NDR2 Acts as the Upstream Kinase of ARK5 during Insulin-like Growth Factor-1 Signaling*

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ARK5 is a tumor progression-associated factor that is directly phosphorylated by AKT at serine 600 in the regulatory domain, but phosphorylation at the conserved threonine residue on the active T loop has been found to be required for its full activation. In this study, we identified serine/threonine protein kinase NDR2 as a protein kinase that phosphorylates and activates ARK5 during insulin-like growth factor (IGF)-1 signaling. Upon stimulation with IGF-1, NDR2 was found to directly phosphorylate the conserved threonine 211 on the active T loop of ARK5 and to promote cell survival and invasion of colorectal cancer cell lines through ARK5. During IGF-1 signaling, phosphorylation at three residues (threonine 75, serine 282, and threonine 442) was also found to be required for NDR2 activation. Among these three residues, phosphorylation of serine 282 seemed to be the most important for NDR2 activation (the same as for the mouse homologue) because its aspartic acid-converted mutant (NDR2/S282D) induced ARK5-mediated cell survival and invasion activities even in the absence of IGF-1. As in the mouse homologue, threonine 75 in NDR2 was required for interaction with S100B, and binding was in a calcium ion- and phospholipase C-dependent manner. We also found that PDK-1 plays an important role in NDR2 activation especially in the phosphorylation of threonine 442. Based on the results of this study, we report here that NDR2 is an upstream kinase of ARK5 that plays an essential role in tumor progression through ARK5.

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ARK5, also known as KIAA0537/NUAK1, is a member of the AMP-activated protein kinase (AMPK) catalytic subunit family and is closely involved in tumor progression, e.g., in colorectal cancer and multiple myeloma, upon stimulation with insulin-like growth factor (IGF)-1 (1-4). ARK5 is unique among AMPK catalytic subunit family members in that its activation is directly regulated by AKT through the phosphorylation of a serine residue on the regulatory domain (1). AKT-activated ARK5 promotes tumor invasion and metastasis through induction of membrane-type-I matrix metalloproteinase (2), and it protects tumor cells from cell death caused by nutrient starvation (1,5) and activation of death receptors, such as tumor necrosis factor-α (5), tumor necrosis factor-related apoptosis-inducing ligand receptor (5), and FAS/CD95 (6), as a result of an inactivation of caspase-6 (6). We recently detected a stage-dependent increase in the amount of ARK5 mRNA in clinical specimens from patients with colorectal cancer (3), and we have demonstrated that expression of ARK5 mRNA is strongly correlated with a poor prognosis of multiple myeloma (4). Thus, ARK5 appears to be an important factor for tumor progression.

Serine/threonine protein kinase AMPK acts as the metabolic sensor in a wide variety of species, for example in mammalian cells, insect cells, and yeast, and its activation has usually been observed in cells exposed to metabolic stress, such as hypoxia and nutrient starvation, and in skeletal muscle cells during exercise (7-10). In addition to metabolic stress, AMPK activation has also been observed in cells stimulated with leptin (11) and adiponectin (12), and thus its molecular regulation has been the focus of diabetes and obesity research (11,12). In addition to their role in these disease states, we recently reported that AMPKs and related kinases play an essential role in tumor progression (1,13-15). AMPKs, especially AMPK-α1, have been reported to be activated by the upstream kinase through the phosphorylation of the threonine residue on the active T loop. Calmodulin-dependent protein kinase kinases (CaMKks) have been reported to be the upstream kinases that activate AMPKs (16,17). Another kinase was recently purified as a result of yeast studies, and serine/threonine protein kinase LKB1 has been identified as its mammalian homologue (18-21). It has also been reported that LKB1 acts as the master kinase of all AMPK catalytic subunit family members in cells under metabolic stress (22). We and other research groups, however, recently reported LKB1-independent phosphorylation of AMPK catalytic subunit family members in cells stimulated with growth factor (23) or with 5-amino-4-imidazolecarboxamide ribose (24).

