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Mapping of Epitopes in Discoidin Domain Receptor 1 Critical for Collagen Binding*

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The binding and activation of the discoidin domain receptor 1 by collagen has led to the conclusion that proteins from the extracellular matrix can directly induce receptor tyrosine kinase-mediated signaling cascades. A region in the extracellular domain of DDR1 homologous to the *Dictyostelium discoideum* protein discoidin-I is also present in the secreted human protein RS1. Mutations in RS1 cause retinoschisis, a genetic disorder characterized by ablation of the retina. By introducing point mutations into the discoidin domain of DDR1 at positions homologous to the retinoschisis mutations, ligand binding epitopes in the discoidin domain of DDR1 were mapped. Surprisingly, some residues only affected receptor phosphorylation, whereas others influenced both collagen-binding and receptor activation. Furthermore, two truncated DDR1 variants, lacking either the discoidin domain or the stalk region between the discoidin and transmembrane domain, were generated. We showed that (i) the discoidin domain was necessary and sufficient for collagen binding, (ii) only the region between discoidin and transmembrane domain was glycosylated, and (iii) the entire extracellular domain was essential for transmembrane signaling. Using these results, we were able to predict key sites in the collagen-binding epitope of DDR1 and to suggest a potential mechanism of signaling.

Discoidin domain receptors 1 and 2 (DDR1 and DDR2)[1] have been recognized as a distinct tyrosine kinase receptor subfamily because of structural and functional homologies. In their extracellular region, both receptors show a domain homologous to the *Dictyostelium discoideum* protein discoidin I. DDR1 and DDR2 are also functionally related by the observation that collagen acts as cognate ligand for both receptors. Whereas DDR1 activation is achieved by all collagens so far tested (types I–VI and VIII), DDR2 is only activated by fibrillar collagens, in particular by collagen type I and type III. In contrast to most other tyrosine kinase receptors, activation of DDRs can take several hours (1, 2).

The cDNA coding for human DDR1 has been cloned from several tissues or carcinoma cells (3–7). Gene orthologs to human DDR1 have been identified in mice, rats, and *Caenorhabditis elegans* (8–10). Expression of human DDR1 is predominantly seen in epithelial cells, particularly from kidney, lung, gastrointestinal tract, and brain, but also in corneal and dermal fibroblasts (7, 9, 11–13). DDR1 seems to be also involved in the differentiation of cerebellar granular neurons (14). Upregulated DDR1 expression has been reported from breast, ovarian, esophagus and brain tumors (5, 15–19). Human DDR1 is located on chromosome 6p21.3 in close proximity to HLA genes, which belong to the telomeric region (class I) of the major histocompatibility complex (20). The juxtamembrane regions in DDR1 and DDR2 are much longer than in other receptor tyrosine kinases (176 and 147 amino acids, respectively). Furthermore, the extracellular domain of DDR1 is shed by an unidentified protease, resulting in a 63-kDa membrane-anchored β-subunit and a 54-kDa soluble α-subunit (7).

Thus far, five isoforms of DDR1 have been cloned as a result of alternative splicing, designated with the suffixes "a" to "e" (21). The longest transcript codes for DDR1c and translates to a protein with 919 amino acids. Compared with the c isoform, the b isoform lacks 6 amino acids inserted in the kinase domain between exons 13 and 14 (7). The a, d, and e isoforms arise through alternative splicing in the juxtamembrane region. The deletion of exon 11 coding for 37 amino acids gives rise to DDR1a, and the deletion of exons 11 and 12 results in DDR1d. In DDR1e, the first half of exon 10 and all of exons 11 and 12 are missing (21). Whereas DDR1a retains the reading frame and is therefore an active kinase, the coding sequence of DDR1d and DDR1e goes out of frame and renders both isoforms kinase-dead. The 37-amino acid insert in DDR1b shows the motif LL*X*NP*X*Y, which can interact with the phosphotyrosine-binding domain of the Shc adapter protein upon collagen-induced tyrosine phosphorylation (1). Binding of the protein FRS2 has been shown to a chimeric molecule containing the juxtamembrane region of DDR1a (22). Furthermore, recent data imply that the Wnt-5a pathway may overlap with DDR1 signaling (23). Deletion of DDR1 in the mouse germ line resulted in viable animals that are significantly smaller than their littermates (24). Female DDR1-null mice show defects in blastocyst implantation and mammary gland development. Decreased proliferation, collagen attachment, and migration have been observed in primary vascular smooth muscle cells cultivated from DDR1-null mice (25).

So far, about 20 other proteins with one or two discoidin homology regions have been described from lower invertebrates up to mammals (26). During the cell aggregation of *Dictyostelium*, discoidin I is secreted, functions as a lectin, and is thought to be important in maintenance of morphology, cytoskeletal organization, and the ability to align with other cells during aggregation (27). In mammals, discoidin-homologous regions are found in membrane-bound and -secreted proteins.

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[1] The abbreviations used are: DDR, discoidin domain receptor; ELISA, enzyme-linked immunosorbent assay; RS1, retinoschisin.

Like the DDR, the neurexin receptor has a single N-terminal discoidin domain, whereas neuropilins have a tandem discoidin domain in the center of their extracellular domain. A C-terminally located tandem discoidin repeat is found in blood clotting factors V and VIII, in the milk proteins MFG-E8 and BA46, and in the endothelial cell-specific Del-1 (28).

