Discoidin Domain Receptor 2 Interacts with Src and Shc following Its Activation by Type I Collagen*

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Discoid domain receptor 2 (DDR2) is an unusual receptor tyrosine kinase in that its ligand is fibrillar collagen rather than a growth factor-like peptide. We examined signal transduction pathways of DDR2. Here we show that DDR2 is also unusual in that it requires Src activity to be maximally tyrosine-phosphorylated, and that Src activity also promotes association of DDR2 with Shc. The interaction with Shc involves a portion of DDR2 that is not previously implicated in interaction with receptor tyrosine kinases. These results identify Src kinase and the adaptor protein Shc as key signaling intermediates in DDR2 signal transduction. Furthermore, Src is required for DDR2-mediated transactivation of the matrix metalloproteinase-2 promoter. The data support a model in which Src and the DDR2 receptor cooperate in a regulated fashion to direct the phosphorylation of both the receptor and its targets.

Receptor tyrosine kinases (RTKs)† of the discoid domain receptor (DDR) family are unlike most RTKs, in that they do not use typical peptide growth factors as ligands; instead, they signal in response to fibrillar collagens (1, 2), establishing the DDR family as receptors for extracellular matrix molecules. Thus far, two DDR receptors have been identified, DDR1 and DDR2. DDR1 is primarily expressed in epithelial cells in the brain, gastrointestinal tract, lung, and kidney, whereas DDR2 is expressed in interstitial cells in the heart, skeletal muscle, lung, brain, and kidney (3). DDR1 and DDR2 are differentially activated by collagens. DDR1 is activated primarily by collagen types I, II, III, V, and XI, whereas DDR2 is activated mainly by collagen types I and III (1, 2, 4).

In addition to their unique ligand specification, several other features distinguish DDR receptors from other RTKs. The kinetics of DDR receptor activation by collagens differs significantly from other RTKs in response to their cognate ligands. For example, platelet-derived growth factor (PDGF) or epidermal growth factor stimulate receptor activation within seconds (4). In contrast, tyrosine phosphorylation of DDR receptors can be detected only after prolonged exposure to collagen (approximately 30 min), and then phosphorylation is sustained for an extended period (more than 16 h) (2, 5). This unique slow-on, slow-off phenomenon and receptor specificity raise important questions about the nature of downstream intracellular signals mediating the effects of DDR2.

Receptor tyrosine kinases contain a catalytic domain that can autophosphorylate one or more tyrosine residues typically located in the noncatalytic region of the receptor. These phosphorylations lead to generation of docking sites for SH2 and PTB domains of signaling molecules that associate with the receptors (6). For example, PDGF receptor and fibroblast growth factor receptor associate with signaling molecules such as phospholipase C-γ, Src, Shc, and phosphatidylinositol 3-kinase. However, it is still unknown which molecules interact with DDR2.

In this study, we have explored intracellular pathways mediating DDR2 signaling, and report that DDR2 signaling is propagated through interaction with both the Src non-receptor tyrosine kinase and the adaptor molecule Shc. Furthermore, we demonstrate that these signals mediate transactivation of the matrix metalloproteinase-2 (MMP-2) promoter.

EXPERIMENTAL PROCEDURES

Plasmids and Cell Lines—We used HSC-T6 cells, an activated rat hepatic stellate cell (i.e. myofibroblast) line (7), COS7 cells, SrcYes/Fyn triple-null cells (SYF) (8), and skin fibroblasts that were isolated from other RTKs in response to their cognate ligands. These results identify Src kinase and the adaptor protein Shc as key signaling intermediates in DDR2 signal transduction. Furthermore, Src is required for DDR2-mediated transactivation of the matrix metalloproteinase-2 (MMP-2) promoter.

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The abbreviations used are: RTK, receptor tyrosine kinase; CA Src, constitutively active Src; DDr, discoid domain receptor; DN Src, dominant negative Src; MMP-2, matrix metalloproteinase-2; pMalC, mouse protein expressed in activated lymphocytes; PDGF, platelet-derived growth factor; WB, Western blot; Ab, antibody; PBTA, phosphotyrosine binding; SH, Src homology; CH, collagen homology.

