Tyrosine 740 Phosphorylation of Discoidin Domain Receptor 2 by Src Stimulates Intramolecular Autophosphorylation and Shc Signaling Complex Formation*

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DDR2 is a receptor tyrosine kinase whose activating ligands are various collagens. DDR2-mediated cellular signaling has been shown to require Src activity. However, the precise mechanism underlying the Src dependence of DDR2 signaling is unknown. Here, using baculoviral co-expression of the DDR2 cytosolic domain and Src, we show that Src targets three tyrosine residues (Tyr-736, Tyr-740, and Tyr-741) in the activation loop of DDR2 for phosphorylation. This phosphorylation by Src stimulates DDR2 autophosphorylation of additional tyrosine residues. In vitro Shc binding assays demonstrate that phosphotyrosines resulting from DDR2 autophosphorylation are involved in Shc binding to the DDR2 cytosolic domain. Mutating tyrosine 740 of DDR2 to phenylalanine stimulates autophosphorylation of DDR2 to an extent similar to that resulting from Src phosphorylation of DDR2. In addition, the DDR2 Y740F mutant protein displays collagen-independent, constitutively activated signaling. These findings suggest that tyrosine 740 inhibits DDR2 autophosphorylation. Collectively, our findings are consistent with the following mechanism for Src-dependent DDR2 activation and signaling: 1) ligand binding promotes phosphorylation of Tyr-740 in the DDR2 activation loop by Src; 2) Tyr-740 phosphorylation stimulates intramolecular autophosphorylation of DDR2; 3) DDR2 autophosphorylation generates cytosolic domain phosphotyrosines that promote the formation of DDR2 cytosolic domain-Shc signaling complexes.

The discoidin domain receptor (DDR) family, including DDR1 and DDR2, belongs to receptor tyrosine kinases (RTKs) family. Its extracellular part, containing the so-called discoidin domain, binds to various collagen proteins as their activating ligands (1–3), and its intracellular part possesses a domain of tyrosine kinase, which shares ~50% sequence homology with that of the Trk family of neurotrophin receptors as well as with insulin receptor (4–6).

Involvement of DDR proteins in the proliferation of various cell types has been reported. Increased DDR1 expression is observed in keratinocytes of the skin and smooth muscle cells around blood vessels when the tissues are injured (7, 8). DDR1 is also expressed in monocyte-derived cells where it is believed to play a role in collagen binding and cell differentiation (9, 10). DDR2 expression is observed in mesenchymal cells and is involved in bone growth (11). During liver fibrosis, induction and activation of DDR2 occur in liver stellate cells, and its tyrosine kinase activity is necessary for the proliferation of stellate cells and for the increase of collagen and MMP-2 synthesis (12, 13). In rheumatoid arthritis, DDR2 induction is also observed in activated synovial fibroblasts and is thought to stimulate the growth of these cells and MMP-1 synthesis (14). In addition, the induction of DDR proteins is implicated in breast and ovarian cancer, and is correlated with metastasis (15, 16).

Autophosphorylation of the cytosolic domain of RTKs is typically a critical event for the activation of RTK-mediated cellular signaling (17). Three-dimensional structure determination as well as associated biochemical and genetic analysis of several RTKs have helped to reveal the detailed molecular mechanisms of RTK autophosphorylation (18–20, 25, 26). For the insulin and fibroblast growth factor receptors, ligand binding triggers the trans-autophosphorylation of two or three tyrosine residues in a so-called activation loop between subdomain VII and VIII of the kinase catalytic core. This phosphorylation causes a considerable change in the three-dimensional structure of the activation loop and stimulates RTK activity by providing ATP and peptide substrate unrestricted access to the active site pocket (17, 19). Further autophosphorylation of tyrosine residues in the juxtamembrane and C-terminal region occurs by the stimulated kinase activity of these receptors (17, 21, 22). Treatment of cells with the ligand collagen triggers tyrosine phosphorylation of the cytosolic domain of DDR1 and DDR2 (1, 2). Potential phosphatase activity notwithstanding, the appearance of tyrosine phosphorylation in DDR proteins upon ligand treatment requires hours. In contrast, tyrosine phosphorylation of most other RTKs occurs on the order of minutes after ligand binding. The reason for this apparent kinetic difference is unclear. In addition it has been suggested that DDR2-mediated signaling requires Src tyrosine kinase activity (23).

In this study, we explore the precise mechanism for activation of DDR2 cytostatic signaling using in vitro analysis.

