Identification of Sites Required for Down-regulation of Na\(^{+}/H^{+}\) Exchanger NHE3 Activity by cAMP-dependent Protein Kinase

PHOSPHORYLATION-DEPENDENT AND -INDEPENDENT MECHANISMS

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We recently identified a region within the cytoplasmic C-terminal tail of the Na\(^{+}/H^{+}\) exchanger NHE3 isof orm (residues 579 to 684) which is essential for inhibition of transport activity by cAMP-dependent protein kinase (PKA) (Cabado, A. G., Yu, F. H., Kapus, A., Gergely, L., Grinstein, S., and Orlowski, J. (1996) J. Biol. Chem. 271, 3590–3599). To further define determinants of PKA regulation, six serine residues located in potential recognition sequences for PKA within, or adjacent to, this region (positions 552, 605, 634, 661, 690, and 691) were altered either independently or in various combinations using site-directed mutagenesis. Wild type and mutant NHE3s tagged with the influenza virus hemagglutinin epitope were stably expressed in exchanger-deficient Chinese hamster ovary cells (AP-1) for functional studies. Of the individual mutations examined, only substitutions at Ser\(^{605}\) or Ser\(^{634}\) affected sensitivity to forskolin, an activator of adenylyl cyclase, although partial inhibition of NHE3 activity by forskolin remained. By contrast, simultaneous mutation of both these serines completely abolished cAMP-mediated inhibition of NHE3 without greatly affecting basal transport activity. Two-dimensional analysis of tryptic digests of immunoprecipitated NHE3 labeled in vivo with \(^{32}\)Porthophosphate revealed several phosphopeptides under basal conditions. Phosphorylation was increased approximately 3-fold in one of these peptides following forskolin treatment, and this change was eliminated by mutation of residue Ser\(^{605}\). Thus, phosphorylation of Ser\(^{605}\) is essential for cAMP-mediated inhibition of NHE3. In addition, Ser\(^{634}\) is also required for the effect of cAMP, even though this residue does not become phosphorylated upon activation of PKA.

Na\(^{+}/H^{+}\) exchange (NHE) activity is thought to be essential for pH homeostasis, transepithelial ion and water transport, and cell volume regulation (1–3), and may also play a role in cell proliferation (2) and adhesion (4, 5). In mammalian cells, six NHE isofoms (NHE1 to NHE6) have been identified (6). NHE1 is the “housekeeping” isoform present in nearly all cells. The other isofoms have a more restricted tissue distribution. Of these, NHE3 is confined to the apical (i.e. brush-border) membranes of some epithelial cells of the renal and gastrointestinal tracts, where it participates in transepithelial Na\(^{+}\) and HCO\(_3\)\(^{-}\) absorption. Bacterial enterotoxins and neurotransmitters that elevate intracellular cAMP (cAMP) levels are known to inhibit NaCl absorption which, together with a concomitant stimulation of net Cl\(^{-}\) secretion, promotes diarrhea (7–10). The effects of these toxins and transmitters on Cl\(^{-}\) secretion have been studied extensively (7). However, little is known about the molecular mechanisms by which cAMP inhibits the absorptive process.

Recent studies of epithelial cells expressing NHE3 as well as exchange-deficient Chinese hamster ovary cells (AP-1) transfected with NHE3 showed that this isoform is inhibited following activation of cAMP-dependent protein kinase (PKA) (11, 12). Functional analysis of NHE chimeras and C-terminally truncated mutants revealed that the cytoplasmic domain of NHE3, particularly the region between amino acids 579 and 684, is essential for the cAMP response (13). This region contains several potential sites for phosphorylation by PKA. Furthermore, recent work by Moe et al. (14) showed that acute inhibition by PKA is accompanied by an increase in the total phosphorylation of NHE3 expressed in AP-1 cells. However, it is unclear whether direct phosphorylation of NHE3 by PKA mediates the inhibition of this transporter. Other studies suggest that an ancillary protein is required for cAMP-induced inhibition and may itself be the relevant PKA substrate (15, 16).

To further investigate the mechanism of NHE3 regulation by PKA, a series of point mutations were made to selectively eliminate possible phosphorylation sites recognized by PKA using site-directed mutagenesis. The data identify two sites in NHE3 that confer responsiveness to cAMP.

