Regulatory Phosphorylation of Serine 703 of Na\(^+\)/H\(^+\) Exchanger Isoform-1 Kinase

(Received for publication, March 12, 1999, and in revised form, May 1, 1999)

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The Na\(^+\)/H\(^+\) exchanger isoform-1 (NHE-1) is the key member of a family of exchangers that regulates intracellular pH and cell volume. Activation of NHE-1 by growth factors is rapid, correlates with increased NHE-1 phosphorylation and cell alkalinization, and plays a role in cell cycle progression. By two-dimensional tryptic peptide mapping of immunoprecipitated NHE-1, we identify serine 703 as the major serum-stimulated amino acid. Mutation of serine 703 to alanine had no effect on acid-stimulated Na\(^+\)/H\(^+\) exchange but completely prevented the growth factor-mediated increase in NHE-1 affinity for H\(^+\). In addition, we show that p90 ribosomal S6 kinase (p90\(^{RSK}\)) is a key NHE-1 kinase since p90\(^{RSK}\) phosphorylates NHE-1 serine 703 stoichiometrically in vitro, and transfection with kinase-inactive p90\(^{RSK}\) inhibits serum-induced phosphorylation of NHE-1 serine 703 in transfected 293 cells. These findings establish p90\(^{RSK}\) as a serum-stimulated NHE-1 kinase and a mediator of increased Na\(^+\)/H\(^+\) exchange in vivo.

Intracellular pH (pH\(_i\)) and cell volume are regulated in part by a family of ion exchangers termed the Na\(^+\)/H\(^+\) exchangers. Among these proteins, Na\(^+\)/H\(^+\) exchanger isoform-1 (NHE-1) is ubiquitous (1, 2). NHE-1 is activated by growth factors that alter its affinity for intracellular H\(^+\) and thereby cause cell alkalinization (1). Intracellular alkalinization mediated by NHE-1 (defined by amiloride-sensitive inhibition) is required for cell growth (3). Abnormalities in NHE-1 function may contribute to disease pathogenesis. In the NHE-1 knockout mouse, seizures develop at age 19 days (4). Increased NHE-1 activity has been demonstrated in cells and tissues of hypertensive humans and animals (5). The spontaneously hypertensive rat (SHR), a genetic model of hypertension, exhibits increased Na\(^+\)/H\(^+\) exchange and increased vascular smooth muscle cell (VSMC) growth compared with the normotensive Wistar Kyoto rat (WKY) (6). In SHR there is no significant change in NHE-1 cDNA sequence (7), steady state mRNA levels (8), or protein expression (9). However, there is a significant increase in NHE-1 phosphorylation in response to growth factors in VSMC derived from the SHR compared with WKY (10). These results suggest that alterations in NHE-1 phosphorylation (and the responsible kinases) contribute to the SHR phenotype.

NHE-1 has been shown to be constitutively phosphorylated in growth-arrested cells. In response to growth factors, multiple sites show increased phosphorylation based on two-dimensional tryptic peptide maps (11). The amino acids phosphorylated by growth factors are located in the carboxyl-terminal 300 amino acids of the exchanger (12). Because changes in NHE-1 phosphorylation may regulate Na\(^+\)/H\(^+\) exchange, these findings suggest that increased Na\(^+\)/H\(^+\) exchange in the SHR is caused by an alteration in the activity of kinases that phosphorylate NHE-1. Thus, identification of growth factor-stimulated kinases that phosphorylate NHE-1 may provide insight into the pathogenesis of hypertension.

Several protein kinases have been proposed to regulate NHE-1, including Ca\(^2+\)-calmodulin-dependent kinases (13), protein kinase C (14), p160 Rho-associated kinase (p160\(^{ROCK}\)) (15), and members of the mitogen-activated protein (MAP) kinase family including ERK1/2 (1, 16), c-Jun amino-terminal kinase, and p38 (17). Data implicating a key role for ERK1/2 are strongest, since pharmacological inhibition of the MEK1-ERK1/2 pathway with PD98059 (18) or transfection with dominant-negative ERK1/2 (19–21) dramatically decreased growth factor-stimulated Na\(^+\)/H\(^+\) exchange. It is not clear that ERK1/2 directly phosphorylate NHE-1, and it is possible that other kinase(s) downstream of ERK1/2 are responsible for NHE-1 phosphorylation.

We proposed previously that p90 ribosomal S6 kinase (p90\(^{RSK}\)), a downstream substrate of ERK1/2 (22), was a physiologically relevant NHE-1 kinase (23, 24). This hypothesis was based on the following findings: 1) a 90-kDa kinase exhibited increased activity toward an NHE-1-GST fusion protein in vitro; 2) immunodepletion of p90\(^{RSK}\) decreased the activity of the 90-kDa kinase; 3) PD98059 blocked growth factor-mediated activation of p90\(^{RSK}\) and decreased NHE-1 phosphorylation; 4) p90\(^{RSK}\) immunoprecipitated from angiotensin II stimulated VSMC-phosphorylated recombinant NHE-1 in vitro; 5) the time courses for stimulation of p90\(^{RSK}\) and Na\(^+\)/H\(^+\) exchange activity by angiotensin II were also similar (23). In the present study, we performed experiments with mutated NHE-1 and p90\(^{RSK}\) proteins to assess their effects on growth factor-stimulated Na\(^+\)/H\(^+\) exchange. The results indicate that p90\(^{RSK}\) phos-
p90RSK Is an NHE-1 Kinase

phorylates serine 703 of NHE-1, and this phosphorylation is required for growth factor stimulation of Na+/H+ exchange.

