THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 45, Issue of November 9, pp. 41559–41565, 2001
© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

Phosphorylation and Cell Cycle-dependent Regulation of Na^+/H^+ Exchanger Regulatory Factor-1 by Cdc2 Kinase*

Received for publication, July 20, 2001, and in revised form, August 20, 2001
Published, JBC Papers in Press, August 30, 2001, DOI 10.1074/jbc.M106859200

Junqi He‡, Anthony G. Lau‡, Michael B. Yaffe§, and Randy A. Hall‡¶

From the ‡Department of Pharmacology, Rollins Research Center, Emory University School of Medicine, Atlanta, Georgia 30322 and the §Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Na^+/H^+ exchanger regulatory factor (NHERF)-1 is a PDZ domain-containing adaptor protein known to bind to various receptors, channels, cytoskeletal elements, and cytoplasmic signaling proteins. We report here that the phosphorylation state of NHERF-1 is profoundly regulated by the cell cycle: NHERF-1 in HeLa cells is hyperphosphorylated in mitosis phase and much less phosphorylated at other points of the cell cycle. This mitosis phase-dependent phosphorylation of NHERF-1 could be blocked by roscovitine, consistent with phosphorylation by cyclin-dependent kinases. *In vitro* studies with purified NHERF-1 fusion proteins and purified kinases revealed that NHERF-1 was robustly phosphorylated by the cyclin-dependent kinase Cdc2. In contrast, the NHERF-1 relative NHERF-2 was not phosphorylated at all by Cdc2. NHERF-1 possesses two serines (Ser^{279} and Ser^{301}) that conform to the SP*X*(K/R) motif preferred for phosphorylation by Cdc2. Mutation of either of these serines reduced Cdc2-mediated phosphorylation of NHERF-1 *in vitro*, and mutation of both residues together completely abolished Cdc2-mediated phosphorylation. When the S279A/S301A NHERF-1 mutant was expressed in cells, it failed to exhibit the mitosis phase-dependent phosphorylation observed with wild-type NHERF-1. Mutation of both Ser^{279} and Ser^{301} to aspartate, to mimic Cdc2 phosphorylation of NHERF-1, resulted in a NHERF-1 mutant with a markedly impaired ability to oligomerize *in vitro*. Similarly, endogenous NHERF-1 from lysates of mitosis phase HeLa cells exhibited a markedly reduced ability to oligomerize relative to endogenous NHERF-1 from lysates of interphase HeLa cells. Mitosis phase NHERF-1 furthermore exhibited the ability to associate with Pin1, a WW domain-containing peptidylprolyl isomerase that does not detectably bind to NHERF-1 in interphase lysates. The association of NHERF-1 with Pin1 facilitated dephosphorylation of NHERF-1, as shown in experiments in which cellular Pin1 activity was blocked by the selective inhibitor juglone. These data reveal that cellular NHERF-1 is phosphorylated during mitosis phase by Cdc2 at Ser^{279} and Ser^{301} and that this phosphorylation regulates NHERF-1 oligomerization and association with Pin1.

Many cell-surface receptors and channels are directly coupled to intracellular signaling proteins through interactions with scaffolding or adaptor proteins. Most adaptor proteins contain a number of modular interaction domains to facilitate the associations of multiple target proteins (1). One common example of such a module is the PDZ[1] domain. PDZ domains can associate with specific carboxyl-terminal motifs on target proteins, and many PDZ domains can also oligomerize with other PDZ domains to enhance the scaffolding or adaptor function of PDZ domain-containing proteins (2).

Na^+/H^+ exchanger regulatory factor-1 (NHERF-1) is a PDZ domain-containing protein that was first identified as a cofactor necessary for regulation of Na^+/H^+ exchanger-3 (NHE3) function by phosphorylation (3). Subsequently, NHERF-1 was identified as a binding partner for the actin-associated ERM proteins (ezrin, radixin, moesin, and merlin) and thus is also known as the ERM-binding phosphoprotein of 50 kDa (EBP50) (4, 5). A close relative of NHERF-1 has been identified and is known variously as SIP-1 (6), E3KARP (7), and NHERF-2 (8). NHERF-1 and NHERF-2 share 52% identity and a conserved domain architecture, possessing two PDZ domains followed by a unique carboxyl-terminal region that mediates association with the ERM proteins (9).

The PDZ domains of NHERF-1 and NHERF-2 specifically recognize the carboxyl-terminal motif (S/T)*X*L (8, 10, 11) and are known to associate with a small number of transmembrane proteins other than Na^+/H^+ exchangers, including the β_2-adrenergic receptor (8, 10, 12), the cystic fibrosis transmembrane conductance regulator (8, 11, 13–15), the sodium bicarbonate cotransporter (16), the platelet-derived growth factor receptor (17), the B1 subunit of the H^+-ATPase (18), the TRP4 calcium channel (19), and the type IIa sodium phosphate cotransporter (20). The NHERF proteins also bind via their PDZ domains to a variety of intracellular signaling proteins, including SRY (6), phospholipase Cβ1/2 (19), phospholipase Cβ3 (21), GRK6A (22), YAP65 (23), TAZ (24), and Epi64 (25). Moreover, it has recently been shown that the PDZ domains of NHERF-1 can homo-oligomerize (17, 26–28) and also hetero-oligomerize with the PDZ domains of NHERF-2 (28).

Since the NHERF proteins have such a large number of binding partners, a primary question of interest is how these interactions might be regulated in a cellular environment. NHERF-1 is known to be phosphorylated in cells (4, 22, 29–31),

* This work was supported by National Institutes of Health Grants RO1-HL64713 (to R. A. H.) and RO1-GM60594 (to M. B. Y.) and by a faculty development award from the Pharmaceutical Research and Manufacturers of America Foundation (to R. A. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Pharmacology, Emory University School of Medicine, 5113 Rollins Research Center, 1510 Clifton Rd., Atlanta, GA 30322. Tel.: 404-727-3699; Fax: 404-727-0365; E-mail: rhall@pharm.emory.edu.

[1] The abbreviations used are: PDZ, PSD-95/Discs-large/ZO-1 homology; NHERF, Na^+/H^+ exchanger regulatory factor; NHE3, Na^+/H^+ exchanger-3; ERM, ezrin, radixin, moesin, and merlin; EBP50, ezrin-binding phosphoprotein of 50 kDa; SIP-1, SRY-interacting protein-1; E3KARP, NHE3 kinase A regulatory protein; GRK, G protein-coupled receptor kinase; GST, glutathione *S*-transferase; MAGI, membrane-associated guanylate kinase-like protein with an inverted domain structure; HEK, human embryonic kidney; PAGE, polyacrylamide gel electrophoresis.

and Ser[289] has been identified as a major site of phosphorylation (22, 29, 31). Although Ser[289] of NHERF-1 can be phosphorylated *in vitro* by several kinases, the kinase responsible for phosphorylation of this site in most cells seems to be GRK6A, which avidly associates with the NHERF PDZ domains through its carboxyl terminus and efficiently phosphorylates NHERF-1 at Ser[289] (22). Mutation of Ser[289] to alanine, however, does not block all cellular phosphorylation of NHERF-1 (22, 29), suggesting that there are other unidentified sites of phosphorylation on NHERF-1. Here, we report that the NHERF-1 phosphorylation state varies profoundly with the cell cycle. More specifically, we report that NHERF-1 is phosphorylated by the cyclin-dependent kinase Cdc2 at Ser[279] and Ser[301] and that this phosphorylation regulates NHERF-1 oligomerization and association with the peptidylprolyl isomerase Pin1.