EXPERIMENTAL PROCEDURES

Materials and Solutions—Carrier-free \(^{22}\)NaCl (range of specific activity, 300–900 mCi/mg; concentration, ~5 mCi/ml) was obtained from NEN Life Science Products (Mandel Scientific, Guelph, ON). Amiloride, ouabain, forskolin, deoxyglucose, pepstatin A, phenylmethylsulfonyl fluoride, and iodoacetamide were purchased from Sigma. Nigericin and amide gel electrophoresis; PBS, phosphate-buffered saline; pH, intracellular pH; HA, hemagglutinin; WT, wild type; TKA-1, tyrosine kinase activator protein 1.
2', 7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) acetoxymethylester were from Molecular Probes, Inc. (Eugene, OR). Enhanced chemiluminescence reagents were from Amersham Corp. Monoclonal mouse antibodies to the influenza virus hemagglutinin epitope (HA) were from BAILCO (clone 16B12; Berkeley, CA). α-Minimal essential medium (αMEM), antibiotics and sera were purchased from BioWhittaker. trypsin-EDTA were purchased from Life Technologies (Burlington, Ontario). Cell culture dishes and flasks were purchased from Becton-Dickinson and/or Corning (Montréal, Québec). All other chemicals and reagents used in these experiments were purchased from BDH Inc. (St. Laurent, Québec) or Fisher Scientific and were of the highest grade available.

Bicarbonate-free medium RPMI 1640 was buffered to varying pH values (between 7.45 and 5.85) in the presence of the K⁺/H⁺-ionophore nigericin (5 μM). Calibration curves were constructed by plotting the extracellular pH, which is assumed to be identical to the internal pH, against the corresponding fluorescence ratio (23).

In Vivo Labeling of Cells with [32P]Orthophosphate—Stably transfected cells were grown to 60–70% confluence on 10-cm dishes and serum-starved for at least 16 h. Cells were then labeled in vivo for 4 h with 4 ml of phosphate-free α-minimal essential medium containing 1 mCi of [32P]orthophosphate. At the end of the incubation period, cells were washed twice with PBS, and treated with or without 10 μg forskolin in Na⁺-rich medium for 10 min. Cells were then washed 3 times with ice-cold PBS and processed immediately for immunoprecipitation.

Immunoprecipitation—The confluent layer of cells labeled with [32P]orthophosphate as described above were scraped into a hypotonic solution containing 10 mm HEPES, 18 mm potassium acetate, 1 mm EDTA, 5 μg/ml trypsin, and 1 μg/ml leupeptin. The cell suspension (1 5 × 10⁶ cells) was treated with 10 mM sodium-methylsulfonyl fluoride, and 1 mM iodoacetamide, pH 7.2. Cells were centrifuged by precipitation for 5 min at 12,000 × g in an Eppendorf centrifuge, and resuspended in 1 ml of 50% RIPA buffer containing 150 mm NaCl, 20 mm Tris-HCl, 0.1% SDS, 0.5% deoxycholate, and 1% Triton X-100, mixed by pipetting, and agitated on a rotating rocking at 4 °C for 1 h. After centrifugation at 12,000 × g for 30 min to remove insoluble cellular debris, the supernatants were cleared by ultracentrifugation with 7 mS Clinical grade agarose beads by rocking for 2 h to reduce nonspecific binding. The beads were spun down and the supernatants incubated overnight with 4 μl of the anti-HA monoclonal antibody. Protein G-Sepharose beads were added and allowed to rock for a further 2 h, and then washed eight times with RIPA buffer. Immunoprecipitated proteins were eluted by boiling in 50 μl of Laemmli sample buffer. For each sample, an aliquot of 40 μl was analyzed by electrophoresis on a 7.5% SDS-polyacrylamide gel, electrophoretically transferred to an Immobilon membrane (Millipore, Bedford, MA), and analyzed by autoradiography. The phosphoprotein corresponding to NHE3 was further characterized by two-dimensional trypsin phosphopeptide mapping.

Two-dimensional Tryptic Phosphopeptide Mapping—The band corresponding to NHE3, which was identified by autoradiography and its gel mobility in relation to prestained low molecular weight markers (Bio-Rad), was excised and soaked in 0.5% polyvinylpyrrolidone (PVP-360; Sigma) in 100 mM acetic acid at 37 °C for 30 min to block nonspecific absorption of trypsin, washed briefly in water, and then with fresh 50 mM ammonium bicarbonate. Digestion was achieved by incubating the piece of membrane in 200 μl of 50 mM ammonium bicarbonate with 10 μg of trypsin overnight at 37 °C, followed by addition of 10 μg of trypsin and a second 2-h incubation at 37 °C. The digested proteins were lyophilized, washed once with 100 μl of water and re-lyophilized, resuspended in 10 μl of H₂O, and spotted at the origin on thin-layer cellulose plates. Phosphopeptides were separated by electrophoresis in formic acid/glacial acetic acid/H₂O (25:78:979) (pH 1.9, 1000 V, 40 min) in the first dimension, and then by ascending chromatography.
phosphorylation in glacial acetic acid/pyridine/H2O (10:1:189) for 20 h in the second dimension. The incorporation of radioactive 32P in the phosphopeptides was quantified using a PhosphorImager (Molecular Dynamics).