Retinoschisin (RS1) is the only protein with a discoidin homology repeat, where a relevance in human disease has been observed. The X-linked inherited disease retinoschisis results in degeneration of the retina, ultimately leading to early blindness in affected males. Retinoschisis is diagnosed as maculopathy, peripheral retinal lesions, and alterations of the vitreous body and has an incidence of 5,000–25,000 in newborns (29). The splitting of the inner layers of the retina is believed to be caused by aberrant development of the Müller glia cells in the retina. Recently, the retinoschisis locus was identified on chromosome Xp22 by positional cloning, and the responsible gene was named *XLRS-1* (30). The *XLRS-1* gene consists of six exons coding for the 224-amino acid-long RS1 protein. A putative leader sequence (amino acids 1–23) is followed by the 157-amino acid-long discoidin repeat. No homology to other proteins is found in the 39- or 5-amino acid-long stretches flanking the discoidin domain N- or C-terminally. Sequence analysis of the *XLRS-1* gene in the majority of patients with retinoschisis led to the identification of a variety of mutations in the coding region, mostly in the discoidin repeat.

About 125 mutations in the *XLRS-1* gene have been described so far, including point mutations as well as deletions of single or multiple nucleotides, leading to frame shifts or truncations (31, 32). More than 80% of these mutations are located in the conserved discoidin repeat. Northern and Western blot analysis in mouse and human tissues found RS1 specifically and only expressed in the eye (33, 34). Whereas *XLRS-1* transcripts were found in the inner layer of the retina, the protein was detected further away in the photoreceptor layer and in bipolar cells. Therefore, RS1 seems to be secreted by underlying cells and then migrates to and potentially exerts its function in close proximity to the photoreceptors. Increased expression of RS1 is detected in human retinoblastoma Weri-Rb1 cells after stimulation with fibronectin and dibutyryl cyclic AMP (35).

Here, we provide evidence that the discoidin domain fold of RS1 is structurally related to the discoidin domain of DDR1. We further show that the discoidin domain of DDR1 is essential for collagen-binding, receptor dimerization, and tyrosine phosphorylation.

EXPERIMENTAL PROCEDURES

Reagents, Cell Lines, and Plasmids—Oligonucleotides were obtained from MWG-Biotech (Ebersberg, Germany). Single point mutations were introduced using 19-mers with the mutated nucleotide in the middle of the primer. Mutations were introduced into the DDR1 cDNA by polymerase chain reaction using the QuikChange Kit and isolated from *Escherichia coli* XL1-blue cells (Stratagene, La Jolla, CA). The vectors pRK5, used for the expression of DDR1 in human cells, and pET30, used for the expression of recombinant DDR1 discoidin domain in bacteria, have been described earlier (1, 7). Human embryonic kidney fibroblast 293 cells were obtained from the American Tissue Culture Collection (Manassas, VA) and cultivated under the recommended conditions. Rat type I collagen was purchased from Collaborative Biomedical Products (Bedford, MA). All other reagents were from Sigma. Antibodies to DDR1 (amino acids 894–913) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and monoclonal anti-phosphotyrosine antibody 4G10 was from Upstate Biotechnology, Inc. (Lake Placid, NY).

Expression and Analysis of Recombinant DDR1 Discoidin Domain—E. coli BL 21 super codon plus competent cells (Stratagene) were transformed with wild type and mutant DDR1 cDNA in the pET30 vector. A single colony from each plate was inoculated in LB medium. Cultures were incubated at 37 °C with vigorous shaking (250 rpm) until A_{600} = 0.8–1.0 was reached and stimulated with 1 mM isopropyl-β-D-thiogalactopyranoside for 2 h. Cells were pelleted by centrifugation and resuspended in buffer A (0,1 M NaH_2PO_4, 10 mM Tris-HCl (pH 8.0), 6 M guanidine HCl (pH 8.0), 10 mM imidazole) on a rocker at 4 °C for 1 h. Lysates were centrifuged at 13,000 rpm at 4 °C for 15 min, and the supernatant was incubated with Ni^{2+}-chelating Sepharose (Amersham Pharmacia Biotech) for 15 min. The Sepharose was washed three times with buffer A, and the bound material was eluted with 0.2 M acetic acid, 6 M guanidine HCl. The purified proteins were analyzed by SDS gel electrophoresis and detected by Coomassie staining. CD spectroscopy was performed using a Jasco J-720 spectropolarimeter (36).

Transient Expression in 293 Cells and Western Blot Analysis—Semiconfluent 293 cells were transfected by calcium phosphate precipitation. Sixteen hours later, cells were transferred to serum-free media for another 24 h. Cells were stimulated with 10 μg/ml collagen for 90 min (or 10, 30, and 90 min for the kinetic analysis) and lysed with 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl (pH 7.5), 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 μg/ml aprotinin. The cellular lysates were centrifuged at 4 °C and 13,000 rpm for 10 min, and aliquots of the supernatant were subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred on to nitrocellulose membrane (Schleicher & Schuell) and immunoblotted with antibodies diluted 1:1,000 (4G10) and 1:500 (DDR1) in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.5% gelatin overnight. Western blots were incubated with mouse and rabbit peroxidase-coupled secondary antibodies, respectively (Bio-Rad), and enhanced chemiluminescence (Amersham Pharmacia Biotech). For reprobing, the membrane was stripped in 70 mM Tris-HCl (pH 6.8), 2% SDS, 0.1% β-mercaptoethanol at 50 °C for 15 min.

