Collagen I Induces Discoidin Domain Receptor (DDR) 1 Expression through DDR2 and a JAK2-ERK1/2-mediated Mechanism in Primary Human Lung Fibroblasts*

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Discoidin domain receptors (DDRs) DDR1 and DDR2 are receptor tyrosine kinases with the unique ability among receptor tyrosine kinases to respond to collagen. Several signaling molecules have been implicated in DDR signaling, including Shp-2, Src, and MAPK pathways, but a detailed understanding of these pathways and their transcriptional targets is still lacking. Similarly, the regulation of the expression of DDs is poorly characterized with only a few inflammatory mediators, such as lipopolysaccharide and interleukin-1β identified as playing a role in DDR1 expression. DDs have been reported to induce the expression of various genes including matrix metalloproteinases and bone morphogenetic proteins, but the regulatory mechanisms underlying DDR-induced gene expression remain to be determined. The aim of the present work was to elucidate the molecular mechanisms implicated in the expression of DDs and to identify DDR-induced signaling pathways and target genes. Our data show that collagen I induces the expression of DDR1 in a dose- and time-dependent manner in primary human lung fibroblasts. Furthermore, activation of DDR2, JAK2, and ERK1/2 MAPK signaling pathways was essential for collagen I-induced DDR1 and matrix metalloproteinase 10 expression. Finally, inhibition of the ERK1/2 pathway abrogated DDR1 expression by blocking the recruitment of the transcription factor polyoma enhancer A-binding protein 3 to the DDR1 promoter. Our data provide new insights into the molecular mechanisms of collagen I-induced DDR1 expression and demonstrate an important role for ERK1/2 activation and the recruitment of polyoma enhancer-A binding protein 3 to the DDR1 promoter. Particularly, the lung, kidney, mammary gland, and gastrointestinal tract, whereas DDR2 is primarily found in cells of mesenchymal origin, such as fibroblasts and smooth muscle cells (1, 2). DDR1 is primarily activated by collagen I—IV and VIII, whereas DDR2 responds to collagen I and to a lesser extent to collagen II, III, and V but does not interact with collagen IV (3, 4). DDR2 has one isoform, whereas five DDR1 isoforms are generated by alternative splicing: DDR1a, b, c, d, and e. DDs have been implicated in the expression of pro-inflammatory mediators and matrix-degrading enzymes and play an important role in processes such as migration, proliferation, extracellular matrix (ECM) remodeling and wound repair (2, 5–7). Moreover, studies in vivo and in vitro have implicated DDs in various fibrotic and fibroproliferative conditions such as cancer, atherosclerosis, inflammation, arthritis, and fibrosis of the kidney, liver, skin, and lung (1, 2, 8–13).

The wide range of processes regulated upon DDR stimulation suggests that DDs may exert their effects via multiple pathways. Thus, DDR1 has been proposed to interact with several cofactors, including ShcA, Nck2, and Shp-2 (14, 15), and STAT5, NF-κB, and p38 MAPK (5, 16, 17). DDR2 appears to require the recruitment of Src for complete phosphorylation (5, 18, 19) and has been shown to signal through the ERK1/2 MAPK and activator protein (AP)-1 (20, 21). However, detailed studies on the pathways activated by DDR 1 and 2 in different cell types and their transcriptional targets have not yet been described. Similarly, very little is known about the regulation of DDR expression, and so far, only a few inflammatory mediators including TNF-α, IL-1β, and LPS have been shown to increase DDR1 expression (22), but the regulatory mechanisms governing the expression of DDs, particularly in structural cells, remain to be elucidated.

Genes reported to be activated upon DDR stimulation include cyclooxygenase-2, monocyte chemotactic protein (MCP)-1, and N-cadherin for DDR1 in fibroblasts, macrophages, and epithelial cells, respectively (5, 17, 23), and bone morphogenetic protein (BMP)-2, BMP-5 and BMP-7, for both DDR1 and DDR2 in fibroblasts (24). Studies with knock-out mice and human carcinoma cells have shown that DDR1 and DDR2 also play a crucial role in the expression of MPP-1, MPP-2, MPP-9, and MPP-10 in fibroblasts (3, 7, 24, 25). MMPs, because of their ECM remodeling activity, have been associated with several pathologic processes including osteoarthritis, autoimmune diseases, cancer, and fibrosis (26–28). Although MMPs in general are differentially regulated, promoters of most MMPs contain binding sites for both AP-1 and

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2 The abbreviations used are: DDR, discoidin domain receptor; MMP, matrix metalloproteinase; BMP, bone morphogenetic protein; PEA, polyoma enhancer-A binding protein; ECM, extracellular matrix; NHFL, normal human lung fibroblast; AP, activator protein; MCP, monocyte chemotactic protein; MOPS, 4-morpholinepropanesulfonic acid; ACh, acetic acid; PE, phycoerythrin; MBG, minor groove binding; ECM, extracellular matrix.
polymyxin B-binding protein (PEA) 3. Furthermore, cooperation between both nuclear factors seems to be essential for the transcripitional activation of those MMPs (29, 30).