**Immunoblotting**—To assess the expression of the gene products, immunoblotting was carried out using monoclonal antibodies against HA. Samples were subjected to electrophoresis in 7.5% polyacrylamide gels and transferred to Immobilon membranes as described above. Mouse monoclonal antibodies against HA were used at a 1:10,000 dilution. The second antibody, goat anti-mouse coupled to horseradish peroxidase, was applied at a 1:5000 dilution. Immunoreactive bands were visualized using enhanced chemiluminescence (Amersham Corp.).

**Immunofluorescence**—To assess the distribution of the expressed gene products, immunofluorescence microscopy was performed. AP-1 cells stably expressing HA epitope-tagged wild type and mutant NHE3s were plated onto glass coverslips and grown to 80% confluence. They were washed three times with PBS, and then fixed for 15 min at room temperature using 4% paraformaldehyde in PBS. Following fixation, the cells were washed 3–4 times with PBS and incubated with 100 mM glycine in PBS for 15 min. The coverslips were washed again and the cells permeabilized with 0.1% Triton X-100 in PBS for 20 min at room temperature. Cells were blocked with 5% donkey serum for 1 h, then incubated with a mouse anti-HA antibody (1:5000 dilution) for 1 h. After this period, the coverslips were washed 4–5 times with PBS and then incubated for 1 h with a donkey anti-mouse antibody conjugated with Cy3 (1:1000 dilution). The coverslips were finally washed and mounted onto glass slides using DAKO Fluorescent mounting medium (DAKO Corp., Carpinteria, CA).

**Statistical Analyses**—Data were analyzed by one-way ANOVA to test for statistically significant differences between control and treated cells.

## RESULTS

**Phosphorylation of NHE3: Basal and PKA-dependent Regulation**—The molecular basis for transducing the inhibitory effect of PKA on NHE3 activity is equivocal, with some evidence supporting direct phosphorylation (14) and other data suggesting indirect regulation through phosphorylation of ancillary PKA-sensitive regulatory protein(s) (15, 16).

To distinguish these proposed mechanisms, rat wild type NHE3 tagged at its C-terminal end with the influenza virus hemagglutinin HA epitope (NHE3HA) was stably expressed in AP-1 cells devoid of endogenous NHE activity. The state of phosphorylation of NHE3HA was evaluated under basal conditions, and following acute exposure to 10 μM forskolin, a direct stimulator of adenylate cyclase. This latter treatment increases the intracellular cAMP (cAMP) concentration severalfold and significantly depresses NHE3 activity via a PKA-dependent pathway in AP-1 cells (24). Cells expressing NHE3HA were pulse-labeled in vivo with [32P]orthophosphate for a 4-h period. NHE3HA was then immunoprecipitated using a monoclonal anti-HA antibody and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by blotting to Immobilon membranes and autoradiography. As shown in Fig. 1A, NHE3HA migrates largely as a phosphoprotein band of ~85 kDa under basal conditions, but small amounts of a dimerized form are often discernible. However, contrary to a previous study (14) using very similar experimental conditions, no apparent change in the degree of total phosphorylation was observed. Western blot analysis (Fig. 1B) of the same membrane filter confirmed that equal amounts of NHE3HA were loaded on the gels, thereby eliminating the possibility that any potential differences in phosphorylation could have been masked by unequal efficiency of immunoprecipitation in basal and cAMP-stimulated cells. In three independent experiments, the average phosphorylation of 32P-labeled NHE3HA normalized for the amount of NHE3HA immunoprecipitated showed no significant difference between control and forskolin-treated cells (Fig. 1C). In summary, inhibition of NHE3 transport activity by PKA is not associated with observable changes in total phosphorylation of the protein when evaluated by one-dimensional SDS-PAGE. At present, we cannot account for the difference between our results and those of Moe et al. (14).