The same as other AMPK family members, ARK5 contains a highly conserved active T loop, suggesting the presence of an upstream kinase that phosphorylates the threonine residue in the catalytic domain. Activation of ARK5 by growth factors, as well as metabolic stress, is important to understand the mechanisms of ARK5 regulation under physiological and pathological conditions. In the current study, we therefore attempted to identify the upstream kinase of ARK5 during IGF-1 signaling. Based on the result of a bioinformatic analysis using the active site of CaMKks, we identified a protein kinase as a candidate and demonstrated that the kinase acts as the upstream kinase of ARK5 during IGF-1 signaling. Here we report NDR2 as a novel ARK5 kinase that acts in cells during IGF-1 stimulation.
NDR2, an Upstream Kinase of ARK5

MATERIALS AND METHODS

Cell Lines and Culture—Human colorectal cancer cell lines HCT-116, DLD-1, and SW480 were purchased from ATCC and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Sigma). For transfection, cells were seeded into a 24-well plate at 5 × 10^4/well, and transfection was performed with TransFast transfection reagent (Promega Corp., Madison, WI). The transfection yield was assessed with a green fluorescent protein expression vector, and the yields in the current study were 97.3% for HCT-116 cells, 98% for DLD-1 cells, and 98.6% for SW480 cells.

Chemicals, Antibodies, Plasmids, and Recombinant Proteins—LY294002, BAPTA, genistein, U-73122, and bisindolylmaleimide-1 were purchased from Calbiochem-Novabiochem, and U0126 was purchased from Upstate (Charlottesville, VA). Antibodies against HA, S100B, PDK-1, phospho-AMPK (Thr(P)-172), and phospho-PDK-1 docking motif were purchased from Sigma and Cell Signaling Technology, Inc. (Beverly, MA). S100B protein and human recombinant IGF-1 were purchased from Sigma and Immuno-Biological Laboratories Co., Ltd. (Gunma, Japan). The expression vectors of ARK5 and DN-ARK5, which was prepared by conversion of the AKT phosphorylating serine residue (serine 600) to an alanine residue (1), were used in the experiments. The expression vectors of DN-AKT1 (K179M: ATP-unbinding mutant) were purchased from Upstate.

Preparation of HA-tagged NDR2—HA-tagged NDR2 was prepared from full-length KIAA0965-ligated pBluescript II SK+ vector supplied by KAZUSA DNA Research Institute with LA PCR (Takara Biomedicals, Kyoto, Japan): upstream primer, 5'-GAGGCAAGTTCGGTTACTG-3'ATATTCAATCCTTATGATGTCCTGATTATGCTGCATG-3'; deletion mutant primer-1, 5'-TCCAAAGACT-3'; deletion mutant primer-2, 5'-TCCAAAGACT-3'; downstream primer, 5'-ATGTTGTAAGCTTCTCTTATCGATGTCCTGATTATGCTGCATG-3'. PCR was performed with using mutagenesis primers (deletion mutant primer-1, 5'-CCCTCACAACCTTATACCCAGTCTTTGGA-3'; deletion mutant primer-2, 5'-TCCAAAGACTGTTTCCGTTACTG-3'; downstream primer, 5'-ATGTTGTAAGCTTCTCTTATCGATGTCCTGATTATGCTGCATG-3'). Each PCR product was electrophoresed on a 1% agarose gel and then subjected to in vitro transcription with T7 RNA polymerase. The efficacy of the siRNA was assessed by the disappearance of the target factor in Western blotting and/or RT-PCR procedure. As a control for the siRNA, double-stranded RNA prepared by the in vitro transcription of empty 28i-pLITMUS vector was used as recommended in the instruction manual.

Immunoprecipitation and Western Blotting—Antibody against HA was mixed with 1 mg of protein from each cell extract prepared by lysing cells with 0.1% Nonidet P-40 containing PBS for 30 min. After mixing, protein G-Sepharose was added and incubated for 2 h. The immunoprecipitates were washed six times with 0.1% Nonidet P-40 containing PBS and then heated in SDS sample buffer for Western blotting.

Proteins were prepared for Western blotting analysis by lysing cells for 30 min with 1% Nonidet P-40 containing PBS. All procedures were carried out at 4 °C. Supernatants were collected by centrifugation at 15,000 rpm for 15 min, and their protein concentrations were determined with a BCA protein assay kit (Pierce) and bovine serum albumin as the standard. Sample proteins separated by SDS-PAGE were transferred onto nitrocellulose membranes by a semidyblotting system. The membranes were blocked at room temperature for 1 h with PBS containing 5% (w/v) skim milk, washed with a mixture of PBS and 0.05% Tween 20 (Sigma, Tween-PBS), and then incubated overnight at room temperature with antibody diluted with PBS. After washing with Tween-PBS, the membranes were incubated with 2000-fold diluted biotinylated horseradish peroxidase-conjugated anti-rabbit IgG antibody. The membranes were then washed with Tween-PBS and developed with the ECL system (Amersham Biosciences).