In the present work, we aimed to understand the molecular mechanisms in the regulation of DDR expression and to identify DDR-associated signaling pathways and transcriptional targets. Our results show a novel mechanism by which collagen I is able to induce DDR1 and MMP-10 expression through integrin independent activation of DDR2 in primary normal human lung fibroblasts (NHLFs). Furthermore, we show that collagen I-induced DDR1 gene expression requires recruitment of phospho-JAK2 to DDR2 and ERK1/2 activation and involves the recruitment of the nuclear factor PEA3 to the DDR1 promoter.

**Experimental Procedures**

**Cell Culture and Reagents**—NHLFs were grown in Dulbecco’s modified Eagle’s medium supplemented with 15% FBS. DDR-overexpressing HEK293 cells were grown in minimal essential medium supplemented with 20% FBS, 2 mM L-glutamine, and 0.25 mg/ml geneticin (all reagents from Invitrogen). DDR-overexpressing HEK293 cells were seeded in poly-D-lysine-coated plates (Sigma-Aldrich) and allowed to attach to a total volume of 20 µl of reaction buffer containing RT-PCR buffer, MgCl2 (5.5 mM), desoxyribonucleoside triphosphate mixture (500 µM), random hexamers (2.5 µM), RNase inhibitors (0.4 unit/µl), and MultiScribe reverse transcriptase (1.25 units/µl) (all of the reagents were from Applied Biosystems) and incubated for 10 min at 25 °C followed by 30 min at 48 °C and 5 min at 95 °C. Real time PCR was performed using TaqMan system 7900HT (Applied Biosystems). TaqMan probes and primers used for the reaction are provided in Table 1. For real time quantitative RT-PCR, 1 µl of total RNA was added to a total volume of 20 µl of reaction buffer containing TaqMan Fast Universal master mix (Applied Biosystems), TaqMan probes (0.3 µM), and forward and reverse primers (0.9 µM). PCR was performed by denaturation at 95 °C for 20 s, followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 s. The expression changes (fold increases) were calculated relative to unstimulated control samples. Where indicated, PCR products (10 µl) were subjected to electrophoresis on 2% agarose gels.

**siRNA and Transfection**—Negative control 2 siRNA and human DDR2-specific siRNA were synthesized by Applied Biosystems. The sequence of DDR2 siRNA is as follows: 5′-GCACUGUCGUAUCACCAATT-3′ (sense) and 5′-UUG-GUGUACUGACAGUGCGT-3′ (antisense). JAK2- and ERK1/2-specific siRNAs were obtained from Cell Signaling Technologies. For siRNA delivery, NHLFs were reverse transfected with 10 nM siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. 48 h after transfection, the cells were starved for 24 h prior to treatment with collagen I (25 µg/ml) or vehicle (ACh, 0.1 mM).