While the above analysis did not reveal an apparent change in the total phosphorylation of NHE3HA in response to PKA, it was conceivable that several phosphorylation sites exist which might mask minor changes in phosphorylation of one or a few sites in response to PKA. To examine this possibility, two-dimensional phosphopeptide mapping was performed on immunoprecipitated NHE3HA. As shown in Fig. 2, A–C, seven major phosphopeptides (P1–P7) were identified together with additional minor spots (a, b, and c). The major phosphopeptides always appeared in different sets of experiments, but the minor spots were not consistently observed. To calculate the levels of phosphorylation, the radioactivity associated with each major spot was counted and then expressed relative to that of P1, which was normalized to a value of 100 (Fig. 2D). Based on the analysis of three separate experiments, only one phosphopeptide (P5) showed a significant increase (3-fold; p < 0.01) in its incorporation of 32P following activation of PKA. Taken together with previous phosphoamino acid analysis showing that only serine residues are phosphorylated in NHE3 (14), these findings strongly suggest that PKA phosphorylates at least 1 serine residue in NHE3 during down-regulation of its activity.

**Identification of Serine Residues in NHE3 That Confer Responsiveness to PKA**—We previously identified a small segment in the cytoplasmic C-terminal region of NHE3 (residues 579–684) that is essential for the cAMP response (13). Within...
and adjacent to this region are several serine residues located in potential recognition sequences for PKA. Of these, six serines at positions 552, 605, 634, 661, 690, and 691 (Fig. 3A) were considered good candidates for further structural studies. These residues were substituted either singly or in various combinations by site-directed mutagenesis and the resulting mutants assayed for altered regulation by PKA.

Na+/H+ exchange activity was initially assessed by measuring rates of amiloride-inhibitable 22Na+ influx at pH 6.3 in cells pretreated with or without 10 μM forskolin. As illustrated in Fig. 3B, activation of adenylate cyclase reduced wild type (WT) NHE3 activity by nearly 50%, consistent with previous observations (11, 13). The data also indicate that the regulatory properties of NHE3 are not influenced by the presence of the C-terminal HA-tag. The behavior of one triple mutant, S661A/S690G/S691G, was indistinguishable from WT, whereas the other triple mutant, S552A/S605G/S634A, was completely unresponsive to activated PKA. Further dissection of these latter residues showed that the S552A mutant mirrored wild type responsiveness to PKA, whereas single mutations at either Ser605 (S605G) or Ser634 (S634A) significantly reduced (p < 0.01) but did not eliminate, inhibition of 22Na+ influx by forskolin. Simultaneous substitutions at both positions completely abolished cAMP-mediated inhibition of NHE3 activity (p < 0.01) without appreciably affecting basal transport activity (Table I), indicating additive interactions between these two sites.

Additional analyses (Fig. 3C) showed that a conservative substitution of Ser605 with Thr maintained the wild type behavior, whereas replacement with Arg caused a significant reduction in the response to PKA. Preservation of the cAMP-inhibitory response in the S605T mutant is consistent with this modified site (i.e. RRRT605(R)) retaining an optimal recognition motif (R/R/KX(S/T)) for PKA (25). In contrast, substitution of Ser634 with Thr, Arg, or Asn resulted in either a partial or a complete loss of responsiveness to CAMP. Again, these substitutions did not appreciably affect basal transport activity (Table I). One caveat to comparing basal transport rates using stably transfected cells is that they are affected by secondary events unrelated to protein structure, such as protein expression levels which are influenced by the number of cDNA copies incorporated into the genome and the sites of integration. When compared with Ser605, Ser634 is in a much weaker context (KHLYS634RH) for recognition by PKA.

FIG. 2. Two-dimensional phosphopeptide mapping of wild type NHE3. A and B, AP-1 cells expressing NHE3 were labeled in vivo with [32P]orthophosphate and then treated with or without 10 μM forskolin, as indicated. NHE3 was then immunoprecipitated with the mouse monoclonal anti-HA antibody, separated by SDS-PAGE, and transferred to Immobilon membranes. The membranes were examined by autoradiography and the phosphorylated protein band corresponding to NHE3 was cut out and digested with trypsin. The tryptic phosphopeptides were spotted onto thin-layer cellulose plates and analyzed in the first dimension by electrophoresis and in the second dimension by thin-layer ascending chromatography. The two-dimensional phosphopeptide maps were analyzed using a PhosphorImager. C, illustration of the location of the phosphopeptides. Peptides P1–P7 were consistently observed. Peptides a–c were seen occasionally. D, [32P]Phosphate content of each phosphopeptide, expressed as a percentage of the counts relative to those for phosphopeptide P1 in control (untreated) cells. The data represent the means ± S.E. of four to six experiments, each performed in quadruplicate. Significant difference from control values was determined by one-way ANOVA and is indicated by an asterisk (p < 0.01).