MATERIALS AND METHODS

Plasmid Construction—All baculoviral expression vectors were constructed using pBacPAK8 vector (Clontech). For convenient glutathione S-transferase (GST) tagging of expressed proteins, the pBacPAK8 vector was modified to contain the GST gene by subcloning a PCR-amplified cDNA fragment of GST, from pGEX4T1 plasmid having a BglII restriction site at the 5’ position and BamHI or Xhol sites at the 3’ position, into BamHI or BamHI-Xhol cutting sites in pBacPAK8 plasmid (pBacPAK 8-GST-BamHI or pBacPAK 8-GST-Xhol). For the baculoviral expression vector of GST-tagged DDR2 cytosolic kinase domain (GST-DDR2 CKD), a PCR-amplified cDNA fragment covering human...
DDR2 from amino acids 441–815 was subcloned into the Xhol-NotI site of pBacPAK8-GST-Xhol vector. Baculoviral expression vectors of GST-tagged full-length human CDK4 (GST-CDK4), DDR1 cytosolic kinase domain (amino acids from 454–914, GST-DR1 CKD), and full-length mouse Akt1 (GST-Akt1) were made by subcloning each PCR-amplified cDNA into a BamHl-NotI site of pBacPAK 8-GST-BamHI vector. Full-length human c-Src gene and kinase-inactive c-Src gene were PCR-amplified from the vector of pUSE human c-Src wild-type or pUSE human c-Src-negative (Upstate Biotechnology) and were subcloned into the Xhol-EcoRI site of pBacPAK 8, respectively, to obtain their baculoviral expression vectors. Mammalian expression vector bearing human full-length DDR2 under the control of the CMV promoter (CMV-DDR2) was constructed by ligating two DNA fragments of Ncol/8srDI-digested N-terminal half and BsrDI/EcoRI-digested C-terminal half of human DDR2 cDNA amplified by PCR with primers bearing human full-length DDR2 under the control of the CMV promoter (CMV-DDR2-K608A), lysine 608 to alanine 608 mutation was introduced into a GST-DDR2 cytosolic domain by replacing Ncol-BamHI fragment within DDR2 cytosolic domain gene with a PCR-amplified fragment to contain the mutation of K608A from overlapping PCR technique using four primers of ccggttacctgacgtc (forward primer containing an Ncol site), gcccggcctggtacgg (reverse primer containing a BamHI site), gtaggctgactttcagc (forward primer containing a K608A mutation), and tgcagctgatccacgac (reverse primer containing a K608A mutation).

To introduce site-directed mutations to the three tyrosine residues (Tyr-736/Tyr-740 and Tyr-741) in the activation segment region of wild-type DDR2 tyrosine kinase domain or kinase-defective DDR2 tyrosine kinase domain, we PCR-amplified fourteen Ncol/BamHI fragments, seven from DDR2 CKD and seven from kinase-defective DDR2 CKD-K608A cDNA, using seven sets of primer pairs consisting of 5′-primer (actcagtgcctgccgtcacc) containing Ncol site and each of seven different 3′-primers containing each mutation as well as a BamHI site such as Y740F primer (ccgcgtctctgacgactgacgcagcgtc), Y736F primer (ccgcgtctctgacgactgacgcagcgtc), Y471F primer (ccgcgtctctgacgactgacgcagcgtc), Y740F/Y741F primer (ccgcgtctctgacgactgacgcagcgtc), and Y736F/Y740F/Y741F primer (ccgcgtctctgacgactgacgcagcgtc), respectively. Each of fourteen PCR fragments was subcloned into the Ncol/BamHI site in pBacPAK8-GST-DDR2 CKD. The K608A and Y740F mutations were introduced into the full-length DDR2 mammalian expression vector (CMV-DDR2) to generate CMV-DDR2-K608A and CMV-DDR2-Y740F expression vector by replacing the wild-type sequence with the cDNA fragment containing each corresponding mutation from the mutated GST-DDR2 CKD baculoviral expression vector.

Cell Lines and Culture—Spodoptera frugiperda SF9 insect cells (Clontech) were maintained in TMN-FH insect medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum, 50 μg/ml gentamycin (Sigma), and 2 mM glutamine (Invitrogen) at 27 °C. HSC T6 and NIH 3T3 cells were cultured using Dulbecco’s modified Eagle’s medium, and SK-N-SH cells were maintained using RPMI in the presence of 10% fetal bovine serum, 150 μg/ml penicillin-streptomycin (Invitrogen), under 5% CO2 at 37 °C. HSC T6 and SK-N-SH cells were kindly provided by Dr. S. L. Friedman (Mount Sinai School of Medicine) and Dr. Y.-H. Seo (Medical College of Seoul National University, Korea) respectively.

