Ndr Protein Kinase Is Regulated by Phosphorylation on Two Conserved Sequence Motifs*

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Ndr is a nuclear serine/threonine protein kinase that belongs to a subfamily of kinases implicated in the regulation of cell division and cell morphology. This subfamily includes the kinases LAT5, Orb6, Cot-1, and Dbf2. We show here that Ndr is potently activated when intact cells are treated with okadaic acid, suggesting that Ndr is normally held in a state of low activity by protein phosphatase 2A. We mapped the regulatory phosphorylation sites of Ndr protein kinase and found that active Ndr is phosphorylated on Ser-281 and Thr-444. Mutation of either site to alanine strongly reduced both basal and okadaic acid-stimulated Ndr activity, while combined mutation abolished Ndr activity completely. Importantly, each of these sites (and also the surrounding sequences) are conserved in the kinase relatives of Ndr, suggesting a general mechanism of activation for kinases of this subfamily. Ser-281 and Thr-444 are also similar to the regulatory phosphorylation sites in several targets of the phosphoinositide-dependent protein kinase PDK1. However, PDK1 does not appear to function as an upstream kinase for Ndr. Thus, Ndr and its close relatives may operate in a novel signaling pathway downstream of an as-yet-unidentified kinase with specificity similar to, but distinct from, PDK1.

Human Ndr is a nuclear serine/threonine protein kinase that has been highly conserved during evolution and which is expressed in almost all cell types of the body (1). Sequence comparisons show that, within the protein kinase superfamily, Ndr is most closely related to a subgroup of kinases known to be important in the control of cell growth, cell division, and cell morphology. This subgroup is exemplified by the kinases LAT5, Orb6, Cot-1, and Dbf2 (50–60% catalytic domain identity with Ndr) and Dbf2 (40% catalytic domain identity with Ndr). Several indications suggest that kinases of this type function, either directly or indirectly, as negative regulators of cyclin/cyclin-dependent kinase complexes. The mammalian LAT5 kinase (also called Wts) is the product of a tumor suppressor gene and possesses an NH2-terminal domain that is able to directly interact with and inhibit Cdc2 (2, 3). Consistent with this, LAT5 interacts genetically with Cdc2 in Drosophila. Orb6 overexpression delays the onset of mitosis in fission yeast, and this effect is dependent upon the presence of a functional Wee1 protein, implying that Orb6 can signal through Wee1 to down-regulate Cdc2 activity (4). Dbf2 is a part of a network of genes required for down-regulation of Cdc28-cyclin B at the end of mitosis in the budding yeast S. cerevisiae (5). Dbf2 is transiently activated at the metaphase/anaphase transition, coincident with changes in its phosphorylation status, and mutation of Dbf2 causes cells to arrest in late mitosis with high Cdc28 activity (6). Finally, the Cot-1 protein of Neurospora crassa is required for hyphal elongation, although it is not known how Cot-1 functions in this process (7). Ndr thus belongs to a subfamily of kinases that are important in cell growth control. Because of the high degree of homology between the members of this kinase subgroup, it is probable that they share, at least to some extent, related substrates and that they are subject to similar regulation.