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§§ The abbreviations used are: RTK, receptor tyrosine kinase; CA Src, constitutively active Src; DDR2, discoid domain receptor; SH2, Src homology; CH, collagen homology.

1 The abbreviations used are: RTK, receptor tyrosine kinase; CA Src, constitutively active Src; DD1, discoid domain receptor; DN Src, dominant negative Src; MMP-2, matrix metalloproteinase-2; pMalC, mouse protein expressed in activated lymphocytes; PDGF, platelet-derived growth fact; WB, Western blot; Ab, antibody; PTB, phosphotyrosine binding; SH, Src homology; CH, collagen homology.

2 The abbreviations used are: RTK, receptor tyrosine kinase; CA Src, constitutively active Src; DD1, discoid domain receptor; DN Src, dominant negative Src; MMP-2, matrix metalloproteinase-2; pMalC, mouse protein expressed in activated lymphocytes; PDGF, platelet-derived growth factor; WB, Western blot; Ab, antibody; PTB, phosphotyrosine binding; SH, Src homology; CH, collagen homology.

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mented with 10% fetal calf serum and grown in the presence of penicillin and streptomycin. Fc-DDR2 and CA or DN Src were cotransfected in COS7 cells using LipofectAMINE (Invitrogen) in Opti-MEM serum-free medium at 37 °C for 24 h. HSC-T6 cells and COS7 cells were serum-starved in Dulbecco's modified Eagle's medium for 16 h and then stimulated with collagen type I (Becton Dickinson) for 2 h.

Cells on 10-cm diameter culture dishes were rinsed twice in PBS (4 °C) and lysed in 0.5 ml of the lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 0.5% Nonidet P-40, 10 mM NaF, 1 mM Na₃VO₄, and protease inhibitor mixture (Roche Molecular Biochemicals)), and insoluble material was removed by centrifugation (15,000 × g, 10 min) at 4 °C. After total cell protein in lysates from serum-starved cells was determined by a Bradford assay (Bio-Rad), antibodies were added to the cell lysates and incubated for 2 h at 4 °C, followed by precipitation on protein A-agarose beads (Sigma). The immunoprecipitated proteins were washed at 4 °C in the lysis buffer prior to direct analysis by SDS-polyacrylamide gel electrophoresis (PAGE) (8% acrylamide gel). For immunoblotting, proteins were transferred to membranes overnight at 50 mA. The membranes were blotted in Tris-buffered saline plus Tween (25 mM Tris-HCl, pH 7.6, 50 mM NaCl, 0.05% or 0.1% Tween 20) and placed in blocking buffer (Tris-buffered saline plus Tween containing 5% fetal calf serum or 5% milk) for 1 h at room temperature. The blots were incubated with 1:20,000 dilution of anti-phosphotyrosine Ab (4G10, Upstate Biotechnology, Inc.), 1:1,000 dilution of anti-DDR2 Ab (R2-JM, R2B), anti-Shc Ab (Upstate Biotechnology, Inc.), and anti-Src Ab (Upstate Biotechnology, Inc.) for 1 h at room temperature. Bound primary antibody was visualized by chemiluminescent horseradish peroxidase substrate (Pierce) with horseradish peroxidase-conjugated antibody to mouse IgG (Upstate Biotechnology, Inc.) for 1 h at room temperature. Antibody to β-actin was visualized by chemiluminescent horseradish peroxidase (Upstate Biotechnology, Inc.) for 1 h at room temperature. Bound primary antibody was visualized by chemiluminescent horseradish peroxidase substrate (Pierce) with horseradish peroxidase-conjugated antibody to mouse IgG (Upstate Biotechnology, Inc.) for 1 h at room temperature.

