Mechanism of Activation of NDR (Nuclear Dbf2-related) Protein Kinase by the hMOB1 Protein*

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NDR (nuclear Dbf2-related) kinase belongs to a family of kinases that is highly conserved throughout the eukaryotic world. We showed previously that NDR is regulated by phosphorylation and by the Ca\textsuperscript{2+}-binding protein, S100B. The budding yeast relatives of Homo sapiens NDR, Cbk1, and Dbf2, were shown to interact with Mob2 (Mps one binder 2) and Mob1, respectively. This interaction is required for the activity and biological function of these kinases. In this study, we show that hMOB1, the closest relative of yeast Mob1 and Mob2, stimulates NDR kinase activity and interacts with NDR both in vivo and in vitro. The point mutations of highly conserved residues within the N-terminal domain of NDR reduced NDR kinase activity as well as human MOB1 binding. A novel feature of NDR kinases is an insert within the catalytic domain between subdomains VII and VIII. The amino acid sequence within this insert shows a high basic amino acid content in all of the kinases of the NDR family known to interact with MOB proteins. We show that this sequence is autoinhibitory, and our data indicate that the binding of human MOB1 to the N-terminal domain of NDR induces the release of this autoinhibition.

NDR\textsuperscript{1} kinase belongs to a highly conserved family of kinases, a subclass of the AGC family of protein kinases (1, 2). The NDR family consists of the mammalian protein kinases NDR1 and NDR2, Drosophila melanogaster NDR, Caenorhabditis elegans SAX1, mammalian, D. melanogaster and C. elegans large tumor suppressor kinases, Neuronal Clara cell COT1, Ustilago maydis UKC1, Saccharomyces cerevisiae Cbk1, Dbf2, and Dbf20, Schizosaccharomyces pombe Orb6 and Sid2, and several plant kinases (1, 2). These kinases share a high sequence conservation, and some possess conserved functions, mainly involving regulation of cell morphology and the cell cycle (2–15).

The kinase domain sequence of NDR is related to that of other members of the AGC group of kinases, e.g. protein kinases A, B, C, and G, PRK, p70\textsuperscript{S6K}, p90\textsuperscript{RSK}, and phosphoinositide-dependent kinase 1 (1). The catalytic domain of the NDR catalytic domain as described by Hanks and Hunter (16). However, the catalytic domains of all of the members of the NDR family are interrupted by an insert of 30–60 amino acids between subdomains VII and VIII. This inserted sequence is not well conserved but is always rich in the basic amino acids, arginine and lysine. The catalytic domain insert has been shown to act as a non-consensus nuclear localization signal in the case of NDR1. NDR1 localizes predominantly to the nucleus in COS-1 cells, whereas mutant NDR1 with a deletion in the insert is localized to the cytosol (17). An additional special feature of the NDR family of kinases is a highly conserved N-terminal domain. In the case of NDR1, this domain consists of 81 amino acids and encompasses a region predicted to form an amphipathic \alpha-helix that binds to the EF-hand Ca\textsuperscript{2+}-binding protein, S100B (18). Finally, the C-terminal extension of NDR kinase contains a broadly conserved hydrophobic motif phosphorylation site that is an important regulatory site in the AGC group of kinases (19).

NDR kinase is efficiently (20–100-fold) activated upon treatment of cells with the protein phosphatase 2A inhibitor, okadaic acid (OA). OA treatment induces the phosphorylation of the activation segment site, Ser-281, and in vivo and in vitro. NDR activation is Ca\textsuperscript{2+}-dependent as shown by the treatment of COS-1 cells with the Ca\textsuperscript{2+} chelator, BAPTA-AM, which abolishes NDR activation. It has been shown that the EF-hand Ca\textsuperscript{2+}-binding protein, S100B, activates NDR in vitro. NDR activation is Ca\textsuperscript{2+}-dependent as shown by the treatment of COS-1 cells with the Ca\textsuperscript{2+} chelator, BAPTA-AM, which abolishes NDR activation. It has been shown that the EF-hand Ca\textsuperscript{2+}-binding protein, S100B, binds to the N-terminal domain of NDR in vivo and in vitro and that Ca\textsuperscript{2+}/S100B activates NDR in vitro. S100B induces increased autophosphorylation on Ser-281. During investigations of the mechanism of S100B-induced autophosphorylation, a third autophosphorylation site, Thr-74, in the N-terminal domain was discovered (21). This site is also crucial for NDR activation, because its mutation to alanine affected NDR activity in vivo.