Collagen Binding, Glycosylation, and Cross-linking Analysis—Transfected 293 cells were starved overnight. Total cell lysates were mixed with an equal volume of HNTG buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) and incubated with collagen-agarose (Sigma) on a rotating wheel at 4 °C for 2 h. Samples were washed three times with HNTG buffer, and bound material was analyzed by Western blotting with an antibody against the C terminus of DDR1 (α-DDR1). To analyze glycosylation, transfected 293 cells were starved for 12 h and treated overnight with 10 μg/ml tunicamycin. Cells were stimulated with 10 μg/ml collagen for 90 min, and total cellular lysates were analyzed by Western blotting. To capture receptor dimers, transfected cells were stimulated with 10 μg/ml collagen for 90 min, washed twice with phosphate-buffered saline, and then incubated with 50 mM sodium bis(sulfosuccinimidyl) suberate for 20 min. DDR1 was immunoprecipitated and analyzed by Western blotting with DDR1 antibody. Western blots were quantified using Quantity-One software (Bio-Rad). The S.D. value was calculated from three independent experiments.

Receptor-Ligand Binding Assay—Assay were modified according to a previously published protocol (37). Tissue culture 96-well plates were coated with different concentrations of type I collagen or bovine serum albumin overnight. Plates were washed twice with phosphate-buffered saline, 0.05% Tween 20 and blocked with 3% bovine serum albumin. A range of concentrations (1 ng to 10 μg) of total lysate from 293 cells expressing DDR1 were applied. After incubation for 2 h at 37 °C, plates were washed three times and then incubated with 5 ng of DDR1 antibody/well for 1 h. Wells were washed three times, and anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:100 dilution) was added for 1 h. Finally, wells were washed five times and developed using the chromogenic substance *o*-phenylenediamine dihydrochloride in 12% H_2O_2. After incubation for 6 min at room temperature, the reaction was stopped with one volume of 0.5 M H_2SO_4. The OD at 492 and 620 nm was determined, and the $A_{492} - A_{620}$ value was calculated. All measurements were done in triplicate. One representative result from three independent measurements is shown.

RESULTS

Sequence Comparison of the Discoidin Domains from RS1 and DDR1—The sequence of the DDR1 discoidin domain shows 41% homology to the discoidin domain of RS1. An alignment of all known discoidin domains indicated that 18 amino acids of the ~160-amino acid-long homology region were invariant in 17 out of 20 sequences (data not shown). From these 18 highly conserved residues, 13 were found to be mutated in patients with retinoschisis (Fig. 1). From these 13 amino acids, we selected nine positions and introduced the respective point mutation found in RS1 into the full-length cDNA of DDR1b.

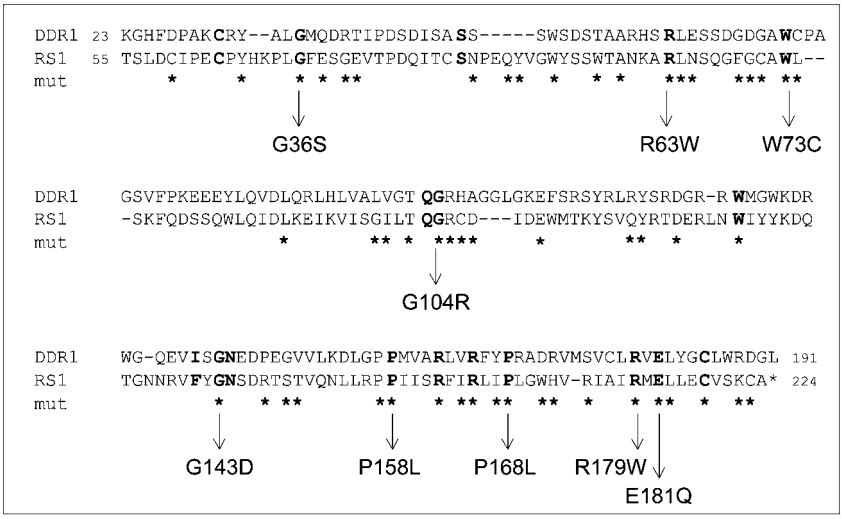

FIG. 1. **Alignment of human DDR1 and RS1 protein sequences and location of point mutants.** Amino acid sequences of the discoidin domains of DDR1 and RS1 were aligned using the program BestFit (Genetics Computer Group). The amino acids conserved in 17 of 20 known discoidin sequences are shown in *boldface type*. Residues mutated in RS1 are indicated with an *asterisk*. Nine point mutations introduced into the full-length cDNA of DDR1 are indicated *below* the sequence alignment.

Purification and Analysis of Recombinant Mutant Discoidin Domain from DDR1—To compare the folding of wild type and mutated DDR1 discoidin domains, the respective cDNAs coding for amino acids 28–186 of wild type and of the G36S mutant were cloned into the pET30 vector. Recombinant discoidin domains were expressed and purified from bacteria. The integrity of the proteins was confirmed by SDS-polyacrylamide gel electrophoresis. Both proteins appeared as ~25-kDa single bands (Fig. 2*A*). CD spectroscopy was used to determine the folding structure. In the wild type, the predicted β-sheet content was 44.7%, and the predicted α-helix content was 15.2% (Fig. 2*B*). The G36S mutant had almost identical β-sheet content and a slightly higher percentage of α-helices. We concluded from this result that site-directed mutagenesis of a single residue in the discoidin domain of DDR1 most likely will not change the overall folding structure.