EXPERIMENTAL PROCEDURES

Preparation of Plasmids and Fusion Proteins—Hexahistidine- and S-tagged NHERF fusion proteins, for both full-length NHERF-1 and NHERF-2 and for various NHERF truncations, were created via insertion of polymerase chain reaction products derived from a rabbit NHERF cDNA into pET-30A (Novagen), followed by expression and purification as previously described (8). NHERF-1 point mutants S279A, S301A, S279A/S301A, S279D, S301D, and S279D/S301D were created by polymerase chain reaction amplification from the native rabbit NHERF cDNA using mutant sequence oligonucleotides; the point mutations at positions 279 and/or 301 were confirmed by ABI sequencing. The NHERF-1 point mutants were inserted into both the pET-30A and pGEX-4T1 vectors for expression as hexahistidine/S-tagged and GST-tagged fusion proteins, respectively. GST-Pin1 fusion protein was prepared using the human Pin1 cDNA inserted into pGEX-4T1 as previously described (32). GST-MAGI-WW was prepared via polymerase chain reaction from a DNA template kindly supplied by Jonathan Wood and Christopher Ross (Johns Hopkins University). The DNA sequence encoding for amino acids 1–355 of human MAGI-2 (encoding both of the MAGI-2 WW domains) was amplified and inserted into the vector pGEX-4T1 for expression as a GST-tagged fusion protein.

Cell Culture and Transfection—All tissue culture media and related reagents were purchased from Life Technologies, Inc. HEK-293, HeLa, and ES2 cells were maintained in complete medium (Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and 1% penicillin/streptomycin) in a 5% CO_2 incubator at 37 °C. To express FLAG- and/or hemagglutinin-tagged versions of NHERF-1 or NHERF-1 mutants, 2 μg of total DNA (in a modified version of the vector pBK-CMV from Stratagene) was mixed with LipofectAMINE (15 μl) and Plus reagent (20 μl) (Life Technologies, Inc.) and added to 5 ml of complete medium in 10-cm tissue cultures plates containing cells at ~50–80% confluency. Following a 4-h incubation, the medium was removed, and 10 ml of fresh complete medium was added. After another 12–16 h, the medium was changed again, and the cells were harvested 24 h later.

HeLa cells were synchronized at the G_1/S phase boundary using the thymidine-aphidicolin double block method as previously described (33). Cells in S phase were obtained 3 h after release from the G_1/S phase block. Mitosis and G_2 phase cells were obtained following a 14–18 h treatment with nocodazole (50 ng/ml; Sigma) as previously described (34). The cell cycle state of the cells harvested following these various protocols was confirmed using flow cytometry.

Western Blotting—Samples (5 μg/lane) were run on 4–20% SDS-polyacrylamide gels (Novex) for 1 h at 150 V and then transferred to nitrocellulose. The blots were blocked in blot buffer (2% nonfat dry milk, 0.1% Tween 20, 50 mM NaCl, and 10 mM HEPES, pH 7.4) for at least 30 min and then incubated with primary antibody in blot buffer for 1 h at room temperature. The blots were washed three times with 10 ml of blot buffer and incubated for 1 h at room temperature with a horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech) in blot buffer. Finally, the blots were washed three more times with 10 ml of blot buffer and visualized via enzyme-linked chemiluminescence using the ECL kit from Amersham Pharmacia Biotech.

Kinase Assays—*In vitro* phosphorylation experiments were carried out at room temperature in a final volume of 25 μl with 10 mM HEPES, pH 7.4, 10 mM $MgCl_2$, and 2 mM EDTA. In this buffer, purified fusion proteins, either His-NHERF-1 (2 μg) or a mutant version of His-NHERF-1, were incubated with purified recombinant human Cdc2/cyclin B (10 units; Calbiochem). Reactions were initiated via the addition of [γ-^{32}P]ATP (1 μCi; Amersham Pharmacia Biotech) and were allowed to proceed for 30 min at room temperature before being stopped with SDS-PAGE sample buffer. Phosphorylated samples were run on 4–20% SDS-polyacrylamide gels, fixed, dried, and exposed to film. The extent of phosphorylation was quantified by scanning of films and densitometric analysis of bands.

Plate Assays—GST-NHERF-1 and GST alone were expressed as described above and purified on glutathione-agarose beads (Sigma). The GST fusion proteins were then eluted from the beads using 250 mM reduced glutathione (Sigma) in harvest buffer (10 mM HEPES, 50 mM NaCl, 5 mM EDTA, and protease inhibitor mixture (Roche Molecular Biochemicals)). The excess glutathione was removed via multiple rounds of concentration and dilution with fresh harvest buffer. The eluted GST fusion proteins were used to coat the wells of 96-well high affinity binding dishes (Fisher). Each well was coated overnight at 4 °C with 1 μg of fusion protein in a 100-μl final volume of harvest buffer. After the coating solution was removed, the wells were washed twice with harvest buffer and then blocked with blot buffer for 30 min at room temperature. His/S-tagged NHERF-1 fusion proteins diluted in blot buffer were added to the precoated wells at the indicated concentrations and incubated for 1 h at room temperature. The wells were then washed five times with 200 μl of blot buffer. For detection, S protein-alkaline phosphatase conjugate (Novagen) diluted 1:4000 in blot buffer was added to the wells (100 μl/well) and incubated for 1 h at room temperature. The wells were then washed four times with blot buffer and twice with harvest buffer. Following the final wash, each well was incubated with 100 μl of alkaline phosphatase substrate (Bio-Rad), and the absorbance at 405 nm was determined using a Thermomax microplate reader (Molecular Devices) to provide quantitative values for the amount of His-NHERF fusion protein bound to each well. Specific binding is defined as the binding to wells coated with a GST-NHERF-1 fusion protein minus the binding to wells coated with GST alone on the same plate.

Pull-down Assays—For GST-NHERF-1 pull-down assays of purified fusion proteins, GST-NHERF-1 fusion proteins and GST alone were purified on glutathione-agarose beads as described above. Aliquots of the fusion protein/bead mixture (50 μl) in 1.5-ml microcentrifuge tubes were blocked for 30 min with 1 ml of 3% bovine serum albumin blocking buffer (also containing 10 mM HEPES, 50 mM NaCl, and 0.1% Tween 20). Equal concentrations of various His-tagged fusion proteins were then incubated with the beads in 1 ml of 3% bovine serum albumin blocking buffer at 4 °C with end-over-end rotation for 1 h. The beads were washed four times with ice-cold 3% bovine serum albumin blocking buffer and twice with harvest buffer. The proteins were eluted from the beads with 1× SDS-PAGE sample buffer, resolved via SDS-PAGE, and transferred to nitrocellulose. The His-tagged fusion proteins were detected via Western blotting with S protein-horseradish peroxidase conjugate (1:4000 dilution; Novagen), and bands were visualized via chemiluminescence as described above.

