Mechanism of Ca\textsuperscript{2+}-mediated Regulation of NDR Protein Kinase through Autophosphorylation and Phosphorylation by an Upstream Kinase*  

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NDR1 (nuclear Dbf2-related) is a serine/threonine protein kinase belonging to subfamily of kinases implicated in the regulation of cell division and morphology. Previously, we demonstrated that the activity of NDR1 is controlled by phosphorylation of two regulatory residues, Ser-281 and Thr-444. Moreover, we found that NDR1 becomes activated through a direct interaction with EF-hand Ca\textsuperscript{2+}-binding proteins of the S100 family. In this work, we characterize this regulatory mechanism in detail. We found that NDR1 autophosphorylates in vitro predominantly on Ser-281 and to a lesser extent on Thr-74 and Thr-444. All of these residues proved to be crucial also for NDR1 activity in vivo; however, in contrast to Ser-281 and Thr-444, Thr-74 seems to be involved only in binding to S100B rather than directly regulating NDR1 activity per se. When we added Ca\textsuperscript{2+}/S100B, we observed an increased autophosphorylation on Ser-281 and Thr-444, resulting in stimulation of NDR1 activity in vitro. Using phosphospecific antibodies, we found that Ser-281 also becomes autophosphorylated in vivo, whereas Thr-444 is targeted predominantly by an as yet unidentified upstream kinase. Significantly, the Ca\textsuperscript{2+}-chelating agent BAPTA-AM suppressed the activity and phosphorylation of NDR1 on both Ser-281 and Thr-444, and specifically, these effects were reversed when we added the sarcoplasmic-endoplasmic reticulum Ca\textsuperscript{2+} ATPase pump inhibitor thapsigargin.

NDR1 (nuclear Dbf2-related) is a conserved and widely expressed nuclear serine/threonine kinase that belongs to a recently identified subfamily of kinases that play a crucial role in cell division and cell morphogenesis. We originally cloned this kinase from a human fetal brain cDNA library using Caenorhabditis elegans expressed sequence tag clone cm11b8 (1). Later, it was mapped to chromosome 12p11 next to the K-ras gene. The two isoforms display an identity of more than 87% at the protein and 77% at the DNA level. In addition to mammalian NDR1 and NDR2, the NDR family of protein kinases comprises orthologous proteins Tricornered (trc gene) from Drosophila melanogaster; Sax-1 from C. elegans; and several closely related kinases such as Warts/Lats kinases from mammals, D. melanogaster, and C. elegans; Cbk1, Dbf2, and Dbf20 from Saccharomyces cerevisiae; Orb6 from Schizosaccharomyces pombe; Ukc1 from Ustilago maydis; and Cot-1 from Neurospora crassa (for review, see Ref. 3). These isoforms share 40–60% amino acid identity within their catalytic domains as well as conserved regions in their regulatory domains. The structural similarity within this family suggests that these isoforms might perform related functions even in evolutionary distant organisms.

In fact, recent reports confirmed the involvement of this group of kinases in various aspects of regulation of cell division and cell morphogenesis. For instance, mutations in trc result in splitting of surface projections such as epidermal hairs, shafts of sensory bristles, larval denticles, and the lateral branches of arista (4). The sax-1 mutants display expanded cell bodies and ectopic neurites in several classes of neurons (5). Mutation in cot-1 results in excessive numbers of branched hyphal tips at restrictive temperature, but these tips fail to elongate (6). The cbk1 mutants show profound defects in cell morphogenesis, including changes from ellipsoidal to round morphology, random budding patterns, and abnormal mating projections (7). Likewise, disruption of ukc1 leads to a change of cell shape from elongated to rounded form, formation of hyphal extensions, and prevents cells from forming filamentous colonies (8). The orb6 mutants lose growth polarity and display altered, spherical morphology with disorganized microtubuli and actin filaments; and moreover, they enter mitosis prematurely (9). On the other hand, cells carrying temperature-sensitive alleles of dbf2 arrest at the end of anaphase with uniform, large budded “dumbbell” morphology (10). Taken together, the functional and structural relatedness within this group of kinases suggests that they may be regulated by similar mechanisms, including, e.g. stimulatory phosphorylation and association with activating proteins, and they may also partially share the same substrates.

The NDR group kinases contain all 12 subdomains of the kinase catalytic domain described by Hanks et al. (11). Regarding their kinase domain sequence, they are most closely related to the members of AGC family of protein kinases comprising...
PKA, PKB, PKCs, PRK, p70S6K, p90RSK, and PDK1. A unique feature of NDR group kinases is an insert of about 30 amino acids between subdomains VII and VIII, which, in the case of NDR1, accommodates a nonconsensus nuclear localization signal (1). All members of this group also contain a poorly characterized N-terminal regulatory domain of different lengths. In NDR1, this domain consists of 87 amino acids, and it encompasses a region that is predicted to form an amphiphilic $\alpha$-helix responsible for binding to S100B (12).