Tyrosine Phosphorylation of DDR1 Mutants—The nine mutations shown in Fig. 1 were introduced into the full-length cDNA of DDR1 and transiently transfected into human embryonic fibroblast 293 cells. To activate the tyrosine kinase in DDR1, cells were stimulated with 10 μg/ml rat collagen type I for 90 min and lysed. Total cellular lysates were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. Probing the blot with an antibody against phosphotyrosine showed collagen-dependent phosphorylation of the wild type (Fig. 3*A*). The blot was stripped and reprobed with an antibody against the C terminus of DDR1. Analysis of this blot indicated that all mutants were expressed in equal amounts compared with the wild type (Fig. 3*B*). The signal intensities of receptor phosphorylation were quantified from three independent experiments and normalized with the receptor expression. We found that four of the nine mutants (G36S, W73C, R179W, and E181Q) showed markedly reduced phosphorylation compared with the wild type (Table I). A slight reduction of about 30% was observed with G104R, G143D, and P168L. In contrast, mutation of the residues R63W and P158L did not significantly affect tyrosine phosphorylation.

Next, we asked if any of the point mutations result in an alteration of the activation kinetics. The mutants were expressed in 293 cells and stimulated for 10, 30, or 90 min (Fig. 3*C*). The time course of two of the mutants tested (P158L and P168L) showed a similar pattern of activation compared with the wild type. Moreover, phosphorylation of the mutants G36S and R179W were significantly reduced but had no effect on the time course of activation. Finally, the mutant E181Q had no detectable increase of phosphorylation during the time period studied.

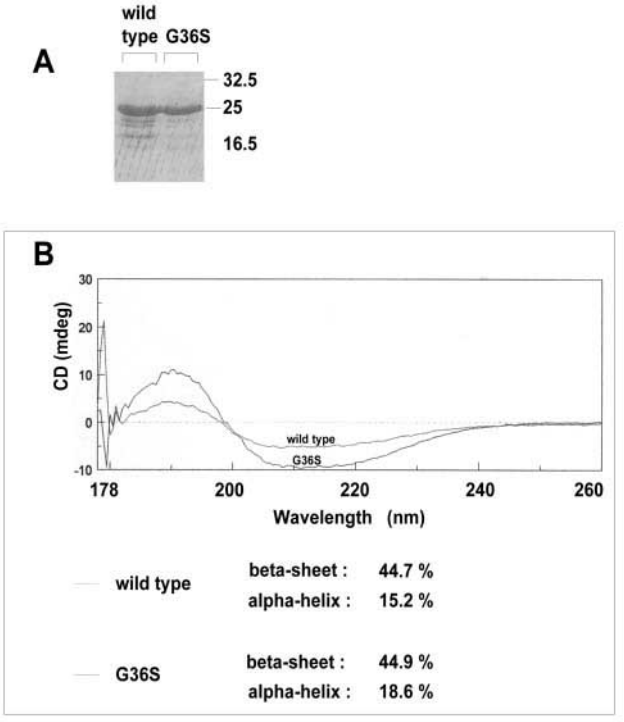

FIG. 2. **Point mutants in DDR1 do not affect the folding of the discoidin domain.** The discoidin domain of wild type and G36S mutant were expressed as His tag fusion protein in bacteria. 10 μg of affinity-purified protein were analyzed by SDS-PAGE and Coomassie staining (*A*). Wild type and G36S mutant proteins were analyzed by CD spectroscopy (*B*). *Graphs* are shown in one diagram, and secondary structure predictions are given *below*.

Collagen Binding of DDR1 Mutants—Equal amounts of protein lysates obtained from 293 cells transfected with either wild type or mutant DDR1 were incubated with collagen covalently coupled to agarose beads. The beads were washed three times with HNTG buffer. Affinity-purified proteins were eluted with Laemmli buffer and analyzed by Western blotting with the C-terminal antibody against DDR1. The signal intensities of three independent experiments were quantified and calculated relative to the wild type signal (Table I). Two of the nine mutants (G36S and R63W) bound equally well to collagen beads as the wild type (Fig. 4*A*). Signals for the mutants W73C, G104R, and P168L were slightly lower compared with the wild type (Table I). However, the amounts of G143D and P158L retained on the beads