Baculoviral Expression and Purification of Proteins—Each generated baculoviral expression vector plasmid DNA was transfected into sf9 cells along with viral genomic DNA from a baculovirus generation kit purchased from Clontech, according to the manufacturer’s manual. The viral stock was amplified to a titer of ~10⁶ plaque-forming units/ml. sf9 cells were infected with multiplicity of infection 10 and left for 48 h before harvest. GST-tagged DDR2 kinase domain proteins were purified using a glutathione-agarose bead affinity column and subsequent Superdex 200 prep grade gel filtration (Amersham Biosciences) by fast-protein liquid chromatography system. Infected sf9 cells from 400-ml cultures were suspended in 20 ml of lysis buffer consisting of 20 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 1 mM diithiothreitol, 10 mM NaF, 0.1 mM EDTA, 0.1 mM sodium vanadate, 0.02% IGE-PAL (Sigma), a proteinase inhibitor mixture tablet (Roche Applied Science) and lysed by sonication for 1 min. The lysate was centrifuged at 12,000 rpm for 30 min using a Sorval SS34 rotor (Beckmann) and the supernatant was applied to 1-ml bed volume of a glutathione-agarose bead column (Amersham Biosciences) pre-equilibrated with 20 mM Tris-HCl (pH 8.0) containing 0.15 M NaCl buffer and washed with the column equilibration buffer. The bound proteins were eluted with 10 ml of washing buffer containing 20 mM reduced glutathione and subsequently concentrated to 1 ml using Vivaspin concentrator (Vivascience, Germany). The concentrated sample was applied to a HiLoad 16/60 Superdex 200 prep grade column pre-equilibrated with a buffer of 20 mM Tris-HCl (pH 8.0) containing 0.15 M NaCl, 1 mM diithiothreitol, 10 mM NaF, and 0.1 mM EDTA and separated with a flow rate of 0.5 ml/min using a fast-protein liquid chromatography system. The eluted GST-DDR2 kinase domain protein fractions were combined and concentrated using Vivaspin concentrator. For a brief purification of GST-tagged proteins bound to glutathione beads, infected sf9 cells were lysed in 500 μl of the lysis buffer described above by sonication and centrifuged at 12,000 rpm using microcentrifuge for 10 min. 50 μl of 50% slurry of glutathione bead was added to the supernatant, and the mixture was slowly rotated for 10 min at 4 °C. Finally the GST-tagged protein-bound bead was obtained by washing with 1× TBS for three times.

Autophosphorylation and Tyrosine Kinase Activity of DDR2—To measure the autophosphorylation activity of DDR2, the reaction was performed using 200 ng of DDR2 kinase domain protein in 20 μl of reaction mixture containing 20 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 0.5 mM diithiothreitol, 0.01 mM ATP, and 0.2 μCi of θP ATP. After 15-min incubation at 30 °C, the reaction was stopped by adding a half volume of 3× Laemml buffer with subsequent boiling for 2 min. The stopped mixture was run in 10% SDS-PAGE gel, the portion of gel containing unreacted free ATP was removed, and the remaining gel was stained using Coomassie Brilliant Blue (Sigma) and dried. The 32P radioactivity in the stained DDR2 kinase protein band was visualized by autoradiography and quantitated using a BAS θP-image analyzer. For measuring the autophosphorylation rate (the phosphate transfer rate from ATP to DDR2 cytosolic domain), the radioactivities in the total reaction mixture and DDR2 band stained by Coomassie Brilliant Blue in the SDS-PAGE gel were measured, respectively, by scintillation counting, and the transferred mole number of phosphate was calculated from the ratio of the two radioactivities as described previously (24). Measurement of DDR2 tyrosine kinase activity toward heterologous peptide substrates was performed using 100 ng of the purified kinase in 20 μl of
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reaction mixture containing 20 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 0.5 mM dithiothreitol, 0.01 mM ATP, 4 μg of peptide substrate such as histone H2B (Sigma) or poly(D₄Y)₉ (Promega) and 0.2 μCi of [³²P]ATP. After 15-min incubation at 30 °C, the reaction was stopped by adding a half volume of 30% phosphoric acid. When H2B was used as a peptide substrate, the reaction mixture was spotted onto p81 paper (Millipore). For poly(D₄Y)₉ as a substrate, reactions were spotted on avidin-coated membrane (Promega). The spotted filter was washed with 0.1 M Tris-HCl (pH 8.0) five times for 10 min each, and the radioactivity of each spot was visualized and quantitated using a BAS image analyzer (Fuji).

Antibodies and Immunoblotting—Phosphotyrosine-specific antibody and antibody against human DDR2 were purchased from Cell Signaling Biotechnology and abcam (UK), respectively. Human c-Src specific antibody and monoclonal antibody against Shc were obtained from Upstate Biotechnology. Samples were boiled for 2 min in 1× Laemmli sample buffer and loaded in 10% SDS-PAGE. Proteins were transferred with horseradish peroxidase-conjugated second-ary antibodies for 1 h, and protein bands were detected by chemiluminescence (Amersham Biosciences).

Shc Binding Assay—As a source of Shc protein, total lysate of HSC T6 cells was used. For the preparation of the lysate, 2 × 10⁶ HSC T6 cells were harvested and lysed by sonication in 5 ml of lysis buffer consisting of 20 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 10 mM NaF, 0.1 M orthovanadate, 0.02% IGE-PAL (Sigma) and a proteinase inhibitor mixture pellet (Roche Applied Science). The supernatant was obtained by centrifugation at 15,000 rpm for 10 min at 4 °C and used for Shc binding assays as follows. Various GST-DDR2 CKD proteins bound to glutathione beads were obtained as described above. For the preparation of autophosphorylated GST-DDR2 CKD bound to beads, kinase reaction mixtures containing Tris-HCl (pH 7.5), 0.1 M NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, and 100 μM of ATP were added to the protein-bound glutathione beads, incubated at 30 °C for 15 min, and washed three times with cold 1× TBS. Mock autophosphorylation was carried using γ-S-ATP instead of ATP. For Shc binding, HSC T6 cell lysate was added to the protein-bound beads, and the mixture was slowly rotated for 1 h at 4 °C. Next, the beads were washed with cold 1× TBS for three times, and the bound proteins on the bead were eluted by boiling in 1× Laemmli buffer for 2 min. The eluted proteins were resolved by 10% SDS-PAGE. Western blotting using Shc-specific antibody was carried out to detect Shc protein.