**Table 1**

| Forward primer | Reverse primer | TaqMan probe |
|----------------|----------------|--------------|
| DDR1-all       | 5′-AGCGCCCATGAAATTATCGATCG-3′ | 6-FAM-AGGATGACTCTCTTCC-3′-MGB |
| DDR1a          | 5′-ACGAATCTGAGCCGCTG-3′ | 6-FAM-ACTTTATGGGAGGCG-3′-MGB |
| DDR1b          | 5′-ACAATGTCGAGCCGCTG-3′ | 6-FAM-ACTTTATGGGAGGCG-3′-MGB |
| DDR1c          | 5′-ACCAAGATGTCTGATGTCAA-3′ | 6-FAM-ACCATGAGGGGTCGTT-3′-MGB |
| DDR1d          | 5′-CTGGTCTGAGGGTCA-3′ | 6-FAM-TGGAGCAGGAGGTTG-3′-MGB |
| DDR1e          | 5′-TTCCTGAGGGTCA-3′ | 6-FAM-CCCTTAATGGTGGTCAAGGA-3′-MGB |
| DDR2-all       | 5′-TCTTCCGAGTAGTTTTGGATCCA-3′ | 6-FAM-CTTTCCAGAACATGAGT-3′-MGB |
| MCP-1          | 5′-TGGCCGAAATGTTGTTAG-3′ | 6-FAM-TCAGATGCTGCCG-3′-MGB |
| MMP-2          | 5′-GCTGCCCTTGGAGTCA-3′ | 6-FAM-AGGACATTACGACG-3′-MGB |
| MMP-9          | 5′-GCCGAGGCTCACCCTTCTAT-3′ | 6-FAM-CCACGAGGCTACCATC-3′-MGB |
| MMP-10         | 5′-CTGGTCTGAGGGTGCA-3′ | 6-FAM-CTTTCCAGAACATGAGT-3′-MGB |
| BMP-2          | 5′-TGGTAGAGTCTTATGGAGGCTG-3′ | 6-FAM-AGGACATTACGACG-3′-MGB |
| BMP-5          | 5′-TGAGGAGGCTACCATC-3′ | 6-FAM-CAATCTGAGTTCTAG-3′ |
| ERK1           | 5′-TCCACCCGTCCCGTCTCAACA-3′ | 6-FAM-AGGATGGATCAGTGTATG-3′-MGB |
| JAK2           | 5′-TGGATTCGCGGTTGTCTGCA-3′ | 6-FAM-AGGATGGATCAGTGTATG-3′-MGB |
| Cyclin D1      | 5′-CAGGTCGCGGCTCTC-3′ | 6-FAM-AGGATGGATCAGTGTATG-3′-MGB |
Flow Cytometry (FACS)—NHLFs collected by EDTA treatment were washed in PBS containing 1% BSA and incubated with biotinylated goat anti-DDR1 antibody or isotype control biotinylated normal goat IgG (both from R & D Systems) for 1 h at 4°C. After washing with PBS containing 1% BSA, the cells were incubated with phycoerythrin-conjugated streptavidin (R & D Systems) for another 1 h at 4°C. The data were acquired on FACSCanto (BD Biosciences) and analyzed using FlowJo software.

Western Blotting and Immunoprecipitation—NHLFs or DDR-overexpressing HEK293 cells were lysed in radioimmune
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ELISA Analysis—The concentration of MMP-10 in NHLFs culture supernatants was determined by MMP-10 Quantikine ELISA (R & D Systems) according to the manufacturer’s instructions.

ChIP Analysis—After the treatment of NHLFs cells, chromatin immunoprecipitation was performed by using the ChIP-IT Express Enzymatic Kit (Active Motif) according to the manufacturer’s instructions. Immunoprecipitation was carried out overnight at 4 °C using 10 μl of anti-PEA3 (Abcam), anti-c-Fos (Abcam), and anti-c-Jun (Active Motif) antibodies. Immune complexes were collected with magnetic beads for 1 h. DNA cross-links of the immune complexes were reverted by heating followed by proteinase K digestion. DNA isolated from an aliquot of the total nuclear extract was used as a loading control for the PCR (input control). PCR was performed with total DNA and immunoprecipitated DNA using the following promoter-specific primers, DDR1 promoter-binding site for PEA3: 5′-TGTTCTTACTTCTGGTGCTCTCT-3′ (forward), 5′-AATATAAATGCAATATGCCTTCTCGA-3′ (reverse), and 6-FAM-TCGAGGAATGCTGG-MGB (TaqMan probe) (67 bp); and MMP-10 promoter-binding site for AP-1: 5′-TGGGTGGTGCGTCTGTCTGTTGCT-3′ (forward), 5′-TGTTCTTACTTCTGGTGCTCTCT-3′ (reverse), and 6-FAM-ATATCTTTAGCTATGAC-3′ (reverse). 12915

Precipitation assay buffer containing 50 mM Tris (pH 8), 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate acid, 0.1% SDS, protease inhibitors (Roche Applied Science), and protease inhibitors (Roche Applied Science). The cell debris was removed by centrifugation, and the supernatants were denatured at 94 °C in 4× NuPAGE sample buffer (Invitrogen) containing 5% β-mercaptoethanol. 20 μg of protein was subjected to electrophoresis on a 4–10% Bis-Tris (MOPS) NuPAGE gel (Invitrogen) and blotted onto a nitrocellulose membrane (Invitrogen). The membranes were blocked for 1 h in TBS, 0.1% (v/v) Tween 20, and 5% (w/v) nonfat dried milk. Immunodetection was carried out using anti-phospho-ERK1/2 (T185/Y187) (Invitrogen), anti-ERK1/2 (Cell Signaling Technologies), anti-phospho-JAK2 (Y1007/Y1008) (Abcam), anti-JAK2 (Santa Cruz), anti-DDR1 (R & D Systems), and anti-GAPDH (Santa Cruz). The bands were visualized using the ECL system (Amer sham Biosciences). Immunoprecipitation was performed overnight at 4 °C using anti-DDR2 (R & D Systems). Immunocomplexes were collected with 100 μl of protein A/G Plus-agarose beads (Santa Cruz) for 1 h at 4 °C and washed with radioimmunprecipitation assay buffer. Protein was extracted by 5 min of incubation in 4× NuPAGE sample buffer (Invitrogen) containing 5% β-mercaptoethanol at 94 °C prior to Western blot analysis. Densitometry of bands was performed using Image J.