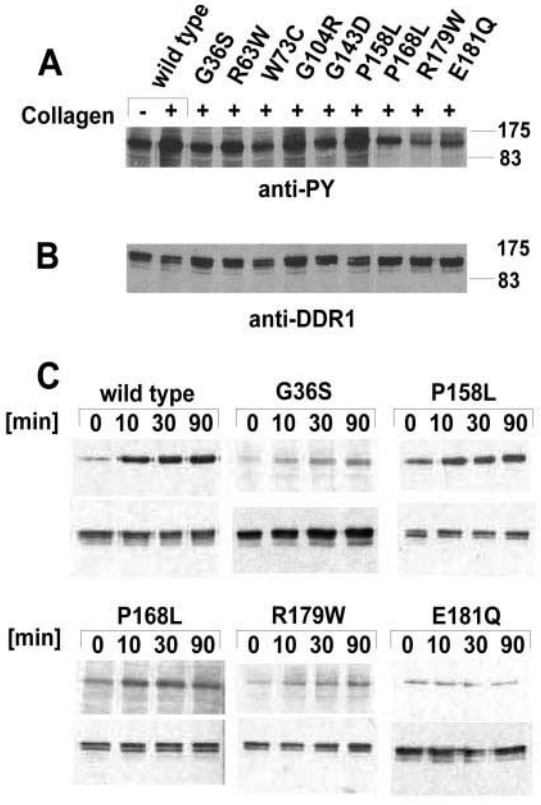

FIG. 3. **Reduced tyrosine phosphorylation of DDR1 point mutants.** DDR1 mutants with single amino acid exchanges in the discoidin domain were expressed in 293 cells, stimulated with collagen, and analyzed by anti-phosphotyrosine Western blotting (*A*). The blot was stripped and reprobed with an antibody against the C terminus of DDR1 (*B*). The time course of phosphorylation for five representative mutants was compared with the wild type (*C*, *upper blot*, anti-phosphotyrosine; *lower blot*, anti-DDR1). One representative result from three independent experiments is given. Molecular weight markers are indicated in *A* and *B*.

TABLE I

Comparison of collagen activation and binding of various mutants

The signal intensity of the Western blots in Figs. 3, *A* and *B*, and 4*A* was quantified from three independent experiments, and the S.D. was calculated. The relative receptor activation was normalized with the receptor expression. Values are given as percentages relative to the wild type.

	Receptor expression	Receptor activation	Collagen binding
	%	%	%
Wild type	100	100	100
G36S	108.3 ± 5.9	54.4 ± 3.8	83.6 ± 9.9
R63W	94.9 ± 3.0	93.2 ± 7.7	98.1 ± 9.0
W73C	95.3 ± 1.5	43.4 ± 5.9	65.6 ± 5.8
G104R	112.2 ± 1.7	70.5 ± 5.0	74.1 ± 0.8
G143D	101.6 ± 5.6	68.2 ± 6.2	58.9 ± 7.0
P158L	91.4 ± 1.9	114.7 ± 11.7	47.4 ± 4.3
P168L	109.7 ± 10.9	73.8 ± 9.8	73.2 ± 3.8
R179W	103.7 ± 5.9	34.1 ± 3.2	187.6 ± 5.9
E181Q	112.7 ± 3.4	40.9 ± 6.3	183.7 ± 2.9

were much lower, indicating a loss of collagen binding affinity. In contrast, the mutants R179W and E181Q showed about 2-fold higher binding to collagen (Fig. 4*A*).

In order to quantify the differences in collagen affinity between wild type and mutant DDR1, we established a receptor-ligand binding assay. First, specific binding of DDR1 was shown by incubating ELISA plates coated with various concentrations of type I collagen together with lysates from 293 cells overexpressing DDR1. Concentration-dependent binding of

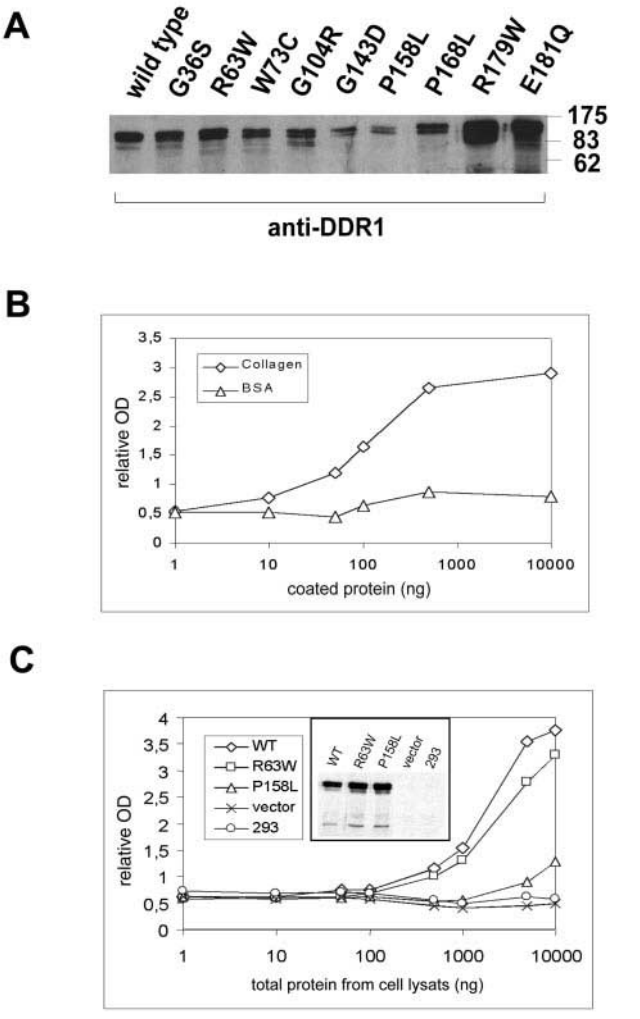

FIG. 4. **Point mutants have altered binding capacity toward collagen.** Equal amounts of lysate from 293 cells expressing DDR1 mutants were incubated with collagen-agarose. Bound material was analyzed by Western blotting with an antibody against the C terminus of DDR1. Molecular weight markers are indicated (*A*). An ELISA-based receptor-ligand binding assay was performed using plates coated with a range of different concentrations of collagen or bovine serum albumin (*B*). A range of different concentrations of protein lysates from cells expressing wild type (*WT*) DDR1 or R63W and P158L mutant were used for the receptor-ligand binding assay. The *inset* shows a Western blot analysis of aliquots of cell lysates, confirming equal expression of DDR1 wild type and mutant protein (*C*).