In our previous work, we described the basic mechanisms of activation of NDR1 protein kinase (13). We found that NDR1 is potently activated by treatment of cells with the protein phosphatase 2A inhibitor okadaic acid (OA), indicating involvement of phosphorylation of serine and/or threonine residues in the regulation of NDR1 activity. Indeed, we demonstrated that activation of NDR1 involves phosphorylation of two regulatory residues, Ser-281 of the activation segment and Thr-444. We also investigated the dependence of NDR1 phosphorylation events on sulfoxide (Alomone) 24 h after transfection. NDR1 was constructed by PCR using primers 5\'-H9262

Moreover, we described a mechanism of in vitro activation of NDR1 through a direct interaction with EF-hand Ca\(^{2+}\)-binding protein S100B, which augments NDR1 autophosphorylation (12). Notably, NDR1 also interacts with S100B in vivo, its activity is rapidly stimulated by treatment with the Ca\(^{2+}\) ionophore A23187, and this activation is dependent upon the N-terminally located S100B binding domain. Intriguingly, we observed that overexpression of S100B in several melanoma cell lines leads to hyperactivation of NDR1 and that NDR1 activity can be inhibited in those cells by W7, a cell-permeable inhibitor of CaM and S100 proteins. Altogether, these results point to the involvement of Ca\(^{2+}\) signaling in regulation of NDR1.

In this study, we have examined the mechanism of Ca\(^{2+}\)/S100B-induced autophosphorylation and activation of NDR1 by means of electrospary ionization mass spectrometry (ESI-MS) and phosphospecific antibodies raised against the regulatory phosphorylation sites Ser-281 and Thr-444. We also investigated the dependence of NDR1 phosphorylation events on intracellular Ca\(^{2+}\). Our findings delineate a Ca\(^{2+}\)-dependent mechanism for activation of NDR1, involving S100B-mediated stimulation of autophosphorylation on Ser-281 and phosphorylation of Thr-444 by an unknown Ca\(^{2+}\)-dependent upstream kinase.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**COS-1 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 units/ml penicillin, 100 $\mu$g/ml streptomycin. Cells were transfected at the subconfluent stage with FuGENE 6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. In some experiments, the cells were treated for 60 min with 1 $\mu$M OA in 0.1% N,N-dimethylformamide (Alexis Corp.), 50 $\mu$A BAPTA-AM in 0.1% dimethyl sulfoxide (Sigma), or 20 $\mu$M thapsigargin in 0.1% dimethyl sulfoxide (Alomone) 24 h after transfection.

**Plasmids—**Mammalian expression vector pcMV5 with HA-tagged NDR1 was constructed by PCR using primers 5\'-CAC TAT CCT TCT GCT ATG TTC TAC CCC TAT TTT GCT GGC ACT CCT GAC TAC ATT GC-3' and 5\'-CGG GAT CCC TAT TTT GCT GCG AAG GCT AGC TGA CGT CTA TTT CTT TTC C-3'. The PCR product has been subcloned into the HindIII and BamHI sites of pcMV5, pGEX-2T_NDR1 plasmid has been described previously (1). The pcMV5_HA-NDR1 and pGEX-2T_NDR1 plasmids for kinase-dead (K118A) and phosphorylation site alanine mutants (T74A, S291A, and T444A) were generated from wild-type vectors using the QuikChange site mutagenesis protocol (Strategene) with the following primers: 5\'-GGA CAT GTG TAT GAA ATG CAT ATA CTC CGT AAA GGA GAT ATG CTT GAA AAA GAG CAG G-3' and 5\'-GCA TAT CGT CTT TAC GGA GTA TAG CAA TTG CAT ACA CAT GTC CGG CAT TAT CTT TCT GCT GAA CGA GC-3' (K118A), 5\'-GCT CGG AAG GAA GCA GAG TTT CCT GCT TGT AAG ACA ACA AGA CGG GG-3' and 5\'-GCA AGA AAC TCT GCT GTC TCT TTC CGA GCA TGT GCT GAT CTC CG-3' (T74A), 5\'-GCT CAG CTA GCA TTC GCT GCA ACT GCT GAC GCT GTC ATT ACT GTA GCA GGT AGC TGA CTG CTA TTT CCT TTC C-3' (S291A), and 5\'-GCT CGG CAT CAA TTA CGT TTA CAA GCG TCT TGA GGG CCT CCT GAC GAC GAC TGT TGT GAT CTA TTT CCT TTC TCT-3' and 5\'-GCT GAC GCT GAT TGT GAT GCT TTA CTC CGT GCT TCC CGT TGT TTT CAA CTA CCG AGC ACC AGT TGT TTC-3' (T444A). pECE_S100B plasmid has been described previously (12). Sequence of all plasmids was confirmed by DNA sequencing.