The results of several recent studies indicate a novel activated signaling pathway involving NDR kinase family members. It has been shown in D. melanogaster that NDR genetically interacts with FURRY, a 300-kDa protein of unknown function (22). In S. cerevisiae, the FURRY relative, Tao3/Pag1, lies on the Cbk1 pathway. Furthermore, Tao3/Pag1 and Cbk1 interact physically and their localization is interdependent (23). In S. pombe, the FURRY-like protein, Morn2/Cps12, interacts physically with Orb6, the S. pombe NDR orthologue (24). The FURRY-like proteins are conserved in mammals, and thus, it is likely that other proteins interacting genetically and/or physically with S. cerevisiae Cbk1 or Dbf2 also play a role in the NDR kinase family pathway in higher eukaryotes. Most of

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1 The abbreviations used are: NDR, nuclear Dbf2-related (for abbreviations of other kinases see Ref. 1); BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid tetraacetoxymethylester, h, human; GST, glutathione S-transferase; HA, hemagglutinin; OA, okadaic acid; SMA, S100B and MOB association; AIS, autoinhibitory sequence.
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these proteins are fairly well conserved throughout evolution. S. cerevisiae Mob1 is a member of the mitotic exit network (25, 26). Dbf2 associates with Mob1, and Mob1 is required for phosphorylation and activation of Dbf2 (27). S. cerevisiae Mob2, a close relative of Mob1, is a member of the Cbk1 pathway. Mob2 is required for the biological function of Cbk1 in the motherdaughter separation after cytokinesis and maintenance of polarized cell growth. Mob2 associates physically with Cbk1, and Cbk1 kinase activity is dependent on Mob2. Furthermore, Mob2 and Cbk1 show interdependent localization (28, 29). Similarly, S. pombe Mob2 interacts physically with the protein kinase Orb6 and is required for Orb6 function in the coordination of cell polarity with the cell cycle (30). Multicellular organisms possess highly conserved MOB proteins. hMOB1α shares a sequence identity/similarity of 50/65% with S. cerevisiae Mob2 and of 57/78% with S. pombe Mob1. The human MOB protein family consists of two almost identical proteins, hMOB1α and hMOB1β (NCBI accession numbers Gi8922671 and 27735029), sharing a sequence identity/similarity of 95/97%; a more distantly related protein, hMOB2 (NCBI accession numbers Gi38091156), that is 41/60% identical/similar to hMOB1α; three other related proteins, hMOB3 α, β, and γ (Gi18677731, 41350330, and 38091155), with an identity/similarity of ~50/73% to hMOB1α; and the weakly similar protein, phocein (Gi41349451), that is 24/45% identical/similar to hMOB1α. Because the nomenclature of MOB proteins in the data bases is rather confusing, we use the above terminology based on homology as also proposed recently by Stravridi et al. (31). To date, no functional domains have been identified in the MOB proteins and the hMOB proteins have no known functions. It has been shown that the MOB relative, phocein, interacts with the protein phosphatase 2A regulatory subunit, striatin, and with proteins involved in vesicular traffic (32, 33).

Here, we characterize the interaction of hMOB1α, the closest relative of yeast Mob1 and Mob2, with human NDR kinase. We show that hMOB1 binding is dependent on the N-terminal domain of NDR and that hMOB1 stimulates NDR kinase activity both in vivo and in vitro. Furthermore, we show that a basic sequence within the insert in the catalytic domain of NDR has an autoinhibitory function and that hMOB1 may stimulate NDR activity by releasing the autoinhibitory effect of this sequence.