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Collagen I induces MCP-1, BMP-2, MMP-2, and MMP-10 mRNA expression in a time-dependent manner in NHLFs. NHLFs were serum-starved overnight and incubated with collagen I (25 μg/ml) or vehicle (Ach, 0.1 μl) at various time points. Total RNA was isolated and reverse transcribed, and real time quantitative PCR was performed using the TaqMan system with specific primers and TaqMan Probes for human MCP-1 (A), BMP-2 (B), MMP-2 (C), MMP-10 (D), MMP-9 (E), BMP-5 (F), and GAPDH. The expression changes (fold increases) were calculated relative to unstimulated control cells after normalizing with GAPDH. The results are representative of mean fold increases ± S.D. of three independent experiments done in triplicate (n = 9; *, p < 0.05; **, p < 0.01). C, MMP-10 protein was measured in the culture supernatant of stimulated NHLFs by ELISA. The results are representative of mean fold increases ± S.D. of three independent experiments done in triplicate (n = 9; *, p < 0.05).

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TGATGGCTGAATCACCATTAC-3′ (forward), 5′-ATC-ACCAACAGTATGATTGCAGC-3′ (reverse), and 6-FAM-CATTCAGACTTTAATAAACAC-MGB (TaqMan Probe) (90 bp). The PCR products (10 μl) were subjected to electrophoresis on 2% agarose gels.

Statistical Analysis—The data were expressed as the means ± S.D. of nine or more independent experiments. Statistical analysis was performed using one-way analysis of variance followed by Tukey test. Significance was set as p < 0.05.

RESULTS

Collagen I, but Not Collagen IV, Induces DDR1 Expression in NHLFs—To determine whether collagen can induce the expression of DDR1 or DDR2 in NHLFs, we stimulated NHLFs with two structurally different collagens, fibrillar collagen I or network-forming collagen IV, for 16 h. Collagen I induced DDR1 mRNA expression in a dose-dependent manner reaching maximal expression with 25 μg/ml (Fig. 1A), whereas constitutive DDR2 mRNA expression was not increased by collagen I (Fig. 1B). In contrast, network-forming collagen IV was unable to induce either DDR1 or DDR2 mRNA expression. Because DDR2 is not able to recognize collagen IV, whereas DDR1 can recognize both collagen I and collagen IV (3, 4) and NHLFs only showed an increased DDR1 expression upon collagen I stimulation, our results suggest a DDR2-mediated response. Collagen I-induced DDR1 protein expression was then assessed using flow cytometry analysis. As shown in Fig. 1C, the percentage of positive NHLFs expressing DDR1 on the cell surface increased significantly after 16 h of collagen I stimulation. To identify which of the five DDR1 isoforms generated by alternative splicing was responsible for the observed response, we first determined which isoforms were expressed in NHLFs. Real time quantitative RT-PCR performed using RNA isolated from untreated NHLFs showed that only the DDR1a and DDR1b isoforms were expressed (Fig. 1D). We then tested which of these two isoforms was induced by collagen I and showed that collagen I was able to induce mRNA expression of both isoforms in a time-dependent manner with maximal expression after 16 h (Fig. 1E).

Collagen I Induces DDR1 and MMP-10 Expression through DDR Activation Independently of β1-Integrin—Collagen I has been shown to induce the expression of several genes including pro-inflammatory and fibro-proliferative factors as well as matrix-degrading enzymes in DDR1- and DDR2-overexpressing human fibrosarcoma cells and mouse fibroblasts (24). We examined a selection of these profibrotic factors to determine whether they were induced by collagen I in NHLFs. Our data demonstrated that MCP-1, BMP-2, MPP-2, and MMP-10 expression was induced in NHLFs in a time-dependent manner reaching maximum expression after 16 h of collagen I stimulation (Fig. 2, A–D), whereas the mRNA expression of the profibrotic factors BMP-5 and MMP-9 was not increased upon collagen I stimulation (Fig. 2, E and F). To elucidate whether the induction of DDR1 and MMP-10 by collagen I was mediated by integrins or DDRs, NHLFs were preincubated with the neutralizing anti-β1 integrin antibody (32) or the tyrosine kinase inhibitor AMN107 (nilotinib) prior to collagen I stimulation. AMN107 is a tyrosine kinase inhibitor of the breakpoint cluster region-Abelson kinase, and earlier work in our laboratory has shown that it is also a very potent inhibitor of DDR activation (31). Our data showed that collagen I-induced DDR1 and MMP-10 mRNA expression was completely abrogated in the presence of AMN107, whereas blocking β1 integrins had no effect on message level (Fig. 3, A and B). Similarly, collagen I-induced MMP-10 protein secretion was inhibited by AMN107, but not by the anti-β1 integrin blocking antibody (Fig. 3C). Expression of cyclin D1, a major regulator of G1 phase cell cycle progression, requires β1 integrin-mediated adhesion to the ECM (33). Anti-β1 integrin antibody inhibited collagen I-induced cyclin D1 mRNA expression in NHLFs (Fig. 3D), showing that it is an effective blocking antibody at a concentration of 10 μg/ml.

