Exploring the Collagen-binding Site of the DDR1 Tyrosine Kinase Receptor*

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Discoidin domain receptors 1 and 2 (DDR1 and DDR2) are tyrosine kinase receptors activated by triple-helical collagens. Aberrant expression and signaling of these receptors have been implicated in several human diseases linked to accelerated matrix degradation and remodeling including tumor invasion, atherosclerosis and liver fibrosis. The objective of this study is to characterize the collagen-binding sites in the discoidin domains of DDR1 and DDR2 at a molecular level. We expressed glutathione S-transferase fusion proteins containing the discoidin and extracellular domains of DDR1 and DDR2 in insect cells and subjected them to a solid-phase collagen-binding assay. We found high affinity binding of the DDR extracellular domains to immobilized type I collagen and confirmed the discoidin-collagen interaction with an enzyme-linked immunosorbsent assay-based read-out. Furthermore, we created a three-dimensional model of the DDR1 discoidin domain based on the related domains of blood coagulation factors V and VIII. This model predicts the presence of four neighboring, surface-exposed loops that are topologically equivalent to a major phospholipid-binding site in factors V and VIII. To test the involvement of these loops in collagen binding, we mutated individual amino acid residues to alanine or deleted short sequence stretches within these loops. We found that several residues within loop 1 (Ser-52–Thr-57) and loop 3 (Arg-105–Lys-112) as well as Ser-175 in loop 4 are critically involved in collagen binding. Our structure-function analysis of the DDR discoidin domains provides new insights into this non-integrin-mediated collagen-signaling mechanism and may ultimately lead to the design of small molecule inhibitors that interfere with aberrant DDR function.

Collagens are the most abundant proteins found in the animal kingdom. Whereas some collagens are key structural components in load-bearing tissues, others are essential elements of basement membranes. Collagens have a pivotal role in regulating cellular differentiation and pattern formation during embryogenesis and postnatal development. Increased synthesis of fibrillar collagens or perturbed turnover correlates with a variety of human diseases, including liver fibrosis, glomerulonephritis, vascular diseases, or tumor angiogenesis (1). Three different types of collagen-receptors are currently known: the tyrosine kinases discoidin domain receptor 1 and 2 (DDR1 and DDR2),† four integrin heterodimers containing the β1 subunit, and glycoprotein VI (2). Although glycoprotein VI is only found on platelets, both integrins and DDR are widely expressed and trigger an array of signaling pathways upon collagen binding.

DDR1 and DDR2 are characterized by a ~155-amino acid discoidin homology domain (DiscD) in the extracellular region of the protein. The discoidin domain is followed by a 200-amino acid stretch termed the stalk region, a single transmembrane peptide, a juxtamembrane region, and the catalytic tyrosine kinase domain (Fig. 1A). DDR1 was isolated from a number of different human tissues and carcinoma cell lines (3–8). RNA in situ hybridization analysis showed specific expression of human DDR1 in epithelial cells, particularly in the mammary gland, brain, kidney, lung and the mucosa of the colon (3). DDR2 is also widely expressed, particularly in skeletal and heart muscle, kidney, and skin.

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† Abbreviations used are: DDR, discoidin domain receptors; DiscD, discoidin domain; Npn-1, neuropilin-1; HA, hemagglutinin; ELISA, enzyme-linked immunosorbsent assay; GST, glutathione S-transferase; HRP, horseradish peroxidase; ExD, extracellular domain.
Collagen-binding Site in DDR1

GST Fusion Protein Purification—DDR1 and DDR2 disso concluded from human cDNAs. Inserts were cloned in-frame into pAcG2T (BD Biosciences), resulting in an N-terminal fusion with glutathione S-transferase (GST). The pAcG2T constructs functioned as transfer vectors for the BaculoGold expression system (BD Biosciences). Attenuated BaculoGold DNA was co-transfected with each transfer vector into Sf9 (Spodoptera frugiperda) cells grown in SFM-FH media (Invitrogen) with 10% heat-inactivated fetal calf serum, 2% glutamine, 50 μg/ml gentamicin, and 2.5 μg/ml Fungizone at 27 °C. Viruses were amplified for three rounds in sequentially larger monolayer culture dishes (25, 75, and 150 cm²). Five days after infection, viral supernatants were harvested for the next round of amplification. Recombinant viruses were used to infect HIV (Trichoplusia ni) cells grown in monolayers in the above medium. Cells were harvested at 27 °C for 2 days and then washed using SD buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 0.5 mM EDTA, 0.5 mM MgCl₂, 10 mM NaF, 1 mM phenylmethanesulfonyl fluoride, 1 mM sodium orthovanadate, and 10 μg/ml aprotinin) with 2.5% Triton X-100. Cell lysates were added to glutathione-linked-agarose beads (BD Biosciences Clontech), incubated for 2 h at 4 °C and subsequently washed using SD buffer. To elute protein, 1 bead volume of glutathione elution buffer (100 mM glutathione, 500 mM NaCl, 1 mM EDTA, 10% glycerol, and 100 mM Tris, pH 8.5) was incubated with the beads at 4 °C for 15 min.