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, and 100 μg/ml streptomycin. Cells were transfected at the subconfluent stage with FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer’s instructions. In some experiments, the cells were treated for 80 min with 1 μM OA in 0.1% N,N-dimethylformamide or 50 μM BAPTA-AM in 0.1% Me2SO 48 h after transfection.

Plasmids—Mammalian expression vector pCMV5 encoding HA-tagged NDR1 was described previously (21). pCMV5-hMOB1α was constructed by PCR using the Image clone 4854541 (BG 754690) as template and the primers 5′-GGG GTA CCA CCA TGG AGA AAC TCA TCT CGT AAG AGG ATC TGA GCT TCT TCT TCA GCA GCC GCT C-3′ and 5′-GCT GTA GAC ATT ATT CTG CTT TTT GAT CCA AGT ATC ATT TAT TGA TGA AGG-3′ and subcloned into the KpnI and XbaI sites of the vector. pGEX2T-hMOB1 was constructed by PCR using the primers 5′-GGG GTA CCA CCA TGG ATC TCA GCT TCT TCT TCA GCA GCC GCT C-3′ and 5′-GTT CTC CAG CAT GTA TCT CTT GTC CCA TAA AAT TTA TGA GAG GTT TTT GTA TCT GTA TCT TAT TAA TTT GTA AAG AGG-3′ and subcloned into the BamHI and XhoI sites of the vector. For the bacterial production of the NDR protein kinase, NDR2 was fused to a capsid-stabilizing protein of lambdoid phage 21 (SHP). The cloning details and vector maps are available upon request. pCMV5 HA-NDR1 and pSHP-NDR2 point mutations were generated from wild-type vectors using the QuickChange site mutagenesis protocol (Stratagene) and the appropriate primers (primer sequences are available upon request). The sequences of all of the plasmids were confirmed by DNA sequencing.

Antibodies—Phosphorylated anti-Ser-281 and anti-Thr-444 antibodies were as described previously (21). Phosphorylated anti-Thr-74 rabbit polyclonal antiserum was raised against the synthetic peptide, AHARKEPTPOEFLRKL. The 12CA5 (HA) and the 9E10 (Myc) monoclonal antibody hybridomas supernatants were used for detection of HA-NDR1 and Myc-hMOB1. Anti-GST-NDR polyclonal antibody was as described previously (17).

Western Blotting—To detect HA-NDR, SHP-NDR, and Myc-hMOB1, samples were resolved by 10 or 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes. 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 then probed overnight at 4 °C with anti-HA modified NDR rabbit polyclonal antibody, 12CA5 (HA) monoclonal antibody supernatant, 9E10 anti-Myc monoclonal antibody supernatant, anti-Thr-444P, anti-Thr-74P. Bound antibodies were detected with horseradish peroxidase-linked secondary antibodies, or Myc-hMOB1 in HA immunoprecipitations were detected with horseradish peroxidase-conjugated protein A/G and ECL.

Bacterial Expression of Human GST-fused AMO1 and Human Schizosaccharomyces NDR2—XL-1 Blue Escherichia coli was transformed with the pGEX-2T-hMOB1 plasmid. Mid-logarithmic phase cells were induced with 0.1 mM isopropyl β-D-thiogalactopyranoside overnight at 20 °C. Bacteria were disrupted using a French press in the presence of 1 mg/ml lysozyme, and the fusion proteins were purified on glutathione-agarose. SHP-NDR2 wild-type and mutant plasmids were transformed into XL-1 Blue E. coli, and the protein was produced as described for GST-NDR1 and purified on glutathione-agarose.