RESULTS AND DISCUSSION

Phosphorylation of Threonine 211 Is Essential for ARK5 Activation during IGF-1 Signaling—We have reported direct ARK5 activation by AKT through phosphorylation of serine 600 in the regulatory domain during IGF-1 signaling in tumor cells (1). Although phosphorylation of the serine residue is essential for ARK5 activation, the phosphorylation of a serine or threonine residue on the active T loop in the catalytic domain is also suspected of being necessary for ARK5 activation. Because phosphorylation of threonine 211 by LKB1 during metabolic stress has recently been reported (22), in the present study we investigated whether phosphorylation of threonine 211 is essential for ARK5 activation during IGF-1 signaling.

Phosphorylation of threonine 172/174 on AMPK-α1/α2 has been reported to be essential for activation (16, 22, 25), and multiple alignment of the catalytic domains of AMPK-α1, AMPK-α2, maternal embryonic leucine zipper kinase (MELK), SNF-1/AMPK-related kinase (SNARK), and ARK5 revealed that threonine 211 and the surrounding amino acid sequence of ARK5 is well conserved within AMPK catalytic subunit family members (Fig. 1A). To determine whether phosphorylation at threonine 211 is essential for activation of ARK5, we investigated the effect of alanine substitution of the threonine residue by a cell invasion assay because we previously found that ARK5 activation is crucial for IGF-1-induced tumor invasion of colorectal cancer cell lines (2). As previously reported, human colorectal cancer cell line SW480...
Identification of NDR2 as a Novel Upstream Kinase of ARK5 during IGF-1 Signaling—In the present study, we found that threonine 211 must be phosphorylated during ARK5 activation upon stimulation with IGF-1. In addition, our current investigation showed that cell extracts from IGF-1-exposed HCT-116 cells phosphorylated threonine 211 of ARK5 as shown in Fig. 2A. Because only wild type, but not threonine 211 mutants (T211D and T211A), was phosphorylated by cell extracts of IGF-1-exposed HCT-116 cells (Fig. 2B), we suggest that these cell extracts contain the upstream kinase of ARK5 that is activated during IGF-1 signaling. Two serine/threonine protein kinases, CaMKK-2a and LKB1, have been identified as upstream kinases phosphorylating a threonine residue on the active T loop in the catalytic domain of the AMPK catalytic subunit family (16, 17, 19–21). It has also been reported that both protein kinases exhibit a well conserved region in the catalytic domain (18). We first investigated whether other unknown protein kinases carry a similar sequence using a BLAST search (versus protein sequences listed in the National Center for Biotechnology Information library) and identified NDR1 (also called STK38) and NDR2 (also called STK38L/KIAA0965) as candidates for the upstream kinase of ARK5. Interestingly NDR2 mRNA was strongly expressed in SW480 cells that exhibited ARK5-mediated tumor invasion (Fig. 2B). By contrast, mRNA expression of LKB1, CaMKK-2a, CaMKK-2b, and NDR1 was very weak in SW480 cells. To identify the kinase responsible for ARK5 activation induced by IGF-1, we constructed a series of siRNAs. As shown in Fig. 2C, each siRNA suppressed expression of each target factor. As shown in Fig. 2D, when HCT-116 cells were exposed to IGF-1, the cell extracts phosphorylated the recombinant protein of human ARK5 catalytic domain (rARK5/CD). Interestingly complete suppression of the IGF-1-induced rARK5/CD phosphorylation resulted when the siRNA for NDR2 was introduced into HCT-116 cells (Fig. 2D). Although a decrease in phosphorylation by about half occurred when siRNA for NDR1 was introduced, the siRNA for other factors did not suppress it at all (Fig. 2D). In addition, cell extracts from IGF-1-exposed HCT-116 cells phosphorylated the recombinant protein of human AMPK-α1 catalytic domain (rAMPK/CD). Interestingly complete suppression of the IGF-1-induced rAMPK/CD phosphorylation resulted when the siRNA for NDR2 was introduced into HCT-116 cells (Fig. 2D). Although a decrease in phosphorylation by about half occurred when siRNA for NDR1 was introduced, the siRNA for other factors did not suppress it at all (Fig. 2D). In addition, cell extracts from IGF-1-exposed HCT-116 cells phosphorylated the recombinant protein of human AMPK-α1 catalytic domain (rAMPK/CD), but none of the siRNA tested in this study suppressed it (Fig. 2D). By contrast, siRNA for other factors did not suppress it at all (Fig. 2D). In addition, cell extracts from IGF-1-exposed HCT-116 cells phosphorylated the recombinant protein of human AMPK-α1 catalytic domain (rAMPK/CD), but none of the siRNA tested in this study suppressed it (Fig. 2D). By contrast, siRNA for other factors did not suppress it at all (Fig. 2D). In addition, cell extracts from IGF-1-exposed HCT-116 cells phosphorylated the recombinant protein of human AMPK-α1 catalytic domain (rAMPK/CD), but none of the siRNA tested in this study suppressed it (Fig. 2D). By contrast, siRNA for other factors did not suppress it at all (Fig. 2D). In addition, cell extracts from IGF-1-exposed HCT-116 cells phosphorylated the recombinant protein of human AMPK-α1 catalytic domain (rAMPK/CD), but none of the siRNA tested in this study suppressed it (Fig. 2D). By contrast, siRNA for other factors did not suppress it at all (Fig. 2D). In addition, cell extracts from IGF-1-exposed HCT-116 cells phosphorylated the recombinant protein of human AMPK-α1 catalytic domain (rAMPK/CD), but none of the siRNA tested in this study suppressed it (Fig. 2D). By contrast, siRNA for other factors did not suppress it at all (Fig. 2D). In addition, cell extracts from IGF-1-exposed HCT-116 cells phosphorylated the recombinant protein of human AMPK-α1 catalytic domain (rAMPK/CD), but none of the siRNA tested in this study suppressed it (Fig. 2D). By contrast, siRNA for other factors did not suppress it at all (Fig. 2D).
As shown in Fig. 2E, tumor invasion activity of SW480 cells stimulated with IGF-1 was suppressed by introduction of the siRNA for NDR2 but not by introduction of the others. Based on these results, we concluded that NDR2 is a novel upstream kinase of ARK5 during IGF-1 signaling, and because no involvement of NDR2 in AMPK-α1 phosphorylation was observed in this study, NDR2 seemed to be a specific kinase for ARK5.