In the current work, we have analyzed the regulation of Ndr protein kinase with regard to phosphorylation. Although some members of the kinase family to which Ndr belongs are known to exist as phosphoproteins, their putative regulatory phosphorylation sites have not been mapped. We demonstrate here that the kinase activity of Ndr depends upon phosphorylation of Ndr on two sites, Ser-281 and Thr-444. Both of these sites, as well as the sequences surrounding them, are highly conserved in LAT5, Orb6, and Cot-1 and are also present (albeit with slightly lower homology) in Dbf2. Ser-281 and Thr-444 of Ndr are also similar to phosphorylation sites in several PDK1 substrates, but despite this Ndr does not appear to be a target of PDK1. Thus, Ndr and its kinase relatives may be regulated by an upstream kinase distinct from PDK1, but with PDK1-like specificity.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—** COS-1 and 293 cells were transfected as described previously (8, 9). Cells were treated for 1 h with 1 μM okadaic acid (Alexis Corp.) or with solvent alone (0.8% N,N-dimethylformamide) in serum-containing medium. Alternatively, cells were starved in Dulbecco’s modified Eagle’s medium lacking serum for 24 h and then stimulated with 100 nM insulin or 50 ng/ml IGF-1 (Roche Molecular Biochemicals) for 10 min. The following plasmids were used for transfection: pCEV.HA-Ndr-WT (10), pcDNA3.HA-Ndr-WT, and various derivative plasmids encoding phosphorylation site mutants (see below). pCMV5.myc-PDK1 (11) and pECE.HA-ΔPH-PKB (12). Immune complex kinase assays for HA-Ndr and for endogenous Ndr were carried out as described (10). HA-ΔPH-PKB was immunoprecipitated and assayed for kinase activity in the same way as HA-Ndr, except that 50 μg (0.1 mg/ml) cell extract was used, and the kinase substrate used was Cros1tide (GRPRTSSFAEG; 30 μM). Immunoblotting was carried out as described (10), using monoclonal antibodies 12CA5 or 9E10 or a polyclonal antibody against phosphorylated Ser-473 of PKB (New England Biolabs).

**Purification of HA-Ndr—** Extracts from transfected COS-1 cells (10–15 mg of protein) were mixed with ~200 μg of 12CA5 prebound to 100 μl of protein A-Sepharose beads. After washing, bound proteins were eluted with 1 ml of buffer containing 50 mM Tris-HCl, pH 7.5, 0.1% NP-40, 4 μM leupeptin, 1 mM benzamidine, 1 mM microcystin, and 1 mg/ml HA peptide (YPYDVPDYA) and then precipitated by the addition of 125 μg/ml sodium deoxycholate and 10% trichloroacetic acid (13). Precipitated proteins were run on a 7.5% preparative SDS-polyacrylamide gel which was then stained with Coomasie Blue. Bands corresponding to HA-Ndr were excised.

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**Mass Spectrometry**—HA-Ndr in gel slices was reduced with dithiothreitol, alkylated with iodoacetamide, and digested with trypsin (14). NanoESI mass spectrometry (ESI-MS-MS) was performed according to Wilm and Mann (15), and phosphopeptides were detected by m/z = 79 precursor ion scanning (16). The mass spectra were acquired on an API 300 triple quadrupole mass spectrometer (PE Sciex, Toronto, Ontario, Canada) equipped with a NanoESI source (Protana, Odense, Denmark). To estimate phosphorylation stoichiometry, peptides were fractionated by LC-MS (17). Phospho- and dephosphopeptides were detected by extraction of the MS data for the corresponding ions.

**Metabolic Cell Labeling**—COS-1 cells in 10-cm dishes were transfected with pECE.HA-Ndr-WT. After 72 h the medium was changed to phosphate-free Dulbecco’s modified Eagle’s medium containing 10% dialyzed fetal calf serum and 1 ml/plate of [32P] (Amersham Pharmacia Biotech). After a further 5 h the cells were stimulated with okadaic acid, harvested, and HA-Ndr purified as described above.

**Phosphoamino Acid Analysis and Phosphate Release**—Tryptic phosphopeptides were separated by LC-MS and the [32P]-phosphopeptides P1 and P4 identified by their molecular masses (17). An aliquot (200 cpm) of each purified [32P]-phosphopeptide was lyophilized and then hydrolyzed in 6M HCl containing 0.2 mg/ml bovine serum albumin at 110 °C for 45 min. The hydrolysate was separated by thin layer electrophoresis at pH 3.5 (18) and radioactivity detected using a PhosphorImager (Molecular Dynamics). In parallel, 400 cpm of each [32P]-phosphopeptide was subjected to solid-phase Edman degradation using an automated model 477A sequenator (Applied Biosystems). Fractions from each cycle of Edman degradation were lyophilized, resuspended in 50% acetonitrile, and spotted onto a thin layer chromatography plate, before exposure to a PhosphorImager screen.