FIG. 3. Effect of forskolin on the activities of wild type and mutant NHE3: 22Na+ uptake determinations. AP-1 cells were transfected with HA-tagged wild type NHE3 (WT) or with various NHE3 mutants altered at serine residues located in potential recognition sequences for PKA (positions 552, 605, 634, 661, 690, and 691). The mutations were generated either independently or in various combinations. A, illustration of the C-terminal region of NHE3 previously shown to be required for cAMP-dependent inhibition of transport activity and potential recognition sequences for PKA. B and C, rates of amiloride-inhibitable 22Na+ influx (a measure of NHE3 activity) at pH 6.3 were measured in transfected cells pretreated for 15 min with or without 10 μM forskolin. The data are calculated as the percentage of forskolin-mediated inhibition of transport activity. Results are presented as the mean ± S.E. of four to six experiments, each performed in quadruplicate. Significant difference from control values was determined by one-way ANOVA and is indicated by an asterisk (p < 0.01).
While the above results supported the structural importance of these specific serine residues (S605G and S634A) in conferring responsiveness to PKA, it was conceivable that the serine mutations might disrupt PKA regulation nonspecifically by altering the production and/or processing of NHE3 HA. To assess this possibility, Western blot analysis and immunofluorescence microscopy were performed. As shown in Fig. 4A, the immunoreactive band corresponding to WT or various NHE3 HA mutants was sharp and had a molecular mass of ~85 kDa. The level of total protein expression was also similar among the various transfectants, with the exceptions of the S552A/S634A and S605G/S634A mutants, which were about 1.5-fold and half, respectively, that of WT. However, these differences are unlikely to be related to the mutations since the triple mutant S552A/S605G/S634A was produced at levels similar to WT. More likely, the observed difference in stable expression of the various constructs reflects other parameters mentioned previously, such as location of the site of cDNA integration into the genome and the number of copies incorporated per cell. Thus, on a gross level, steady-state protein accumulation does not appear to be greatly affected by these serine mutations.

The cellular distribution of wild type NHE3 HA and two PKA regulation-defective mutants (S605G and S552A/S605G/S634A) was examined by immunofluorescence microscopy. As shown in Fig. 4B, the majority of immunoreactive WT protein accumulated in an intracellular compartment near the nucleus with lesser amounts in a punctate distribution throughout the cell and in the plasma membrane. The intracellular distribution of NHE3 HA was recently found to correspond to that of recycling endosomes and is quite distinct from that of heterologously expressed NHE1 HA which is located predominantly at the cell surface. Likewise, the distributions of the S605G and S552A/S605G/S634A mutants in the resting state were indistinguishable from WT. These data suggest that production and membrane targeting of NHE3 were not noticeably affected by these serine mutations in the cytoplasmic domain, at least within the resolution of this technique.

The inhibitory effect of PKA on amiloride-inhibitable 22Na uptake by cells containing WT or mutant NHE3s was validated fluorimetrically by measuring the relative rates of Na+-dependent pHi recovery in acid-loaded cells pretreated with or without forskolin. As shown in Fig. 5, in acidified cells expressing the S552A mutant, the rate of Na+-dependent alkaliniza-

| NHE3 constructs          | Rates of transport (nmol/min/mg protein) |
|-------------------------|-----------------------------------------|
| WT                      | 3.33 ± 0.31                             |
| S616A/S690G/S661G       | 1.61 ± 0.42                             |
| S552A/S605G/S633A       | 3.31 ± 0.40                             |
| S634A                   | 2.20 ± 0.26                             |
| S605G                   | 5.20 ± 0.74                             |
| S605G/S634A             | 4.07 ± 0.90                             |
| S552A                   | 1.43 ± 0.20                             |
| S605G                   | 3.32 ± 0.27                             |
| S605G                   | 2.88 ± 0.28                             |
| S605G                   | 2.93 ± 0.81                             |
| S634A                   | 6.04 ± 0.76                             |
| S634A                   | 4.08 ± 0.56                             |
| S634A                   | 4.17 ± 0.98                             |
| S634A                   | 2.19 ± 0.95                             |

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| Table I | Basal rates of amiloride-inhibitable 22Na uptake at pH 6.3 in AP-1 cells stably expressing wild type (WT) and mutant NHE3s |
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icked in Chinese hamster ovary (AP-1) cells transfected with NHE3 (11, 13), thereby making them a useful model for investigating the structural basis of cAMP regulation of this transporter. The present results support a dual mechanism for regulation of NHE3 by PKA that involves both phosphorylation-dependent and -independent events.