HA-NDR Kinase Assay—Transfected COS-1 cells were washed once with ice-cold phosphate-buffered saline and harvested in 1 ml of ice-cold phosphate-buffered saline containing 1 mM Na3VO4 and 20 mM β-glycerophosphate before lysis in 500 μl of IP buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM Na3VO4, 20 mM β-glycerophosphate, 1 μM microcystin, 50 mM phenylmethylsulfonyl fluoride, 4 μM leupeptin, and 1 μM benzamidine). Lysates were centrifuged at 20,000 × g for 20 min, and duplicate aliquots (250 μg) of the 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. The beads were then washed twice with IP buffer, once with 10 mM IP buffer containing 1 μM Na3VO4, and finally twice with 20 mM Tris-HCl, pH 7.5, containing 4 μM leupeptin and 1 mM benzamidine. Thereafter, the beads were resuspended in 30 μl of buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol, 100 μM γ-[32P]ATP (~1,000 cpm/pmol), 1 μM cAMP-dependent protein kinase inhibitor peptide, 4 μM leupeptin, 1 μM benzamidine, 1 μM microcystin, and 1 mM NDR1 substrate peptide (KKRRNRLSLVA). After a 60-min incubation at 30 °C, the reactions were processed as described previously (21).

SHP-NDR Kinase Assays—1 μg of purified recombinant SHP-NDR wild type and mutants (without further treatment or pre-autophosphorylation in the presence of 10 μM GST-hMOB1 or GST) were assayed in a 30-μl reaction mixture containing 20 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol, 100 μM γ-[32P]ATP (~1,000 cpm/pmol), and 1 mM NDR1 substrate peptide (KKRRNRLSLVA). After incubation at 30 °C, the reactions were processed and kinase activity was determined as described for the HA-NDR kinase assay.

Immunoprecipitations—Cos-1 cells transfected with HA-NDR wild type or mutants and Myc-hMOB1 were harvested as described above. Cell lysate protein (0.5 mg) was precleared with protein A- or G-Sepharose and mixed subsequently for 3 h at 4 °C with 12CA5 antibody prebound to protein A-Sepharose or with 9E10 antibody prebound to protein G-Sepharose. The beads were then washed twice with IP buffer, once with IP buffer containing 1 μM NaCl, once again with IP buffer, and finally twice with 20 mM Tris-HCl, pH 7.5, containing 4 μM leupeptin and 1 mM benzamidine. Samples were resolved by 12% SDS-PAGE, and Myc-hMOB1 and HA-NDR were detected by Western blotting.

GST Pull-down Assay—25 μg aliquots of GST or GST-hMOB1 were incubated with glutathione-Sepharose for 2 h at 4 °C. The beads were washed three times with Tris-buffered saline, then 3-μl aliquots of SHP-NDR wild type or mutants were added and incubated for 3 h at 4 °C. The beads were washed five times with Tris-buffered saline and resuspended in 30 μl of 1× SDS sample buffer, and the samples were resolved by 12% SDS-PAGE. NDR bound to GST-hMOB1 was detected by Western blotting.
Activation of NDR Kinase by hMOB1

**RESULTS**

hMOB1 Activates NDR—Previous work by other groups has shown in yeast that Mob1 and Mob2 are required for the activity of the NDR-related kinases Dbf2 and Cbk1, respectively (26, 27). To test whether hMOB1 also plays a role in NDR activation, we cotransfected Myc-hMOB1 or the empty vector with HA-NDR1 into COS-1 cells. The cells were treated with the protein phosphatase 2A inhibitor, OA. At day 3, OA is the only known potent activator of NDR in vivo (20, 21) and it has been shown that yeast Mob1 becomes phosphorylated after OA treatment (27). The kinase activity of immunoprecipitated HA-NDR1 was stimulated 35-fold by a 45-min treatment of 1 μM OA (Fig. 1A). Co-expression of Myc-hMOB1 induced an additional 2–3-fold increase in NDR kinase activity (Fig. 1A). At the 45-min OA time point, HA-NDR1 co-expressed with Myc-hMOB1 was stimulated 100-fold compared with the control. This indicates a role for hMOB1 in NDR activation. To test whether hMOB1 acts directly on NDR, we performed in vitro kinase assays of bacterially expressed SHP-NDR2 in the presence of GST-hMOB1 or GST. We used NDR2 for in vitro experiments, because it can be readily produced in sufficient amounts. NDR2, which is 86% identical to NDR1, has been shown to be regulated in the same way as NDR1 (34). Furthermore, we confirmed with NDR1 produced in SF9 cells that wild-type NDR1 behaves in vitro similar to NDR2 with respect to hMOB1 (data not shown). GST-hMOB1 stimulated SHP-NDR2 autophosphorylation ~2-fold (data not shown). Furthermore, SHP-NDR2, which was pre-autophosphorylated in the presence of GST-hMOB1, has up to a 6-fold higher kinase activity against the NDR substrate peptide than SHP-NDR pre-autophosphorylated in the presence of GST (Fig. 1B). These results show that hMOB1 has a direct positive effect on NDR kinase activity. We tested the effect of hMOB1 on the phosphorylation state of the NDR phosphorylation sites Thr-74, Ser-281, and Thr-444 (Thr-75, Ser-282, and Thr-442 in the case of NDR2; the phosphorylation sites of NDR2 were recognized by phosphospecific antibodies generated against the corresponding phosphorylation sites of NDR1). SHP-NDR2 phosphorylation on the autophosphorylation sites Ser-282 and Thr-75 was slightly increased, whereas Thr-442 of SHP-NDR2, which is known to be targeted by an upstream kinase in vivo, showed no autophosphorylation (Fig. 1B).