NDR2 Is Activated in a PDK-1-dependent Manner during IGF-1 Signaling—NDR2 was also identified by an analysis of cDNA synthesized from human brain at the KAZUSA DNA Research Institute (Japan), and they numbered it KIAA0965 (GenBank access number AB023182). Regulation of the cell cycle and morphogenesis have been reported to be the roles of the NDR family AGC kinases (28). To investigate the mechanism of NDR2 activation during IGF-1 signaling, we constructed a mammalian expression plasmid with HA tag (HA-NDR2) and MBP fusion protein produced in E. coli (MBP-NDR2). Because the amino acid sequence revealed that NDR2 contains a PDK-1 docking motif on its C-terminal side (amino acids 438–443) on which PDK-1 can bind and phosphorylate the target factor, just like in the case of AKT activation by PDK-1, a mutant lacking the PDK-1 docking motif was also constructed in mammalian expression vector (Fig. 3A), and the HA-tagged wild-type NDR2 and PDK-1 docking motif (PDM)-deleted mutant (HA-NDR2/PDM) were successfully expressed in HCT-116 cells (Fig. 3B). As shown in Fig. 3C, our current results showed that IGF-1-stimulated cell extracts induced the phosphorylation of the PDM region in NDR2. In addition, the phosphorylation was suppressed when cells were pretreated with LY294002 but not with U0126 (Fig. 3C), suggesting that the intracellular signaling is mediated by LY294022-sensitive factors, such as phosphatidylinositol 3-kinase and PDK-1. To further investigate an interaction between