A recent study using this cell line showed that acute inhibition of NHE3 by PKA is associated with a 3-fold increase in total phosphorylation of the exchanger as assessed by one-dimensional SDS-PAGE (14), although whether phosphorylation was the cause of the reduction in transport activity was not addressed. Using very similar experimental conditions, we were unable to detect significant increases in the apparent total amount of phosphorylation of NHE3 following a 10-min incubation with 10 μM forskolin, despite the fact that this treatment elevated cAMP levels severalfold and reproducibly inhibited transport activity. The reason for this discrepancy between studies remains obscure. The most likely explanation for our inability to detect a change in total phosphoprotein content is that phosphorylation of one or a few residues by PKA may have been masked by constitutive phosphorylation of other sites, or by concomitant decreases in phosphorylation of other sites. Nonetheless, the present results reopened the possibility that inhibition of NHE3 activity by PKA might be indirect, as suggested by investigators that advocate the essential role of ancillary proteins such as NHE-RF (16). To address this, phosphopeptides generated by tryptic digestion of in vivo 32P-labeled NHE3 were analyzed in two dimensions by electrophoresis and thin-layer chromatography. This approach revealed the existence of multiple phosphorylation sites on NHE3 in unstimulated cells. Importantly, only one of seven tryptic phosphopeptides showed increased labeling (3-fold) upon cellular activation of PKA. Thus, the data are most consistent with cAMP-mediated phosphorylation at a single site. Together with the high background phosphorylation of NHE3 in resting cells, this may account for the lack of a detectable change in total phosphorylation of the exchanger when determined by one-dimensional SDS-PAGE.

In a previous study, we identified a small segment in the cytoplasmic C-terminal region of NHE3 (residues 579–684) which is essential for the cAMP response (13). Therefore, this region was targeted for mutagenesis in an effort to identify the relevant phosphorylation site(s), and to ascertain whether there is a causal relationship between elevated PKA phosphorylation and decreased transport activity of NHE3. Six serine

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**Fig. 5.** Effect of forskolin on the activities of wild type and mutant NHE3s: pH determinations. AP-1 cells transfected with WT and mutant NHE3s were loaded with BCECF in bicarbonate-free medium. The cells were acid-loaded by an ammonium prepulse and treated with (solid circles) or without (open circles) 10 μM forskolin during the final 10 min of incubation with the dye. Next, the pH recovery induced by the addition of Na+ was measured fluorimetrically. Results in each panel are the mean ± S.E. of at least 12 cells from three separate experiments.

**Fig. 6.** Two-dimensional phosphopeptide mapping of NHE3 mutants. AP-1 cells expressing single mutants of NHE3 (S552A, S605G, or S634A) were labeled in vivo with [32P]orthophosphate and then treated with or without 10 μM forskolin for 10 min. The NHE3 mutants were then immunoprecipitated with a mouse anti-HA antibody. The immunoprecipitated protein was separated by SDS-PAGE and then transferred to Immobilon membranes. The membranes were exposed for autoradiography, and the phosphoprotein band was cut out and digested with trypsin. The tryptic phosphopeptides were spotted onto thin-layer cellulose plates and analyzed in the first dimension by electrophoresis and in the second dimension by thin-layer ascending chromatography. The two-dimensional phosphopeptide maps were analyzed using a PhosphorImager. Radiograms are representative of three experiments of each type.

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icked in Chinese hamster ovary (AP-1) cells transfected with NHE3 (11, 13), thereby making them a useful model for investigating the structural basis of cAMP regulation of this transporter. The present results support a dual mechanism for regulation of NHE3 by PKA that involves both phosphorylation-dependent and -independent events.