Immunoprecipitation and Western Blotting—Lysates from 293 cells were diluted 1:1 (v/v) in HNTG buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol) and incubated with anti-DDR1 antibody (15) coupled to protein A-Sepharose beads (Amersham Biosciences) for 3 h. Beads were washed four times with wash buffer (187.5 mM Tris, pH 6.8, 6% SDS, 30% glycerol, 0.1% bromphenol blue, and 15% 2-mercaptoethanol). Proteins were separated on 7.5% polyacrylamide gels and subjected to Western blotting. Samples were probed using a monoclonal anti-phosphotyrosine antibody (4G10; Upstate Biotechnology) diluted 1:500 in NET-Gel (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 0.05% gelatin). Western blots were developed using secondary horseradish peroxidase-linked anti-mouse IgG (Bio-Rad Laboratories) and enhanced chemiluminescence (Amersham Biosciences). Blots were stripped (65 mM Tris, pH 6.8, 2% SDS, and 50 μM 2-mercaptoethanol) and re-probed with anti-DDR1 polyclonal (Santa Cruz Biotechnology) or anti-HA monoclonal (Sigma) antibody and secondary HRP-linked anti-rabbit or -mouse IgG. Lysates from HIV cells were subjected to SDS-PAGE and analyzed by Western blotting with polyclonal antibodies raised against the N-terminal regions of DDR1 and DDR2, respectively (11). The blots were stripped and re-probed with an anti-GST-linker-HRP monoclonal antibody (Santa Cruz).

For membrane fractionation, cells were lysed with Tris-sucrose buffer (10 mM Tris, pH 7.4, 1 mM EDTA, and 0.1 mM sucrose), homogenized, and centrifuged at 3000 × g for 5 min. The supernatant containing cytoplasmic and transmembrane fractions was isolated and centrifuged at 100,000 × g for 1 h. The pellet was resuspended in Tris-sucrose buffer and immunoprecipitated overnight with an anti-DDR1 antibody. Beads were washed three times with Tris-sucrose buffer and subjected to SDS-PAGE. DDR1 was detected using a monoclonal anti-HA antibody (Sigma).

Collagen-DDR1 ELISA—Type I collagen was diluted in phosphate-buffered saline to a concentration of 50 μg/ml and added to 96-well microtiter plates (50 μl/well). Plates were incubated in 1 h at room temperature and washed twice with phosphate-buffered saline. Wells were incubated with 150 μl of polyclonal IgG in phosphate-buffered saline containing 0.05% Tween 20 for 1 h. Wells were washed once with 150 μl of wash buffer (0.5 mg/ml SBA in phosphate-buffered saline containing 0.05% Tween 20). DDR1-transfected 293 cells were lysed with Tween 20-based lysis buffer, and lysate was added to each well and incubated at room temperature overnight (lysates were normalized for cell number by Western blot analysis before ELISA). Wells were washed six times with wash buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, containing 0.012% hydrogen peroxide). After 30 min at room temperature, the reaction was stopped using 50 μl/well of 3% sulfuric acid. Plates were analyzed in an ELISA reader at 490 nm, and values were subjected to statistical analysis using Microsoft Excel. All experiments were performed in triplicate.