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**FIGURE 2. Phosphorylation of ARK5 by NDR2 upon stimulation with IGF-1.** A, HCT-116 cells were exposed (+) to 100 ng/ml IGF-1 for 2 h, and cell extracts were then mixed with recombinant protein coding the catalytic domain of ARK5 (wild-type, WT; T211D mutant, TD; T211A mutant, TA) with specific primer pairs for NDR2 or Western blotting with anti-MBP antibody after purification on an amylose resin column. B, RT-PCR (30 cycles) was performed on RNAs extracted from HCT-116, DLD-1, and SW480 cell lines with specific primer pairs for LKB1, CaMKM-2α (CaMK2α), CaMKM-2β (CaMK2β), NDR1 (N1), NDR2 (N2), or glyceraldehyde-3-phosphate dehydrogenase (GH). C, RNAs were extracted from HCT-116 cells into which no siRNA (P), control siRNA (RNNings), or specific siRNA (siNDR2) for LKB1 (LKB1), CaMKM-2α (CaMK2α), CaMKM-2β (CaMK2β), NDR1 (N1), NDR2 (N2), or glyceraldehyde-3-phosphate dehydrogenase (GH) were introduced, and RT-PCR (32 cycles) was performed using specific primer pairs for LKB1, CaMKM-2α (CaMK2α), CaMKM-2β (CaMK2β), NDR1 (N1), NDR2 (N2), or glyceraldehyde-3-phosphate dehydrogenase (GH). D, HCT-116 cells into which no siRNA (Control), control siRNA (Control), or specific siRNA for LKB1 (LKB1), CaMKM-2α (CaMK2α), CaMKM-2β (CaMK2β), NDR1 (N1), or NDR2 (N2) had been introduced were exposed (+) or not exposed (−) to 100 ng/ml IGF-1 or glucose (Glc)-free medium for 2 h. Cell extracts were then mixed with recombinant protein coding the catalytic domain of ARK5 or AMPK-α1 in the presence of [γ-32P]ATP. Phosphorylated (pARK5 or pαARK5) total (tARK5) recombinant proteins were detected by autoradiography or Western blotting with anti-MBP antibody after purification on an amylose resin column. E, tumor invasion activity of HCT-116 cells into which no siRNA (Control), control siRNA (Control), or specific siRNA for NDR2 (NDR2) had been introduced was investigated in the presence (closed) or absence (open) of 100 ng/ml IGF-1. The numbers of invaded cells shown are the means of three experiments, and the bars represent the S.E. values.

**FIGURE 3. NDR2 activation regulated by PDK-1 during IGF-1 signaling.** A, schematic model of NDR2 and its construction. Dotted boxes, HA or MBP tag region; closed boxes, catalytic domain; shaded box, PDK-1 docking motif. B, HCT-116 cells were transfected with expression vector containing (+) or not containing (−) wild-type (WT) or deletion mutant (ΔPDM) HA-NDR2, and cell extracts were used for Western blotting with antibody to HA (HA-NDR2) or actin. C, HCT-116 cells pretreated (+) or not pretreated (−) with 20 μM LY294002 (LY) or 20 μM U0126 were exposed (+) or not exposed (−) to 100 ng/ml IGF-1, cell extracts were mixed with MBP-NDR2, and then PDK-1 docking motif-phosphorylated NDR2 (pPDM) and total NDR2 (tNDR2) were detected by Western blotting with anti-MBP antibody after purification on an amylose resin column. D, cell transfected with expression vector containing wild-type or threonine 442-mutated (Ala-converted, TA; Asp-converted, TD) HA-NDR2 (Ndr2-7442) were exposed to medium containing (+) or not containing (−) 100 ng/ml IGF-1, and immunoprecipitates with anti-HA antibody (HA) or PDK-1, phospho-PDK-1, or HA. IB, immunoblot; Ab, antibody. E, expression vector containing wild-type or deletion mutant (ΔPDM) HA-NDR2 was introduced into HCT-116 cells, and immunoprecipitation with anti-HA antibody was performed after IGF-1 treatment (100 ng/ml for 2 h). Immunoprecipitates were blotted with antibody against HA (pHA), or HA (HA), or phospho-A5 (pA5) and actin. F, HCT-116 cells transfected with expression vector containing HA-NDR2 were exposed or not exposed to 100 ng/ml IGF-1 for 2 h followed by pretreatment with (+) or without (−) 20 μM LY294002 or 20 μM U0126, and immunoprecipitates with anti-HA antibody were blotted with antibody against human PDK-1 or HA (Ndr2). G, tumor invasion activity was investigated in HCT-116 or DLD-1 cells transfected with or not transfected (Parental) with expression vector containing or not containing (Empty) ARK5, HA-NDR2 (Ndr2), ARK5/T211D (DN-ARK5), and/or HA-NDR2/ΔPDM (Ndr2/ΔPDM) in the presence (closed) or absence (open) of 100 ng/ml IGF-1. The numbers of invaded cells shown are the means of three experiments, and the bars represent the S.E. values.
PDK-1 and NDR2 during IGF-1 signaling, wild-type, T442A mutant, and T442D mutant NDR2 were overexpressed in cells. As shown in Fig. 3D, wild-type but not T442D-mutated NDR2 showed an interaction with PDK-1 and PDM site phosphorylation. Interestingly any interaction between T442D mutant and PDK-1 was not observed; however, T442A-mutated NDR2 exhibited an interaction but not PDM site phosphorylation. AKT1 is also one PDK-1 substrate, and it is well known that the activation is caused by PDK-1 through binding via the PDM sequence of AKT and phosphorylation (29–31). Thus, it seems to be that phosphorylation is promoted after interaction. Therefore, we suggest that PDK binds with NDR2 and phosphorylates the threonine residue within the PDM sequence during IGF-1 signaling.