**EXPERIMENTAL PROCEDURES**

**Materials—**Anti-NHE-1 antisera (G116) was a gift from Dr. L. Ng (Leicester Royal Infirmary, UK); anti-p90RSK antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA); NHE-1 synthetic peptides EP-1 (RRARIGSDPLA) and EP-2 (MARIARIGSD-PLAYEKP) were from PeptidoGenic Research (Livermore, CA). The plasmid (pDNA3.1) was obtained from Invitrogen (Carlsbad, CA). Human NHE-1 cDNA was a gift from Dr. L. Fliegel (University of British Columbia, Canada). DN-RSK and WT-RSK have been described (25). Neomycin and PD98059 were from Calbiochem. Acetoxyethyl ester of 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF)-AM was from Molecular Probes (Eugene, OR). Activated p90RSK2 was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Sequencing grade trypsin was from Promega (Madison, WI). Thin layer cellulose plates were from Eastman Kodak Co.

**Cell Culture—**VSMC were isolated from the thoracic aorta of 200–250-g male Harlan Sprague-Dawley rats (Harlan Sprague-Dawley) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (FCS) for 5 min and were harvested. NHE-1 was immunoprecipitated, washed six times with lysing buffer, and doubled in Laemmli sample buffer containing 100 mM dithiothreitol. For phosphopeptide mapping, the samples were fractionated by 7% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The phosphorilated NHE-1 was identified by autoradiography, excised and digested with trypsin following established protocols (29). The resulting peptides were fractionated by two-dimensional phosphopeptide mapping (29). In brief, the peptides were separated in the first dimension by electrophoresis (1,000 V for 1.5 h, 10% acetic acid, 1% pyridine in water, pH 3.3) and in the second dimension by ascending chromatography (37% 1-butanol, 25% pyridine, 9% acetic acid in water, pH 5.0) (30) for 2 h. The phosphopeptides were detected by autoradiography, and the radioactivity was analyzed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). To permit comparison of fold change in 32P incorporation among different experiments, the following calculation was performed. The normalization constant, k, was calculated for each experiment by Equation 1: P1 densitometric volume in 0.5% FCS times k = 1.0. The normalized value for each phosphopeptide was then determined by Equation 2: P, densitometric volume in 20% FCS sample divided by P1, densitometric value in 0.5% FCS times k. In Vitro NHE-1 Phosphorylation by p90RSK—NHE-1 was immunoprecipitated from a lysate of 500 μg of serum-deprived PS127A cells with the G116 NHE-1 antibody and protein A-agarose beads. The immunoprecipitant was incubated at 30°C for 30 min in 50 μl of kinase reaction buffer containing 0.5 μg of active p90RSK2. The reaction was stopped by washing 6 times with the lysis buffer and then subjected to tryptic two-dimensional phosphopeptide mapping as described above.

**Phosphorylation and Purification of the NHE-1 Peptide-1 (EP-1)—**Immunoprecipitated p90RSK was incubated at 30°C for 5 min in 200 μl of kinase reaction buffer (30 mM HEPES, pH 7.4, 10 mM MgCl2, 50 μM ATP, 50 μCi/ml [γ-32P]ATP) with various concentrations of EP-1. The reactions were centrifuged for 10 at 9,000 rpm, and 1 ml of the supernatant was collected and applied to 1 × 1-cm P-81 paper. The P-81 paper was washed 4 times for 2 min with 0.1% phosphoric acid and incorporated 32P measured by scintillation counting (26). Non-specific phosphorylation was determined by subtraction of the counts from reactions containing the same concentration of EP-1 without p90RSK. For the analysis of the stoichiometry of phosphorylation of EP-1, four immunoprecipitates were incubated together at 30°C in 400 μl of kinase reaction buffer as described above with 10 μM EP-1, and samples were removed at the times indicated.

**Phosphorylation and Purification of the NHE-1 Peptide-2 (EP-2)—**Immunoprecipitated p90RSK was incubated at 30°C for 30 min in 200 μl of kinase reaction buffer containing 100 μM EP-2. The reaction was centrifuged for 5 min at 5,000 rpm, and the supernatant was collected and digested with 2 μg of trypsin at 37°C overnight. The pH was adjusted to 8.0, and ammonium bicarbonate (100 mM) was added. Tryptic phosphopeptides were purified on a C18 reverse phase high pressure liquid chromatography column. The mass and sequence of the peptides were determined using a triple quadrupole mass spectrometer (Finnigan MAT, San Jose, CA) as described previously (27).