Yeast Two-hybrid Screening—The Saccharomyces cerevisiae yeast reporter strain, L40, has the following genotype: MATα his3 leu2 lys2 trp1 238 leu2·his3::LEU2-HIS3 URA3::LEU2-HIS3 LYS2::LEX-A-LEU2 L.40 were grown at 30 °C in YPAD (1% yeast extract, 2% peptone, 2% glucose, and 0.1% NaCl) supplemented with 10% serum to an OD of 0.5. After total cell protein in lysates from serum-starved cells was determined by a Bradford assay (Bio-Rad), antibodies were added to the cell lysates and incubated for 2 h at 4 °C, followed by precipitation on protein A-agarose beads (Sigma). The immunoprecipitated proteins were washed at 4 °C in the lysis buffer prior to direct analysis by SDS-polyacrylamide gel electrophoresis (PAGE) (8% acrylamide gel). For immunoblotting, proteins were transferred to membranes overnight at 50 mA. The membranes were blotted in Tris-buffered saline plus Tween (25 mM Tris-HCl, pH 7.6, 2% SDS, 100 mM β-mercaptoethanol) at 55 °C for 30 min prior to reprobing with different primary antibodies.

RESULTS

DDR2 Associates with Src, and Its Phosphorylation Is Regulated by Src—To identify endogenous DDR2 expression and its signaling pathway, we initially utilized a rat hepatocyte cell line (HSC-T6), as we previously identified DDR2 expression in this cell type by homology PCR for receptor tyrosine kinases (12). These cells were cultured with or without collagen type I for 2 h. Immunoprecipitation of cell lysates was performed using a polyclonal antibody (R2-JM) to the DDR2 juxtamembrane domain, followed by Western blot (WB) with either anti-DDR2 or an anti-phosphotyrosine antibody (Fig. 1, lane 1). Immunoprecipitation/WB for anti-DDR2 confirmed DDR2 expression with and without collagen, and WB with anti-phosphotyrosine demonstrated that DDR2 is phosphorylated by exposure to type I collagen (arrowhead). Interestingly, WB for anti-phosphotyrosine revealed another band of ~60 kDa (asterisk). Furthermore, WB for anti-Src demonstrated that Src associates with DDR2 in stellate cells stimulated with type I collagen (arrow).

FIG. 1. Expression of endogenous DDR2 and its association with c-Src in hepatic stellate cells (HSC-T6). Cultured HSC-T6 cells were stimulated with (lanes 2, 4, 6, and 8) or without (lanes 1, 3, 5, and 7) collagen type I (20 μg/ml) for 2 h. Cells lysates were immunoprecipitated (IP) by a polyclonal anti-DDR2 antibody (lanes 1–6) or control rabbit serum (lanes 7 and 8), followed by WB with anti-DDR2 (lanes 1 and 2), anti-phosphotyrosine (lanes 3 and 4), or anti-Src (lanes 5 and 6) as described under “Experimental Procedures.” Immunoprecipitation/WB for anti-DDR2 confirmed DDR2 expression with and without collagen, and WB with anti-phosphotyrosine demonstrated that DDR2 is phosphorylated by exposure to type I collagen (arrowhead). Interestingly, WB for anti-phosphotyrosine revealed another band of ~60 kDa (asterisk). Furthermore, WB for anti-Src demonstrated that Src associates with DDR2 in stellate cells stimulated with type I collagen (arrow).
we performed experiments in which the phosphorylation state of a constitutively dimerized form of DDR2 (FcDDR2, a dimerized DDR kinase resulting from the replacement of the DDR2 extracellular domain with the Fc region of human IgG) was analyzed in the presence of CA or DN Src. As shown in Fig. 3D, FcDDR2 is much more active than unstimulated DDR2 and appears to be as active as fully stimulated DDR2. In COS7 cells that were cotransfected with FcDDR2 and CA Src, markedly increased tyrosine phosphorylation of FcDDR2 was detectable (Fig. 3A, lane 4), compared with that in COS7 cells transfected with only FcDDR2 (Fig. 3A, lane 3). Some phosphorylation was still detected in cells transfected only with FcDDR2 without CA Src, which could reflect autophosphorylation because of its dimeric structure and/or the activity of endogenous Src kinase. Importantly, the ability of Src to promote tyrosine phosphorylation of DDR2 depends on intact kinase activity of the DDR2 receptor, because CA Src promoted much less tyrosine phosphorylation of a kinase-dead form of DDR2 (Fig. 3A, lane 6). Thus, the catalytic activity of DDR2 and Src seem to synergistically promote tyrosine phosphorylation of DDR2.