Collagen I Induces MMP-10 mRNA Expression through DDR2 by a JAK2-ERK1/2-dependent Mechanism in NHLFs and DDR2-overexpressing HEK293 Cells—To address the question as to whether DDR1, DDR2, or both receptors were involved in mediating collagen I induction of DDR1 and MMP-10, we utilized HEK293 stable transfected cell lines overexpressing the
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Collagen I induces MMP-10 mRNA expression by a JAK2-ERK1/2-dependent mechanism in NHLFs and DDR2-overexpressing HEK293 cells. A and B, NHLFs (A) and DDR2-overexpressing HEK293 cells (B) were serum-starved overnight. The cells were then incubated with AG490 (25 μM), PD98059 (20 μM), or SB203580 (20 μM) for 1 h prior to 16 h of stimulation with collagen I (25 μg/ml). Total protein was isolated and reverse transcribed, and real-time quantitative PCR was performed using the TaqMan system with specific primers and TaqMan Probes for human MMP-10 and GAPDH. The expression changes (fold increases) were calculated relative to unstimulated control cells after normalization to GAPDH or total protein is presented. The results are representative of mean fold increase ± S.D. of three independent experiments done in triplicate (n = 9; *, p < 0.05). C, DDR2-overexpressing HEK293 cells were serum-starved overnight and incubated with collagen I (25 μg/ml) at various time points. Total protein was isolated and subjected to SDS-PAGE followed by anti-phospho-JAK2, anti-JAK2, anti-phospho-ERK1/2, anti-ERK1/2 MAPK, and anti-DDR1 immunoblotting. Anti-GAPDH was used as loading control. Quantification relative to control cells after normalization to GAPDH or total protein is presented. The results are representative of mean fold increase ± S.D. of three independent experiments (n = 3; *, p < 0.05; **, p < 0.01). D and E, DDR2-overexpressing HEK293 cells were serum-starved overnight. The cells were then incubated with AG490 (25 μM) (D) or PD98059 (20 μM) (E) for 1 h prior to 16 h of stimulation with collagen I (25 μg/ml). Total protein was isolated and subjected to SDS-PAGE followed by anti-phospho-JAK2, anti-JAK2, anti-phospho-ERK1/2 MAPK, and anti-DDR1 immunoblotting. Anti-GAPDH was used as loading control. Quantification relative to control cells after normalization to GAPDH or total protein is presented. The results are representative of mean fold increase ± S.D. of three independent experiments (n = 3; *, p < 0.05; **, p < 0.01; ***, p < 0.001).