In Vitro p90RSK Reaction Using GST-NHE-1 Fusion Protein as a Substrate—Immunoprecipitated p90RSK from PS127A cells stimulated by 20% FCS for 5 min was incubated at 30°C for 10 min in 200 μl of kinase reaction buffer containing 10 μg of GST-NHE-1 fusion protein (which contains amino acids 675–725 of human NHE-1 (23)). In Vivo Labeling and Phosphopeptide Mapping—PS127A cells, transfected PS120 cells, and transfected 293 cells were metabolically labeled with 32Porthophosphate, using previously published protocols (29). At the same stage of phosphorylation, the cells were washed twice with phosphate-free medium and then labeled with 250 μCi/ml [32P]orthophosphate for 3 h. Cells were stimulated with 20% FCS for 5 min and were harvested. NHE-1 was immunoprecipitated, washed six times with lysis buffer, and boiled in Laemmli sample buffer containing 100 mM dithiothreitol. For phosphopeptide mapping, the samples were fractionated by 7% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The phosphorylated NHE-1 was identified by autoradiography, excised and digested with trypsin following established protocols (29). The resulting peptides were fractionated by two-dimensional phosphopeptide mapping (29). In brief, the peptides were separated in the first dimension by electrophoresis (1,000 V for 1.5 h, 10% acetic acid, 1% pyridine in water, pH 3.3) and in the second dimension by ascending chromatography (37% 1-butanol, 25% pyridine, 9% acetic acid in water, pH 5.0) (30) for 2 h. The phosphopeptides were detected by autoradiography, and the radioactivity was analyzed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). To permit comparison of fold change in 32P incorporation among different experiments, the following calculation was performed. The normalization constant, k, was calculated for each experiment by Equation 1: P1 densitometric volume in 0.5% FCS times k = 1.0. The normalized value for each phosphopeptide was then determined by Equation 2: P, densitometric volume in 20% FCS sample divided by P1, densitometric value in 0.5% FCS times k.
acid-loaded with 20 μM nigericin in KCl solution (135 mM KCl, 10 mM HEPES, pH 6.5). After the pH, decreased to 6.5, the solution was changed to TBSS, pH 7.4. The nigericin/high K⁺ technique was used to calibrate the relationship between excitation ratio (F500/450) and pH. The rate of pH recovery was converted to nmol of H⁻/min/liter cells (ΔpH) by multiplying by the buffering power. Buffering power was determined by stepwise reduction of NH₄Cl under conditions in which ion fluxes were completely inhibited (5 mM BaCl₂, 30 μM ethyl isopropyl amiloride). The data were then plotted as nmol of H⁻/min/liter cells (ΔpH) versus pH.

Statistics—Values presented are means ± S.D. Student's t test was used when appropriate. p values < 0.05 were considered statistically significant.

RESULTS

Serum Stimulates Phosphorylation of NHE-1 in Cultured Cells—It has been shown previously that serum stimulates phosphorylation of NHE-1 and that the majority of the de novo incorporation occurs in a single tryptic peptide of NHE-1 (30). We performed two-dimensional tryptic phosphopeptide mapping of immunoprecipitated NHE-1 from PS127A fibroblasts that were maintained in 0.5% FCS (Fig. 1A) or stimulated with 20% FCS for 5 min (Fig. 1B). Five major phosphopeptides were identified (see scheme in Fig. 1C). To analyze changes in phosphorylation among different experiments, the intensity changes of each spot was normalized to spot P1 using the calculations described under "Experimental Procedures." This normalization obviated the need to demonstrate that equal quantities of NHE-1 were expressed and immunoprecipitated for different experiments and cell preparations. Unlike previous investigations that showed growth factors increase the phosphorylation of only one peptide (30), we found two serum-stimulated NHE-1 phosphopeptides (Fig. 1, A and B). These phosphopeptides, termed P4 and P5 (Fig. 1C), showed 4.2 ± 0.8-fold and 11.3 ± 0.7-fold increases (n = 3, all analyses are mean ± S.D.), respectively, in response to 20% FCS (Fig. 1D). Based on the magnitude of the serum-stimulated increase in phosphorylation and electrophoretic mobility, we believe that phosphopeptide P5 is the same tryptic peptide described by other investigators.