In this study, we showed that the IGF-1-induced phosphorylation of ARK5, especially threonine 211, was suppressed by the siRNA for NDR2 and that NDR2 was also phosphorylated during IGF-1 signaling. This strongly suggests that NDR2 phosphorylates threonine 211 of ARK5 upon stimulation by IGF-1. A pull-down assay using HA-tagged NDR2 was performed to examine the interaction between NDR2 and PDK-1 during IGF-1 signaling. As shown in Fig. 3E, PDK-1 was detected in anti-HA antibody immunoprecipitates from wild-type HA-NDR2-transfected HCT-116 cells but not from HA-NDR2/ΔPDM-transfected HCT-116 cells, and the interaction was IGF-1-dependent. The IGF-1-induced interaction between NDR2 and PDK-1 was found to be phosphatidylinositol 3-kinase-dependent and mitogen-activated protein kinase-independent because LY294002, but not U0126, suppressed it (Fig. 3F). These results suggest that PDK-1 plays an essential role in NDR2 activation through binding to its docking motif on NDR2 during IGF-1 signaling. We have demonstrated previously that IGF-1 stimulates tumor invasion through ARK5 activation, and the role of PDK-1/NDR2/ARK5 activation in tumor invasion was examined. As shown in Fig. 3G, HCT-116 and DLD-1 cells, in which ARK5 expression is weak, exhibited increased invasion activity when ARK5 or NDR2 was introduced, and IGF-1 accelerated the invasion activity. In addition, the invasion activity was suppressed by introduction of DN-ARK5 or NDR2/ΔPDM, and the acceleration of activity by IGF-1 in ARK5-overexpressing cells was suppressed by NDR2/ΔPDM (Fig. 3G). When SW480 cells, in which ARK5 mRNA is strongly expressed, were used, NDR2/ΔPDM had a similar inhibitory effect (Fig. 3H). These results suggest that NDR2 regulates the ARK5-mediated tumor invasion activity during IGF-1 signaling, and we identified NDR2 as a novel upstream kinase of ARK5 during IGF-1 signaling.

The regulation of NDR kinases has been well investigated, and the phosphorylation of threonine residues in the C-terminal region by unidentified upstream kinase(s) is well known (28, 32–34). Although we raised the possibility that PDK-1 activates NDR2 in cancer cells during IGF-1 signaling in this study, PDK-1 is not involved in the phosphorylation of the C-terminal threonine in NDR1 (35). The amino acid sequence of NDR2 shows high similarity to that of NDR1, but the subcellular localization is quite different (NDR1 is found in nuclei; NDR2 is found in cytoplasm) (33), suggesting the presence of different mechanisms activating NDR kinases. In addition, recent progressive investigations revealed MOB protein kinases (33) and STE20-like kinase family MST3 (34) as candidates of the upstream kinases(s), but these kinases in cancer cells seem to act as tumor suppressors (36). ARK5, which is the substrate candidate of NDR2 in cancer cells during IGF-1 signaling, is a tumor progression-associated factor (2), and the role of NDR kinases in non-tumor cells, such as neuronal cells, has been reported recently (37). Although the NDR kinases may be regulated by STE20-like kinases and MOB kinases in non-tumor cells, we suggest that the regulation of NDR kinases, especially NDR2, is initiated by PDK-1 as one of the signal transduction pathways, such as tumor progression signaling stimulated with IGF-1.