hNDR1 Interacts with hMOB1—To investigate whether hMOB1, the closest relative of yeast MOB2 and MOB1 in mammals, is able to interact with NDR1, epitope-tagged NDR1 and hMOB1 were cotransfected into COS-1 cells. In co-immunoprecipitation experiments of Myc-hMOB1 with HA-NDR1 (Fig. 2A) as well as those of HA-NDR1 with Myc-hMOB1 (Fig. 2B), hMOB1 associated with NDR1. The protein level of Myc-hMOB1 was dramatically increased upon OA stimulation, whereas Myc-hMOB1 transfected without NDR1 was present at similar levels in OA-treated and OA-untreated cells. We did not address the molecular basis of this phenomenon. The kinase-dead mutant with the mutated catalytic lysine in the ATP binding site, K118A, was still able to interact with hMOB1 after OA stimulation (Fig. 2A). Thus, the interaction was not dependent on NDR kinase activity. Furthermore, we tested whether the two important in vivo phosphorylation sites of NDR, Ser-281 and Thr-444, as well as a recently identified in vitro autophosphorylation site, Thr-74, play a role in NDR-MOB interaction. The NDR mutants S281A and T444A still interacted with hMOB1 after OA stimulation, whereas the T74A mutant showed almost complete absence of interaction with hMOB1 (data not shown).

NDR-hMOB1 Interaction Depends on OA-induced Modification on hMOB1, whereas Phosphorylation of NDR Is Not Required—Myc-hMOB1 interacted with HA-NDR1 in OA-treated COS-1 cells, but it was not clear whether OA acted just by increasing the Myc-hMOB1 protein level or whether OA-induced modification of NDR1, hMOB1, or both promoted the interaction. To address this question, we transfected HA-NDR1 and Myc-hMOB1 separately into COS-1 cells and stimulated them with 1 μM OA or left them unstimulated. We then pooled the lysates (NDR1 ± OA with hMOB1 ± OA) and immunoprecipitated with α-HA antibody. In the two combinations containing Myc-hMOB1 of OA-treated cells, Myc-hMOB1 strongly associated with HA-NDR, regardless of whether the HA-NDR was expressed in OA-treated or unstimulated cells. Myc-hMOB1 from unstimulated cells showed only a weak interaction with HA-NDR from both unstimulated and OA-stimulated cells (Fig. 2C). Thus, OA-induced phosphorylation of NDR is not required for interaction but hMOB1 modification is necessary. Furthermore, GST-hMOB1 was phosphorylated by immunoprecipitated HA-NDR. The phosphorylation of GST-hMOB1 increased 3–4-fold when HA-NDR was immunoprecipitated from OA-stimulated COS-1 cells. However, the phosphorylation of GST-hMOB1 was also observed when kinase-dead HA-NDR was immunoprecipitated (Fig. 2D). We conclude that a kinase that co-immunoprecipitates with NDR is able to phosphorylate hMOB1.
**The Highly Conserved N-terminal Domain of NDR Is Required for Kinase Activation**—The N-terminal regulatory domain of NDR kinase is highly conserved in the closest relatives of NDR throughout the eukaryotic world (Fig. 3A). Several residues are completely invariant throughout evolution from single cell organisms to humans. This prompted us to test the functional significance of these residues with respect to NDR kinase activity. Mutations of the highly conserved residues induced strong inhibition of OA-stimulated kinase activity (Fig. 3B). The first part of the N-terminal domain covering amino acids 1–33 and containing a predicted β-sheet in hNDR proved to be important for kinase activation. The deletion of the first 30 amino acids completely abolished kinase activation (data not shown). The point mutations in this region strongly reduced kinase activity. The mutation of Thr-16, Glu-18, and Glu-28 reduced activity to ~40%, whereas the mutation of Lys-24 and Tyr-31 reduced activity to ~20%. Mutation to alanine of Arg-41, Arg-44, or Leu-48, all of which lie in a predicted first α-helix covering the amino acids 40–55 and are situated close together on the same side of the predicted α-helix, reduced kinase activity to below 20% of wild-type activity. Mutation of the residues in a predicted second α-helix situated in the previously described S100B binding region of NDR and covering amino acids 60–80 also led to the inhibition of kinase activity. Mutation of Lys-72, Glu-73, Thr-74, Arg-78, and Leu-79 to alanine reduced kinase activity to 20% or lower. Taken together, the results imply that the high conservation of the N-terminal domain in the following termed SMA (S100B and MOB Association) domain is due to an absolute requirement of the conserved residues for proper kinase function, either by ensuring the correct structural conformation of the protein or being directly involved in binding to interacting proteins.