The MEK1 Inhibitor PD98059 Decreases Phosphorylation of Phosphopeptide P5—We and others (24) have observed that phosphorylation of NHE-1 in response to serum is inhibited by PD98059 suggesting that at least one NHE-1 kinase is regulated by the MEK1 and ERK1/2 pathway. Because we also found that ERK1/2 did not readily phosphorylate recombinant NHE-1-(625–747) as shown by a stoichiometry of only <0.05 (mol of phosphate/mol of total substrate) in an immune complex kinase reaction (17), it is unlikely that ERK1/2 are NHE-1 kinases. However, we previously reported that a 90-kDa NHE-1 kinase (with characteristics consistent with p90RSK) was a possible NHE-1 kinase based on the intensity of phosphorylation and immunodepletion experiments. Activation of this 90-kDa kinase by serum was inhibited by PD98059, consistent with reports that p90RSK is activated by the MEK1-ERK1/2 pathway. To identify which NHE-1 phosphopeptides might be substrates for p90RSK, we studied the effect of MEK1 inhibition on serum stimulation of NHE-1 phosphorylation. The concentration of PD98059 required to inhibit p90RSK-dependent phosphorylation of NHE-1 was determined by incubating PS127A cells with PD98059 (10, 50, and 100 μM) for 1 h. Cells were then stimulated with 20% FCS for 5 min followed by p90RSK immunoprecipitation. An immune complex kinase assay was then performed using GST-NHE-1-(625–747) as substrate (23). There was a >10-fold stimulation of p90RSK by 20% FCS measured by GST-NHE-1 phosphorylation (Fig. 2A, 1st and 3rd lanes). PD98059 inhibited p90RSK activity in a concentration-dependent manner (Fig. 2A, 4th to 6th lanes) which was significant (58 ± 7% inhibition, n = 3) at 100 μM (Fig. 2B). To determine the effect of MEK1 inhibition in vivo, PS127A cells were maintained in 0.5% FCS and incubated with 0.2% Me₂SO (vehicle) or 100 μM PD98059 for 1 h. The cells were then kept in 0.5% serum (Fig. 2C) or stimulated with 20% FCS for 5 min. NHE-1 was immunoprecipitated and two-dimensional tryptic phosphopeptide analysis performed. Phosphorylation of phosphopeptide P5 was significantly inhibited by 100 μM PD98059 (62 ± 7%, n = 3), whereas phosphorylation of P4 was not significantly changed in subsequent experiments (Fig. 2D). Based on the findings that phosphopeptide P5 showed the greatest increase in phosphorylation in response to serum, was dependent on MEK1, and co-migrated with a serum-stimulated peptide previously reported, subsequent studies focused on this phosphopeptide.

Immunoprecipitated p90RSK Phosphorylates NHE-1 Peptide-1 (EP-1) to High Stoichiometry—Previous results from our laboratory showed that p90RSK specifically phosphorylates serine and/or threonine residues located between amino acids 670 and 714 of NHE-1 in vitro (23). Sequence analysis revealed a consensus p90RSK phosphorylation motif (RXXS) (34) at amino acids 700–703 (Fig. 3A). To characterize this site of NHE-1 as a substrate for p90RSK, we used phosphopeptide analysis performed. Phosphorylation of phosphopeptide P5 was significantly inhibited by 100 μM PD98059 (62 ± 7%, n = 3), whereas phosphorylation of P4 was not significantly changed in subsequent experiments (Fig. 2D). Based on the findings that phosphopeptide P5 showed the greatest increase in phosphorylation in response to serum, was dependent on MEK1, and co-migrated with a serum-stimulated peptide previously reported, subsequent studies focused on this phosphopeptide.

**FIG. 1.** Serum-stimulated phosphorylation of NHE-1: presence of two serum-stimulated phosphopeptides. Two-dimensional tryptic phosphopeptide maps of NHE-1 were prepared from PS127A cells maintained in 0.5% FCS (A) or stimulated with 20% FCS for 5 min (B). The origins are shown as black circles at the left lower corners. C, scheme for two-dimensional phosphopeptide map of NHE-1 identifies 5 major phosphopeptides. D, PhosphorImager analysis of the relative volume of each phosphopeptide spot. Data were normalized to spot P1 as described under "Experimental Procedures." Results are the mean ± S.D., n = 3.
Fig. 3. Incubation of 10 μM EP-1 with activated, immunoprecipitated p90RSK for 30 min produced a stoichiometric phosphorylation of EP-1 (Fig. 3D). The relatively high affinity of p90RSK for the EP-1 peptide and a stoichiometry of 1.0 suggests that serine 703 of NHE-1 is a physiological site of phosphorylation by p90RSK.

A Synthetic NHE-1 Phosphopeptide Containing Serine 703 Co-migrates with Phosphopeptide P5—To confirm that serine 703 is the amino acid phosphorylated in response to serum, we performed co-migration studies of synthetic and endogenous NHE-1 tryptic phosphopeptides. We designed a synthetic peptide that included serine 703 and the surrounding amino acid sequence of NHE-1 (EP-2, Fig. 3A). Based on this sequence, we predicted that EP-2 would generate the endogenous phosphopeptide P5 when cleaved by trypsin. To generate phosphorylated EP-2, activated p90RSK was immunoprecipitated from rat VSMC stimulated with 200 nM angiotensin II for 5 min. An immune complex kinase reaction was then performed with EP-2 as substrate. The phosphorylated peptide was then cleaved with trypsin, purified by reverse phase high pressure liquid chromatography, and analyzed by mass spectrometry. Tryptic cleaved, phospho-EP-2 (tryptic pEP-2, sequence, IGpSDPLYEPK, where pS is phosphoserine) was then subjected to two-dimensional peptide mapping and shown to migrate as a single spot (Fig. 4A). The electrophoretic mobility of tryptic pEP-2 was very similar to P5 of endogenous NHE-1 immunoprecipitated from PS127A cells stimulated by 20% FCS (Fig. 4B). To confirm that P5 and tryptic pEP-2 were identical, tryptic pEP-2 (sample in Fig. 4A) and tryptic endogenous NHE-1 peptides (sample in Fig. 4B) were mixed and subjected to two-dimensional peptide mapping. Tryptic pEP-2 co-migrated with endogenous phosphopeptide P5 (Fig. 4C).