**Site-directed Mutagenesis**—The insert of the plasmid pECE.HA-Ndr-WT was cloned between the Kan and XbaI sites of pCDNA3 (Invitrogen) to generate pCDNA3.HA-Ndr-WT. Ser-281 and Thr-444 were mutated by oligonucleotide-directed mutagenesis using QuickChange (Stratagene).

**RESULTS**

**Mapping of Regulatory Phosphorylation Sites in Ndr**—We observed that the activity of both transfected and endogenous Ndr was potently stimulated upon treatment of intact cells with okadaic acid (OA) at concentrations that would result in specific inhibition of protein phosphatase 2A (19). Moreover, in vitro incubation of Ndr with purified protein phosphatase 2A resulted in a complete loss of Ndr activity (data not shown). These findings prompted us to map the regulatory phosphorylation sites in Ndr that are essential for its activity. COS-1 cells expressing HA-Ndr were treated with OA or with solvent alone, and HA-Ndr was then immunopurified using the 12CA5 monoclonal antibody. Approximately 1 µg of purified HA-Ndr was digested with trypsin, and the resultant mixture of peptides was analyzed by ESI-MS-MS in a 979 precursor scan (16). This procedure measures the mass:charge ratio (m/z) of each peptide in the mixture that, upon fragmentation, liberates a species with a m/z of 79 (corresponding to a single phosphate group).

When HA-Ndr from control cells was analyzed in this way, only weak phosphopeptide signals could be detected (Fig. 1A). In HA-Ndr from OA-treated cells, however, the abundance of phosphopeptides was markedly increased (Fig. 1B). The observed m/z of each of these peaks could be accounted for by five Ndr-derived peptides, each of which was 79 Da heavier than would be expected for the nonphosphorylated state (Fig. 1C). The identities of several of these phosphopeptides were also confirmed by partial sequencing (data not shown). The five phosphopeptides derive from two regions of the Ndr polypeptide. The first group of phosphopeptides (P1, P2, and P3) are from the N-terminus of the protein (Ndr amino acids 437–446), and the second group of phosphopeptides (P4 and P5) come from the carboxyl-terminal region of the Ndr polypeptide (~60 amino acids COOH-terminal to the catalytic domain (Ndr amino acids 437–447)). Each of these regions contains three amino acids that could potentially be phosphorylated (marked in bold in Fig. 1C). To precisely locate each phosphorylation site, HA-Ndr was isolated from cells that had been metabolically labeled with [32P] prior to OA treatment, and tryptic peptides were purified chromatographically; one phosphopeptide from each region (P1 from Ndr amino acids 277–294, and P4 from Ndr amino acids 437–446) was then selected for further analysis. As shown in Fig. 1D, phosphopeptide P1 gave rise exclusively to phosphoserine upon acid hydrolysis, and released radioactivity in the fifth cycle of Edman degradation, demonstrating phosphorylation of Ser-281. In contrast, phosphopeptide P4 contained phosphothreonine, and released [32P] in cycle 8 of Edman degradation, showing that Thr-444 is phosphorylated (Fig. 1E). These results demonstrate that cell stimulation with OA results in the phosphorylation of two sites in Ndr and that these two sites are Ser-281 and Thr-444.

**Phosphorylation Stoichiometry of Ser-281 and Thr-444**—A portion of the tryptic peptides from Fig. 1, A and B, were analyzed by LC-MS to estimate the relative abundance of phosphorylated and dephosphorylated forms of Ser-281- and Thr-444-containing peptides. Assuming equal elution efficiency of phospho- and dephospho-forms, both Ser-281 and Thr-444 in Ndr from control cells were phosphorylated to a stoichiometry of ~0.1 mol/mol, consistent with the fact that HA-Ndr from unstimulated cells has a low but measurable level of basal activity (see Fig. 2). Upon OA treatment, Ser-281 phosphoryl-
Ndr activity. COS-1 cells expressing either wild-type HA-Ndr or the indicated mutants were treated for 1 h with solvent alone (open bars) or with 1 μM OA (filled bars). HA-tagged kinases were then immunoprecipitated and assayed for kinase activity (upper panel). Data are the mean ± S.D. of duplicate immunoprecipitations. In the lower panel, protein extracts (10 μg) were analyzed by immunoblotting with 12CA5 to verify similar expression levels of each Ndr construct.