Collagen I-induced MMP-10 expression was specifically driven by DDR2 activation, we used a siRNA approach to knock down DDR2 expression. The DDR2 mRNA and protein levels were significantly reduced in NHLFs transfected with DDR2-specific siRNA (Fig. 6A and B) 72 h after transfection. Collagen I-induced DDR1 (Fig. 6C) and MMP-10 (Fig. 6D) mRNA expression, as well as MMP-10 protein secretion (Fig. 6E) was completely abrogated in the presence of DDR2-specific siRNA. Furthermore, NHLFs transfected with DDR2 siRNA showed lower levels of constitutive DDR1 protein expression as well as JAK2 and ERK1/2 phosphorylation (Fig. 6F). Subsequently, we knocked down JAK2 and ERK1/2 expression to further demonstrate that collagen I induced DDR1 and MMP-10 expression by a JAK2/ERK1/2 mechanism. The mRNA and protein expression of different DDRs (DDR1a, DDR1b, and DDR2) present in NHLFs. As shown in Fig. 4, collagen I was able to induce the mRNA expression of both DDR1 and MMP-10 in DDR2- but not DDR1a- or DDR1b-overexpressing HEK293 cells in a time-dependent manner, indicating a DDR2-mediated effect. Several pathways have been implicated in DDR signaling particularly for DDR1 (5, 18); however, signaling pathways activated by DDR2 have only been poorly elucidated. To identify key signaling pathways downstream of DDR2 in NHLFs, we tested the effect of pharmacological inhibitors of three important pathways, (JAK/STAT, MEK/ERK, and p38 MAPK) upon collagen I-induced MMP-10 expression. Both NHLFs and DDR2-overexpressing HEK293 cells were incubated with AG490, PD98059, and SB203580, selective pharmacological inhibitors of JAK2, MEK1, and p38 MAPK, respectively, prior to 16 h of collagen I stimulation. Collagen I-induced MMP-10 mRNA expression was significantly inhibited in the presence of JAK2 and MEK1, but not p38 MAPK inhibitors in both NHLFs (Fig. 5A) and DDR2-overexpressing HEK293 cells (Fig. 5B). These data suggest that both JAK2 and ERK1/2 signaling pathways are involved in collagen I-induced MMP-10 mRNA expression. Time course experiments revealed that collagen I induced ERK1/2 MAPK and JAK2 phosphorylation in a time-dependent manner reaching maximum phosphorylation after 16 h of stimulation in DDR2-overexpressing HEK293 cells (Fig. 5C). The inhibition of JAK2 or ERK1/2 MAPK activity in turn leads to the inhibition of DDR1 protein expression (Fig. 5D and E). To further prove that collagen I-mediated induction of DDR1 and MMP-10 expression was specifically driven by DDR2 activation, we used a siRNA approach to knock down DDR2 expression. The DDR2 mRNA and protein levels were significantly reduced in NHLFs transfected with DDR2-specific siRNA (Fig. 6A and B) 72 h after transfection. Collagen I-induced DDR1 (Fig. 6C) and MMP-10 (Fig. 6D) mRNA expression, as well as MMP-10 protein secretion (Fig. 6E) was completely abrogated in the presence of DDR2-specific siRNA. Furthermore, NHLFs transfected with DDR2 siRNA showed lower levels of constitutive DDR1 protein expression as well as JAK2 and ERK1/2 phosphorylation (Fig. 6F). Subsequently, we knocked down JAK2 and ERK1/2 expression to further demonstrate that collagen I induced DDR1 and MMP-10 expression by a JAK2/ERK1/2 mechanism. The mRNA and protein expression of...
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A

B

C

D

E

F
JAK2 and ERK1/2 was significantly reduced in NHLFs transfected with JAK2-specific (Fig. 7, A and B) or ERK1/2-specific (Fig. 8, A and B) siRNA, respectively, 72 h after transfection. Collagen I-induced DDR1 and MMP-10 mRNA expression, as well as MMP-10 protein secretion was inhibited in the presence of JAK2-specific (Fig. 7, C–E) or ERK1/2-specific (Fig. 8, C–E) siRNA. Moreover, HNLFs transfected with JAK2-specific (Fig. 7F) or ERK1/2-specific (Fig. 8F) siRNA showed a significant reduction of constitutive DDR1 protein expression.

Collagen I Induces DDR2 Tyrosine Phosphorylation Prior to Recruitment of Phospho-JAK2 to the Receptor—DDR2 has 13 tyrosine residues in its cytoplasmic region, and phosphorylation of some of these residues by autophosphorylation or by Src kinase has been shown to be involved in receptor activation (5). Because the activation of the JAK/STAT pathway is often strongly associated with receptor tyrosine kinases, we wanted to determine whether JAK directly interacted with DDR2 or its tyrosine-phosphorylated active form. We immunoprecipitated DDR2 after collagen I stimulation at different time points. Collagen I was able to induce sustained DDR2 tyrosine phosphorylation within an hour in both NHLFs (Fig. 9A) and DDR2-overexpressing HEK293 cells (Fig. 9B). Furthermore, tyrosine phosphorylation was followed by the recruitment of phospho-JAK2 to DDR2 after 4 h of collagen I stimulation (Fig. 9, A and B). Taken together, our results show that collagen I triggers MMP-10 expression through activation of DDR2 and the recruitment of phospho-JAK2 to the signaling complex in NHLFs.

The Transcription of DDR1 Is Mediated by PEA3 and Inhibition of the ERK1/2 MAPK Signaling Pathway Blocks Recruitment of PEA3 to the DDR1 Promoter—There is very little known about the transcription factors involved in the expression of the target genes of DDR2 activation, and transcription factors governing DDR1 expression are also poorly described. Our data demonstrate that the expression of both DDR1 and MMP-10 was induced upon collagen I stimulation. Furthermore, nuclear factors AP-1 and PEA3 have been shown to have binding sites in most MMP promoters, often having a cooperative effect on gene expression (30, 34). We performed sequence analysis of the DDR1 promoter and showed that one putative binding site for AP-1 and two for PEA3 are also present in the DDR1 promoter (Fig. 10A). Because we showed the involvement of the MEK/ERK pathway in DDR1 and MMP-10 expression and because both nuclear factors have been proposed to be activated by ERK1/2 MAPK, we sought to investigate the effects of collagen I-induced ERK1/2 MAPK activation on the binding activity of AP-1 and PEA3 to the promoters of DDR1 and MMP-10. ChIP analysis showed that inhibition of ERK1/2 blocked constitutive recruitment of PEA3, but not AP-1, to the promoter of DDR1 in DDR2-overexpressing HEK293 cells (Fig. 10B). Because the MEK1 inhibitor inhibited DDR1 expression, this result suggests that recruitment of PEA3 to the DDR1 promoter is essential for the induction of DDR1 expression. Interestingly, constitutive recruitment of PEA3 to the promoter of MMP-10 was not inhibited in the presence of the MEK1 inhibitor (Fig. 10C), suggesting that different transcriptional mechanisms regulate the expression of the two genes.