To show that phosphopeptide P5 is a good substrate for p90RSK, NHE-1 was immunoprecipitated from PS127A cells, and an in vitro kinase reaction was performed with activated p90RSK. Phosphorylated endogenous NHE-1 was then trypsinized and subjected to two-dimensional peptide mapping (Fig. 4D). Only a single phosphopeptide that co-migrated with P5 and tryptic pEP-2 was observed. Thus, only serine 703 appears to be phosphorylated by p90RSK in vitro, despite the fact that NHE-1 has five potential p90RSK phosphorylation sites based on the RXS motif (RERS (56), RFTS (325), RLRS (648), RIGS (703), and RCLS (796)).

Mutation of Serine 703 Specifically Inhibits Serum Stimulation of Phosphopeptide P5—To provide additional evidence that serine 703 is phosphorylated in response to serum, we mutated serine 703 to alanine (S703A) in NHE-1. The mutated NHE-1 (NHE-1(S703A)) or wild type NHE-1 (NHE-1(WT)) proteins were then stably expressed in PS120 fibroblasts that lack all known NHE isoforms (35). In brief, PS120 cells were trans-
fected with appropriate cDNAs and selected for expression of NHE-1(S703A) or NHE-1(WT) by maintenance in neomycin. To select for clones with high levels of protein expression, acid loading and recovery was performed weekly (31). Equal expression of full-length Na\(_{\text{1}}\)/H\(_{\text{1}}\) exchanger was confirmed by immunoprecipitation and Western blot analysis with anti-NHE-1 antibody (G116) (36) for at least three different clones (not shown). Transfected PS120 cells were maintained in 0.5% FCS or stimulated with 20% FCS for 5 min. NHE-1 was then immunoprecipitated, and two-dimensional tryptic phosphopeptide mapping was performed. The phosphopeptide map of NHE-1 from PS120 expressing NHE-1(WT) maintained in 0.5% FCS (Fig. 5A) or stimulated with 20% FCS for 5 min (Fig. 5B) was similar to the map of NHE-1 from PS127A cells (Fig. 1A). However, in the NHE-1 map from PS120 cells expressing NHE-1(S703A), phosphopeptide P5 was completely absent in both cells maintained in 0.5% FCS (Fig. 5C) or stimulated 20% FCS for 5 min (Fig. 5D), consistent with serine 703 being the phosphoamino acid present in this tryptic peptide.

Serum-stimulated Na\(^+\)/H\(^+\) Exchange Is Inhibited in Cells Transfected with NHE-1(S703A)—To determine the functional consequences of mutating NHE-1 serine 703, stably transfected PS120 cells were analyzed for serum-stimulated Na\(^+\)/H\(^+\) exchange. Transfected PS120 cells were acid-loaded to an intracellular pH of 6.5 by exposure to 20 \(\mu\)M nigericin (a K\(^+\)/H\(^+\) ionophore) in the presence of 135 mM KCl (pH 6.5) as described previously (33). In non-transfected PS120 cells, which lack functional NHE-1, there was a very slow recovery from the acid load that was not altered by treatment with 20% FCS (Fig. 6A). In PS120 cells transfected with NHE-1(WT) there was a rapid pH\(_{\text{i}}\) recovery to a resting pH\(_{\text{i}}\) of 7.0 within 300 s (Fig. 6B). When these cells were treated with 20% FCS, there was an increase in both the rate and extent of intracellular alkalinization (Fig. 6B). Both the pH\(_{\text{i}}\) recovery and serum-stimulated recovery were due to expression of NHE-1 as there was 100% inhibi-