A recent study using this cell line showed that acute inhibition of NHE3 by PKA is associated with a 3-fold increase in total phosphorylation of the exchanger as assessed by one-dimensional SDS-PAGE (14), although whether phosphorylation was the cause of the reduction in transport activity was not addressed. Using very similar experimental conditions, we were unable to detect significant increases in the apparent total amount of phosphorylation of NHE3 following a 10-min incubation with 10 μM forskolin, despite the fact that this treatment elevated cAMP levels severalfold and reproducibly inhibited transport activity. The reason for this discrepancy between studies remains obscure. The most likely explanation for our inability to detect a change in total phosphoprotein content is that phosphorylation of one or a few residues by PKA may have been masked by constitutive phosphorylation of other sites, or by concomitant decreases in phosphorylation of other sites. Nonetheless, the present results reopened the possibility that inhibition of NHE3 activity by PKA might be indirect, as suggested by investigators that advocate the essential role of ancillary proteins such as NHE-RF (16). To address this, phosphopeptides generated by tryptic digestion of in vivo 32P-labeled NHE3 were analyzed in two dimensions by electrophoresis and thin-layer chromatography. This approach revealed the existence of multiple phosphorylation sites on NHE3 in unstimulated cells. Importantly, only one of seven tryptic phosphopeptides showed increased labeling (3-fold) upon cellular activation of PKA. Thus, the data are most consistent with cAMP-mediated phosphorylation at a single site. Together with the high background phosphorylation of NHE3 in resting cells, this may account for the lack of a detectable change in total phosphorylation of the exchanger when determined by one-dimensional SDS-PAGE.

In a previous study, we identified a small segment in the cytoplasmic C-terminal region of NHE3 (residues 579–684) which is essential for the cAMP response (13). Therefore, this region was targeted for mutagenesis in an effort to identify the relevant phosphorylation site(s), and to ascertain whether there is a causal relationship between elevated PKA phosphorylation and decreased transport activity of NHE3. Six serine
residues located in potential recognition sequences for PKA within, and adjacent to, amino acids 579 and 684 (positions 552, 605, 634, 661, 690, and 691), were altered independently and in various combinations. Only serine residues were targeted since a previous study indicated that all phosphoamino acids in NHE3 are phosphoseresines (14). The data revealed that only Ser605 and Ser634 were essential for PKA-mediated inhibition of NHE3, thereby highlighting the functional importance of these specific residues.

Proposed Role of Ser605—The present analysis revealed that PKA-mediated phosphorylation of NHE3 was only affected by mutations at Ser605. This site is in a context (i.e. Arg-Arg-Arg-Ser605-Ile-Arg) that conforms to a canonical consensus sequence for phosphorylation by PKA (R/R/Kx(S/T+)x). Replacement of Ser605 with Thr preserved the responsiveness to forskolin, consistent with this site being a target for PKA. On the other hand, other substitutions (i.e. S605G or S605R) only diminished the inhibition of NHE3 activity by forskolin by approximately half, even though forskolin-induced phosphorylation was abolished (as shown for the S605G mutant). The results suggest that while direct phosphorylation of Ser605 is a significant mechanism responsible for inhibition of NHE3 by PKA, an additional phosphorylation-independent process must also be required to attain the full inhibitory effect of PKA.

A similar hypothesis regarding phosphorylation-independent regulation was proposed earlier by Weinman and colleagues (15, 16) who used cellular fractionation and reconstitution experiments to identify an ancillary phosphoprotein (NHE-RF) which is required for cAMP-induced inhibition of the rabbit renal proximal tubule Na+/H+ exchanger (i.e. NHE3). Phosphorylation of this ancillary protein may confer regulation in addition to that provided by direct phosphorylation of NHE3. Moreover, recent studies using the yeast two-hybrid system have identified a second regulatory protein, E3KARP (NHE3 kinase A regulatory protein), which interacts with NHE3 (27). This protein was identified earlier by K. Seedorf and A. Ullrich as TKA-1 (tyrosine kinase activator protein 1; GenBank accession number Z50150) based on its ability to bind and activate the platelet-derived growth factor receptor. NHE-RF and TKA-1/E3KARP share ~50% identity in their amino acid sequences. Both of these proteins contain two highly conserved regions that share significant identity (~50%) to PDZ domains in adaptor proteins which recognize the motif (S/T)XV in target proteins and have been implicated in the clustering of ion channels or junctional proteins at the plasma membrane (28, 29). Transfection of either regulatory protein into PS120 fibroblasts stably expressing NHE3 partially restored cAMP-induced inhibition (~25–30%) of NHE3 (27). This cell line, which seemingly lacks endogenous expression of both NHE-RF and TKA-1/E3KARP, does not appear to exhibit cAMP-mediated inhibition of NHE3 even though it contains a functional adenylate cyclase-PKA pathway. It is not clear why in these studies PKA-mediated phosphorylation of NHE3 itself failed to cause a partial inhibitory response, as would be expected from the analysis of the S605G mutant. However, contrary to the data of Yun et al. (27), recent preliminary experiments3 showed a significant forskolin-induced decrease (~50–60%) in rat NHE3 activity when stably expressed in PS120 cells, consistent with results obtained using AP-1 cells. Reasons for the discrepancy between studies are unclear. Nevertheless, the roles of direct phosphorylation of NHE3 and of an ancillary regulator are not mutually exclusive and may in fact be complementary.