DDR1 was observed with collagen-coated plates but not with control plates and was observed with as little as 10 ng of collagen/well (Fig. 4*B*). Next, we selected two mutants, R63W and P158L, for the receptor-ligand binding assay. Both mutants do not affect DDR1 tyrosine phosphorylation, but they vary in collagen binding. The mutant R63W showed no significant difference in collagen affinity compared with the wild type, whereas P158L had a drastically impaired collagen-binding capacity. Lysates from cells expressing either of these mutants, wild type DDR1, or control vector were subjected to the ELISA-based receptor-ligand binding assay (Fig. 4*C*). Over a range of 4 orders of magnitude in protein concentration (1 ng to 10 μg), the R63W mutant showed only an approximately 10% reduction in collagen affinity, whereas the binding of P158L was about 3–4-fold lower than the wild type. The ELISA data strongly support the binding results obtained by the solid-phase collagen-binding assay shown in Fig. 4*A*.

A summary of the responses by all mutants is given in Table I. The mutation in one of nine conserved residues (R63W) did

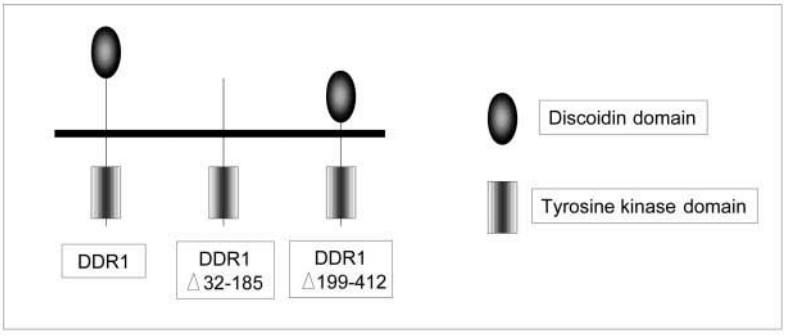

FIG. 5. **Schematic representation of DDR1 deletion mutagenesis.** The mutant Δ32–185 lacks the discoidin domain, and the mutant Δ199–412 lacks the stalk region between discoidin and the transmembrane region.

not drastically alter ligand binding or activation. The two mutants G36S and P168L were still able to significantly bind to collagen, but they failed to be fully tyrosine-phosphorylated. Mutation of W73C, G104R, or G143D simultaneously affected collagen binding and receptor activation. Two mutants (R179W and E181Q) showed reduced kinase activation but increased collagen binding. The opposite seemed to be the case for P158L, which is equally strong activated by collagen but has significantly less affinity to collagen compared with the wild type.

Deletion Mutagenesis of the Extracellular Domain—Next, we created two deletion mutants in the DDR1 extracellular domain. By joining amino acid 31 with 186, we generated a mutant lacking the discoidin domain, called Δ32–185 (Fig. 5). The second mutant was a deletion of amino acids 199–412, which retains the discoidin domain but lacks the stalk region (Δ199–412). The mutants and wild type DDR1 were expressed in 293 cells and stimulated with collagen. Total lysates were analyzed by anti-phosphotyrosine Western blotting (Fig. 6*A*). Whereas wild type DDR1 showed collagen-dependent phosphorylation, both mutants were not phosphorylated on tyrosine. Reprobing of the blot indicated that both deletion mutants were expressed at levels comparable with those seen in the wild type (Fig. 6*B*). Western blot analysis of affinity-purified protein lysates was used to compare the ability of the mutants to bind to collagen agarose with that of the wild type. The results indicated that the wild type and the Δ199–412 mutant bound to collagen-agarose (Fig. 6*C*). In contrast, the Δ32–185 mutant did not bind, suggesting that the discoidin domain is essential for collagen binding and that discoidin and stalk region are necessary for receptor activation.

Glycosylation of the Stalk Region—Due to differential glycosylation, DDR1 appears as a doublet of bands in Western blots. This doublet is seen for wild type DDR1 and the Δ32–185 mutant in Fig. 6*A*. In contrast, the Δ199–412 mutant ran as a single band. To test for glycosylation, wild type and deletion mutants were expressed in 293 cells and treated with tunicamycin, an inhibitor of *N*-linked glycosylation. Total cell lysates were analyzed by Western blotting with an antibody specific for the DDR1. In response to tunicamycin, the majority of DDR1 is found in the Western blot as a protein with an apparent molecular mass of 100 kDa, while the glycosylated protein is seen as a 125-kDa protein (Fig. 7). Collagen treatment had no effect on the glycosylation of DDR1. The Δ32–185 mutant appeared as a 105-/95-kDa doublet on the Western blot without tunicamycin and is reduced to a single 84 kDa in the presence of tunicamycin. In contrast, the Δ199–412 mutant ran as a 77-kDa protein with or without tunicamycin. These data demonstrate that glycosylation of DDR1 is only found in the stalk region (amino acids 199–412) but not in the discoidin domain (amino acids 32–185).