Transfections, MMP-1 Promoter Assay, and Enzyme-linked Immunosorbent Assay of MMP-2—1 × 10⁶ NIH 3T3 cells were plated into 24-well dishes and left overnight. Cells were transfected with plasmid DNAs of 0.2 μg of MMP-1 promoter-Luciferase vector along with 1 μg of CMV-DDR2 (wild-type) or CMV-DDR2-Y740F or CMV-DDR2-K608A, respectively, per well using CytoPure transfection reagent (Q Biogene) according to the manufacturer’s protocol. After 24 h, cells were treated with 20 μg/ml type I collagen (BD Biosciences). 12 h later, cells were harvested and luciferase activity was measured (Promega). To construct stable cell lines expressing wild-type DDR2 or DDR2-Y740F, 10 μg of each expression plasmid was transfected into 10⁶ SK-N-SH cells in a 6-well plate along with 1 μg of hygromycin resistance gene plasmid using CytoPure transfection reagent. After 48 h, transfected cells were transferred to 10-cm diameter culture dish and subject to selection with a gradual increase of hygromycin B (Invitrogen) from 200 to 600 μg/ml until visible colonies appeared. Isolated stable cell colonies were tested for the expression of transfected DDR2 by Western blotting using the antibody specific to DDR2. For measuring MMP-2 expression, 1 × 10⁶ cells of each clone as well as parental SK-N-SH cells were plated into both of pre-collagen coated and non-coated 24-well dishes in serum-free RPMI medium and further incubated for 36 h. The culture medium from each well was harvested and tested for the level of MMP-2 using an enzyme-linked immunosorbent assay kit purchased from R&D Systems according to the manufacturer’s protocol.

RESULTS

Src Phosphorylates the DDR2 Tyrosine Kinase Domain—When we expressed the human DDR2 tyrosine kinase domain (amino acids 441–815) as a GST fusion in s9f cells by baculoviral infection, the expressed GST-DDR2 CKD did not undergo detectable tyrosine phosphorylation in s9f cells as shown by Western blotting using phosphotyrosine-specific antibody. However, when GST-DDR2 CKD was co-expressed with human c-Src tyrosine kinase in s9f cells, significant induction of GST-DDR2 CKD tyrosine phosphorylation was detected (Fig. 1A). Control GST-tagged proteins, including GST-CDK4, did not display detectable tyrosine phosphorylation in the same experiment (Fig. 1B), indicating that tyrosine phosphorylation occurred within the DDR2 tyrosine kinase domain. In contrast, co-expression of GST-DDR2 CKD with a kinase-negative Src did not induce tyrosine phosphorylation of GST-DDR2 CKD (Fig. 1C). The degree of tyrosine phosphorylation in GST-DDR2 CKD and its kinase-defective mutants (Kd-GST-DDR2 CKD) was estimated without (-) or with co-expression of Src (+).
proteins in sf9 cells was confirmed by Western blotting (data not shown).

Tyrosine phosphorylation by Src shows some specificity toward the DDR2 kinase domain among other tested kinases, including the human DDR1b kinase domain (amino acids 545–913), human CDK4, and murine Akt1 (Fig. 1B). When GST-tagged forms of each of these kinases were co-expressed with Src in sf9 cells, Src-dependent tyrosine phosphorylation of GST-CDK4 and GST-Akt1 was undetectable, and such phosphorylation of GST-DDR1b CKD was much weaker than Src-dependent tyrosine phosphorylation of GST-DDR2 CKD (Fig. 1B). Similar amounts of each GST-tagged kinase were used in these experiments, and the identity of each purified protein was confirmed by Western blotting with specific antibodies (data not shown).

To determine whether the GST-DDR2 CKD tyrosine phosphorylation we detected upon Src co-expression was due to Src activity, GST-DDR2 CKD autophosphorylation, or both, a parallel experiment was performed with a GST-tagged kinase-defective mutant of the DDR2 kinase domain (kd-GST-DDR2 CKD). This kinase-defective mutant was generated by converting lysine 608, a conserved catalytic amino acid in the active site, to alanine. This point mutation completely abolished the tyrosine kinase activity of the purified protein (data not shown). After co-expression with Src in sf9 cells, the kd-GST-DDR2 CKD protein displayed considerably less tyrosine phosphorylation than GST-DDR2 CKD, but tyrosine phosphorylation of kd-GST-DDR2 CKD was not abolished (Fig. 1C). This finding suggested that initial tyrosine phosphorylation of the DDR2 tyrosine kinase domain by Src occurs, and this in turn stimulates autophosphorylation of the DDR2 tyrosine kinase domain. The purified c-Src could phosphorylate the purified kd-GST-DDR2 CKD protein in an in vitro phosphorylation reaction as well (data not shown). This is consistent with the idea that Src directly phosphorylates the DDR2 tyrosine kinase domain in our sf9 co-expression experiments.