Next, COS7 cells were cotransfected with a dominant negative Src and FcDDR2, which inhibited tyrosine phosphorylation of FcDDR2 compared with that in COS7 cells transfected with only FcDDR2 (Fig. 3B). Importantly, cotransfection of dominant negative Src also inhibited the tyrosine phosphorylation of full-length DDR2 stimulated by type I collagen (Fig. 3C, lanes 7 and 8).

To further establish the physiological relevance of the interaction between DDR2 and Src kinase, SYF and wild type fibroblasts were stimulated by collagen type I. As shown in Fig. 4, DDR2 phosphorylation by collagen type I stimulation was delayed and decreased in SYF cells. In particular, the upper band in WB for anti-phosphotyrosine, which represents a highly phosphorylated form of DDR2, is diminished in SYF knockout cells (Fig. 4, lanes 7 and 8).

**DDR2 Associates with Shc in a Src-dependent Manner**—To further explore interactions between DDR2 and Src, we next utilized the yeast two-hybrid system to clone proteins that bind to phosphorylated DDR2 cytoplasmic domain in the presence of Src kinase. The bait vector encoded the cytoplasmic domain of DDR2 (fused to LexA) but was also modified to include the expression of constitutively active Src to phosphorylate the expressed LexA-DDR2 fusion protein (Fig. 5A). To first test whether Src tyrosine kinase induced phosphorylation of the DDR2 cytoplasmic domain in yeast, we performed an antiphosphotyrosine blot. Lysates of yeast cells transformed with control bait (pBTM116-lamin, pBTM116[src], and pBTM116-DDR2) and cells expressing LexA-DDR2 fusion together with Src (pBTM116 [src] DDR2) were precipitated by anti-DDR2 (R2B), followed by WB with anti-DDR2, anti-LexA, and/or anti-phosphotyrosine (Fig. 5A). Immunoblot with anti-DDR2 or anti-LexA demonstrated a band corresponding to the expected size of the LexA-DDR2 cytoplasmic domain fusion protein in the yeast transformed with either pBTM116-DDR2 or pBTM116[src]-DDR2 bait vector; however, phosphorylation was confined to the fusion protein co-expressed with Src kinase.
To identify potential interacting prey proteins by yeast two-hybrid, we chose a commercial library from skeletal muscle cells, where DDR2 is expressed. From the $6 \times 10^6$ transformants that were screened with the DDR2 bait, 46 colonies were positive for transcriptional activation. Among these was a 2.5-kbp cDNA encoding part of the Shc adaptor protein. As shown in Fig. 5A, Shc has three isoforms (46, 52, and 66 kDa) (16, 17). CH1, SH2, and a part of PTB domains are common to each of these. The region of Shc identified by yeast two-hybrid overlapped almost exactly with CH1 and SH2 domains, which are included in all three isoforms. C, to determine whether Src tyrosine kinase is necessary for Shc association with DDR2, cloned Shc vector was cotransformed with each control bait vector or original bait vector (lanes 3 and 4); however, phosphorylation was confined to the fusion protein co-expressed with Src kinase (lane 4). By yeast two-hybrid screening with DDR2 bait from human skeletal muscle cDNA library, the Shc adaptor protein was cloned. B, Shc has three isoforms (46, 52, and 66 kDa). The region of Shc identified by yeast two-hybrid overlapped almost exactly with CH1 and SH2 domains, which are included in all three isoforms. C, to determine whether Src tyrosine kinase is necessary for Shc association with DDR2, cloned Shc vector was cotransformed with each control bait vector or original bait vector in L40 yeast and was cotransformed with each control bait vector or original bait vector (PTB, CH1, SH2), and full-length Shc were inserted into the pGAD vector as an in-frame fusion with a hemagglutinin tag to create a panel of putative prey molecules. Three control bait vectors and the original vector were cotransformed into L40 yeast. As shown in Table I, -galactosidase assay demonstrated that full-length Shc and (CH1+SH2) domains interacted with DDR2 in the presence of active Src kinase. This finding established that the portion of Shc containing both the (CH1+SH2) domains is the minimal region required to interact with phosphorylated DDR2. To eliminate the possibility that SH2 domain is misfolded and does not associate with DDR2 in yeast, we used a truncated mouse protein expressed in activated lymphocytes (mPAL) as a positive control bait, which is known to associates with Shc SH2 (18). -Galactosidase assay demonstrated that truncated mPAL associated with full-length Shc and Shc-SH2 (data not shown).