For GST, GST-NHERF-1, GST-Pin1, and GST-MAGI-WW pull-down assays of cell lysates, cells were harvested from 10-cm plates and lysed in 500 μl of ice-cold lysis buffer (10 mM HEPES, 50 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, and protease inhibitor mixture). The lysate was solubilized via end-over-end rotation at 4 °C for 30 min and clarified via centrifugation at 14,000 rpm for 15 min. A small fraction of the supernatant was taken at this point and incubated with SDS-PAGE sample buffer to examine expression of proteins in the whole cell extract. The remaining supernatant was incubated with 50 μl of GST, GST-NHERF-1, or GST-Pin1 fusion protein adsorbed onto glutathione-agarose. These incubations were carried out for 2 h with end-over-end rotation at 4 °C. After five washes with 1.0 ml of lysis buffer, the pulled-down proteins were eluted from the beads with 1× SDS-PAGE sample buffer, resolved by SDS-PAGE, and subjected to Western blot analysis with an anti-NHERF-1 (anti-EBP50) antibody (BD Transduction Laboratories). In some cases, the amount of NHERF-1 remaining in the lysate was also quantified using the anti-NHERF-1 antibody to assess the percentage of total cellular NHERF-1 that had been pulled down by the fusion protein.

Immunoprecipitation—Cells were harvested and lysed in 500 μl of ice-cold lysis buffer. The lysate was solubilized via end-over-end rotation at 4 °C for 30 min and clarified via centrifugation at 14,000 rpm for 15 min. A small fraction of the supernatant was taken at this point and incubated with SDS-PAGE sample buffer to examine expression of proteins in the whole cell extract. The remaining supernatant was incubated with 10 μl of anti-Cdc2 antibody (Oncogene Research Products) and 30 μl of protein A/G-agarose (Calbiochem) for 2 h with end-over-end rotation at 4 °C. After five washes with 1.0 ml of lysis buffer,

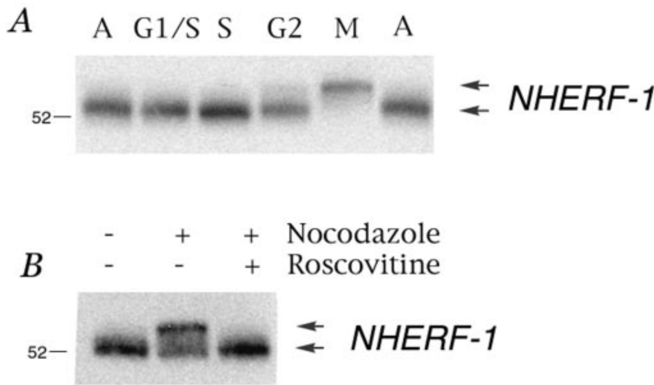

Fig. 1. **NHERF-1 exhibits a cell cycle-dependent mobility shift upon SDS-PAGE.** *A*, NHERF-1 mobility shift at mitosis phase. Lysates of asynchronous HeLa cells (*A lanes*) or HeLa cells synchronized at G_1/S phase (*G1/S lane*), S phase (*S lane*), G_2 phase (*G2 lane*), and mitosis phase (*M lane*) were separated on 12% SDS-polyacrylamide gels and transferred to nitrocellulose. The blots were then probed with the anti-NHERF-1 antibody. Almost all NHERF-1 protein shifted to a larger apparent size (~4 kDa larger) during mitosis phase. The blot shown is representative of five independent experiments. *B*, the mitosis phase NHERF-1 mobility shift is due to phosphorylation by cyclin-dependent kinases. HeLa cells were arrested in mitosis phase with nocodazole (50 ng/ml, 14–18 h) with or without roscovitine (20 nM), a selective inhibitor of cyclin-dependent kinases. Roscovitine completely blocked the NHERF-1 SDS-PAGE mobility shift that is normally observed during mitosis phase. This blot is representative of three independent experiments. Molecular mass standards (in kilodaltons) are shown on the left.

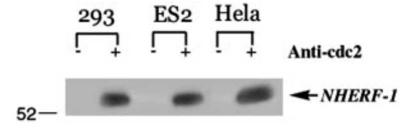

Fig. 2. **Cdc2 associates with cellular NHERF-1.** Cell lysates prepared from three different cell types (HEK-293, ES2, and HeLa) were incubated with protein A/G-agarose in the absence (−) or presence (+) of the anti-Cdc2 antibody to immunoprecipitate Cdc2. The immunoprecipitates were fractionated on 4–20% SDS-polyacrylamide gels for analysis by Western blotting with the anti-NHERF-1 antibody. Co-immunoprecipitation of endogenous NHERF-1 with endogenous Cdc2 was observed in all three cell types examined. This blot is representative of three independent experiments.

the immunoprecipitated proteins were eluted from the beads with 1× SDS-PAGE sample buffer, resolved by SDS-PAGE, and subjected to Western blot analysis with the anti-NHERF-1 (anti-EBP50) antibody.

RESULTS

To examine if the phosphorylation state of NHERF-1 might be regulated during the cell cycle, HeLa cells were synchronized and harvested at different cell cycle time points. Previous studies have shown that phosphorylation of NHERF-1 can significantly increase the apparent size of the NHERF-1 polypeptide on SDS-polyacrylamide gels (4, 22, 29–31). Thus, NHERF-1 phosphorylation was assessed in these initial studies via Western blotting with the anti-NHERF-1 antibody. As shown in Fig. 1*A*, NHERF-1 from mitosis phase cells exhibited an ~4-kDa increase in apparent size upon SDS-PAGE relative to NHERF-1 from cells in any other stage of the cell cycle. This mitosis phase-dependent shift in NHERF-1 mobility upon SDS-PAGE was completely blocked by treatment of mitosis phase cells with roscovitine, a selective inhibitor of cyclin-dependent kinase activity (Fig. 1*B*). These findings reveal that endogenous NHERF-1 is phosphorylated by cyclin-dependent kinases during mitosis phase in HeLa cells.

Cyclin-dependent kinases typically bind to their substrates with high affinity (35). Thus, we next examined whether we could detect a physical complex in cells between NHERF-1 and the cyclin-dependent kinase Cdc2. Cell lysates were prepared from three different cell types (HEK-293, ES2, and HeLa), and endogenous Cdc2 was immunoprecipitated from each lysate using the anti-Cdc2 antibody. Anti-NHERF-1 Western blots of the immunoprecipitates revealed robust co-immunoprecipitation of endogenous NHERF-1 with Cdc2 from all three of the cell lysates examined (Fig. 2). These data demonstrate that endogenous NHERF-1 and endogenous Cdc2 physically associate in a variety of cell types.

We next performed *in vitro* phosphorylation assays to examine whether Cdc2 might be able to directly phosphorylate NHERF-1. As shown in Fig. 3*A*, incubation of purified Cdc2/cyclin B with purified His-NHERF-1 or His-NHERF-2 fusion proteins in the presence of [^{32}P]ATP resulted in specific phosphorylation of NHERF-1, but not NHERF-2. The normalized levels of the fusion proteins are shown in Fig. 3*B*. NHERF-1 contains two serine residues (Ser^{279} and Ser^{301}) that roughly conform to the consensus motif (SP*X*(R/K)) for phosphorylation by Cdc2 (35, 36). These two serines are not conserved in NHERF-2, and their relative positions within the NHERF-1 polypeptide are schematically illustrated in Fig. 3*C*. Mutation of Ser^{279} to an amino acid that cannot be phosphorylated, either alanine or aspartate, resulted in a significant decrease in Cdc2-mediated *in vitro* phosphorylation of NHERF-1 (Fig. 3, *A* and *B*, *third lanes*). Similarly, mutation of Ser^{301} also reduced Cdc2-mediated phosphorylation of NHERF-1 (Fig. 3, *A* and *B*, *fourth lanes*). Mutation of both residues together completely abolished Cdc2-mediated phosphorylation of NHERF-1 (Fig. 3, *A* and *B*, *fifth lanes*), indicating that these two serines are the primary sites at which NHERF-1 is phosphorylated by Cdc2.