**NDR2 Interacts with S100B in a PLC-γ-dependent Manner during IGF-1 Signaling**—As described above, a BLAST search analysis revealed NDR2 as a novel ARK5 kinase, and the biochemical properties of NDR2, especially the phosphorylation sites required for activation, have been well characterized. The threonine residue on the N-terminal side of mouse NDR2 is phosphorylated during interaction with S100B in the presence of calcium ion, and an unknown upstream kinase phosphorylates the threonine residue on the C-terminal side (32). After phosphorylation of both threonine residues, the serine residue on the catalytic domain is autophosphorylated, and activated mouse NDR2 transduces signaling to the next factor (32). A comparison of the amino acid sequence of human and mouse NDR2 revealed that all phosphorylation sites and surrounding sequences are well conserved (data not shown). Next we investigated whether an interaction between S100B and the threonine residue on the N-terminal side of human NDR2 in the presence of calcium ion is triggered by IGF-1 the same as in mouse homologue. Because the sequence around threonine 75 of human NDR2 was highly homologous with the S100B binding site of mouse NDR2, we constructed alanine- (T75A/HA-NDR2) or aspartic acid (T75D/HA-NDR2)-substituted mutants in this study. As shown in Fig. 4A, wild-type and mutated NDR2 were expressed in HCT-116 cells, and S100B was detected in immunoprecipitates with anti-HA antibody from cells transfected with wild-type vector and exposed to IGF-1. The same results were obtained in cells transfected with T75D/HA-NDR2 but not with T75A/HA-NDR2 (Fig. 4A). Because T75D/HA-NDR2 interacted with S100B even in the absence of IGF-1 stimulation and no interaction between T75A/HA-NDR2 and S100B was observed (Fig. 4A), we concluded that S100B interacts with NDR2 through threonine 75, the same as in mouse NDR2, and that phosphorylation of the threonine residue is required for their interaction. The effects of BAPTA (chelator of intracellular calcium ion), U-73122 (inhibitor of PLC-γ), and LY294002 (inhibitor of phosphatidylinositol 3-kinase) were examined in relation to the mechanism of the interaction between S100B and NDR2 during IGF-1 signaling. As shown in Fig. 4B, the interaction between S100B and NDR2 induced by IGF-1 was sup-
pressed by BAPTA and U-73122 but not by the others. These findings indicate that an increase in free intracellular calcium ion concentration is required for binding of S100B to NDR2. PLC-γ has been reported to mobilize calcium ions upon stimulation of growth factor receptors, such as epidermal growth factor receptor, platelet-dependent growth factor receptor, and IGF receptor (38–40). Thus, our findings suggest that the calcium ion required for the interaction between NDR2 and S100B is supplied by the PLC-γ-mediated pathway as a result of IGF receptor activation.