**Antibodies—**Anti-Ser-281P and anti-Thr-444P rabbit polyclonal antisera were raised against the synthetic peptides NRRQAFS/PDK1/T- YGTPD for the Ser-281 phosphorylation site and KDWFYINYT- (P0)/LYKRFEG for the Thr-444 phosphorylation site (Neosystem, Strasbourg, France). The peptides had been conjugated to keyhole limpet hemocyanin. Rabbit injections and bleed collection were done by Strategic Biosolutions. The anti-Ser-281P antiserum was used without further purification, but the anti-Thr-444P antibody was purified on protein A-Sepharose (Amersham Biosciences) from synthetic peptide coupled to cyanogen bromide-activated Sepharose (Amersham Biosciences). Antibodies were eluted with 0.1 M glycine, pH 2.5. The 12CA5 HA monoclonal antibody hybridoma supernatant was used for immunodetection and immunoprecipitation of HA-NDR1 variants. A rabbit anti-NDR1, C-terminal polyclonal antiserum directed against a synthetic peptide TARGAIYSMKAAK (corresponding to NDR1 amino acids 452–465) has been described previously (1). A rabbit polyclonal antiserum that recognizes S100B was raised against recombinant human S100B and was used without further purification (14).

**Bacterial Expression of Human GST-fused NDR1—**BL21-DE3 Escherichia coli strain (Novagen) was transformed with the pGEX-KIF protein expression plasmids and the pGEX-LacI* repressor (Qiagen). Mid-logarithmic phase cells were induced with 0.5 mM isopropyl-$\beta$-D-thiogalactopyranoside for 4 h at 30 °C. Bacteria were lysed by a French press in presence of 1 mg/ml lysozyme, and the fusion proteins were purified on glutathione-agarose (Amersham Biosciences) as described. Recombinant proteins were assayed for kinase activity as described below, and autophosphorylation was determined after SDS-PAGE separation either by Cerenkov counting or by exposure to a PhosphorImager screen followed by analysis with ImageQuant software (Molecular Dynamics).

**GST-NDR1 Kinase Assay—**1 μg of purified recombinant GST-NDR1 wild-type and mutants (without further treatment or autophosphorylation for CAcL) and 10 μM absence of 1 mM CAcL, and 100 μM S100B (Sigma) were assayed in a 20-μl reaction containing 20 mM Tris- HCl pH 7.5, 10 mM MgCl₂, 1 μM dithiothreitol, 100 μM γ-32P]ATP (–2,000 cpm/pmol; Amersham Biosciences), and 1 mM NDR1 substrate peptide (KRRNRRL5VA). After a 30-min incubation at 30 °C, reactions were stopped with 50 mM EDTA, and 10-μl reaction solutions were spotted onto 2-cm² squares of P-81 phosphocellulose paper (Whatman). These were subsequently washed 4 × 5 min and 3 × 20 min in 1% phosphoric acid and once in acetone before counting in a liquid scintillation counter. One unit of NDR1 activity was defined as the amount that catalyzed the phosphorylation of 1 nmol of peptide substrate in 1 min.

**HA-NDR1 Kinase Assay—**S-transfected and treated COS-1 cells were washed once with ice-cold phosphate-buffered saline and harvested by scraping into 1 ml of ice-cold phosphate-buffered saline containing 1 mM Na₃VO₄ and 20 mM $\beta$-glycerophosphate before lysis in 500 μl of immunoprecipitation (IP) buffer (20 mM Tris- HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM Na₃VO₄, 20 mM $\beta$-glycerophosphate, 0.5 mM phenylmethylsulfonyl fluoride, 4 μM leupeptin, 1 mM benzamidine, and 1 tablet of Complete protease inhibitors/10 ml of IP buffer (Roche)). Lysates were centrifuged at 20,000 × $g$ for 20 min, and triplicate aliquots (200 μg) of supernatant were precleared with protein A-Sepharose for 60 min and mixed subsequently for 3 h at 4 °C with 12CA5 antibody prebound to protein A-Sepharose (-1 μg antibody per 1 ml of IP buffer). The beads were then washed twice with IP buffer, once for 10 min with IP buffer containing 1 mM NaCl, once again for 10 min with IP buffer, and finally twice with 20 mM Tris- HCl pH 7.5, containing 4 μM leupeptin and 1 mM benzamidine. Thereafter, beads were resuspended in 30 μl of

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3 The abbreviations used are: PKA, PKB, PKC, protein kinase A, B, and C, respectively; BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane,N,N,N',N'-tetraacetic acid tetraacetylmethyl; CaM, calmodulin; ESI-MS, electrospray ionization-mass spectrometry; GST, glutathione S-transferase; HA, hemagglutinin; HPLC, high performance liquid chromatography; IP, immunoprecipitation; LC-MS, liquid chromatography-mass spectrometry; MSMS, tandem mass spectrometry; OA, okadaic acid; PKD, phosphoinositide-dependent kinase.
buffer containing 20 mM Tris-HCl pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol, 100 μM [γ-32P]ATP (~1,000 cpm/μmol), 1 μM cAMP-dependent protein kinase inhibitor peptide (Bachem), 4 μM leupeptin, 1 mM benzamide, 1 μM microcystin-LR, and 1 mM NDR1 substrate peptide (KKRRNRRSLVA). After a 60-min incubation at 30 °C, 15 μl of supernatant was removed, and the substrate incorporation into the phosphate peptide was determined as described for GST-NDR1.