To confirm the association of Shc with DDR2 in mammalian cells, we used skin fibroblasts from DDR2 knockout mice (9), which were reconstituted by retroviral infection with green fluorescent protein, wild type mouse DDR2, or a kinase-dead DDR2 mutant (Fig. 6). Each of these cell populations was cultured with or without collagen type I (20 μg/ml). Cells lysates were precipitated by anti-DDR2 polyclonal antibody, followed by Western blot with anti-DDR2, anti-phosphotyrosine, and/or anti-Shc. Immunoprecipitation/WB demonstrated that Shc associated with DDR2 when cells expressing wild type DDR2 were stimulated with collagen type I (Fig. 6, lane 4). Shc association with DDR2 did not occur when cells expressing the kinase-dead mutant were stimulated with collagen type I (Fig. 6, lane 6).

We further investigated the requirement for Src kinase for Shc to associate with DDR2. Fc-DDR2 and dominant negative Src were cotransfected in COS7 cells. Cells lysates were precipitated with protein A, followed by Western blot with anti-DDR2, anti-phosphotyrosine, and/or anti-Shc. As shown in Fig. 7A, the phosphorylated Fc-DDR2 was dephosphorylated by dominant negative Src. Autophosphorylated Fc-DDR2 associated with Shc (Fig. 7A, lane 3), whereas Fc-DDR2 dephosphorylated by dominant negative Src did not support the Shc-DDR2 interaction (Fig. 7A, lane 4). Furthermore, the Src kinase inhibitor, SU6656, partially inhibited phosphorylation of DDR2 by collagen I stimulation, while completely inhibiting Shc association with DDR2 (Fig. 7B, lanes 4 and 5). On the other hand, Fc-DDR2 was highly phosphorylated by overexpressed Src, which with each prey vector into L40 yeast. As shown in Table I, yeast-two-hybrid system was utilized to clone proteins that bind to the phosphorylated DDR2 cytoplasmic domain in the presence of Src kinase. A, lysates of yeast cells transformed control baits (pBTM116-lamin (lane 1), pBTM116(src) (lane 2), and pBTM116-DDR2 (lane 3)), and cells expressing LexA-DDR2 cytoplasmic domain together with Src (lane 4) were precipitated by anti-DDR2, followed by WB with anti-LexA, anti-DDR2, and/or anti-phosphotyrosine. Immunoblot with anti-LexA or anti-DDR2 demonstrated a band corresponding to the expected size of the LexA-DDR2 cytoplasmic domain fusion protein in the yeast transformed with either pBTM116-DDR2 or pBTM116[src]-DDR2 bait vector (lanes 3 and 4); however, phosphorylation was confined to the fusion protein co-expressed with Src kinase (lane 4). By yeast two-hybrid screening with DDR2 bait from human skeletal muscle cDNA library, the Shc adaptor protein was cloned.