Tyrosine Phosphorylation by Src Stimulates the Autophosphorylation Activity of DDR2 and Its Tyrosine Kinase Activity toward Exogenous Substrates—To explore the functional significance of the observed DDR2 CKD phosphorylation by Src, we tested whether this modification stimulates the autophosphorylation activity of DDR2. To obtain tyrosine-phosphorylated GST-DDR2 CKD by Src (GST-DDR2 CKD-pY), sf9 cells were co-infected with baculoviruses encoding Src and GST-DDR2 CKD. In preliminary experiments, when the ratio of Src to GST-DDR2 CKD baculoviral titer was 1:3 or greater, the level of tyrosine phosphorylation in a specific amount of co-expressed GST-DDR2 CKD remained constant (data not shown). Therefore, we reasoned that saturation of GST-DDR2 CKD tyrosine phosphorylation by Src could be achieved by co-infecting the viruses in a ratio of 1:1 with a multiplicity of infection of 10. Expressed GST-DDR2 CKD was purified in to excess of 90% using glutathione-agarose affinity chromatography and a subsequent Superdex 200 fast-protein liquid chromatography. Src protein was undetectable in the purified GST-DDR2 CKD-pY, as judged by Western blotting (data not shown). Unphosphorylated GST-DDR2 CKD was purified from sf9 cells in the same manner after 2 days of baculovirus infection with only GST-DDR2 CKD.

Following the purifications outlined above, the autophosphorylation activities of Src phosphorylated DDR2 (GST-DDR2 CKD-pY) and non-Src phosphorylated DDR2 (GST-DDR2 CKD) were assayed in kinase reactions containing [γ-32P]ATP. Reactions were carried out at 4 °C for 4 h or at 30 °C for 30 min. Non-Src-phosphorylated GST-DDR2 CKD displayed negligible autophosphorylation at 4 °C and weak autophosphorylation at 30 °C. In contrast, the autophosphorylation activity of Src-phosphorylated GST-DDR2 CKD-pY was increased ~3-fold at 4 °C and 7-fold at 30 °C relative to that of GST-DDR2 CKD (Fig. 2A). These data suggest that phosphorylation by Src reduces a thermodynamic barrier that otherwise inhibits DDR2 kinase domain autophosphorylation. Next, we tested the effect of Src phosphorylation on the activity of the DDR2 kinase toward the exogenous peptide substrates histone H2B and poly(D4Y). We found that the DDR2 kinase phosphorylated by Src has 5- to 6-fold greater activity toward these substrates relative to that of the non-Src-phosphorylated DDR2 kinase (Fig. 2B).

Next, we examined whether accumulated autophosphorylation on the DDR2 kinase domain affects its tyrosine kinase activity. We prepared two DDR2 kinase domain proteins with different degrees of tyrosine phosphorylation by performing in vitro autophosphorylation reactions for 10 min at 30 °C in the presence or absence of 100 μM ATP. DDR2 kinase domain purified after co-expression with Src in sf9 cells was used for this preparation. Although the two proteins showed about 2-fold difference in tyrosine phosphorylation as estimated by p-Tyr Western blotting, they had approximately equal tyrosine kinase activity (Fig. 2C). This result suggests that tyrosine phosphorylation of DDR2 by Src, rather than DDR2 autophosphorylation, may be the primary determinant of DDR2 tyrosine kinase activity.

When we compared the enzyme kinetics of Src-phosphorylated and non-Src-phosphorylated purified GST-DDR2 CKD using histone H2B as a peptide substrate, tyrosine phosphorylation of the DDR2 kinase by Src reduced the $K_m$ of DDR2 for ATP from 15 μM (GST-DDR2 CKD) to 5.5 μM (GST-DDR2 CKD-pY) and increased $V_{max}$ by ~5-fold. This
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indicates that Src phosphorylation of the DDR2 cytosolic kinase domain increases both its affinity for ATP as well as its turnover rate.

**Autophosphorylation of the DDR2 Kinase Domain Occurs via an Intramolecular Mechanism**—Our results described above indicate that phosphorylation by Src precedes and stimulates autophosphorylation of the DDR2 receptor tyrosine kinase cytoplasmic domain. In an effort to decipher the autophosphorylation step more in detail, we examined whether DDR2 autophosphorylation occurs via intramolecular, cis-autophosphorylation or through intermolecular, trans-autophosphorylation.

When we measured the autophosphorylation rate (rate of phosphate transfer from ATP to DDR2 CKD) in the autophosphorylation reaction as varying the concentration of GST-DDR2 CKD-pY from 10.3 to 835 nM, the estimated rate (picomoles of transferred phosphate per minute per picomole of GST-DDR2 CKD-pY) at each different concentration of GST-DDR2 CKD-pY remains almost constant as giving the values between 0.29 and 0.32 with a maximum difference of only ~10% even though the GST-DDR2 CKD-pY concentration was changed by up to 81-fold (Fig. 3). The 10% variation might happen by some experimental errors in dealing with the highly diluted GST-DDR2 CKD-pY protein. GST-DDR2 CKD-pY exhibited a monomeric form of molecular weight in the purification step of fast-protein liquid chromatography gel-filtration chromatography (data not shown). This fact indicates that autophosphorylation of DDR2 cytosolic domain occurs via an intramolecular, cis-phosphorylation mechanism.