Western Blotting—To detect GST-NDR1 or HA-NDR1, samples were resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore). Membranes were blocked in TBST (50 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.05% Tween 20) containing 5% skimmed milk powder and were then probed for 2 h with 4 μg/ml anti-NDR1 C-term or 1:100 12CA5 monoclonal antibody supernatant. Bound antibodies were detected with corresponding horseradish peroxidase-linked secondary antibodies and ECL (Amersham Biosciences). For detection of phosphorylated NDR1, either 1 μg of GST-NDR1 or HA-NDR1 immunoprecipitated from 100 μg of COS-1 detergent extracts as described earlier in this paper was separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked in TBST containing 5% skimmed milk powder and incubated for 2 h at room temperature or overnight at 4 °C with 1:1,000 anti-Ser-281P antiserum in the presence of 50 μg/ml competing dephosphopeptide or 1:500 anti-Thr-444P purified antibody. Both antibodies were detected with horseradish peroxidase-conjugated donkey anti-rabbit Ig antibody (Amersham Biosciences) and ECL. To detect immunoprecipitated S100B, samples were separated on 18% SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membrane was blocked for 2 h with 5% bovine serum albumin and 1% fetal calf serum in TBST and was then probed with anti-S100B antiserum diluted 1:1,000 in the same buffer. Bound antibody was then detected with corresponding horseradish peroxidase-linked secondary antibody in TBST and ECL.

Coimmunoprecipitation of NDR1 and S100B—Cytoplasmic COS-1 cell extracts were prepared by lysing the cells for 10 min in a hypotonic buffer containing 10 mM HEPES pH 7.9, 0.5 mM CaCl2, 1.5 mM MgCl2, 10 mM KCl, 1 mM Na2VO4, 20 μM β-glycerophosphate, 1 μM microcystin-LR, 50 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 4 μM leupeptin, 1 mM benzamide, and 1 tablet of Complete protease inhibitors/10 ml of buffer. After centrifugation, the cells were homogenized in Dounce homogenizer. After centrifugation at 3,300 x g for 15 min, supernatants with cytoplasmic extracts were saved, and isolated nuclei found in the pellets were processed further by lysis for 30 min in a high salt buffer containing 20 mM HEPES pH 7.9, 25% glycerol, 0.5 mM CaCl2, 1.5 mM MgCl2, 0.5 mM KCl, 1 mM Na2VO4, 20 μM β-glycerophosphate, 1 μM microcystin-LR, 50 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 4 μM leupeptin, 1 mM benzamide, and 1 tablet of Complete protease inhibitors/10 ml of buffer. After centrifugation at 20,000 x g for 30 min, the cytoplasmic and nuclear extracts were pooled at a ratio of 4:1. 1 mg of protein extracts was precleared for 60 min with protein-A Sepharose and incubated for 3 h at 4 °C with 5 μl of anti-S100B antiserum immobilized on 10 μl of protein A-Sepharose. Afterward, the beads were washed four times in buffer containing 20 mM HEPES pH 7.9, 25 mM KCl, 5% glycerol, 1 mM MgCl2, 0.1 mM CaCl2, 0.5 mM phenylmethylsulfonyl fluoride, 4 μM leupeptin, 1 mM benzamide, 1 mM Na2VO4, 20 μM β-glycerophosphate, 1 μM microcystin-LR, and 50 mM NaF. Immunoprecipitates were boiled in sample buffer and immunoblotted for S100B or HA-NDR1 as described earlier in this paper.

Mass Spectrometry—10 μg of untreated or autophosphorylated GST-NDR1 (2 h at 30 °C, either in presence or absence of 1 mM CaCl2, and 10 μM bovine S100B (C)). After separation by SDS-PAGE, GST-NDR1 was excised and processed by tryptic cleavage for MS analysis of phosphopeptides by precursor ion scanning of m/z ~79. Phosphopeptides whose m/z could be assigned to NDR1-derived phosphopeptides are labeled P1–P4. Some of the peptides were detected in several charged states designated [M-2H]2–, [M-3H]3–, etc. Peptide P2 results presumably from trace cysteinyltryptic degradation of the trypsin preparation because it overlaps with P1 and terminates with an aromatic residue. Astersk denotes an abundant, double-charged nonphosphorylated NDR1 peptide (amino acids 378–391) with m/z 860. D, the phosphopeptide from A–C which derive from three regions of the NDR1 polypeptide are summarized with the phosphoacceptor residues marked in each peptide in bold.