The yeast two-hybrid system was utilized to clone proteins that bind to the phosphorylated DDR2 cytoplasmic domain in the presence of Src kinase. A, lysates of yeast cells transformed control baits (pBTM116-lamin (lane 1), pBTM116(src) (lane 2), and pBTM116-DDR2 (lane 3)), and cells expressing LexA-DDR2 cytoplasmic domain together with Src (lane 4) were precipitated by anti-DDR2, followed by WB with anti-LexA, anti-DDR2, and/or anti-phosphotyrosine. Immunoblot with anti-LexA or anti-DDR2 demonstrated a band corresponding to the expected size of the LexA-DDR2 cytoplasmic domain fusion protein in the yeast transformed with either pBTM116-DDR2 or pBTM116[src]-DDR2 bait vector (lanes 3 and 4); however, phosphorylation was confined to the fusion protein co-expressed with Src kinase (lane 4). By yeast two-hybrid screening with DDR2 bait from human skeletal muscle cDNA library, the Shc adaptor protein was cloned. B, Shc has three isoforms (46, 52, and 66 kDa). The region of Shc identified by yeast two-hybrid overlapped almost exactly with CH1 and SH2 domains, which are included in all three isoforms. C, to determine whether Src tyrosine kinase is necessary for Shc association with DDR2, cloned Shc vector was cotransformed with each control bait vector or original bait vector in L40 yeast and -galactosidase assay was performed again. The LexA-DDR2 cytoplasmic domain fusion protein was able to interact with Shc clone when Src was present.
in turn was highly associated with Shc (Fig. 8, lane 7).

To identify residues in DDR2 critical for Src/Shc interaction, we generated a Y471F mutant in the juxtamembrane region of DDR2 to investigate whether this site is critical for Src/Shc interaction based on the following reasons. 1) A database search, by using “Scansite” of the Division of Signal Transduction, Harvard Institutes of Medicine (scansite.mit.edu/), identified Tyr-471 as a candidate tyrosine critical for the Src/Shc interaction. 2) Src binds to phosphotyrosine residues in the juxtamembrane region of other RTKs like PDGF-R α and β (19). 3) DDR1 associates with the Shc PTB domain at a similar residue in its juxtamembrane region (20).

Plasmids encoding a point mutation of Y471F and double point mutations of K608E and Y471F of FcDDR2 were cotransfected with or without wild type Src in COS7 cells. Cell lysates were precipitated with protein A, followed by Western blot with anti-phosphotyrosine, or anti-Shc as described under “Experimental Procedures.” One or 3 µM SU6656, which did not completely inhibit the phosphorylation of DDR2 by collagen stimulation, inhibited Shc-DDR2 interaction (lanes 4 and 5).

Fig. 6. Association of DDR2 with Shc in mammalian cells. Skin fibroblasts were isolated from DDR2 knockout mice and immortalized by transfection with SV 40 large T antigen. DDR2 expression by the cells was reconstituted by retroviral infection with either wild type mouse DDR2 (lanes 3 and 4) or a kinase-dead DDR2 mutant (DDR2KE) (lanes 5 and 6). These cells were each stimulated with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) collagen type I (20 µg/ml) for 2 h. Cell lysates were immunoprecipitated (IP) by anti-DDR2, followed by WB with anti-DDR2, anti-phosphotyrosine, or anti-Shc as described under “Experimental Procedures.” Immunoprecipitation/WB demonstrated that Shc associated with DDR2 only when cells expressing wild type DDR2 were stimulated with collagen type I (lane 4). Shc association with DDR2 did not occur when cells expressing the kinase-dead mutant were stimulated with collagen type I (lane 6).

Fig. 7. Src kinase-dependent association of DDR2 with Shc in mammalian cells. A, COS7 cells transfected with empty vector (lane 1). FcDDR2 (lanes 3 and 4) and DN Src (lanes 2 and 4) were transiently expressed in COS7 cells. Cell lysates were precipitated by protein A, followed by Western blotting with anti-DDR2, anti-phosphotyrosine, or anti-Shc as described under “Experimental Procedures.” FcDDR2, which was dephosphorylated by DN Src, did not support the Src-DDR2 interaction (lane 4). B, DDR2−/− (lane 1) and DDR2-reconstituted DDR2−/− (lanes 2–5) skin fibroblasts were stimulated with collagen type I for 2 h in the presence (lanes 4 and 5) or absence (lanes 1–3) of Src kinase inhibitor SU6656. Cells lysates were precipitated by anti-DDR2, followed by Western blotting with anti-DDR2, anti-phosphotyrosine, or anti-Shc as described under “Experimental Procedures.” One or 3 µM SU6656, which did not completely inhibit the phosphorylation of DDR2 by collagen stimulation, inhibited Shc-DDR2 interaction (lanes 4 and 5).