DISCUSSION

Our data show that collagen I induces the expression of DDR1 and MMP-10 through the activation of DDR2 and independently of integrins in a time and concentration-dependent manner in primary NHLFs. To our knowledge, this is the first report showing that DDR1 is a target gene for collagen I-induced DDR2 activation. We also show that of the five known DDR1 isoforms, NHLFs constitutively express DDR1a and DDR1b, and the expression of only these two isoforms is induced in response to collagen I stimulation. Furthermore, the effect appears to be specific for these DDR1 isoforms because collagen I failed to up-regulate DDR2 expression.

In addition to DDR1a and DDR1b, collagen I also induced the mRNA expression of several genes that have been implicated in fibrotic and remodeling conditions including MCP-1, BMP-2, MMP-2, and MMP-10 in a time-dependent manner in NHLFs, reaching maximal expression after 16 h of stimulation. MCP-1 is chemotactic for mononuclear phagocytes and has been identified as a profibrotic mediator playing a role in the development of interstitial fibrosis (35, 36). BMPs have been shown to modulate a wide range of processes including ECM production, cell migration, and mesenchymal cell differentiation and have been implicated in fibrotic conditions as both protective and fibrosis-promoting mediators (37–40). Our results demonstrate that collagen I is able to induce BMP-2 but not BMP-5 expression in human primary lung fibroblasts, suggesting different transcriptional regulatory mechanisms and roles for the different BMPs. A key mechanism, by which DDRs regulate cell adhesion, migration, proliferation, and ECM remodeling, is controlling the expression and activity of MMPs (5, 41). Our data show that collagen I induces MMP-2 and MMP-10 but not MMP-9 expression in NHLFs. This result supports previous reports demonstrating that DDR2 plays an essential role in the induction of mouse fibroblast proliferation and migration con-

![Collagen Induces DDR1 and MMP-10 expression through DDR2 in NHLFs](image)
comitantly with the expression and activity of MMP-2 and without the involvement of MMP-9 (25). MMP-10 has long been known to play a role in physiological and pathological situations in the context of connective tissue remodeling (42–44) and has been associated with lung and liver fibrosis (45, 46).

Integrins are the main receptors for ECM components in mammals playing an important role in the regulation of fibroblast proliferation and the expression of MMPs. The four collagen-specific integrins share the β1 subunit, which forms heterodimers with the α1, 2, 10, and 11 subunits. Although studies on the relevance of integrins on collagen I-induced MMP expression are conflicting (47–49), incubation of NHLFs with integrin β1 blocking antibody prior to collagen I stimulation confirmed that collagen I does not require integrin activation to
trigger DDR1 and MMP-10 expression. This result agrees with previous studies showing that DDR signaling is independent of integrin activation (4, 49, 50). Importantly, we showed that the DDR inhibitor AMN107 (31, 51) was able to block collagen I-induced DDR1 and MMP-10 expression, further confirming that collagen I exerts its effect through DDR independently of integrin activation.

Collagen I is able to activate both DDR1 and DDR2, whereas collagen IV only targets DDR1. Our results demonstrated that collagen IV was unable to induce MMP-10 and DDR1 gene expression, therefore identifying DDR2 as the receptor driving collagen I-induced DDR1 and MMP-10 expression. Additionally, only DDR2, but not DDR1a- or DDR1b-overexpressing HEK293 cells showed increased MMP-10 and DDR1 mRNA expression upon collagen I stimulation, confirming that collagen I-induced MMP-10 and DDR1 expression is mediated via DDR2 and not via the DDR1a or DDR1b isoforms. Finally, the knocking down of DDR2 expression in NHLFs with DDR2-specific siRNA completely abrogated collagen I-induced DDR1 and MMP-10 mRNA expression and MMP-10 protein secretion, further confirming the specific role of DDR2 in collagen I-mediated induction of DDR1 and MMP-10 expression.