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**Fig. 3.** Kinetics and stoichiometry of phosphorylation of the synthetic NHE-1 peptide (EP-1) mediated by p90\(^{RSK}\). A, the amino acid sequence of the NHE-1-predicted phosphorylation site by p90\(^{RSK}\) in vitro and the sequence of synthetic peptides (EP-1 and EP-2) and tryptic phospho-EP-2. B, rate of incorporation of phosphate into EP-1 as a function of EP-1 concentration. Active p90\(^{RSK}\) was immunoprecipitated from angiotensin II-stimulated vascular smooth muscle cells and an in vitro kinase assay performed with the indicated concentrations of EP-1. \(^{32}\)P incorporation was determined by liquid scintillation spectrometry (mean \(\pm\) S.D., \(n = 3\)). C, Lineweaver-Burk plot of data in A. D, stoichiometry of phosphorylation of 10 \(\mu\)M EP-1 by p90\(^{RSK}\).
Fig. 6. NHE-1(S703A)-transfected cells exhibit decreased growth factor-stimulated pH recovery from an acid load. PS120 cells were stably transfected with NHE-1(8703A) or NHE-1(WT) cDNAs. Intracellular pH was lowered by exposure to nigericin and KCl, pH 6.5. The external buffer was changed from the KCl solution, pH 6.5, to TBSS, pH 7.4, at time 0. A, PS120 cells transfected with pcDNA3.1 alone exhibited minimal pH recovery. B, PS120 cells expressing NHE-1(WT) exhibited rapid pH recovery. The rate and extent of pH recovery were stimulated in cells pretreated with 20% FCS for 5 min. C, PS120 cells expressing NHE-1(S703A) exhibited rapid pH recovery. The rate and extent of pH recovery were not changed by pretreatment with 20% FCS. The rate of proton flux (JH+) during acid recovery was calculated for PS120 cells transfected with NHE-1(WT) or NHE-1(S703A) (D). At pH 6.8, only PS120 cells expressing NHE-1(WT) showed increased JH+ (E) (mean ± S.D., n = 3); PS120 cells were stably transfected with NHE-1(S703A) or NHE-1(WT) cDNAs. Cells were incubated in TBSS, pH 7.4, until a stable pH was obtained. Cells were stimulated with 1 unit/ml α-thrombin (F) at the time 0. The magnitude of intracellular alkalinization was compared at 300 s after agonist stimulation (G). The difference between PS120 cells stably transfected with NHE-1(S703A) or NHE-1(WT) cDNAs was significant for α-thrombin (mean ± S.D., n = 3, p < 0.05).

The ability of serum to stimulate Na+/H+ exchange has previously been shown to be due to an increase in affinity for intracellular H+ (37, 38). Kinetic analysis of the H+ flux showed that there was no significant difference in kinetic properties of NHE-1(WT) compared with NHE-1(S703A) when stimulated by acidification alone with maximal JH+ values of 2.07 and 2.42 mmol of H+ /min/liter cells at pH 6.70 and pH 6.80 values (pH i at half-maximal JH+) of 6.82 and 6.84, respectively (Fig. 6D). In cells transfected with NHE-1(WT), 20% FCS increased the maximal JH+ significantly to 4.51 mmol of H+ /min/liter cells at pH 6.70 and shifted the pH50, to 6.96, consistent with a decrease in Km for H+, as shown by a shift to the right in the JH+ versus pH plot (37, 38). In contrast, cells transfected with NHE-1(S703A) had a maximal JH+ of 2.68 mmol of H+/ min/liter cells at pH 6.70 and pH50 of 6.85 which differed significantly from NHE-1(WT) (p < 0.01, n = 4, Fig. 6D).

α-Thrombin-stimulated Na+/H+ Exchange Is Inhibited in Cells Transfected with NHE-1(S703A)—To confirm that the effect of the S703A mutation is a general property for multiple growth factors, transfected PS120 cells were also stimulated with α-thrombin (1 units/ml) which has been reported to increase NHE-1 phosphorylation and stimulate NHE-1 in transfected PS120 cells (12). In these experiments no acid loading was performed which obviated any nonspecific effects of nigericin. In addition, stimulation of Na+/H+ exchange under these conditions can only be due to a change in affinity for intracellular H+, as intracellular pH should be at equilibrium. In PS120 cells transfected with NHE-1(WT), α-thrombin increased pHi by 0.16 ± 0.04 pH units (n = 3) (Fig. 6, F–G). In contrast, a much smaller intracellular alkalinization was elicited by α-thrombin in cells transfected with NHE-1(S703A) (0.05 ± 0.02 pH units, n = 3, p < 0.05 versus NHE-1(WT)). Based on these findings we conclude that phosphorylation of serine 703 is essential for the growth factor-mediated shift in H+ affinity of NHE-1.