Fig. 8. A Y471F mutation reduces the association of DDR2 with Shc and Src. FcDDR2 (lanes 2 and 7), FcDDR2Y471F (lanes 3 and 8), FcDDR2KE (lanes 4 and 9), or FcDDR2KEY471F (lanes 5 and 10) were transiently expressed with (lanes 6–10) or without (lanes 1–5) wild type Src in COS7 cells. Cell lysates were precipitated by protein A, followed by Western blotting with anti-phosphotyrosine, anti-DDR2, and anti-Shc as described under “Experimental Procedures.” The Y471F mutant DDR2 (lanes 3 and 8) slightly decreased its phosphorylation; however, the K608E, Y471F DDR2 double mutants (lanes 5 and 10) dramatically decreased its phosphorylation (arrowhead). Furthermore, Shc association was dramatically decreased by the Y471F mutation (arrow).
were cultured in the presence of collagen type I (30 μg/ml) with or without Src kinase inhibitor, SU6656 (1 μM SU6656; S10, 10 μM SU6656) for 24 h after transfection. As described under Experimental Procedures: Data are expressed as mean ± S.D. of at least three different experiments. *, p < 0.05; **, p < 0.01 versus control of DDR2 +/- skin fibroblasts.

Fig. 9. DDR2 signaling mediates MMP-2 promoter activity. A 1686-bp MMP-2 promoter cDNA driving a luciferase reporter gene was transiently co-transfected along with CA Src (CA), DN Src (DN), or a control vector (C) into DDR2 +/- or DDR2 +/- skin fibroblasts. Cells were cultured in the presence of collagen type I (30 μg/ml) with or without Src kinase inhibitor, SU6656 (S1, 1 μM SU6656; S10, 10 μM SU6656) for 24 h after transfection. Luciferase activity was measured as described under “Experimental Procedures.” Data are expressed as mean ± S.D. of at least three different experiments. *, p < 0.05; **, p < 0.01 versus control of DDR2 +/- skin fibroblasts.

siently cotransfected with CA or DN Src expression plasmid and a luciferase reporter cDNA, which incorporates 1686 bp upstream of the translation start site of the MMP-2 gene. Cells were cultured in the presence of collagen type I with or without Src kinase inhibitor, SU6656 for 24 h after transfection. As shown in Fig. 9, MMP-2 promoter activity in DDR2 +/- cells was 10 times higher than that in DDR2 +/- cells. Furthermore, it was significantly up-regulated by CA Src and down-regulated by DN Src or SU6656 in DDR2 +/- cells. A similar effect on MMP-2 promoter activity was also identified in COS7 cells, which were cotransfected with pMT21 plasmid with or without wild type DDR2.

DISCUSSION

Adhesion to extracellular matrices and to other cells is mediated by a diverse family of receptors, the best characterized being integrins (13), cadherins (22), selectins (23), and cell adhesion molecules (24). Src kinases have been implicated in adhesion events regulated by these receptors (25). Src family kinases also communicate with many different RTKs (14). The biochemical connections between these different receptors and Src family kinases include phosphorylation of Src family kinase, association with the RTK, activation of Src kinases, and phosphorylation of the RTK. Interestingly, the DDR family of RTKs have recently been found to represent a new receptor that responds to the extracellular matrix in particular by binding collagen (1, 2). Thus, the interaction between DDRs and other classes of matrix receptors, as well as the sharing of downstream signaling pathways such as Src, must be explored.

