is not required. Phosphorylation of NHE1 is required for full activation of transport activity by receptor-mediated signaling mechanisms, including the response to thrombin (31, 43, 45), endothelin (45), and lysophosphatidic acid (30). In contrast, activation of NHE1 by osmotic stress (46) or intracellular acidification (31) and inhibition of NHE1 by ATP depletion (47) occur without detectable changes in phosphorylation of the exchanger. Together, these findings suggest that phosphorylation is a major determinant of NHE1 activity in response to receptor-dependent, but not receptor-independent, regulation.

The physiological significance of NHE1 activation by NIK was confirmed by our finding that kinase-inactive NIK-D152N inhibits NHE1 activity by PDGF but not by thrombin. This finding is consistent with the finding that NIK and its *Drosophila* homolog Msn bind to the Grb2-like adaptor proteins Nck and Dock, respectively (13, 15) and that NIK mediates activation of JNK by the EphB1 and EphB2-tyrosine kinase receptors (23). Activation of NHE1 by growth factors acting at receptor-tyrosine kinases is well established (28, 43), although previous findings indicate this is mediated by a Ras-dependent ERK (extracellular signal-regulated kinase) cascade (32, 44). Our current findings suggest that receptor tyrosine kinases couple to the activation of NHE1 through two pathways, one mediated by Ras and Raf and another mediated by NIK (Fig. 9). We also found that activation of JNK, but not NHE1, by NIK requires the C-terminal MEKK1-binding site in NIK and is blocked by kinase-inactive MEKK1, suggesting that NIK also functions to divergently regulate these two downstream effectors.

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