Ndr Requires Phosphorylation on Ser-281 and Thr-444 for Its Activity—Ndr mutants were created in which either Ser-281 or Thr-444 was replaced by alanine. The protein kinase activity of each of these mutants was measured following cell treatment with OA or with solvent alone. HA-Ndr-WT was potently (~25-fold) stimulated by OA (Fig. 2). Both the S281A and T444A mutants had markedly reduced basal kinase activity but were still activated (15–20-fold) by OA. However, combined mutation of the two sites (S281A/T444A) reduced basal activity to near undetectable levels and abolished the ability of Ndr to be stimulated by OA. Western blot analysis confirmed that the various mutants were expressed at comparable levels (Fig. 2). These results confirm that Ndr requires phosphorylation on Ser-281 and Thr-444 for its activity.

We also attempted to create constitutively active mutants of Ndr by replacing Ser-281 and/or Thr-444 with negatively charged amino acids. S281D and S281E mutants had basal and OA-stimulated activities similar to those of the S281A mutant, suggesting that steric constraints in this region of the molecule are too tight to allow effective mimicry of a phosphate group by a negatively charged amino acid side chain (data not shown). T444D and T444E mutants had moderately (1.5–2-fold) elevated basal kinase activity (data not shown).

Ser-281 and Thr-444 Are Conserved in Ndr-related Kinases—Comparison of the sequence of Ndr with those of the closely related kinases LATS, Orb6, and Cot-1 shows that potential phosphorylation sites corresponding to both Ser-281 and Thr-444 are conserved throughout the family (Fig. 3). Moreover, the sequences surrounding these sites are highly conserved. It is therefore probable that Orb6, LATS, and Cot-1 are also regulated by phosphorylation on sites equivalent to Ser-281 and Thr-444 of Ndr. Dbf2 also contains potential phosphorylation sites at positions equivalent to Ser-281 and Thr-444 of Ndr; however, the sequences surrounding these sites are somewhat less well conserved (not shown).

Evidence That Ndr Is Not a Target of PDK1—Ser-281 of Ndr shows significant homology to a site in several protein kinases that are substrates for the phosphoinositide-dependent kinase PDK1 (see “Discussion”). We therefore tested the possibility that Ndr too might be a target of PDK1. Myc-tagged PDK1 was coexpressed with HA-Ndr in HEK-293 cells, and Ndr was then immunoprecipitated and assayed for kinase activity. PDK1 overexpression had very little effect on HA-Ndr activity (Fig. 4A), although it was readily detectable by Western blotting (Fig. 4B). In the same experiment, PDK1 caused a >20-fold activation of ΔPH-PKB, an established PDK1 substrate (12).
We found that endogenous Ndr was activated in vitro (10), although it autophosphorylated efficiently (data not shown).

Several PDK1 substrates are activated when cells are exposed to insulin or IGF-1. We investigated whether these ligands are able to activate endogenous Ndr in HEK-293 cells. We found that endogenous Ndr was activated ~15-fold by OA, but showed no response to either insulin or IGF-1 (Fig. 4C). The same extracts used to measure Ndr kinase activity in Fig. 4C were immunoblotted using a phosphospecific antibody that recognizes phosphorylated Ser-473 of PKB (which is similar to Thr-444 of Ndr). As expected, phosphorylation of PKB Ser-473 was strongly induced by both insulin and IGF-1 (Fig. 4D). The lack of activation of Ndr by insulin or IGF-1 was confirmed using epitope-tagged Ndr overexpressed in HEK-293 cells (data not shown). Since Ndr was not activated under conditions that promote phosphorylation and activation of PKB, it would appear that the kinase that activates Ndr is distinct from the kinase responsible for phosphorylation of PKB.