**The SMA Domain Is Required for NDR-MOB Interaction**—Thr-74, which was previously shown to be important for NDR kinase activity and interaction with S100B, also turned out to be required for NDR-hMOB1 interaction in COS-1 cells. Therefore, we investigated the involvement of the conserved residues of the SMA domain in NDR-hMOB1 interaction. Co-transfection of HA-tagged NDR mutants and Myc-hMOB1 and subsequent co-immunoprecipitation of Myc-MOB with HA-NDR revealed that several of the conserved residues important for kinase activation are also required for NDR-hMOB1 interaction (Fig. 4, A–C). Tyr-31, Arg-41, Thr-74, and Arg-78 seem to be absolutely required for interaction, whereas the Lys-24, Arg-44, and Leu-79 mutants displayed reduced interaction. Only three of the mutants with a strongly reduced kinase activity (Arg-48, Lys-72, and Glu-73) showed no decrease in the ability to bind hMOB1.

**Tyr-32 of NDR2 Kinase Is Essential for Activation by hMOB1 in Vitro**—The residues Tyr-31, Arg-41, Arg-44, Thr-74, and Arg-78 of NDR1 were shown to be important for kinase activation and interaction with hMOB1 in COS-1 cells. We examined whether these residues are involved directly in the interaction of NDR and hMOB1 or indirectly by influencing interactions with other proteins in the cells. We used bacterially produced SHP-NDR2, wild type, and the mutants Y32A, R42A/R45A, E74A, T75A, and R79A (corresponding to the NDR1 mutants Y31A, R42A/R45A, E73A, T74A, and R78A) together with GST-hMOB1 protein to perform *in vitro* kinase assays to measure the direct effect of hMOB1 on NDR kinase activity (Fig. 5A). The mutation of Glu-74, a residue that affects kinase activity but not interaction with hMOB1 in COS-1 cells, led to a reduction in kinase basal activity *in vitro*. This very highly conserved Glu-74 seems to have an important intrinsic role for the function of NDR kinase, although it is not involved in the binding to
hMOB1. The mutation of residues Arg-42 and Arg-45, which lie on the same side of a predicted α-helix and whose mutation affects NDR-hMOB1 interaction as well as kinase activation in COS-1 cells, led to a reduction in kinase activity in vitro but did not completely abolish the activation by hMOB1. NDR with mutated Tyr-32 showed an intact basal kinase activity but was not activated by hMOB1 in vitro at all, pointing to an important role for this residue in the direct interaction with hMOB1. The activation of the T75A and the R79A mutants by hMOB1 was not different to wild-type NDR, suggesting that Thr-75 and Arg-79 are not directly involved in binding to hMOB1 and do not affect the kinase activity of NDR per se. Furthermore, the pull-down assays showed that NDR2 with mutated Tyr-32 does not bind to hMOB1, whereas the binding of NDR2 with mutated Arg-42 and Arg-45 is not abolished (Fig. 5B).