Role of the Extracellular Domain of DDR1 in Dimerization—Ligand-induced receptor dimerization has been shown for several receptor tyrosine kinases, suggesting that collagen-induced signaling of DDR may follow similar mechanisms. To evaluate the role of the extracellular domain of DDR1 during receptor activation, the deletion mutants Δ32–185 and Δ199–412 were expressed in 293 cells, and the collagen-induced dimerization was captured by covalent cross-linking with sodium bis(sulfosuccinimidyl) suberate. Western blot analysis of collagen-stimulated and sodium bis(sulfosuccinimidyl) suberate-treated cells expressing the Δ199–412 mutant showed two dominant bands between 150 and 220 kDa, which were not present in collagen-treated but non-cross-linked cells and to a much lower extent in cross-linked but not collagen-treated cells (Fig. 8). In contrast, collagen-stimulated cells expressing the Δ32–185 mutant only showed a very weak band at ~200 kDa, suggesting severely impaired dimerization. Based on three independent experiments, quantification of the monomer *versus* dimer band revealed that 32.7% ± 3.2% of the Δ199–412

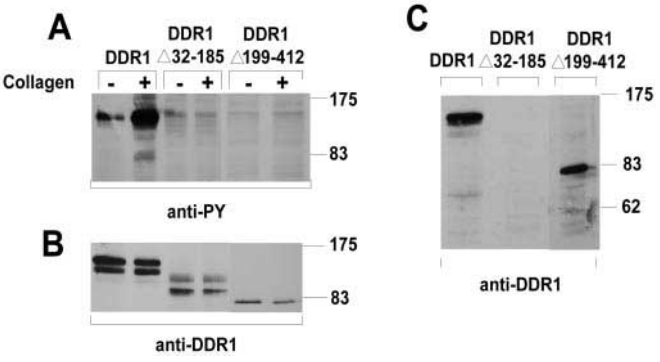

FIG. 6. **The discoidin domain of DDR1 is essential for collagen binding.** Wild type and deletion mutants were expressed in 293 cells and stimulated with collagen. Tyrosine phosphorylation was monitored by Western blotting of total cellular lysates (*A*). The blot was reprobed with an antibody against the C terminus of DDR1 (*B*). Equal amounts of cell lysates were incubated with collagen-agarose. Affinity-purified material was analyzed by Western blotting with an antibody against the C terminus of DDR1 (*C*). Molecular weight markers are indicated.

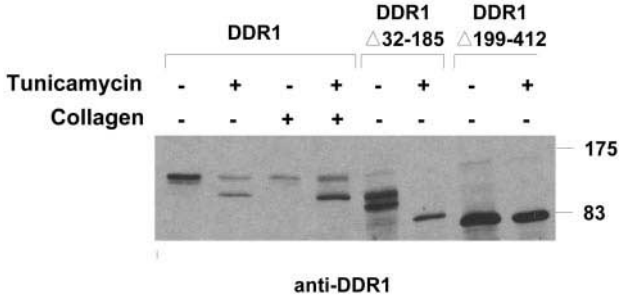

FIG. 7. **DDR1 is only glycosylated in the stalk region.** Wild type and deletion mutants were expressed in 293 cells, treated with tunicamycin overnight, and stimulated with collagen for 90 min. Total cellular lysates were analyzed by Western blotting with an antibody against the C terminus of DDR1. Molecular weight markers are indicated.

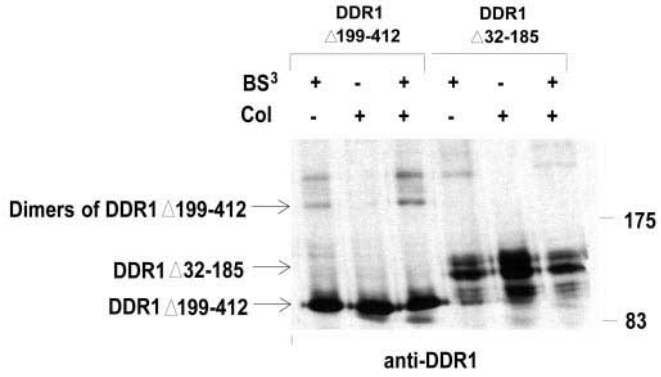

FIG. 8. **The discoidin domain is involved in receptor dimerization.** The DDR1 Δ32–185 and Δ199–412 mutants were expressed in 293 cells, stimulated with collagen, and incubated with sodium bis(sulfosuccinimidyl) suberate cross-linker. Total cellular lysates were analyzed by Western blotting with an antibody against the C terminus of DDR1. One representative result from three independent experiments is shown. Molecular weight markers are indicated.

mutant was captured by the cross-linker in response to collagen activation. Therefore, we conclude that the discoidin domain is necessary and sufficient for collagen-induced receptor dimerization.

DISCUSSION

The extensive homology between the primary sequence of the discoidin domains in DDR1 and RS1 led us to hypothesize that structurally conserved epitopes are present in both domains. However, a functional homology between DDR1 and RS1 has not been demonstrated so far. In fact, DDR1 was found to be overexpressed in human tumors, whereas mutations in RS1 cause early blindness. Throughout the entire sequence of RS1, a large number of different disease-linked mutations have been reported in the past years (31). Surprisingly, the majority of these mutants affect residues in RS1 that are conserved in all of the 20 proteins with discoidin homology repeats characterized thus far. To test if this conservation in the primary sequence results in conserved structural and functional properties, we introduced nine mutations found in RS1 into the respective position in DDR1. Indeed, seven of the DDR1 mutants showed reduced activation of the kinase function in response to collagen (G36S, W73C, G104R, G143D, P168L, R179W, and E181Q). In a direct ligand-binding assay, we detected a significantly lower binding to collagen with two mutants (G143D and P158L) but a higher affinity with the mutants R179W and E181Q. One out of the nine mutated positions remained relatively unchanged in its collagen-binding properties compared with the wild type (R63W).