FIG. 1. Mapping of NDR1 in vitro autophosphorylation sites. A–C, 10 μg of GST-NDR1 was left untreated (A) or was autophosphorylated for 2 h in the absence (B) or presence of 1 mM CaCl2 and 10 μM bovine S100B (C). After separation by SDS-PAGE, GST-NDR1 was excised and processed by tryptic cleavage for MS analysis of phosphopeptides by precursor ion scanning of m/z ~79. Phosphopeptides whose m/z could be assigned to NDR1-derived phosphopeptides are labeled P1–P4. Some of the peptides were detected in several charged states designated [M-2H]2–, [M-3H]3–, etc. Peptide P2 results presumably from trace cysteinyltryptic degradation of the trypsin preparation because it overlaps with P1 and terminates with an aromatic residue. Asterisks denotes an abundant, double-charged nonphosphorylated NDR1 peptide (amino acids 378–391) with m/z 860. D, the phosphopeptide from A–C which derive from three regions of the NDR1 polypeptide are summarized with the phosphoacceptor residues marked in each peptide in bold.
Mechanism of NDR1 regulation by Ca\(^{2+}\)

Mapping of in Vitro Autophosphorylation Residues of NDR1—We observed that NDR kinase becomes potently autophosphorylated on serine and threonine residues \textit{in vitro}, and that the autophosphorylation as well as NDR1 kinase activity markedly increase upon incubation of NDR1 with the EF-hand Ca\(^{2+}\)-binding proteins of the S100 family, in particular S100B (1, 12). These results prompted us to analyze the NDR1 autophosphorylation sites by means of nanospray ECI-MS/MS analysis. 10 \(\mu\)g of purified GST-NDR1 was left untreated (Fig. 1A) or autophosphorylated either in the absence (Fig. 1B) or presence (Fig. 1C) of purified bovine S100B. After tryptic cleavage of the differently treated GST-NDR1 samples, resultant mixtures of peptides were introduced into a triple-quadrupole mass spectrometer and analyzed for phosphorylated peptides by precursor ion scanning for \(\gamma\)-\[^{32}\text{P}]\text{ATP}\) incorporation. We detected ion species corresponding to the phosphorylated peptides increased markedly (Fig. 1, A and C). The observed \(m/z\) value of most of these signals could be assigned in both samples to four NDR1-derived phosphopeptides P1–P4 which, upon liberation of the phosphate group (79.97 Da), give rise to expected tryptic (or in one case chymotryptic) NDR1 dephosphopeptides (Fig. 1D). To confirm the identity of these peptides, we performed a LC-MS analysis of GST-NDR1 autophosphorylated with \(\gamma\)-\[^{32}\text{P}]\text{ATP}\) (data not shown). After collection of the phosphopeptide-enriched radioactive fractions, we again analyzed the samples by ESI-MS using an \(m/z\) – 79 precursor ion scanning method. Having found the same species as in the analysis of the unlabeled GST-NDR1, we subsequently performed a low energy MS/MS analysis in positive ion mode. We detected ion species corresponding to the phosphopeptides listed in Fig. 1D, including the phosphorylated residues, thereby confirming identity of the NDR1 \textit{in vitro} autophosphorylation sites.

The four phosphopeptides derived from three regions of the NDR1 polypeptide. The first three phosphopeptides correspond to sites identified previously also in NDR1 activated \textit{in vivo} by OA. P1 and P2 correspond to phosphopeptides encompassing Ser-281 of the activation segment and P3 represents a phosphopeptide containing the residue Thr-444 from hydrophobic motif located in the C-terminal region of NDR1. Significantly, the phosphopeptide P4 proved to be an as yet unidentified
phosphoresidue Thr-74 from the N-terminal part of NDR1 which has been defined previously as an S100 binding region (residues 62–83; Ref. 12).