The Insert in the Catalytic Domain Acts as an Autoinhibitory Sequence—A common feature of the NDR family of kinases is an insert in the catalytic domain of 30–60 amino acids between subdomains VII and VIII. The insert sequence is not highly conserved but contains in all cases many positively charged amino acids. Therefore, we tested the importance of these positive residues for NDR activity and NDR-hMOB1 interaction. Mutating the amino acid sequence265KRKAETWKRNRR276 to amino acid sequence265AAAAETWAANRR276 to amino acid sequence increased both the kinase activity and phosphorylation of Thr-74, Ser-281, and Thr-444 in COS-1 cells (Fig. 6, A and B) but did not affect NDR-MOB binding (data not shown). To test whether this insert sequence has an autoinhibitory function, we produced recombinant SHP-NDR2 insert mutant and tested its in vitro activity. NDR with the mutated insert sequence had a 4–6-fold...
elevated basal kinase activity, similar to hMOB1-stimulated NDR2, proving that the insert sequence is autoinhibitory. Moreover, NDR2 with a mutated autoinhibitory sequence (AIS) was only slightly (~1.5-fold) stimulated by hMOB1. Furthermore, we examined the effect of the combined mutation of Tyr-32 and the AIS. The mutation of Tyr-32, which abolishes the binding of hMOB1 to NDR, did not affect the activity of the AIS mutant NDR2, and the AIS Y32A mutant was not activated by hMOB1 (Fig. 6C). Altogether, these results indicate that hMOB1 binding induces the release of the autoinhibition caused by the AIS.

The Ca\(^{2+}\) Chelator BAPTA-AM Reduces NDR-MOB Interaction in Cells, but the Action of hMOB1 on NDR in Vitro Is Ca\(^{2+}\)-independent—Previously, we showed that treatment of COS-1 cells with the Ca\(^{2+}\) chelator BAPTA-AM reduces OA-induced NDR kinase activation (21). Therefore, we tested whether the treatment of COS-1 cells with BAPTA-AM influences NDR-hMOB1 interaction. In co-immunoprecipitation experiments of Myc-hMOB1 with HA-NDR of BAPTA- and OA-treated cells, BAPTA-AM reduced NDR-hMOB1 interaction corresponding to the observed reduction in NDR kinase activation (Fig. 7A). Therefore, we tested whether Ca\(^{2+}\) influences the action of hMOB1 on NDR \textit{in vitro}. The addition of Ca\(^{2+}\) to NDR \textit{in vitro} kinase assays in the presence or absence of GST-hMOB1 had no effect on NDR kinase activity (Fig. 7B). Thus, Ca\(^{2+}\) has no direct influence on NDR-hMOB1 interaction.