**Three Tyrosine Residues in the DDR2 Activation Loop Are the Targets of Src Phosphorylation**—The activation mechanism of IRTK has been well characterized (18, 19, 22, 25, 26). These studies have demonstrated that phosphorylation of three tyrosine residues (Tyr-1158, Tyr-1161, and Tyr-1162) within the activation loop of IRTK is critical for the activation of IRTK autophosphorylation as well as for its tyrosine kinase activity toward exogenous substrates (19, 25, 26).

We speculated that three tyrosine residues, Tyr-736, Tyr-740, and Tyr-741 of DDR2, are likely to be involved in the activation of DDR2 kinase activity by Src phosphorylation, because the kinase domain of DDR2 is highly homologous to that of the insulin receptor, and we could determine that the three tyrosine residues correspond to Tyr-1158, Tyr-1161, and Tyr-1162 in the activation loop of IRTK by comparing the conserved kinase domain amino acid sequences of DDR2 and the insulin receptor (Fig. 4A).

To explore the possibility of DDR2 activation loop tyrosine residue phosphorylation by Src, we created seven tyrosine-to-phenylalanine mutations in each residue, in pairwise combinations, and in all the three residues in the context of baculoviral vectors expressing the kinase-defective DDR2 K608A mutant (kd-GST-DDR2 CKD). These seven mutants are depicted in Fig. 4B. The K608A mutation was employed to abolish DDR2 autophosphorylation and thereby unmask DDR2 tyrosine phosphorylation derived solely from Src activity.

When we expressed the aforementioned mutants in s9 cells, we obtained stable, soluble DDR2 expression from five of the seven mutants (kd-Y736F, kd-Y740F, kd-Y736F/Y741F, and kd-Y736F/Y740F/Y741F). Following purification after co-expression with Src in s9 cells, the status of Src phosphorylation of these five mutants proteins was examined by Western blotting using a phosphotyrosine-specific antibody. Tyrosine phosphorylation was undetectable in the Y736F/Y740F/Y741F triple mutant. The Y736F, Y740F, Y736F/Y741F, and Y736F/Y740F/Y741F mutants each displayed tyrosine phosphorylation at levels slightly lower than was observed for DDR2 with no activation loop mutations (Fig. 5). These data indicate that Src can phosphorylate all three activation loop tyrosine residues of DDR2.

**Mutation of Tyrosine 740 to Phenylalanine in the Activation Loop of DDR2 Mimics the Effects of Src Phosphorylation on DDR2 Autophosphorylation**—To elucidate the roles of Tyr-736, Tyr-740, and Tyr-741 in DDR2 autophosphorylation, we generated another seven
tyrosine-to-phenylalanine mutations within the baculoviral expression vector of GST-DDR2 CKD. The wild-type GST-DDR2 CKD and its seven mutant proteins were expressed in sf9 cells, with or without Src co-expression, and were purified by glutathione-agarose bead.

Interestingly, Western blotting with a phosphotyrosine-specific antibody revealed that, in the absence of Src co-expression, all mutant proteins containing phenylalanine 740 substitutions (Y740F, Y736F/Y740F, Y740F/Y741F, and Y736F/Y740F/Y741F) displayed robust tyrosine phosphorylation at a level nearly comparable to that of the wild-type DDR2 co-expressed with Src (Fig. 6A). In contrast, the Y736F and Y741F mutants were weakly tyrosine-phosphorylated (Fig. 6A).

Co-expression of Src with mutant DDR2 proteins possessing Y740F substitutions did not increase the level of DDR2 tyrosine phosphorylation beyond the levels observed in the absence of Src co-expression (data not shown). These data suggest that substituting tyrosine 740 with phenylalanine can in some way mimic some if not much of the effect of Src phosphorylation of the DDR2 kinase domain. In addition, we note that significant phosphorylation occurs on tyrosine residues other than those in the DDR2 activation loop in these experiments, because the triple phenylalanine-substituted mutant (Y736F/Y740F/Y741F) shows a considerable increase in tyrosine phosphorylation (Fig. 6A).

Next, we tested whether the Y740F mutation could stimulate autophosphorylation of the DDR2 tyrosine kinase in vitro. Fig. 6B depicts the in vitro autophosphorylation activities of wild-type and various DDR2 mutant proteins. All mutant proteins possessing the Y740F substitution displayed significantly increased incorporation of 32P by tyrosine phosphorylation relative to wild-type DDR2 and mutant proteins that retain the Tyr-740 residue (Fig. 6B). These data indicate that the Y740F mutation stimulates DDR2 kinase autophosphorylation like Src phosphorylation of wild-type DDR2. Therefore this can be interpreted that tyrosine 740 might inhibit DDR2 cis-autophosphorylation. Note that, as for Src-phosphorylated DDR2, we observed that autophosphorylation of the DDR2 Y740F mutant occurred via an intramolecular mechanism (data not shown).