Transfection of p90RSK cDNA Modulates Phosphorylation of NHE-1 Serine 703—To show that p90RSK phosphorylates NHE-1 serine 703 in vivo, we transiently co-transfected 293 cells with cDNAs for NHE(WT) and either wild type p90RSK (WT-RSK) or a kinase-inactive p90RSK that functions as a dominant-negative (DN-RSK) (25, 39). We utilized 293 cells (which express endogenous human NHE-1) for these experiments because they exhibited much higher transfection efficiency than PS127A cells. Transfected 293 cells were serum-deprived for 24 h, labeled with 32P-orthophosphate, and then stimulated with 20% FCS for 5 min. Labeled NHE-1(WT) was immunoprecipitated and analyzed by two-dimensional trypsin phosphopeptide mapping. To compare changes in phosphorylation of phosphopeptides P4 and P5 among different experiments, the intensity of each spot was normalized to the fold increase observed in response to 20% FCS in LacZ-transfected cells using the calculations described under “Experimental Procedures” (Fig. 7, A and B). In 293 cells transfected with WT-
RSK, phosphopeptide P5 phosphorylation was increased by 34 ± 5% compared with LacZ-transfected cells, whereas there was no significant change in P4 phosphorylation (Fig. 7, C and E). In contrast, in 293 cells transfected with DN-RSK there was a significant inhibition of P5 phosphorylation (−24 ± 6%) compared with LacZ-transfected cells (Fig. 7, D and E). There was no significant change in P4 phosphorylation in DN-RSK-transfected cells. These data indicate that p90RSK is a serum-stimulated kinase that specifically phosphorylates P5 (and hence serine 703) in cultured cells.

DISCUSSION

In the present study we report that p90RSK is a serum-activated NHE-1 kinase, and we identify human NHE-1 serine 703 as an amino acid whose phosphorylation is required for serum stimulation of Na+/H+ exchange. Serine 703 is a part of a consensus p90RSK phosphorylation motif (RXXS) in NHE-1, similar to the p90RSK consensus motifs reported previously (34). Characterization of p90RSK as an NHE-1 kinase represents a new function for this serine-threonine kinase and may have important implications for control of cell proliferation and diseases such as hypertension. Stimulation of Na+/H+ exchange is a universal feature of growth factor receptor activation and is required for cell proliferation (35, 37, 38, 40). Increased Na+/H+ exchange is a common feature in both hypertensive animal models and patients with essential hypertension (5). Because increased NHE-1 activity and phosphorylation are present in the SHR genetic model of hypertension (6, 10), it is possible that growth factor stimulation of p90RSK activity is important in the pathogenesis of hypertension.

Phosphorylation of NHE-1—In fibroblasts, Sardet et al. (40, 41) showed that the phosphorylation state of a transfected Na+/H+ exchanger correlated temporally with growth factor-stimulated intracellular alkalization. Epidermal growth factor, α-thrombin, and okadaic acid phosphorylated a set of common serine sites. However, the role of phosphorylation in regulating NHE-1 activity has been controversial, since phosphorylation is not required for activation by certain mediators such as muscarinic agonists and hyperosmotic stress (28, 42). In fact, truncation of the NHE-1 cytoplasmic tail distal to amino acid 635 abolishes the major sites of phosphorylation and greatly reduces (but does not eliminate) growth factor activation of NHE-1 (43). These results suggest that key serine residues carboxyl to amino acid 635 are required for NHE-1 activation but that other regulatory mechanisms such as binding of associated proteins (44, 45) are also important.

Two-dimensional Tryptic Phosphopeptide Mapping of NHE-1 Demonstrates Two Serum-stimulated Peptides—Two-dimensional tryptic phosphopeptide maps of phosphorylated NHE-1 have been reported by several investigators (10, 12, 15, 30, 41) to show five peptides, at least one of which is increased by serum. It is not possible to compare directly the present study with previous reports because the experimental protocols differ. We observed two peptides, P4 and P5, that were significantly stimulated by serum (Fig. 1). Phosphopeptide P4 is not an insufficiently cleaved P5 peptide since mutation of serine 703 to alanine removed peptide P5 without effect on P4 (Fig. 5). Thus, our results suggest that there are at least two serum-stimulated phosphopeptides in NHE-1. p90RSK Phosphorylates Ser-703 in Vitro—Several results indicate that serine 703 is a physiologically important site for p90RSK-mediated phosphorylation of NHE-1. Sequence analysis demonstrated a consensus p90RSK phosphorylation motif, RXXS (amino acids 700–703 in NHE-1), similar to the p90RSK consensus motif reported previously (34). By using this sequence we designed a synthetic peptide (EP-1) and characterized the kinetics and stoichiometry of phosphorylation by p90RSK (Fig. 3). The calculated Kₘ of 8 μM and stoichiometry 1.0 for EP-1 phosphorylation compares favorably to the Kₘ of 5 μM reported for p90RSK phosphorylation of the 40 S ribosomal subunit S6 protein (46). In addition, serine 703 was the only site in immunoprecipitated endogenous NHE-1 phosphorylated by purified activated p90RSK (Fig. 4D), despite the fact that NHE-1 has several RXXS motifs. We were unable to determine which isoform of p90RSK mediates EP-1 phosphorylation because the available antibodies immunoprecipitate all isoforms of p90RSK (23).

Serine 703 Is the Serum-stimulated Phosphorylation Site in NHE-1—We showed that the serum-stimulated tryptic peptide P5 comigrated with a synthetic phosphorylated peptide (EP-2) designed to mimic endogenous P5 (Fig. 4C). The “tryptic cleaved phospho-EP-2” was confirmed by mass spectroscopic analysis to have the sequence IgpSDPLYEPK. These results indicate that P5 also has the sequence IGpSDPLYEPK and that there is only a single phosphoserine in the endogenous P5 (Ser-703). These conclusions are further supported by the complete loss of P5 phosphorylation when serine 703 was mutated to alanine. It should be noted that P4 is a separate serum-stimulated phosphopeptide whose sequence and physiologic importance remains to be determined.