Reduction of Autophosphorylation Rate and Kinase Activity in GST Phosphorylation Site Mutants—To confirm the in vitro autophosphorylation of NDR1 on the residues Thr-74, Ser-281, and Thr-444 and to estimate the influence of these residues on the autophosphorylation-induced kinase activity of NDR1, we constructed a series of GST-NDR1 alanine mutants at these positions (as well as a GST-NDR1 kinase-dead alanine mutant in the catalytic site residue Lys-118). As shown in Fig. 2, B and C, wild-type GST-NDR1 became efficiently autophosphorylated in vitro (up to 0.4 pmol of phosphorus/pmol of GST-NDR1). The addition of S100B homodimers brought about an approximate 2-fold increase in the autophosphorylation rate accompanied by about a 5-fold increase in kinase activity. Because the kinase-dead mutant did not show any detectable autophosphorylation, the NDR1 kinase activity must account for the observed effects. As shown in Fig. 2, A and B, the S281A mutant displayed a markedly reduced autophosphorylation rate (irrespective of the addition of S100B), thereby confirming this residue as the major in vitro autophosphorylation site of NDR1. As expected, the kinase activity of S281A decreased similarly and was almost undetectable (Fig. 2C). Although not comparable with S281A, the T444A point mutation also led to a significant decrease of both autophosphorylation rate and kinase activity, indicating that this residue is also susceptible to autophosphorylation in vitro. The smaller effects of T444A, compared with the S281A mutation, may be readily explained by the unfavorable, hydrophobic peptide sequence upstream of this residue with only two basic amino acids located at positions –8 and –10 (Table I). Finally, the T74A mutant displayed only a negligible reduction in autophosphorylation rate and kinase activity in absence of Ca\(^{2+}\)/S100B. Although the sequence N-terminal to this site in fact matches the basic NDR1 consensus sequence fairly well, according to the quantitative analyses by LC-MS, the phosphorylation at this site always accounted for less than 5% of the total phosphate incorporation (data not shown). After the addition of Ca\(^{2+}/\)S100B, however, the impact of this point mutation approached that of T444A, and the stimulatory effect of Ca\(^{2+}/\)S100B on autophosphorylation rate and kinase activity was diminished strongly compared with the other GST-NDR1 variants. These facts indicate that Thr-74 represents only a minor autophosphorylation site, and the low responsiveness to the addition of Ca\(^{2+}/\)S100B most likely reflects the inability of T74A mutant to interact with this protein.

**Mechanism of NDR1 Activation by Ca\(^{2+}/\)S100B**—To examine the effect of S100B on NDR1 phosphorylation status, we raised rabbit polyclonal antibodies directed against phosphopeptides of the two known activation phosphorylation sites Ser-281 and Thr-444. As shown in Fig. 2D, these antibodies were specific for the phosphorylated NDR1 because corresponding phosphorylation point mutants S281A, T444A, and kinase-dead were not recognized. Using these reagents, we found that Ca\(^{2+}/\)S100B enhances phosphorylation on both Ser-281 and Thr-444 (similar to the general increase in autophosphorylation as presented in Fig. 2, A and B) without an apparent preference for one of the residues (Fig. 2D, middle and bottom panels). This synergy between the phosphorylation of both activation segment and hydrophobic motif sites may provide a simple explanation for why NDR1 activity increases more robustly than does the overall autophosphorylation after the addition of Ca\(^{2+}/\)S100B. Nevertheless, it is striking that although the S281A mutation entirely abolishes the autophosphorylation on Thr-444, the T444A mutant displayed an almost normal autophosphorylation on Ser-281. Apart from the fact that Thr-444 only was autophosphorylated to a minor extent, these data indicate that the impact of the activation segment phosphoresidue on the NDR1 kinase activity was higher than the impact of the hydrophobic motif residue or that these residues are phosphorylated in a sequential manner as is known for most of other AGC family protein kinases (18). Both alternatives may apply for an intramolecular mechanism of the NDR1 autophosphorylation as defined previously (1). Finally,
Thr-74. This indicates that the Thr-74-dependent, S100B-mediated autophosphorylation may play a crucial role in vivo.

To examine this possibility, we analyzed extracts from transfected COS-1 cells again with the anti-Ser-281P and anti-Thr-444P antisera. As shown in Fig. 3B, the Ser-281 residue was constitutively phosphorylated in vivo (and this phosphorylation was enhanced further after treatment with 1 μM OA), whereas the Thr-444 site only became modified after OA treatment. Intriguingly, we found that the phosphorylation of Ser-281 depends entirely on the activity of NDR1 because the kinase-dead K118A mutant did not display any phosphorylation on this position. This fact implies that Ser-281 is an autophosphorylation residue also in vivo. Significantly, autophosphorylation accounts for the major portion of NDR1 phosphorylation in vivo because wild-type NDR1 incorporated markedly higher amounts of radioactive orthophosphate than its kinase-dead counterpart in metabolically labeled COS-1 cells upon treatment with OA (data not shown). Furthermore, mutation of Thr-444 did not impair the phosphorylation of Ser-281, which again points to the low importance of this residue for NDR1 autophosphorylation (Fig. 3B, middle panel). For the phosphorylation of Thr-444, however, we found that this residue became modified also after abolishing the kinase activity of NDR1 in kinase-dead mutant K118A (Fig. 3B, bottom panel). This fact strongly suggests that, at least in OA-treated cells, this site must be targeted by an as yet unidentified upstream kinase. Finally, mutation of residue Thr-74 led to a marked reduction of both Ser-281 and Thr-444 phosphorylation (both with and without OA treatment), which explains the somewhat unexpected kinase activity data observed for this mutant. This implies that Thr-74 is not only involved in the S100B-mediated autophosphorylation of NDR1, but it probably participates also in targeting the upstream kinase to NDR1.