Proposed Role of Ser634—The sites on NHE3 where these ancillary regulatory proteins interact is unknown. It is tempting to speculate that Ser634, which is immediately downstream of the phosphorylation site identified in this study, may form part of, or interact spatially with, the putative regulator-binding site. Mutation of this residue (S634A) reduced the inhibitory effect of PKA by at least half without altering the phosphorylation of tryptic peptides of NHE3 within detectable limits. The ability of Ser605 to be phosphorylated during forskolin stimulation was also not affected by mutagenesis of Ser634. Nevertheless, the Ser at position 634 is critical since even conservative substitutions (Ser → Thr) diminished the PKA effect. Only after simultaneous substitutions at both positions (S605G/S634A) was PKA-mediated inhibition of NHE3 completely abolished. The results are consistent with these sites acting in an independent, but additive, manner. Hints of such a dual mechanism were obtained in our earlier studies of NHE3 deletion mutants (13). We found that PKA responsiveness was lost in two stages: complete inhibition was intact following C-terminal deletion of NHE3 at position 684, whereas only partial inhibition was seen in a mutant truncated at position 638. Mutants lacking the carboxyl tail distal to position 579 were completely unresponsive to forskolin. Taken together, these results suggest multiple regulatory sites. Ser634 is in a very weak consensus sequence (Lys-His-Leu-Tyr-Ser634; Arg-His) for recognition by PKA. The surrounding sequence also does not conform to a PDZ-binding motif ([S/T]XV), so it is unclear whether Ser634 can directly bind to the putative PDZ domains of NHE-RF or TKA-1/E3KARP. The structural integrity of the region encompassing Ser634 may, however, be necessary to maintain NHE3 in a conformation capable of binding NHE-RF or TKA-1/E3KARP.

One additional mechanism that must be considered when accounting for the distinctive regulation of NHE3 by cAMP pertains to its predominant accumulation in recycling endosomes.2 This observation is consistent with previous reports of a relatively amiloride-insensitive form of the Na+/H+ exchanger (presumably NHE3) present in endosomal-enriched vesicles of the renal cortex (30–32). It raises the possibility that regulation of its activity may be mediated by recruitment of transporters to and from the plasma membrane. A similar paradigm has been proposed for the regulation of epithelial water and H+ transport by aquaporin-2 and H+-ATPases, respectively (33). In accordance with this notion, Mircheff and colleagues (34, 35) have reported a shift in sedimentation density of the vesicular compartment expressing NHE3 following treatment of renal cells with parathyroid hormone or after induction of hypertension. Moreover, stimulation of NHE3 by epidermal growth factor was found to be sensitive towortmannin (36), a specific inhibitor of phosphatidylinositol 3-kinase which has been implicated in various stages of endosomal vesicular trafficking (37). Taken together, these observations are consistent with an intracellular redistribution of the exchangers following treatment with agents that modify the rate of transport.

In this regard, it is particularly noteworthy that the region between Ser605 and Ser634 in NHE3 contains the sequence (Tyr629-Lys-His-Leu), which is compatible with a tyrosine-based endosomal targeting motif (YYYY; where Y is a polar residue and O represents a hydrophobic residue, usually leucine) (38, 39). While the functional importance of the YHKL sequence remains to be demonstrated, it is tempting to speculate that phosphorylation of Ser605 by PKA could induce a conformational change in this region that enhances endocytosis of NHE3. This process may be facilitated by interactions with the PKA-sensitive NHE-RF or TKA-1/E3KARP, leading to a net reduction in the number of transporters (i.e. Vmax) on the

3 E. Szabó and J. Orlowski, unpublished observations.
cell surface. In this scenario, mutation of Ser634, which is immediately adjacent to the endocytic sequence, may disrupt a component of the endocytic process that is influenced by PKA. In addition, phosphorylation by PKA may affect other kinetic properties of NHE3 since the transporters remaining on the plasma membrane also exhibit a reduction in their apparent affinity for intracellular protons (13).

In conclusion, NHE3 stably transfected in AP-1 cells is a phosphoprotein under basal conditions. Activation of PKA reduces NHE3 activity by a dual mechanism involving both direct phosphorylation and indirect events, such as interactions with PKA-sensitive regulatory proteins. A more precise definition of the mechanisms responsible for regulating NHE3 activity is likely to involve intracellular vesicular trafficking.

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