**DDR2 Autophosphorylation Is Required for Shc Binding to the DDR2 Cytosolic Domain**—It has been shown that the PTB domain signaling protein Shc binds to activated DDR2 receptors in the liver hepatic stellate cell line HSC T6 (23). We tested whether Shc binds preferentially to the Src-phosphorylated DDR2 cytosolic domain or to DDR2 that has undergone both Src phosphorylation and autophosphorylation. GST-DDR2 cytosolic domains purified from sf9 cells with and without Src co-infection were used for in vitro Shc binding assays in HSC T6 cell lysates. Both DDR2 proteins were subject to autophosphorylation reactions at 30 °C for 15 min. Mock autophosphorylation reactions contained γ-S-ATP. DDR2 cytoplasmic domains expressed without Src co-expression did not bind appreciably to Shc, irrespective of whether they were subjected to autophosphorylation conditions prior to Shc binding assays. In contrast, DDR2 co-expressed with Src showed some Shc binding, and this binding was significantly increased by the in vitro autophosphorylation reaction (which was also shown to increase DDR2 tyrosine phosphorylation) (Fig. 7A). This result suggests that autophosphorylation of the DDR2 cytosolic domain is necessary for optimal Shc binding.

Next, we performed the Shc binding assay with purified Y740F and Y736F/Y740F/Y741F mutant proteins. Previous experiments in this study (see above) established that the Y740F and Y736F/Y740F/Y741F mutants possess robust autophosphorylation activity without pre-phosphorylation by Src. In Shc binding assays with or without the formal autophosphorylation reaction, both Y740F and Y736F/Y740F/Y741F showed binding profiles similar to that of wild-type DDR2 cytosolic domain pre-phosphorylated by Src and subjected to autophosphorylation (Fig. 8B). This result supports our previous conclusion that the Y740F mutation mimics the action of Src with respect to DDR2 autophosphorylation and (in this experiment) Shc binding and strengthens the conclusion that Src activates DDR2 signaling by phosphorylating Tyr-740.

The fact that even the triple mutant DDR2 protein Y736F/Y740F/Y741F shows Shc binding after autophosphorylation indicates that the three tyrosine residues in the activation loop of DDR2 do not participate in Shc binding per se and that other, autophosphorylated tyrosine residues in DDR2 are directly involved in Shc binding. Because Tyr-471 in the DDR2 juxtapanel region has been previously implicated in Shc binding (23), we tested whether a DDR2 Y471F mutation impairs Shc binding. As with the wild-type protein, the Y471F mutant is tyrosine-phosphorylated by co-expressed Src in sf9 cells, and this phosphoryla-
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FIGURE 7. Autophosphorylation of DDR2 is necessary for Shc binding to DDR2 cytosolic domain. A, glutathione beads loaded with GST-DDR2 CKD or with the GST-DDR2 CKD-pY were autophosphorylated (+) or subjected to mock reaction (−), then subjected to in vitro bead binding assay for Shc. The presence of Shc in the bead eluate was exploited by Western blotting. The presence of an equal amount of GST-DDR2 CKD in each bead eluate and its accumulation of tyrosine phosphorylation by pre-autophosphorylation reaction before the binding assay were confirmed by Coomassie Brilliant Blue (CBB) staining and by Western blotting with p-Tyr antibody, respectively. “Bead only” means no GST-DDR2 CKD protein on the bead in the binding assay. B, the same type of experiment with A was done using various mutants of GST-DDR2 CKD along with GST-DDR2 CKD-pY for comparison. Y740F denotes GST-DDR2 CKD carrying Y740F mutation; other mutants are similarly depicted.

transfection, cell cultures were either treated or not treated with the ligand collagen. Fig. 8A shows that MMP-1 promoter activity was induced ~2.3-fold by collagen treatment in cells co-transfected with the wild-type DDR2 expression plasmid. However, in the case of Y740F-DDR2 mutant co-transfection, the basal MMP-1 promoter activity was ~1.8-fold greater than that of wild-type DDR2 co-transfection, and this level was not increased significantly with collagen treatment. Meanwhile, as expected, we did not observe any activation of MMP-1 promoter activity by collagen treatment in case of DDR2 K608A co-transfection (Fig. 8A).

To confirm further the ligand-independent activation of DDR2 signaling by Y740F mutation, we generated six clonal cells from SK-N-SH cells that express stably transfected either wild-type DDR2 (three clones, DDR2 wild-C1, -C2, and -C3) or DDR2 Y740F (three clones,
DDR2 Y740F-C1, -C2, and -C3) in addition to the low level of endogenous DDR2 protein (Fig. 8B).