Phosphorylation on both Ser-281 and Thr-444 Occurs in a Ca\(^{2+}\)-dependent Manner—Because our current and previous work indicated that Ca\(^{2+}\)/S100B is essential both for NDR1 activity in vivo and for an efficient NDR1 autophosphorylation on Ser-281 and Thr-444 in vitro (Fig. 2D) and because the S100B-NDR1 interaction is known to be fully Ca\(^{2+}\)-dependent (12), we sought to examine the role of intracellular Ca\(^{2+}\) in the activation of NDR1. For this purpose, we treated transfected COS-1 cells with the membrane-permeable agent BAPTA-AM, which is freely taken up into cells, where it is hydrolyzed by cytosolic esterases and trapped intracellularly as active, membrane-impermeable Ca\(^{2+}\) chelator BAPTA (19). As shown in Fig. 4A, 50 μM BAPTA-AM dramatically reduced the OA-stimulated NDR1 activity almost to the basal activity level of the untreated cells. Likewise, examination of the phosphorylation status of NDR1 demonstrated that phosphorylation on both Ser-281 and Thr-444 declined almost to the base line (Fig. 4B).

Notably, both NDR1 activity and the phosphorylation of Ser-281 and Thr-444 were rescued by coincubation of BAPTA-AM with 20 μM thapsigargin, a sesquiterpene lactone capable of inhibiting endoplasmic reticulum Ca\(^{2+}\) stores (20). These results confirm the Ca\(^{2+}\)-specificity of the observed BAPTA-AM effects and combined with the experiments showed in Fig. 3, allow us to conclude that NDR1 is regulated by a Ca\(^{2+}\)-dependent, most likely S100B-mediated autophosphorylation on Ser-281 and by phosphorylation by an as yet unidentified Cu\(^{2+}\)-dependent upstream kinase on Thr-444.

Thr-74 Is Required for the Association of HA-NDR1 with Ca\(^{2+}\)/S100B—We have demonstrated previously that NDR1 forms functional complexes with S100B in vivo and that this interaction depends on an intact N-terminal domain of NDR1.
with anti-S100B immunoprecipitates (and cytoplasmic cell lysates were prepared, pooled, and analyzed for corresponding empty vectors as indicated. 48 h later, nondetergent nuclear phosphorylation residues Thr-74, Ser-281, or Thr-444, and S100B or corresponding mutant did not get phosphorylated on this position, whereas Thr-74 and Thr-444 could merely account for a minor percentage of the incorporated phosphate. Moreover, mutation of Ser-281 led to most dramatic decrease of NDR1 autophosphorylation-induced kinase activity, thereby confirming this residue as the major NDR1 autophosphorylation site.

The addition of Ca\(^{2+}\)/S100B led to a 2-fold increase of NDR1 autophosphorylation (on both Ser-281 and Thr-444) accompanied by about a 5-fold increased kinase activity. As expected, mutation of Ser-281 again led to an almost total decline of both autophosphorylation and kinase activity. Nevertheless, we also observed a marked, about 5-fold reduction of NDR1 kinase activity for the T444A mutant. These facts suggest that both residues are necessary for the full-active NDR1 and point to a synergy between these two residues in activation of NDR1. The outstanding importance of these residues is not surprising because similar observations were made also for other AGC group kinases (for review, see Refs. 21 and 22). Based on structural analysis, the PKA activation segment residue Thr-197 was found in its phosphorylated form to align the catalytic site of that enzyme, thereby generating an active kinase conformation (23). On the other hand, the recently resolved structure of PKB\(\beta\) confirmed the crucial role of the hydrophobic motif with its residue Ser-474 which, upon phosphorylation, undergoes a series of interactions with \(\alpha\)B and \(\alpha\)C helices of the catalytic domain, thereby promoting the disorder to order transition of this part of molecule with concomitant restructuring of the activation segment and reconfiguration of the kinase bivalent structure (24, 47).