In addition to preparing the NHERF-1 Ser^{279}/Ser^{301} mutants as purified fusion proteins, we also prepared these mutants for expression in mammalian cells. As shown in Fig. 4*A*, the mitosis phase-induced shift in SDS-PAGE mobility observed with wild-type NHERF-1 was completely blocked by mutation of Ser^{279} and Ser^{301} to alanine. This finding suggests that Ser^{279} and Ser^{301} are the sites of the mitosis phase-dependent NHERF-1 phosphorylation and furthermore suggests that phosphorylation of these sites induces a conformational change in NHERF-1 that alters the mobility of NHERF-1 on SDS-polyacrylamide gels. This idea is further strengthened by observations that mutation of Ser^{279} and Ser^{301} to aspartate, which should mimic phosphorylation of these residues, resulted in a NHERF-1 mutant that was shifted upward in apparent size on SDS-polyacrylamide gels (Fig. 4*B*) in a manner similar to that observed for wild-type NHERF-1 during mitosis phase.

We next sought to understand the functional significance of NHERF-1 phosphorylation at Ser^{279} and Ser^{301}. To assess whether phosphorylation of these two residues might alter NHERF-1 oligomerization, GST-NHERF-1 immobilized on beads was incubated with lysates from either interphase HeLa cells or mitosis phase HeLa cells. The amount of endogenous NHERF-1 that bound to the immobilized GST-NHERF-1 was then examined via a pull-down assay (Fig. 5*A*). These studies revealed that ~50% of endogenous NHERF-1 could be pulled down from the lysates prepared from interphase cells, but that <10% of endogenous NHERF-1 from mitosis phase cells could be pulled down. These data indicate that hyperphosphorylation of NHERF-1 in mitosis phase impairs its ability to oligomerize. Similarly, the S279D/S301D mutant of NHERF-1 expressed as a fusion protein exhibited significantly reduced oligomerization *in vitro* when analyzed both in plate binding assays (Fig. 5*B*) and in pull-down assays (Fig. 5*C*). Thus, using three independent techniques, it was found that phosphorylation of NHERF-1

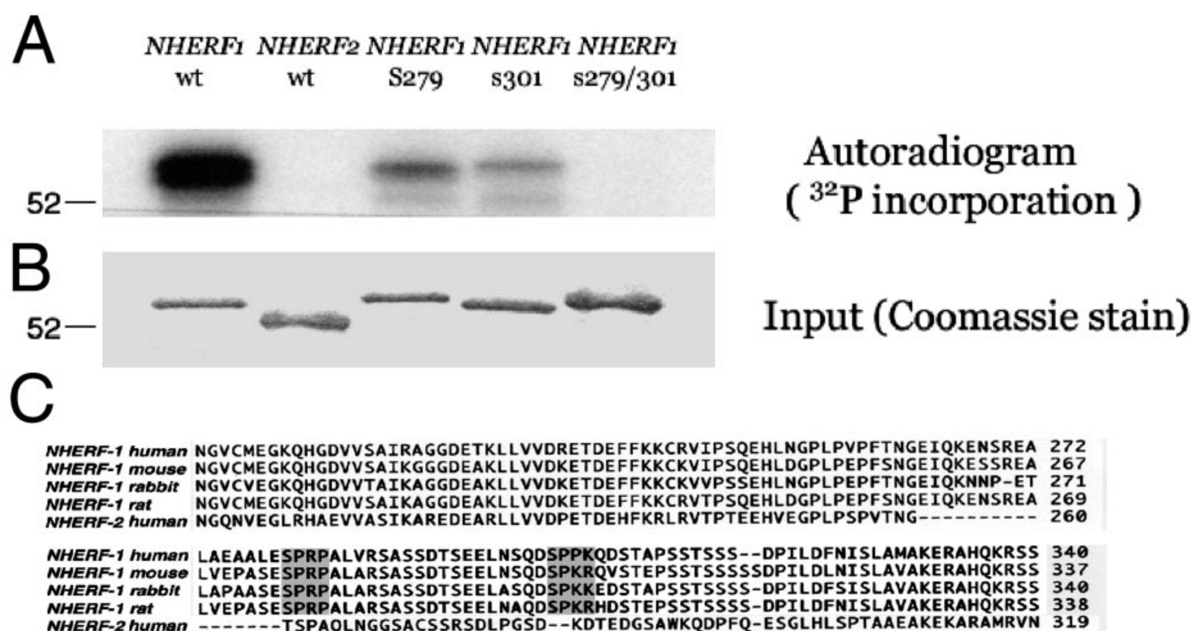

FIG. 3. **NHERF-1 is phosphorylated at Ser^{279} and Ser^{301} by purified Cdc2 kinase.** *A*, wild-type (*wt*) NHERF-1, wild-type NHERF-2, and NHERF-l mutated at Ser^{279} and Ser^{301} and doubly mutated at both Ser^{279} and Ser^{301} were expressed as His-tagged fusion proteins, and 2 μg of each was incubated with purified Cdc2/cyclin B (10 units) for 30 min in the presence of [^{32}P]ATP. The resultant phosphorylation is shown in this autoradiogram, which is representative of four independent experiments. Wild-type NHERF-1 was phosphorylated by purified Cdc2 *in vitro*, whereas wild-type NHERF-2 was not phosphorylated at all. Mutation of either Ser^{279} or Ser^{301} significantly reduced Cdc2-mediated phosphorylation, whereas mutation of both Ser^{279} and Ser^{301} together completely abolished Cdc2-mediated phosphorylation. *B*, shown is the Coomassie Blue-stained SDS-polyacrylamide gel illustrating the relative loading and size of the fusion proteins examined for Cdc2 phosphorylation in the experiment shown in *A*. *C*, shown is the alignment of the deduced amino acid sequences of human, mouse, rabbit, and rat NHERF-1 as well as human NHERF-2. Ser^{279} and Ser^{301}, which roughly conform to the consensus motif (SP*X*(R/K)) for phosphorylation by Cdc2, are conserved in the different species of NHERF-1, but are not conserved in NHERF-2.

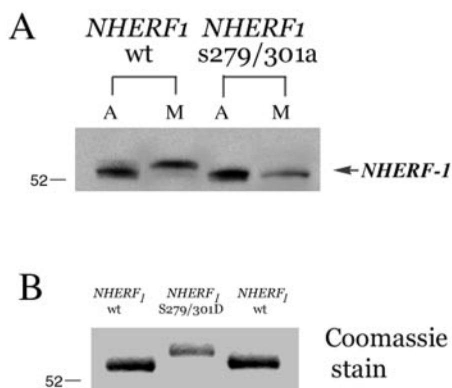

FIG. 4. **Phosphorylation of NHERF-1 during mitosis is abolished by mutation of Ser^{279} and Ser^{301} to alanine.** *A*, HeLa cells transfected with wild-type (*wt*) FLAG-NHERF-1 or FLAG-NHERF-1 carrying mutations at both Ser^{279} and Ser^{301} (*S279/301A*) were harvested 48 h post-transfection as either asynchronous (*A lanes*) or mitosis phase (*M lanes*) lysates. The lysates were then separated on 12% SDS-polyacrylamide gels and analyzed via Western blotting with an anti-FLAG antibody. Wild-type NHERF-1 was significantly phosphorylated during mitosis, as assessed by mobility shift upon SDS-PAGE. In contrast, the S279A/S301A NHERF-1 mutant did not exhibit any mobility shift in mitosis phase. This blot is representative of three independent experiments. *B*, S279D/S301D NHERF-1 fusion protein mimics mitotic NHERF-1 phosphorylation. His-tagged fusion proteins corresponding to wild-type NHERF-1 and NHERF-1 mutated to aspartate at both Ser^{279} and Ser^{301} (*S279/301D*) were run on a 12% SDS-polyacrylamide gel and examined via Coomassie Blue staining. The S279D/S301D mutation induced a mobility shift in NHERF-1 similar to that observed for mitosis phase phosphorylation of cellular NHERF-1 at Ser^{279} and Ser^{301}. Molecular mass standards (in kilodaltons) are shown on the left.

at Ser^{279} and Ser^{301} in cells or mutation of Ser^{279} and Ser^{301} to aspartate (to mimic phosphorylation) markedly reduces NHERF-1 oligomerization.