**DISCUSSION**

We found that NDR kinase is activated by and interacts with hMOB1 \textit{in vitro} and in COS-1 cells. MOB proteins, similar to NDR family kinases, are highly conserved throughout the eukaryotic world. hMOB1 is the closest relative of yeast Mob1 and Mob2. The yeast NDR family kinases, Dbf2 and Cbk1, were both shown to interact with yeast Mob1 and Mob2. The finding that hMOB1 activates and interacts with NDR supports the existence of a highly conserved signaling pathway. The interaction of NDR and hMOB1 in COS-1 cells increased when the cells were treated with OA. We have shown that the interaction of NDR1 and hMOB1 in cell lysates depends on an OA-induced modification of hMOB1 but not that of NDR. A possible explanation for this is that hMOB1 is sequestered by interaction with another protein and hMOB1 is released upon OA treatment and interacts with NDR. Therefore, the observed activation of NDR by OA may not be due solely to the direct inhibition of Ser-281 and Thr-444 dephosphorylation and activation of the pathway leading to Thr-444 phosphorylation but also to an increase in hMOB1 interaction with NDR. The observed phosphorylation of GST-hMOB1 by immunoprecipitated wild-type and kinase-dead HA-NDR1 suggests that OA stimulates a kinase that phosphorylates hMOB1 and is in a complex with NDR. It is conceivable that this phosphorylation is responsible for the binding of NDR to hMOB1 in COS-1 cells. There is now a need to identify the phosphorylation sites on hMOB1 and the kinase that phosphorylates hMOB1. We showed previously that NDR activation depends on Ca\(^{2+}\) (21). The treatment of COS-1 cells with the Ca\(^{2+}\) chelator
BAPTA-AM led to a decrease in NDR-hMOB interaction. However, Ca\textsuperscript{2+}/H11001 had no effect on hMOB1-stimulated NDR activity in vitro. Therefore, Ca\textsuperscript{2+} may be required for MOB modification in vivo but it plays no role in the direct activation of NDR by MOB.

Several residues within the N-terminal SMA domain of NDR are important for NDR-hMOB1 interaction and for the kinase activation in vivo. On the other hand, only one of these residues, Tyr-32, proved to be important for interaction with and activation by hMOB1 in vitro. In contrast, it has been shown by NMR studies that Xenopus laevis MOB1 interacts with a synthetic peptide covering the S100B binding region of NDR (35). This region also contains residues Thr-74 and Arg-78, which are important for NDR activation and interaction with hMOB1 in vivo. Furthermore, the previously resolved crystal (31) and NMR (35) structures of hMOB1 and X. laevis MOB1, respectively, revealed that MOB1 has a negatively charged and exposed potential interaction surface. Thus, it is likely that positively charged residues of the NDR SMA domain such as Arg-78 are involved in the interaction with hMOB1 but that its mutation is not sufficient to disrupt the interaction with NDR under in vitro conditions. The residues Arg-41 and Arg-44, which lie on the same side of a predicted α-helix, may also participate in the interaction, but their mutation is not sufficient to disrupt the interaction in vitro. However, the mutation of Tyr-32 might disrupt the overall structure of the SMA domain, thereby disabling the interaction with hMOB1, or Tyr-32 of NDR might interact directly with hMOB1.

Although the sequence of the insert in the kinase catalytic domain between subdomains VII and VIII is not well conserved between NDR and the yeast kinases Cbk1 and Dbf2, they all have a sequence with a high basic amino acid content. Because this sequence is located just in front of the activation segment phosphorylation site, the question of whether it has a regula-
family (hMOB1 (17). In yeast, Mob2 is important for the localization of the NDR nuclear localization signal in COS-1 cells in the case of NDR1. It was suggested previously that the binding of yeast Mob1 to a phobic motif upstream kinase. In accordance with this finding, disruption of autoinhibition also facilitated phosphorylation by the hydrophobic motif upstream kinase. In contrast, Mob2 binds to the release of the autoinhibition caused by the AIS. Crystallographic studies of MOB-bound and MOB-unbound NDR suggest for the mechanism of OA-induced NDR kinase activation. It was reported that hMOB2 interacts with NDR1 and NDR2 from Jurkat cells and that hMOB2 stimulates NDR kinase activity (36).

S100B, a previously described activator of NDR, is constitutively bound to NDR in cells independent of OA stimulation (21). S100B may constitutively maintain the correct conformation of the SMA domain. It has been shown recently that the NDR-derived S100B-binding peptide adopts its helical conformation after binding to S100B (37). Thus, a high concentration of S100B, a previously described activator of NDR, is constitutively maintained. In contrast, Mob1 in certain cell types (for example, melanoma cells (18)) may lead to constitutively elevated NDR activity. In contrast, MOB proteins may transmit a signal by fluctuation of the MOB protein level during the cell cycle as is reported for S. cerevisiae Mob1 (38) and/or by post-translational modification of MOB that promotes the interaction with NDR as we have suggested for the mechanism of OA-induced NDR kinase activation.

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