**DISCUSSION**

In this paper, we have shown that the activity of Ndr protein kinase is critically dependent on phosphorylation at two sites, Ser-281 and Thr-444. Based on these results, we predict that LATS, Orb6, Cot-1, and possibly Dbf2 are regulated by phosphorylation on homologous sites. Both Ser-281 and Thr-444 must be phosphorylated for full Ndr activity, but phosphorylation of either site alone is sufficient for a more limited degree of activity. Two of the kinases closely related to Ndr, LATS and Dbf2, are already known to exist as phosphoproteins, although their phosphorylation sites have not been mapped (3, 6). Both LATS and Dbf2 are phosphorylated in a cell cycle-dependent manner, as shown by cell cycle stage-dependent changes in their mobility on SDS gels that can be reversed by in vitro treatment with calf intestinal phosphatase. Ndr protein kinase is the closest known relative of the tumor suppressor LATS in mammalian cells, and several tissues and cell types co-express Ndr and LATS. The high level of homology of these kinases in regions targeted for phosphorylation in Ndr suggests the possibility that they could be regulated by the same upstream kinase.

Ser-281 of Ndr shows homology to activation-loop phosphorylation sites that are found in several members of the AGC subgroup of serine/threonine kinases, such as PKB/Akt (20), p70 S6 kinase (11, 21), SGK (22, 23), protein kinase C (24, 25), and a subgroup of serine/threonine kinases, such as PKB/Akt (20), p70 S6 kinase (11, 21), SGK (22, 23), protein kinase C (24, 25), and cAMP-dependent protein kinase (26). The activation-loop site in each of these kinases is a substrate for PDK1. Based on sequence comparisons, Cheng et al. (26) proposed a consensus phosphorylation motif for PDK1 as Thr-Xaa-Cys-Gly-Thr-Xaa-Asp/Glu-Glu-Tyr-Tyr-Xaa-Ala-Pro-Glu, where Xaa is a hydrophobic residue, and the first Thr in the sequence is the phosphoacceptor. The sequence around Ser-281 of Ndr is homologous to this consensus in 9 out of 12 positions. Moreover, most PDK1 targets contain a second phosphorylation site approximately 60 amino acids carboxyl-terminal to the end of the kinase catalytic domain, which is flanked by hydrophobic amino acids (consensus Phe-Xaa-Xaa-Phe-Ser/Thr-Phe/Tyr) and which, at least in the case of PKB, is phosphorylated by a modified form of PDK1 (27). Thr-444 of Ndr shows similarity to this second motif both in terms of the sequence surrounding it and in terms of its positioning within the polypeptide chain. However, our results suggest that Ndr is not phosphorylated by PDK1. One potential explanation for this could be an unusual structural feature present in Ndr. As shown in Fig. 3, Ndr and its close relatives contain an insert of ~40 amino acids between the catalytic subdomains VII and VIII, which is absent from all other known kinases. This insert interrupts the canonical kinase catalytic domain, beginning COOH-terminally to the conserved “DFG” kinase motif and ending directly NH2-terminally to Ser-281. The close proximity of this domain to Ser-281 might influence the recognition of Ser-281 by upstream kinases, such that phosphorylation by PDK1 is prevented. Moreover, in Ndr this domain harbors a nuclear localization signal so that in the cell, Ndr and PDK1 may be separated from each other in distinct compartments (1). Taken together, our results suggest that Ndr might be the target of an as-yet-unidentified nuclear activation loop kinase that has a substrate specificity similar to, but distinct from, PDK1. In addition, the stimulation of Ndr activity by OA in intact cells, as well as the ability of protein phosphatase 2A to inactivate Ndr in vitro, indicate that Ndr may be a physiological substrate of protein phosphatase 2A.

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