For several known Cdc2 substrates, it has been shown that phosphorylation by Cdc2 promotes physical association of the substrate protein with the peptidylprolyl isomerase Pin1 via the Pin1 WW domain (32, 37–43). We therefore examined whether NHERF-1 associates with Pin1 in a phosphorylation-dependent fashion. Lysates from HeLa cells in either interphase or mitosis phase were incubated with beads loaded with control GST, GST-Pin1 fusion protein, or another fusion protein (GST-MAGI-WW) that contains the two WW domains of the scaffolding protein MAGI-2/AIP1 (44). Pull-down assays followed by anti-NHERF-1 Western blotting revealed robust association of GST-Pin1 with NHERF-1 from mitosis phase lysates, but not from interphase lysates (Fig. 6). No NHERF-1 was detectable in any of the control GST pull-down samples or in any of the GST-MAGI-WW pull-down samples. These data indicate that NHERF-1 specifically associates with Pin1, but only when NHERF-1 is hyperphosphorylated by cyclin-dependent kinases in mitosis phase.

It has recently been shown that Pin1 can, in some cases, alter the rate of dephosphorylation of its binding partners (42, 45). We therefore examined the dephosphorylation of NHERF-1 by harvesting synchronized HeLa cells at a number of time points following mitosis phase. As shown in Fig. 7*A*, the SDS-PAGE mobility of NHERF-1 was reduced in a time-dependent fashion following mitosis phase. This reduction in the apparent size of NHERF-1 most likely reflects dephosphorylation of NHERF-1 by protein phosphatase-1 and/or protein phosphatase-2A since the effect could be blocked by the phosphatase inhibitors okadaic acid and calyculin A (Fig. 7*B*). This dephosphorylation of NHERF-1 could be blocked also by juglone, an inhibitor of Pin1 prolyl isomerase activity (Fig. 7*C*). Taken together with the GST-Pin1 pull-down experiments, these data indicate that Pin1 associates with hyperphosphorylated NHERF-1 during mitosis phase and regulates NHERF-1 dephosphorylation.

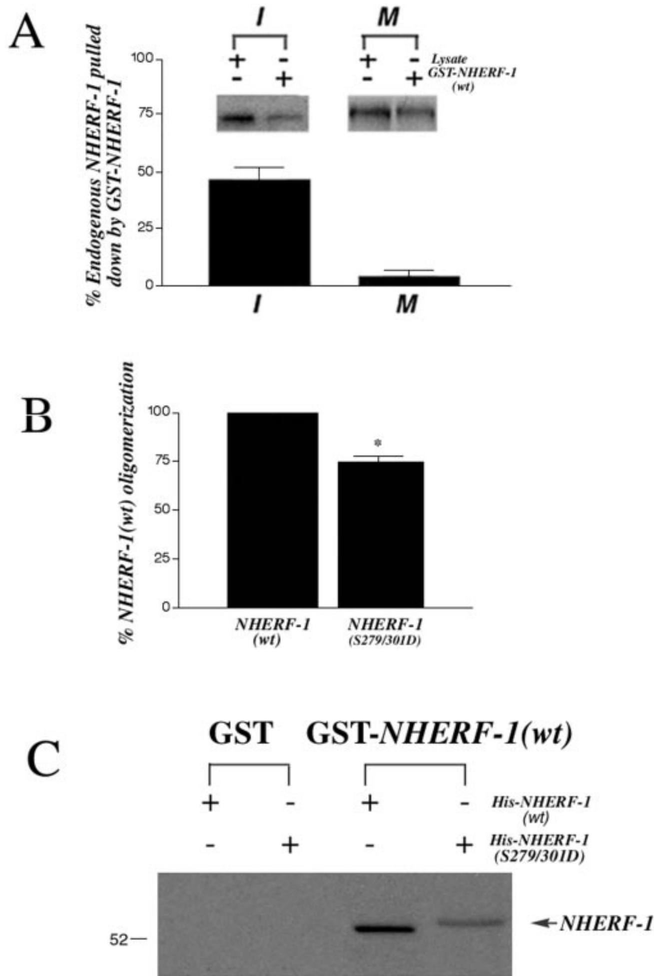

FIG. 5. **NHERF-1 phosphorylation during mitosis phase inhibits NHERF-1 oligomerization.** *A*, NHERF-1 phosphorylation during mitosis phase inhibits NHERF-1/NHERF-1 oligomerization. Lysates derived from either interphase or mitosis phase HeLa cells were incubated with glutathione-agarose beads loaded with GST or wild-type (*wt*) GST-NHERF-1, and the amount of NHERF-1 remaining in the supernatant was assessed following pull-down. Approximately 50% of the cellular NHERF-1 from interphase lysates was pulled down by the GST-NHERF-1 fusion protein. In contrast, <10% of the cellular NHERF-1 in the mitosis phase lysates was pulled down by GST-NHERF-1, indicating that mitosis phase NHERF-1 is markedly impaired in its ability to oligomerize. The *bars* and *error bars* represent the means ± S.E. for three independent experiments, and the *inset* shows the data from a representative experiment. *B*, the S279D/S301D (*S279/301D*) mutation decreases NHERF-1/NHERF-1 oligomerization in a plate binding assay. The binding of wild-type His-NHERF-1 (300 nM) to wells coated with 1 μg of GST, GST-NHERF-1, or GST-S279D/S301D NHERF-1 was quantitated. The S279D/S301D mutant exhibited decreased oligomerization relative to the wild type. The data for each condition are expressed as the percent binding of that observed for wild-type His-NHERF-1 to wild-type GST-NHERF-1 on the same plate. The *bars* and *error bars* represent the means ± S.E. for three independent experiments, each performed in triplicate. *C*, the S279D/S301D mutation decreases NHERF-1/NHERF-1 oligomerization in a pull-down assay. Equal concentrations (100 nM) of wild-type His-NHERF and His-S279D/S301D NHERF-1 were incubated with glutathione-agarose beads loaded with either GST or GST-NHERF-1. No binding of His-NHERF-1 or His-S279D/S301D NHERF-1 to control GST was detectable under these conditions. In contrast, GST-NHERF-1 beads consistently pulled down both His-NHERF-1 and His-S279D/S301D NHERF-1. However, the amount of mutant fusion protein pulled down was markedly decreased relative to the wild type. These data are representative of three independent experiments.