Notably, we also observed a strongly compromised capability of the NDR1T74A mutant to be significantly autophosphorylated and activated by Ca\(^{2+}\)/S100B. As mentioned above, this residue is a part of the S100B binding domain of NDR1 (amino acids 62–86), and it is broadly conserved among protein kinases of the NDR group from different origin (Fig. 6A). However, we cannot unambiguously distinguish between the possibility that solely a presence of a threonine or serine residue at this position is necessary for activation of NDR1 by Ca\(^{2+}\)/S100B and the possibility that their phosphorylation is required as well. Nevertheless, the N-terminal domain of NDR1, which is highly conserved in its S100B binding region within the whole NDR subgroup of AGC kinases, seems to exert an autoinhibitory effect on the kinase catalytic domain which can be relieved by binding S100B or other potential interacting proteins. Analogous intramolecular domain-domain interactions are well known for a number of AGC and other kinases, such as PKB (pleckstrin homology domain), PKCs (C1 domain), PRK (HR1 domain), or calmodulin (CaM)-dependent kinases (AID domain). Intriguingly, the mechanism of NDR1 regulation by Ca\(^{2+}\)/S100B is reminiscent of the regulatory features of CaM kinase II, another Ca\(^{2+}\)-controlled kinase, which is known to undergo an intramolecular (intersubunit) autophosphorylation on two threonine residues within its autoinhibitory and a nearby CaM binding domain upon binding of CaM (25). We have demonstrated previously that NDR1 also possesses the capacity to interact with CaM, although to a lesser extent than S100B, but CaM failed to activate NDR1 (12). Notably, the S100B relative S100A1 and, in part, S100B itself, are known to activate through a direct interaction the invertebrate giant sarcomeric kinase twitchin and its vertebrate counterpart titin; however, the exact molecular mechanism of how this activation occurs has not been elucidated so far (26, 27).

As we reported earlier, the Ser-281 and Thr-444 residues become phosphorylated also in vivo (13). However, it was not known how these two regulatory sites are targeted in living cells. Here, we report that the Ser-281 site is an autophosphorylation residue in vivo because the kinase-inactive K118A mutant did not get phosphorylated on this position, i.e. the kinase activity of NDR1 is indispensable for the phosphoryla-
activation segment and hydrophobic motif residues within the AGC family. The identical residues are the conservation of S100B binding region is shown among several NDR-related kinases from different origin as well as conservation of parts of activation segment and hydrophobic motif residues within the AGC family. The identical residues are boxed in black, and similar residues are in gray. The phosphorylation sites are in bold. B, model of NDR1 regulation. NDR1 becomes autophosphorylated on the three indicated residues, Thr-74, Ser-281, and Thr-444 in vitro. However, only Ser-281 is an autophosphorylation site in vivo. In contrast, Thr-444 is targeted mainly by an as yet unidentified upstream kinase. Nevertheless, both phosphorylation events are Ca\(^{2+}\)-dependent and essential for NDR1 activity in vivo. Although Thr-74 is also a minor autophosphorylation residue in vitro, it is currently not clear whether Thr-74 becomes (auto)phosphorylated also in vivo. Nonetheless, this residue appears to be a crucial component of the S100B binding domain located in N terminus of NDR1 (for details, see ‘Discussion’).

The phosphorylation sites are in bold. B, model of NDR1 regulation. NDR1 becomes autophosphorylated on the three indicated residues, Thr-74, Ser-281, and Thr-444 in vitro. However, only Ser-281 is an autophosphorylation site in vivo. In contrast, Thr-444 is targeted mainly by an as yet unidentified upstream kinase. Nevertheless, both phosphorylation events are Ca\(^{2+}\)-dependent and essential for NDR1 activity in vivo. Although Thr-74 is also a minor autophosphorylation residue in vitro, it is currently not clear whether Thr-74 becomes (auto)phosphorylated also in vivo. Nonetheless, this residue appears to be a crucial component of the S100B binding domain located in N terminus of NDR1 (for details, see ‘Discussion’).
not sensitive to inhibitors of phosphoinositide 3-kinase, \(^4\) it is rather unlikely that Ser-473 kinase will be the same protein as the hydrophobic motif kinase phosphorylating Thr-444 in NDR1. For similar reasons, it is also improbable that NDR1 could be targeted by atypical PKCs such as PKC\(\beta\) II (which curiously possess a phosphate-mimicking, negative-charge glutamate residue in place of Ser/Thr phosphoacceptors). Inactivation of this enzyme and the corresponding signaling pathway.

The Ca\(^{2+}\)-dependent activation of Ca\(^{2+}\)/calmodulin by OA is postulated to target Ca\(^{2+}\)-dependent protein kinases, which includes NDR1. \(^5\) Nevertheless, the Ca\(^{2+}\)-dependent phosphorylation of NDR1 is the result of OA activation of L-type Ca\(^{2+}\) channels, leading to the intracellular free Ca\(^{2+}\) concentration which is postulated to affect the activity of protein kinases. Therefore, it is possible that OA may activate NDR1 indirectly, through some Ca\(^{2+}\)-dependent signaling pathways. For example, OA can activate the expression of Ca\(^{2+}\)/calmodulin-dependent protein kinases, which are involved in the regulation of the phosphatidylinositol signaling pathway. It was also postulated that OA may activate NDR1 directly through the Ca\(^{2+}\)-dependent phosphorylation of NDR1, but this remains to be demonstrated.

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\(^4\) S. J. Bichsel, unpublished results.

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