Our data suggest that distinct sets of epitopes are present in the discoidin domain of DDR1. One set of residues is relevant for collagen binding but not for transmembrane signal transduction, whereas another set of residues appears to be involved in both binding and signaling. Surprisingly, although the mutant P158L was fully activated upon ligand stimulation, it failed to properly adhere to collagen. The reason for the unusual properties of this residue remains to be further investigated. Last, the two residues Arg^{179} and Glu^{181} most likely lie in an epitope that has a low ligand affinity but is necessary for receptor signaling, since mutagenesis of these residues dramatically increased collagen binding but reduced receptor activation. Currently, we cannot exclude the possibility that some of the mutations affect the overall folding of the discoidin domain. Nevertheless, similar expression levels between wild type and mutant molecules on the one hand and expression of the G36S mutant in bacteria followed by CD experiments on the other hand suggested that mutant proteins should be correctly folded. Indeed, mutations leading to folding problems tend to be expressed in very low amounts.

To gain further understanding of the DDR1 discoidin domain folding, structural analysis by nuclear magnetic resonance spectroscopy, x-ray crystallography, or homology modeling will be necessary. Currently, only structural information about related discoidin domains is available. For instance, the structure of a discoidin domain of blood coagulation factor V (also called C domains), was initially predicted using a molecular model based on the x-ray structure of the distant related galactose oxidase (38). The suggested β-barrel structure has been recently confirmed by x-ray crystallography for the second discoidin domain of factor V and VIII (39, 40). Based on these structural data, a surface epitope involved in binding of factor V to membrane-anchored phosphatidylserine has been suggested (41, 42). Such structural and functional information should help for future definition and analysis of regions of functional importance in DDR1.

Our results confirm the separation of the DDR1 extracellular domain into two parts: the N-terminal discoidin domain and the remaining C-terminal part, which we named the stalk region. Whereas the boundaries of the discoidin domain in DDR1 are clearly defined by homologous sequences, so far no homology to other proteins was detected for the remaining 215 amino acids of the stalk region. Here, evidence is presented that both regions are also functionally distinct. Whereas the DDR1 receptor binds collagen using only the discoidin domain, the kinase activation involves both parts of the extracellular domain. Together with the observation that the discoidin domain is essential for dimerization, the following sequence of events is proposed: (i) one or more epitopes in the discoidin domain get in contact with triple helical collagen; (ii) receptor dimers are formed, and this event induces an overall structural rearrangement at least within the stalk region; and (iii) the conformational changes are transmitted through the membrane and allow trans-phosphorylation of the two kinase domains. In the absence of the discoidin domain, receptors fail to bind collagen and therefore do not dimerize, whereas the absence of the stalk region allows collagen binding and dimerization but no transmembrane signaling. We also show that glycosylation of the extracellular domain of DDR1 is restricted to the stalk region and that the presence of the carbohydrate moiety appears to be essential for proper signal transduction.

Due to the high sequence homology, it is tempting to speculate that binding to components of the extracellular matrix is a more general property of discoidin domains. The secreted aortic carboxypeptidase-like protein displays an N-terminal discoidin domain. Immunohistochemistry of aortic cells showed that aortic carboxypeptidase-like protein colocalizes with the extracellular matrix, potentially with collagens (43). Furthermore, the presence of an N-terminal discoidin domain in the mammalian neurexin IV homologue Caspr (also called paranodin) might be necessary to guide neuronal axons along myelinated membranes (44). One could also envision the binding of RS1 to collagen fibers specifically expressed in the eye. By binding to collagens, RS1 could function as molecular "glue" that strengthens the adhesion of the retinal inner and outer layers.

How does the discoidin domain of DDR1 recognize collagen? Our previous work indicated that only native triple helical collagen is able to induce DDR phosphorylation, whereas denatured collagen (gelatin) is not a ligand for DDR (1). Recombinant triple helical collagen formed by the α-chain of collagen type V stimulates DDR1 phosphorylation to a degree comparable with native collagen (45). Nevertheless, short triple helical "minicollagens" formed out of 10 glycine-proline-hydroxypro-

line repeats ($(GPH)_{10}$) peptides fail to activate DDR.[2] This is in contrast to the fact that integrins of the β_1 family and the glycoprotein VI collagen receptor respond well to $(GPH)_{10}$ peptides (46).

Interestingly, the well established interaction between collagen and $\alpha_2\beta_1$ integrin has only very recently been defined on a structural basis (47). The I domain of the α_2 integrin subunit is engaged in binding to two of the three chains of triple helical collagen. A bivalent metal (normally Mg^{2+}) ion is necessary for the collagen/integrin interaction that simultaneously complexes a glutamic acid side chain from the collagen as well as two serines and one threonine from the integrin I domain (47, 48). In principle, a similar mechanism could be proposed for the collagen/discoidin interaction, although the presence of metal ions has not been proven so far. The architecture of the collagen-binding groove of the integrin I domain has been mapped by site-directed mutagenesis allowing the identification of residues that showed different properties under static conditions compared with shear stress conditions (49). These differential interactions could be the molecular basis for high and low affinity binding of integrins induced by conformational changes of the entire integrin molecule (50).

In conclusion, we were able to identify conserved sequence residues in the discoidin domain of DDR1 that are essential for collagen binding. We found an unexpected dichotomy in DDR1 ligand binding, receptor clustering, and tyrosine phosphorylation. The newly identified binding epitopes in DDR1 might be also functionally relevant in homologous proteins with the discoidin domain triggering the interaction with collagen or related matrix ligands.

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