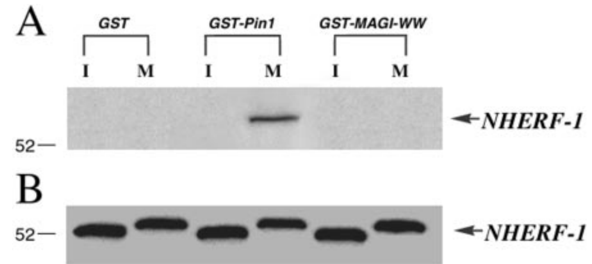

FIG. 6. **Interaction of mitosis phase NHERF-1 with Pin1.** HeLa cell lysates prepared from interphase (*I lanes*) or mitosis phase (*M lanes*) cells were incubated with glutathione-agarose beads loaded with GST, GST-Pin1, or GST-MAGI-WW (a fusion protein containing the WW domains of MAGI-2). After washing, proteins associated with the beads were subjected to Western blotting with the anti-NHERF-1 antibody (*A*), whereas total lysates were also examined as a control (*B*). No binding of NHERF-1 to control GST or GST-MAGI-WW was detectable under any condition. In contrast, the GST-Pin1 beads robustly pulled down NHERF-1 from mitosis phase lysates, but not from interphase lysates. The data shown are representative of four independent experiments.

DISCUSSION

Our findings reveal that cellular NHERF-1 is phosphorylated during mitosis phase at Ser^{279} and Ser^{301} by the cyclin-dependent kinase Cdc2. The only previously identified site of NHERF-1 phosphorylation is Ser^{289}, which can be phosphorylated by several kinases *in vitro*, but is mainly phosphorylated by GRK6A in cells (22). Mutation of Ser^{289} to Ala reduces phosphorylation of cellular NHERF-1, but S289A NHERF-1 mutants still exhibit significant cellular phosphorylation (22, 29). Thus, it has been widely recognized that there must be additional phosphorylation sites on NHERF-1 other than Ser^{289}. The identities of these sites and the kinases that phosphorylate them have remained elusive, however. In the experiments described here, we have identified two novel NHERF-1 phosphorylation sites and a kinase (Cdc2) that efficiently phosphorylates the two identified sites both *in vitro* and in cells.

Phosphorylation of NHERF-1 at Ser^{289} significantly enhances NHERF-1 oligomerization (28). Since Ser^{279} and Ser^{301} flank Ser^{289}, it might reasonably be expected that phosphorylation of these two sites would have an effect on NHERF-1 oligomerization similar to the effect of phosphorylation of Ser^{289}. However, we have found exactly the opposite to be true. Phosphorylation of Ser^{279} and Ser^{301} or mutation of these sites to aspartate to mimic phosphorylation results in significantly decreased oligomerization of NHERF-1. The difference in the functional effects of phosphorylation on Ser^{289} *versus* Ser^{279}/Ser^{301} is mirrored by the difference in the SDS-PAGE size shift induced by phosphorylation of Ser^{289} *versus* Ser^{279}/Ser^{301}: phosphorylation of Ser^{279} and Ser^{301} (or mutation to aspartate) results in a much larger size shift than does phosphorylation or mutation of Ser^{289}. We offer the hypothesis that phosphorylation of Ser^{289} and phosphorylation of Ser^{279} and Ser^{301} induce two distinct conformations of the NHERF-1 polypeptide, one conformation (Ser^{289}-phosphorylated) in which the PDZ domains are more accessible for oligomerization and another (Ser^{279}/Ser^{301}-phosphorylated) in which the PDZ domains are less accessible for oligomerization. This idea could be most directly examined through crystallographic approaches. The crystal structure of NHERF-1 PDZ1 has recently been solved (46), but the crystal structure of full-length NHERF-1 has yet to be described.

We observed that NHERF-1 is hyperphosphorylated during mitosis phase and then becomes dephosphorylated during interphase through the actions of protein phosphatase-1 and/or protein phosphatase-2A. Blockade of these phosphatases with okadaic acid or calyculin A maintains NHERF-1 in a hyperphosphorylated state. Interestingly, Shenolikar *et al.* (27) have reported that treatment of cells with okadaic acid markedly decreases NHERF-1 oligomerization. This finding is consistent

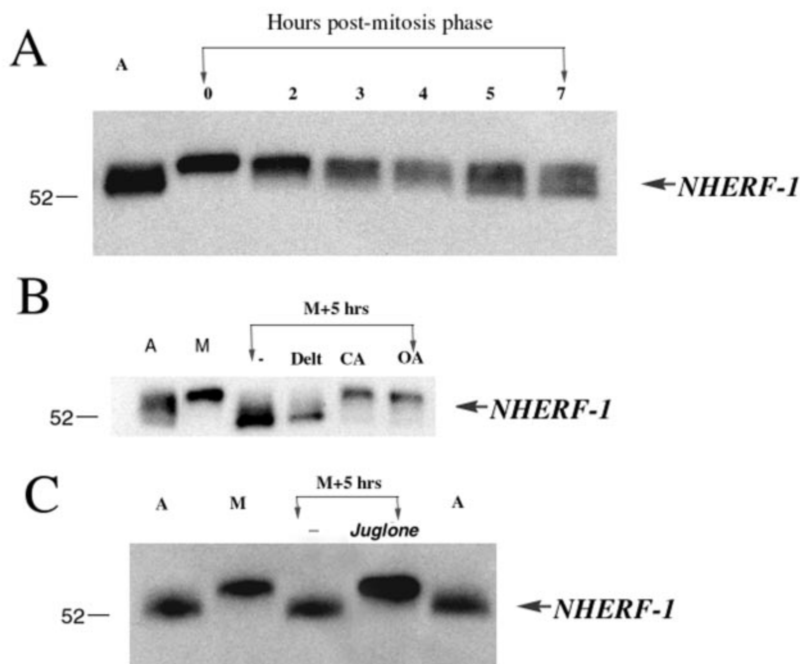

FIG. 7. **Pin1 promotes NHERF-1 dephosphorylation.** *A*, NHERF-1 is gradually dephosphorylated following cell exit from mitosis phase. Mitosis phase HeLa cells were washed three times with Dulbecco's modified Eagle's medium and then reseeded in six-well plates. The cells were harvested at different time points and run on 12% SDS-polyacrylamide gels. NHERF-1 from asynchronous HeLa cells (*A lane*) is shown (*first lane*), and NHERF-1 from mitosis phase cells exhibited an SDS-PAGE mobility shift (*second lane*) that gradually disappeared over several hours following reseeding. *B*, NHERF-1 dephosphorylation is mediated by protein phosphatase-1 or protein phosphatase-2A. Mitosis phase HeLa cells (*M lane*) were reseeded in six-well plates and then harvested after 5 h in the absence or presence of the following phosphatase inhibitors: deltamethrin (*Delt*; 10 μM), calyculin A (*CA*; 2 nM), and okadaic acid (*OA*; 1 μM). The protein phosphatase-1/protein phosphatase-2A-selective inhibitors calyculin A and okadaic acid both blocked NHERF-1 dephosphorylation, whereas the protein phosphatase-2B-selective inhibitor deltamethrin did not. *C*, NHERF-1 dephosphorylation is blocked by the Pin1 inhibitor juglone. Reseeded mitosis phase HeLa cells were harvested after 5 h in the absence or presence of juglone (20 μg/ml). NHERF-1 dephosphorylation was blocked by the juglone treatment, indicating that Pin1 prolyl isomerase activity promotes NHERF-1 dephosphorylation. Molecular mass standards (in kilodaltons) are shown on the left. All experiments shown in this figure are representative of at least three experiments.

with our observation that NHERF-1 oligomerization is impaired by phosphorylation of Ser^{279} and Ser^{301}. If cellular phosphorylation of Ser^{279} and Ser^{301} is prolonged by treatment with okadaic acid, as we have observed, the net result should be a decreased capacity of NHERF-1 for oligomerization, as Shenolikar *et al.* (27) have observed. Superficially, it might seem that inhibition of phosphatase activity could have mixed effects on NHERF-1 oligomerization since phosphorylation of Ser^{279} and Ser^{301} and phosphorylation of Ser^{289} regulate oligomerization of NHERF-1 in different directions. However, Ser^{289} is kept constitutively phosphorylated in most cells by a tightly associated kinase (GRK6A) and is not highly regulated by phosphatases (22). Thus, inhibition of phosphatase activity should preferentially enhance phosphorylation of Ser^{279} and Ser^{301}, leading to a decrease in NHERF-1 oligomerization, as Shenolikar *et al.* (27) have reported.