EXPERIMENTAL PROCEDURES

Molecular Modeling—A preliminary model of the human DDR1 disso concluded from human cDNAs. Inserts were cloned in-frame into pAcG2T (BD Biosciences), resulting in an N-terminal fusion with glutathione S-transferase (GST). The pAcG2T constructs functioned as transfer vectors for the BaculoGold expression system (BD Biosciences). Attenuated BaculoGold DNA was co-transfected with each transfer vector into Sf9 (Spodoptera frugiperda) cells grown in SFM-FH media (Invitrogen) with 10% heat-inactivated fetal calf serum, 2% glutamine, 50 μg/ml gentamicin, and 2.5 μg/ml Fungizone at 27 °C. Viruses were amplified for three rounds in sequentially larger monolayer culture dishes (25, 75, and 150 cm²). Five days after infection, viral supernatants were harvested for the next round of amplification. Recombinant viruses were used to infect HIV (Trichoplusia ni) cells grown in monolayers in the above medium. Cells were harvested at 27 °C for 2 days and then washed using SD buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 0.5 mM EDTA, 0.5 mM MgCl₂, 10 mM NaF, 1 mM phenylmethanesulfonyl fluoride, 1 mM sodium orthovanadate, and 10 μg/ml aprotinin) with 2.5% Triton X-100. Cell lysates were added to glutathione-linked-agarose beads (BD Biosciences Clontech), incubated for 2 h at 4 °C and subsequently washed using SD buffer. To elute protein, 1 bead volume of glutathione elution buffer (100 mM glutathione, 500 mM NaCl, 1 mM EDTA, 10% glycerol, and 100 mM Tris, pH 8.5) was incubated with the beads at 4 °C for 15 min.

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For the ELISA of GST-DDR binding to collagen, purified recombinant proteins were added to collagen-coated wells and incubated at room temperature for 3 h. Wells were washed six times with wash buffer, and 50-μl aliquots of anti-GST monoclonal antibody (diluted 1:1000 in wash buffer) were added for 1 h at room temperature. After six washes, bound protein was detected as above. For collagen-DDR1 loop peptide ELISA, 100 μl of lysate from 293 cells transfected with DDR1 were added to each collagen-coated well along with loop peptides in 1250-fold molar excess (relative to collagen) and incubated at room temperature overnight. Binding was detected using polyclonal rabbit anti-DDR1 C-terminal antibody and secondary HRP-linked anti-rabbit IgG antibody.

RESULTS

DDR1 and DDR2 constitute a unique pair of tyrosine kinase receptors because they are activated not by soluble growth factors but by native, triple-helical collagen. Although previous work has shown that the DDR-collagen interaction is direct, the molecular basis of this interaction, in particular the precise role of the discoidin domain, remains unclear. To study this interaction, we decided to produce the discoidin domain or the entire extracellular domain (ExD) of DDR1 and DDR2 as recombinant proteins in insect cells. The constructs were expressed as GST fusion proteins in HiV insect cells (Fig. 1B).

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Figure 1. Recombinant discoidin and extracellular domains of DDR1 or DDR2 bind to collagen. A, overall topology of DDR1 and DDR2. TM, transmembrane region; JM, juxtamembrane region. B, the cDNAs coding for the DiscD or ExD of DDR1 or DDR2 were cloned in frame with GST. C and D, GST-DDR fusion proteins derived from these constructs were expressed in insect cells, and the presence of proteins with the predicted molecular weight was confirmed by Western blotting with anti-DDR antibodies. E and F, equal amounts of insect cell lysates expressing GST-DDR1 (E) or GST-DDR2 (F) fusion proteins were mixed with collagen immobilized to agarose beads. Bound material was detected by anti-GST Western blotting and compared with Western blots of total insect cell lysates.

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Purification of the GST fusion proteins by glutathione-agarose affinity chromatography yielded significant amounts of all four constructs (0.1–1.0 mg of protein per 10⁸ cells). Proteins were eluted by glutathione displacement and were subjected to an ELISA-based binding assay. Plates coated with 50 μg/ml type I collagen were incubated with various concentrations of purified GST fusion proteins, and the amount of bound material was quantified by anti-GST ELISA (Fig. 2). At a 100 nM protein concentration, the DDR1-DiscD fused to GST had a 14-fold higher affinity to collagen than GST alone. We were surprised to find that DDR1-ExD had about a 10-fold higher affinity to collagen than the DiscD alone, in that DDR1-ExD binding reached a plateau at a protein concentration of ~10 nM compared with 100 nM for DDR1-DiscD. The DDR2-DiscD showed a binding profile similar to that of DDR2-ExD, with no significant increase in binding capacity upon addition of the stalk region. In contrast to DDR1-ExD, the DDR2-ExD had much lower affinity for type I collagen, and a linear increase in binding was observed through all concentrations measured (60–180 nM). These differences between DDR1 and DDR2 are possibly caused by diverse sequence motifs within the stalk region. The overall identity between the two sequences is much lower for the stalk regions than for the discoidin domains (44%...
versus 58%). Furthermore, DDR1 shows a unique stretch of about 15 amino acids near the plasma membrane (amino acids 385–399) that is not present in DDR2.

Whereas previous work indicated that the discoidin domain of DDR1 is essential for binding to triple-helical collagen, the molecular composition of this binding epitope remains unknown (13). To address this question, we exploited the homology between the discoidin domains of human DDR and the following three related human discoidin domains: the C-terminal domain in coagulation factor V, the homologous domain in factor VIII, and the first discoidin domain in Npn-1. We selected these domains, because their three-dimensional structures were recently resolved by x-ray crystallography (16–18).