When cells were plated into normal culture dishes, MMP-2 expression was higher consistently in all the three clonal cells expressing DDR2 Y740F than in the three clones of wild-type DDR2 by an average of 1.6-fold (Fig. 8C). On the other hand, when cells were plated on the collagen-coated wells to examine the collagen-dependent induction of MMP-2 expression, we did not see a notable increase of MMP-2 expression over the level observed in normal culture dishes in the case of the three clonal cells expressing DDR2 Y740F, whereas there was an average 1.8-fold induction in three clonal cells expressing wild-type DDR2. Parental SK-N-SH cells behaved similarly to the clonal cells expressing wild-type DDR2 (Fig. 8C). Collectively, these results support the idea that the Y740F mutant constitutively activates DDR2-dependent signaling and are consistent with the predictions of our biochemical experiments.

**DISCUSSION**

In this study, we used co-expression of Src, the DDR2 kinase domain, and a variety of DDR2 mutant proteins to explore the mechanism of Src-mediated DDR2 receptor tyrosine kinase signaling. This approach enabled us to circumvent many of the difficulties associated with studying Src phosphorylation of DDR2 exclusively in vitro. For example, carrying out phosphorylation reactions in vitro with purified DDR2 kinase domain and Src proteins yields low levels of DDR2 tyrosine phosphorylation. In addition, the tyrosine kinase activity of DDR2 thus prepared is not robust, making subsequent analysis difficult if not impossible. We think this liability is due to a thermal decay of DDR2 enzymatic activity during in vitro phosphorylation reactions at 30 °C: we found that purified DDR2 tyrosine kinase loses >80% of its activity after 30-min incubation at 30 °C.3

Here, we revealed that DDR2 tyrosine kinase strictly requires the initial phosphorylation on the tyrosine residues of its activation loop by Src to show a considerable autophosphorylation activity in our study. Src tyrosine kinase activity was also suggested to enhance signaling by epidermal growth factor receptor, platelet-derived growth factor receptor, and IRTK (27–29). However, when we assayed the autophosphorylation activity of epidermal growth factor receptor and IRTK kinase domains purified from sf9 cells, they showed considerable autophosphorylation activity in the absence of Src co-expression, and this activity was not significantly enhanced even when they were purified after co-expression with Src.4 It has been reported that the activated IRTK with three tyrosine residues phosphorylated in its activation loop can be easily obtained by in vitro autophosphorylation reaction even on ice using recombinant IRTK protein purified from expressed sf9 cells (19). These facts suggest that epidermal growth factor receptor and IRTK might be less dependent on Src activity for their activation, whereas Src kinase activity is indispensable for the activation of the DDR2 tyrosine kinase.

In this study, we have also made progress in elucidating the molecular mechanism underlying the activation, autophosphorylation, and Shc binding of the DDR2 cytosolic domain. We obtained evidence that, among the three activation loop tyrosines, only Tyr-747 is exclusively required for autoinhibiting the autophosphorylation activity of DDR2. Furthermore, we observed that wild-type DDR2 phosphorylated by Src and the Y740F mutant function similarly in terms of autophosphorylation and Shc binding. Therefore our findings indicate that the main function of Src in the activation of DDR2 receptor signaling is the induction of phosphorylation tyrosine 740.

It is unclear whether the three tyrosine residues in the activation loop can be further phosphorylated by autophosphorylation aside from being phosphorylated by Src. However, it is clear that other tyrosine residues in DDR2 can be largely phosphorylated in the autophosphorylation reaction and at least one of these phosphorylated residues is responsible for the binding of Shc protein. Although previous report suggested that tyrosine 471 in the juxtamembrane region of DDR2 may be phosphorylated by Src and that phosphorylated Tyr-471 is the site for Shc binding (23), our data indicate that Tyr-471 phosphorylation does not occur directly by Src, but it may result from autophosphorylation. However, our results support the previous observation that phosphorylated Tyr-471 could be involved in Shc binding.

In most receptor tyrosine kinases, phosphorylation of tyrosine residues in the activation loop is a key event in the stimulation of RTK autophosphorylation activity (17). In crystallographic studies, it was shown that Tyr-1162 (corresponding to Tyr-740 of DDR2) of unactivated IRTK forms a specific hydrogen bond with aspartate 1132 within the active site to stabilize the activation loop into an inhibitory conformation (19). According to our data, it seems that an unphosphorylated, intact tyrosine 740 residue in the DDR2 activation loop also has a critical role for autoinhibitory effect on DDR2 tyrosine kinase activity. Its modification either to phosphotyrosine or phenylalanine could relieve DDR2 tyrosine kinase from a restrained state of autoinhibition to become an active conformation to drive the intramolecular self-phosphorylation.

Abrupt regulation of DDR2 is emerging as a likely cause of various human diseases, including organ fibrosis, rheumatoid and osteoarthritis, and cancer (12–16, 31). Consequently, studies to decipher the precise mechanism of DDR2 tyrosine kinase activation and its signaling partners may prove important for the development of effective therapies (e.g. potent and specific DDR2 inhibitors) for these diseases. Our data suggest that the structure around the active site pocket of the DDR2 tyrosine kinase changes upon its activation by phosphorylation, because the $K_m$ for ATP and $V_{max}$ are different between non-activated and activated DDR2 tyrosine kinases. In fact, it may be possible to identify small molecule inhibitors that preferentially target the ATP binding pocket of the activated DDR2 tyrosine kinase.

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