NHERF-1 and NHERF-2 share 52% sequence identity and have many functional similarities. For example, they both regulate Na^+/H^+ exchange (3, 7); they both bind to the ERM proteins (4, 5, 14, 47, 48); and their PDZ domains have similar binding preferences (6, 8, 10, 11, 13–25). Given this multitude of similarities, it has become a point of significant interest to characterize potential functional differences between the two proteins. This work demonstrates that NHERF-1 is an excellent substrate for Cdc2, but NHERF-2 is not. There are no antibodies against NHERF-2 currently commercially available; and thus, we could not directly determine whether or not endogenous NHERF-2 exhibits hyperphosphorylation during mitosis phase as does NHERF-1. However, we did perform studies expressing hemagglutinin-tagged NHERF-2 in several different cell types, and in no case did we observe any cell cycle-dependent shift in mobility as with NHERF-1 (data not shown). Indeed, it has been reported that NHERF-2 is not phosphorylated in cells at all, under any conditions (30). It therefore seems likely that a key functional difference between NHERF-1 and NHERF-2 is that NHERF-1 is heavily regulated in cells by phosphorylation, whereas NHERF-2 is not.

Phosphorylation of NHERF-1 at Ser^{279} and Ser^{301} not only decreases NHERF-1 oligomerization, but also enhances binding of NHERF-1 to the peptidylprolyl isomerase Pin1. Phosphorylation of NHERF-1 Ser^{279} and Ser^{301} may therefore be viewed as a "switch," enhancing association of NHERF-1 with some proteins (like Pin1) while decreasing association with other proteins (like other NHERF proteins). Furthermore, Pin1 is capable of inducing profound conformational changes in some of its binding partners due to Pin1-mediated isomerization of Ser–Pro bonds (43, 49). Such conformational changes may lead to further alterations in protein-protein interactions, notably changes in interactions with protein phosphatases (42, 45). Regulation of NHERF-1 interactions via phosphorylation and consequent Pin1 association is likely to have a significant impact on NHERF-1 cellular function since NHERF-1 is viewed predominantly as an adaptor protein (9) and is therefore functionally defined by the set of proteins with which it can interact. Moreover, the phosphorylation-dependent interaction of NHERF-1 with Pin1 may have consequences not only for NHERF-1 regulation, but also for regulation of Pin1. Pin1 has been found to associate with a number of other intracellular proteins (32, 37–43, 45) and is known to play a key role in the regulation of the cell cycle and apoptosis (50). It is possible that NHERF-1 may modulate some of these actions of Pin1 in a phosphorylation-dependent fashion.

What are the broader potential physiological implications of the Cdc2-NHERF-1 interaction reported here? NHERF-1 was originally identified as a cofactor necessary for hormonal regulation of NHE3, the renal Na^+/H^+ exchanger (3). Interestingly, hormonal regulation of NHE3 is known to be strongly modulated during the cell cycle (51). The molecular basis of this regulation has not been elucidated, however. Since the mitosis phase-dependent and Cdc2-mediated phosphorylation of NHERF-1 that we have observed alters NHERF-1 oligomerization and NHERF-1 association with other proteins, it might also potentially alter NHERF-1 regulation of NHE3. Furthermore, NHERF-1 has been implicated in a variety of cellular functions other than modulation of Na^+/H^+ exchange (9), and the regulation of NHERF-1 by Cdc2 may therefore have implications for the regulation of the various trafficking and signaling pathways in which NHERF-1 is involved.

In summary, we have found that NHERF-1 is hyperphosphorylated during mitosis phase and that this phosphorylation is mediated by Cdc2. We furthermore have identified two novel sites of NHERF-1 phosphorylation (Ser^{279} and Ser^{301}) that conform to the optimal motif for Cdc2 phosphorylation and that mutagenesis studies have demonstrated are indeed the sites for Cdc2-mediated phosphorylation of NHERF-1. Finally, we have found that phosphorylation of NHERF-1 by Cdc2 inhibits NHERF-1 oligomerization and simultaneously promotes NHERF-1 association with the peptidylprolyl isomerase Pin1. NHERF-1 was first identified as a protein involved in the regulation of Na^+/H^+ exchange by phosphorylation (3); and the alternative name for NHERF-1, ezrin-binding *phosphoprotein* of 50 kDa (4), reflects early recognition that NHERF-1 is highly phosphorylated in most cell types. The findings reported here contribute to the understanding of NHERF-1 regulation by phosphorylation. More specifically, these findings describe a molecular mechanism by which cellular signaling pathways involving NHERF-1 may be regulated by the cyclin-dependent kinase Cdc2 in a cell cycle-dependent fashion.

Acknowledgments—We thank Robert Lefkowitz for support during the early stages of this project, Jeremy Blitzer for contributions to preliminary aspects of these studies, Amanda Castleberry for excellent technical assistance, Jianguo Xu for helpful discussion, Shirish Shenolikar and Ed Weinman for providing the pET-NHERF-1 constructs, and Jonathan Wood and Christopher Ross for providing the MAGI-2/AIP1 cDNA.