Growth Factor Stimulation of NHE-1 Phosphorylation Involves a MEK1-ERK1/2-p90RSK Pathway—This report defines NHE-1 as a new substrate for p90RSK and establishes a novel role for p90RSK in cell function. Identification of p90RSK is not surprising because several recent studies suggest that the
MEK-ERK1/2 pathway is important in activation of NHE-1. These studies include inhibition of serum-stimulated NHE-1 activity in fibroblasts by dominant-negative ERK1/2 (16, 19) and inhibition of NHE-1 activation in platelets with PD98059 (20). Mitogen stimulation of ERK1/2 via MEK1 is required for phosphorylation and activation of p90RSK (47, 48) since PD98059 prevents activation of p90RSK (47). Indeed, MAP kinases directly phosphorylate p90RSK (22) at MAP kinase sites (47). We found that 100 μM PD98059 inhibited p90RSK activity and NHE-1 phosphorylation (specifically peptide P5) by 60%. Since serum-stimulated phosphorylation of NHE-1 occurs at a p90RSK consensus sequence (RXXS) and this phosphorylation is inhibited by PD98059, these data suggest that p90RSK directly phosphorylates NHE-1 in the cell.

Protein kinases reported to phosphorylate NHE-1 include Ca2+-calmodulin-dependent kinases (13), protein kinase C (14), p160 Rho-associated kinase (p160ROCK) (15), ERK1/2 (1, 16), c-Jun amino-terminal kinase (17), and p38 (17). None of these kinases appear to phosphorylate directly NHE-1 based on the kinetics and stoichiometry of phosphorylation. For example, we found that recombinant NHE-1 was a substrate for ERK1/2, p38, and p90RSK, but the stoichiometry of phosphorylation observed for p90RSK was 20-fold greater than the other kinases (17). Bianchini et al. (19) also failed to show significant kinase activity of ERK1/2 toward recombinant NHE-1. Tominaga et al. (15) showed recently that p160ROCK phosphorylated recombinant NHE-1, but two peptides appeared in the two-dimensional tryptic phosphopeptide maps suggesting nonspecific phosphorylation. Finally, we showed that transfection of WT-RSK inhibited phosphorylation of P5 specifically (Fig. 7). Thus, multiple characteristics of NHE-1 phosphorylation and p90RSK function indicate that the MEK1-ERK1/2-p90RSK pathway is involved in serum-stimulated phosphorylation of NHE-1.

Functional Analysis of NHE-1-S703A Mutant—It has been proposed that modulation of the exchanger by phosphorylation involves the pH sensor, because the serum-mediated increase in NHE-1 affinity for H+ was abolished in the S703A mutant (Fig. 6). Our data disagree with some conclusions of previous studies that used deletion-mutation analysis of NHE-1. It was suggested that a negative regulatory domain was lost by deletions carboxyl to amino acid 698 (12, 31). Serine residues essential for activity were proposed to exist within the domain encompassed by amino acids 567–636, as deletions carboxyl to amino acid 636 decreased growth factor activation by ~50% (12, 31, 49). A possible explanation to reconcile these data is that alterations in structure that regulate function are more likely to occur with deletion analysis. Thus we believe that phosphorylation of serine 703 is one of the critical regulatory mechanisms for NHE-1 function.

How might phosphorylation of serine 703 augment NHE-1 activity? One mechanism may involve interactions with the calmodulin binding domain (residues 567–635) which acts as a negative regulatory domain and is critical for growth factor regulation of NHE-1 (43). Recently, a calcineurin B homologous protein termed CHP was shown to interact with NHE-1 by binding to this domain (44). Overexpression of CHP inhibited serum- and GTPase-stimulated NHE-1 activity suggesting that CHP may be released upon growth factor stimulation. It is possible that phosphorylation of serine 703 may change the structure of NHE-1 in this part of the molecule through backbone folding and non-covalent interactions to enhance calmodulin and/or CHP release. In addition to serine 703, other serines are also phosphorylated in response to serum (e.g. serine(s) in the P4 peptide), suggesting that cooperative interactions among two or more serines may be required to activate NHE-1. Another mechanism may involve interactions with regulatory proteins. NHE-RF, a functional regulatory protein that binds to the carboxyl tail of NHE-3 via a PDZ domain, was discovered (50, 51). Although NHE-1 does not have a PDZ domain, Goss et al. (45) identified a 24-kDa protein that co-immunoprecipitated with NHE-1. These data suggest that future efforts to identify proteins that interact specifically with phosphorylated NHE-1 may provide further insight into mechanisms by which the Na+/H+ exchanger is regulated.
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