Studies performed in recent years have lead to the identification of DDR-associated signaling molecules and pathways (5); however, despite these efforts, still very little is known about signaling pathways downstream of DDR2. Our data show that collagen I induced JAK2 and ERK1/2 phosphorylation in a time-dependent manner and that pharmacological inhibition of JAK2 or MEK1, the kinase upstream of ERK1/2, resulted in the inhibition of collagen I-induced DDR1 and MMP-10 expression. Furthermore, the knocking down of JAK2 and ERK1/2 expression with JAK2- and ERK1/2-specific siRNA inhibited both constitutive and collagen I-induced DDR1 and MMP-10 expression in NHLFs, confirming essential roles for both signaling pathways in collagen I-mediated induction of DDR1 and MMP-10 expression. This correlates with a recent report showing that inhibition of ERK1/2 leads to decreased MMP-13 promoter activity and expression upon collagen II stimulation in synovial fibroblasts (20). Similarly, the expression of MMP-9 requires ERK1/2 activation in glioma cells (52).

Importantly, basal protein expression levels of DDR1, as well as constitutive phosphorylation of JAK2 and ERK1/2, were reduced in NHLFs in the presence of the DDR2-specific siRNA, suggesting a link between DDR2 pathway activity and the activation of these signaling pathways leading to DDR1 expression. DDR2 has 13 potential tyrosine phosphorylation sites serving as docking sites for signaling molecules (3–5, 8). In this study, we show that collagen I induced sustained DDR2 tyrosine phosphorylation within the first hour of stimulation followed by recruitment of phospho-JAK2 to DDR2 after 4 h in both NHLFs and DDR2-overexpressing HEK293 cells. Because the inhibition of either JAK2 or ERK1/2 leads to the abrogation of DDR1 and MMP-10 expression, this result suggests that JAK2 recruitment and activation by DDR2 are upstream events that lead to ERK1/2 phosphorylation, which in turn triggers DDR1 and MMP-10 expression.

Promoters of all MMPs share several cis-acting elements, including binding sites for AP-1 and PEA3, a member of the Ets oncoprotein family of transcription factors often juxtaposed and acting cooperatively in the regulation of the expression of MMPs (53, 54). Interestingly, both nuclear factors are downstream targets of ERK1/2 and have been shown to require ERK1/2 activation to induce MMP expression (55–57). We identified one potential binding site for AP-1 at −94 bp and two for PEA3 at −342 and −831 bp in the DDR1 promoter using Genomatix software tools (Fig. 10A). Our finding that expression of both DDR1 and MMP-10 is induced by collagen I and is ERK1/2-dependent led us to hypothesize that recruitment of PEA3 and AP-1 may also play a role in the expression of DDR1. Our results show constitutive recruitment of PEA3, c-Jun, and c-Fos to the promoter of both DDR1 and MMP-10. Inhibition of the ERK1/2 signaling pathway by a MEK1 inhibitor abrogates constitutive recruitment of PEA3 to the DDR1 promoter in DDR2-overexpressing HEK293 cells, suggesting a possible mechanism by which ERK1/2 inhibition leads to the inhibition of DDR1 expression. Importantly, our results indicate that inhibition of PEA3 is sufficient to block DDR1 expression. Interestingly, constitutive recruitment of both AP-1 subunits, c-Jun and c-Fos, to the DDR1 promoter is unaffected by the MEK1 inhibitor, suggesting that AP-1 and PEA3 are under the control...
of different signaling pathways that may also play a role in the transcriptional activation of the DDR1 gene. We have not been able to demonstrate PEA3 recruitment in primary human lung fibroblasts. Because ChIP requires a high level of promoter binding, we believe it is technically not possible to show the differential effect in nuclear factor recruitment in these cells upon collagen stimulation. Alternatively, we cannot rule out that although activation of ERK1/2 is also required for DDR1 expression in NHLFs, transcriptional regulation and transcription factor recruitment may differ between HEK and NHLF cells. Although inhibition of ERK1/2 abrogated collagen I-induced MMP-10 expression, constitutive recruitment of AP-1 and PEA3 to the MMP-10 promoter was not affected. This finding indicates additional complexity in the DDR2 activated transcriptional program and suggests that ERK1/2 may activate additional nuclear factors involved in MMP-10 expression.

In summary, this study provides evidence that collagen I triggers the expression of DDR1 through activation of DDR2 by a JAK2/ERK1/2-mediated mechanism and independently of integrins in primary human lung fibroblasts. In addition, our results demonstrate the MEK1/ERK1/2-dependent recruitment of PEA3 to the DDR1 promoter, which is essential for collagen I-induced DDR1 expression. Although the present work provides a possible molecular mechanism for the induc-
tion of DDR1 expression upon collagen I stimulation, further studies are needed to elucidate the details of the transcriptional and post-transcriptional regulation of DDR1 expression.

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