REFERENCES

1. Pawson, T., and Scott, J. D. (1997) *Science* **278,** 2075–2080
2. Sheng, M., and Sala, C. (2001) *Annu. Rev. Neurosci.* **24,** 1–29
3. Weinman, E. J., Steplock, D., Wang, Y., and Shenolikar, S. (1995) *J. Clin. Invest.* **95,** 2143–2149
4. Reczek, D., Berryman, M., and Bretscher, A. (1997) *J. Cell Biol.* **139,** 169–179
5. Murthy, A., Gonzalez-Agosti, C., Cordero, E., Pinney, D., Candia, C., Solomon, F., Gusella, J., and Ramesh, V. (1998) *J. Biol. Chem.* **273,** 1273–1276
6. Poulat, F., de Santa Barbara, P., Desclozeaux, M., Soullier, S., Moniot, B., Bonneaud, N., Boizet, B., and Berta, P. (1997) *J. Biol. Chem.* **272,** 7167–7172
7. Yun, C. H. C., Oh, S., Zizak, M., Steplock, D., Tsao, S., Tse, C.-M., Weinman, E. J., and Donowitz M. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 3010–3015
8. Hall, R. A., Ostedgaard, L. S., Premont, R. T., Blitzer, J. T., Rahman, N., Welsh, M. J., and Lefkowitz, R. J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 8496–8501
9. Shenolikar, S., and Weinman, E. J. (2001) *Am. J. Physiol. Renal Physiol.* **280,** F389–F395
10. Hall, R. A., Premont, R. T., Chow, C.-W., Blitzer, J. T., Pitcher, J. A., Claing, A., Stoffel, R. H., Barak, L. S., Shenolikar, S., Weinman, E. J., Grinstein, S., and Lefkowitz R. J. (1998) *Nature* **392,** 626–630
11. Wang, S., Raab, R. W., Schatz, P. J., Guggino, W. B., and Li, M. (1998) *FEBS Lett.* **427,** 103–108
12. Cao, T. T., Deacon, H. W., Reczek, D., Bretscher, A., and Von Zastrow, M. (1999) *Nature* **401,** 286–290
13. Short, D. B., Trotter, K. W., Reczek, D., Kreda, S. M., Bretscher, A., Boucher, R. C., Stutts, M. J., and Milgram, S. L. (1998) *J. Biol. Chem.* **273,** 19797–19801
14. Sun, F., Hug, M. J., Lewarchik, C. M., Yun, C. H., Bradbury, N. A., and Frizzell, R. A. (2000) *J. Biol. Chem.* **275,** 29539–29546
15. Raghuram, V., Mak, D. D., and Foskett, J. K. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 1300–1305
16. Bernardo, A. A., Kear, F. T., Santos, A. V., Ma, J., Steplock, D., Robey, R. B., and Weinman, E. J. (1999) *J. Clin. Invest.* **104,** 195–201
17. Maudsley, S., Zamah, A. M., Rahman, N., Blitzer, J. T., Luttrell, L. M., Lefkowitz, R. J., and Hall, R. A. (2000) *Mol. Cell. Biol.* **20,** 8352–8363
18. Breton, S., Wiederhold, T., Marshansky, V., Nsumu, N. N., Ramesh, V., and Brown, D. (2000) *J. Biol. Chem.* **275,** 18219–18224
19. Tang, Y., Tang, J., Chen, Z., Trost, C., Flockerzi, V., Li, M., Ramesh, V., and Zhu, M. X. (2000) *J. Biol. Chem.* **275,** 37559–37564
20. Gisler, S. M., Stagljar, I., Traebert, M., Bacic, D., Biber, J., and Murer, H. (2001) *J. Biol. Chem.* **276,** 9206–9213
21. Hwang, J. I., Heo, K., Shin, K. J., Kim, E., Yun, C., Ryu, S. H., Shin, H. S., and Suh, P. G. (2000) *J. Biol. Chem.* **275,** 16632–16637
22. Hall, R. A., Spurney, R. F., Premont, R. T., Rahman, N., Blitzer, J. T., Pitcher, J. A., and Lefkowitz, R. J. (1999) *J. Biol. Chem.* **274,** 24328–24334
23. Mohler, P. J., Kreda, S. M., Boucher, R. C., Sudol, M., Stutts, M. J., and Milgram, S. L. (1999) *J. Cell Biol.* **147,** 879–890
24. Kanai, F., Marignani, P. A., Sarbassova, D., Yagi, R., Hall, R. A., Donowitz, M., Hisaminato, A., Fujiwara, T., Ito, Y., Cantley, L. C., and Yaffe, M. B. (2000) *EMBO J.* **19,** 6778–6791
25. Reczek, D., and Bretscher, A. (2001) *J. Cell Biol.* **153,** 191–206
26. Fouassier, L., Yun, C. C., Fitz, J. G., and Doctor, R. B. (2000) *J. Biol. Chem.* **275,** 25039–25045
27. Shenolikar, S., Minkoff, C. M., Steplock, D., Evangelista, C., Liu, M.-Z., and Weinman, E. J. (2001) *FEBS Lett.* **489,** 233–236
28. Lau, A. G., and Hall, R. A. (2001) *Biochemistry* **40,** 8572–8580
29. Weinman, E. J., Steplock, D., Tate, K., Hall, R. A, Spurney, R. F., and Shenolikar, S. (1998) *J. Clin. Invest.* **101,** 2199–2206
30. Lamprecht, G., Weinman, E. J., and Yun, C. H. (1998) *J. Biol. Chem.* **273,** 29972–29978
31. Zizak, M., Lamprecht, G., Steplock, D., Tariq, N., Shenolikar, S., Donowitz, M., Yun, C. H., and Weinman, E. J. (1999) *J. Biol. Chem.* **274,** 24753–24758
32. Yaffe, M. B., Schutkowski, M., Shen, M., Zhou, X. Z., Stukenberg, P. T., Rahfeld, J.-U., Xu, J., Kuang, J., Kirschner, M. W., Fischer, G., Cantley, L. C., and Lu, K. P. (1997) *Science* **278,** 1957–1960
33. Heintz, N., Sive, H. L., and Roeder, R. G. (1983) *Mol. Cell. Biol.* **3,** 539–550
34. Patel, R., Holt, M., Philipova, R., Moss, S., Schulman, H., Hidaka, H., and Whitaker, M. (1999) *J. Biol. Chem.* **274,** 7958–7968
35. Endicott, J. A., Noble, M. E. M., and Tucker, J. A. (1999) *Curr. Opin. Struct. Biol.* **9,** 738–744
36. Holmes, J. K., and Solomon, M. J. (1996) *J. Biol. Chem.* **271,** 25240–25246
37. Shen, M., Stukenberg, P. T., Kirschner, M. W., and Lu, K. P. (1998) *Genes Dev.* **12,** 706–720
38. Crenshaw, D. G., Yang, J., Means, A. R., and Kornbluth, S. (1998) *EMBO J.* **17,** 1315–1327
39. Lu, P. J., Zhou, X. Z., Shen, M., and Lu, K. P. (1999) *Science* **283,** 1325–1328
40. Lu, P. J., Wulf, G., Zhou, X. Z., Davies, P., and Lu, K. P. (1999) *Nature* **399,** 784–788
41. Gerez, L., Mohrmann, K., van Raak, M., Jongeneelen, M., Zhou, X. Z., Lu, K. P., and Van Der Sluijs, P. (2000) *Mol. Biol. Cell* **11,** 2201–2211
42. Zhou, X. Z., Kops, O., Werner, A., Lu, K. P., Shen, M., Stoller, G., Kullertz, G., Stark, M., Fischer, G., and Lu, P. K. (2000) *Mol. Cell* **6,** 873–883
43. Stukenberg, P. T., and Kirschner, M. W. (2001) *Mol. Cell* **7,** 1071–1083
44. Wood, J. D., Yuan, J., Margolis, R. L., Colomer, V., Duan, K., Kushi, J., Kaminsky, Z., Kleiderlein, J. J., Sharp, A. H., and Ross, C. A. (1998) *Mol. Cell. Neurosci.* **11,** 149–160
45. Liu, W., Youn, H.-D., Zhou, X. Z., Lu, K. P., and Liu, J. O. (2001) *FEBS Lett.* **496,** 105–108
46. Karthikeyan, S., Leung, T., Birrane, G., Webster, G., and Ladias, J. A. (2001) *J. Mol. Biol.* **308,** 963–973
47. Yun, C. H. C., Lamprecht, G., Forster, D. V., and Sidor, A. (1998) *J. Biol. Chem.* **273,** 25856–25863
48. Nguyen, R., Reczek, D., and Bretscher, A. (2001) *J. Biol. Chem.* **276,** 7621–7629
49. Schutkowski, M., Bernhardt, A., Zhou, X. Z., Shen, M., Reimer, U., Rahfeld, J.-U., Lu, K. P., and Fischer, G. (1998) *Biochemistry* **37,** 5566–5575
50. Lu, K. P. (2000) *Prog. Cell Cycle Res.* **4,** 83–96
51. Chakraborty, M., Chatterjee, D., Gorelick, F. S., and Baron, R. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 2115–2119