ARK5-mediated Tumor Cell Survival and Invasion Is Regulated by NDR2 through Three Point Phosphorylations—We have reported that SW480 cells express ARK5, FAS ligand, and FAS and that the suppression of ARK5 caused by introduction of antisense RNA, siRNA, or dominant-negative mutants leads to cell death through ligation of FAS ligand and FAS (6). In the current study, we further investigated the involvement of NDR2 in suppression of cell death through activation of ARK5 and its mechanism by constructing T75D, T75A, S282D, S282A, T442D, and T442A mutants of NDR2. As shown in Fig. 5A, SW480 cells died after introduction of two ARK5 mutants (ARK5/T211A and ARK5/S600A) that act as dominant negatives or the siRNA for ARK5, consistent with our previous report (6). Because the introduction of DN-AKT1 or siRNA for NDR2 into cells induced cell death (Fig. 5A), FIGURE 5. Tumor cell survival and invasion induced by NDR2/ARK5 pathway. A, cell survival assay (48 h after transfection) was performed using SW480 cells transfected or not transfected (Parental) with expression vector containing or not containing (Empty) ARK5 (wild type (wt), T211A mutant, or S600A mutant), NDR2 (wild type, T75D mutant, T75A mutant, S282D mutant, S282A mutant, T442D mutant, or T442A mutant), DN-AKT1, or the control (CNT/i) or targeting double-stranded RNA for ARK5 (ARK5/i) or NDR2 (Ndr2/i). The numbers of survived cells shown are the means of three experiments, and the bars represent the S.E. values. B, SW480 cells transfected or not transfected (Parental) with expression vector containing or not containing (Empty) ARK5 (T211A mutant or S600A mutant) or NDR2 (wild type, T75D mutant, T75A mutant, S282D mutant, S282A mutant, T442D mutant, or T442A mutant) were exposed for 48 h to medium containing (closed) or not containing (open) 100 ng/ml IGF-1, and the invaded cells were counted. The numbers of invaded cells shown are the means of three experiments, and the bars represent the S.E. values. C, HCT-116 or DLD-1 cells transfected or not-transfected (Parental) with expression vector containing or not containing (Empty) wild-type ARK5 or NDR2 (wild type, T75D mutant, T75A mutant, S282D mutant, S282A mutant, T442D mutant, or T442A mutant) were exposed for 48 h to medium containing (closed) or not containing (open) 100 ng/ml IGF-1, and the invaded cells were counted. The numbers of invaded cells shown are the means of three experiments, and the bars represent the S.E. values.

FIGURE 6. Schematic model of ARK5 activation through NDR2 during IGF-1 signaling. PI-3K, phosphatidylinositol 3-kinase; IGF-R, IGF receptor.
AKT and NDR2 act in the upstream portion of ARK5 for cell survival in SW480 cells. Interestingly three alanine-converted mutants, T75A/HANDR2, S282A/HANDR2, and T442A/HANDR2, also induced cell death (Fig. 5A), suggesting that phosphorylation of three amino acids (threonine 75, serine 282, and threonine 442) is required for NDR2 activation to promote ARK5-mediated cell survival in SW480 cells. In addition, IGF-1 accelerated tumor invasion activity of SW480 cells, and the acceleration was mediated by ARK5 because DN-ARK5 suppressed it (Fig. 5B). The invasion activity of SW480 cells was also suppressed by transfection of three alanine-converted mutants, and the same was true for acceleration of invasion activity by IGF-1 (Fig. 5B). It should be noted that aspartic acid-converted mutant of serine 282 (S282D/HANDR2), but not the other two aspartic acid-converted mutants (T75D/HANDR2 and T442D/HANDR2), significantly accelerated invasion activity even in the absence of IGF-1 (Fig. 5B). The same results were obtained when ARK5 was expressed in HCT-116 and DLD-1 cells (Fig. 5C). In the case of mouse NDR2, serine 282 has been reported as the most important amino acid residue for NDR2 activation (32). It was reported that autophosphorylation of serine 282 is initiated followed by threonine 75 and threonine 442 phosphorylation, and even aspartic acid conversion of serine 282 led to enough activity (32). Therefore, our current results confirm the previous study and suggest that phosphorylation of serine 282 is the most important step for NDR2 activation during IGF-1 signaling.

NDR2: New Function as the Upstream Kinase of ARK5 and Its Activation Mechanism during IGF-1 Signaling—ARK5 is the tumor invasion-associated factor that transduces IGF-1 signaling in tumor cells, and we identified NDR2 as a novel upstream kinase of ARK5 in the present study. Based on the results of this study, we concluded that the mechanism of ARK5 activation through NDR2 during IGF-1 signaling is as follows (Fig. 6). At least two intracellular signals are initiated to activate ARK5 upon stimulation by IGF-1. One signal activates PDK-1 through phosphatidylinositol 3-kinase activation. Activated PDK-1 transduces the signal to AKT, which, in turn, phosphorylates serine 600 on the regulatory domain of ARK5; activated PDK-1 also causes phosphorylation of threonine 442 of NDR2. The other is a calcium ion regulation signal through PLC-γ. Interaction between S100B and NDR2 through threonine 75 is promoted by mobilization of calcium ion as a result of PLC-γ activation. After phosphorylation of these two threonine residues, serine 282 is autoprophosphorylated, and activated NDR2 phosphorylates threonine 211 in the catalytic domain of ARK5. Thus, ARK5 acts as the tumor invasion-associated factor through NDR2 during IGF-1 signaling.

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