In this study, we have shown that endogenously expressed DDR2 receptors in an activated hepatic stellate cell line (HSC-T6) are associated with the non-receptor tyrosine kinase Src. Furthermore, we demonstrate that Src is requisite for maximal DDR2 tyrosine phosphorylation and leads to additional association with the adaptor molecule Shc, resulting in increased MMP-2 promoter activity.

There is minimal information about downstream signaling molecules for DDR receptors. Shc has recently been shown to associate with DDR1 (2), although the interacting region of Shc for DDR1 differs from that used by Shc for DDR2. Shc is expressed as three alternatively spliced adapter proteins, which share an N-terminal PTB domain, a central glycine sequence, and C-terminal SH2 domain as shown in Fig. 5R. Shc proteins are tyrosine-phosphorylated by receptor tyrosine kinases and bind to phosphotyrosine residues on those receptors. The b isoform of DDR1 has an additional 37-amino acid sequence inserted by alternative splicing into the juxtamembrane domain containing an LXNPXY sequence (26). Upon collagen-mediated receptor activation, the tyrosine in the LXNPXY sequence becomes phosphorylated, providing a binding site for PTB domain of Shc. In contrast, we find by yeast two-hybrid system that DDR2 associates with CH1-SH2 domains of Shc but not its PTB, because the portion of Shc containing both the CH1+SH2 domains is the minimal region required to interact with phosphorylated DDR2. Consistent with DDR2 interacting with non-PTB portion of Shc, DDR2 lacks the NPXY site by which Shc PTB domain has been shown to bind DDR1 and other receptors. We additionally established that phosphorylation of DDR2 receptor is necessary for association with Src, and that the Tyr-239/Tyr-240 residues in the CH1 domain of Shc are also phosphorylated by Src (27). Shc phosphorylation at tyrosine residues in the CH1 domain (Tyr-239, Tyr-240, and Tyr-317) may also be necessary for the association with DDR2, particularly because these residues are preserved in all three isoforms of Shc (17).

We have identified Tyr-471 as a critical phosphotyrosine residue of DDR2 required for Shc interaction, among the 14 tyrosines in the intracellular domain. As shown in Fig. 8, a Y471F mutation and/or kinase-dead K608E dramatically decreased Shc association; however, it did not totally inhibit the association, which suggests there are still other minor association sites with Shc.

Tyrosine-phosphorylated Shc can recruit Grb2/Sos through a binding event between the Grb2 SH2 domain and Shc phosphotyrosine residues, ultimately resulting in activation of Ras, the extracellular signal-regulated kinase cascade, and mitogen-activated protein kinase signaling pathway. We have recently demonstrated a marked decrease in MMP-2 expression in DDR2 +/- fibroblasts compared with DDR2 +/- cells (21). Therefore, we performed an MMP-2 promoter assay in the presence of CA or DN Src in each of these cell types. MMP-2 promoter activity in DDR2 +/- cells was significantly higher than that in DDR2 +/- cells, and in DDR2 +/- cells was further up-regulated by CA Src but down-regulated by DN Src or Src kinase inhibitor, SU6656.

Prior to the identification of DDR2 as a collagen receptor, cell interactions with collagen had been ascribed primarily to integrins, a family of heterodimeric receptors composed of α and β chains, which, among other activities, can up-regulate MMP-2 (29). It seems possible that integrins and DDR2 are co-localized and aggregated as they hold collagen as a common ligand, which may enable their signaling pathways to converge downstream. Our findings support this notion by demonstrating that the Src pathway, already known to be involved downstream of integrin signaling, is requisite for maximal DDR2 tyrosine phosphorylation and for engagement of the Shc adaptor protein. However, we could not establish a direct relationship between integrins and the DDR2 signaling pathway (data not shown). Emerging insights into DDR2 signaling pathways will be critical to understanding how this receptor system interacts with other matrix receptors, as well as the as-yet-unclear role of this receptor in mediating cellular responses to matrix molecules in its environment.

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Discoidin Domain Receptor 2 Interacts with Src and Shc following Its Activation by Type I Collagen

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