Whereas all five sequences share less than 20% identity, the eight strands forming the central β-barrel are well conserved (Fig. 3A). Based on this alignment and the structural information, we generated a three-dimensional model for the DDR1-DiscD (Fig. 3B). The conserved N- and C-terminal cysteine residues (Cys-31 and Cys-185 in DDR1, respectively) form a disulfide bridge located at the top of the barrel. In addition, our model predicts that the S atoms of Cys-74 and Cys-177 are optimally positioned to form a second disulfide bridge. More importantly, at the bottom of the barrel, four prominent, finger-like loops protrude (Fig. 3B). Each loop consists of about 7–11 amino acid residues, and the position of each loop within the primary sequence is conserved between DDR1 and other discoidin domains (as highlighted in Fig. 3A). Based on their exposed character in the molecular model and the relevance of the topologically equivalent regions of other discoidin domains for interactions with diverse ligands (19), we hypothesized that these loops may be involved in collagen-binding by DDR1.

To test this hypothesis, we designed and produced mutant proteins with partial deletions of the loop sequences that were expected not to compromise the folding of the discoidin domain (Fig. 4A). In addition, we systematically mutated single amino acid residues located in these loops to alanine (Fig. 4B). In total, we created six deletion mutants and 11 point mutants within the context of the full-length DDR1 sequence. Although the initial set of mutants was created using the unmodified DDR1 cDNA (13), we also introduced an HA tag at the C terminus of the sequence, thereby allowing more accurate normalization of expression levels. We expressed individual mutants in human embryonic kidney 293 cells, stimulated these cells with type I collagen for 90 min, and determined DDR1 tyrosine phosphorylation by immunoprecipitation followed by Western blotting. We found that the mutation of Arg-105 (in loop 3) almost completely abolished DDR1 activation (Fig. 5A), whereas the Ser-175 to alanine mutation in loop 4 entirely lost its responsiveness to collagen (Fig. 5C). No other single amino acid exchange had a measurable effect on DDR1 phosphorylation. Furthermore, modification of loop 1, by either deleting the sequence stretch Ser-52–Thr-57 or by deleting Trp-53 and alanine-mutating residues 54–56 resulted in significant but not complete ablation of DDR1 activity (Fig. 5C). In contrast, deletion of loop 2 (Δ68–70) did not perturb DDR1 function. In agreement with the R105A point mutant, a deletion of loop 3 (Δ105–112) had a detrimental effect on DDR1 phosphorylation. To show that all DDR1 mutant proteins are properly processed
FIG. 3. Molecular modeling of DDR1 discoidin domain. A, alignment of the DDR1 discoidin domain sequence with homologous sequences from blood coagulation factor V, factor VIII, neuropilin-1, and DDR2. Loop regions relevant for protein-protein interactions are boxed in green. Conserved residues are highlighted in red, partially conserved residues in gray. Sequence stretches forming the β-barrel are designated with $S$, extra strands with $\beta$. Swiss-Prot accession numbers are: factor V, NP_000121; factor VIII, NP_000123; neuropilin-1, NP_003864; DDR2, NP_006173; and DDR1, NP_054699. Numbering of amino acid residues refers to the DDR1 sequence. B, ribbon model of DDR1 discoidin domain based on the x-ray structure of factors V/VIII and neuropilin-1. β-Strands are shown in blue, loops in gray, and disulfide bonds in yellow. Notice the presence of the deep groove spanned by loops 1 and 3. Residues Arg-105 and Ser-175 are shown with their non-hydrogen atoms.
within the cell, we prepared membrane fractions of transfected 293 cells and showed equal amounts of DDR1 wild-type and mutant protein localized at the cell surface (Fig. 5E).

To directly quantify collagen-binding of various DDR1 mutants, we used the ELISA-based readout described above. Collagen-coated plates were incubated with lysates from DDR1...
wild-type or mutant overexpressing cells. Bound protein was detected by a primary DDR1-specific antibody followed by a secondary horseradish-conjugated antibody. We found that the two point mutants R105A and S175A, which failed to be activated by collagen, largely failed to bind to collagen as well (Fig. 6). Several other point mutants, such as H106A, V173A, and M174A, had markedly reduced binding in the ELISA although they were still activated by collagen when assessed by antiphosphotyrosine Western blotting (Fig. 5). Deletion of residues within any of the four loops resulted in loss of collagen affinity to varying degrees. It was interesting to note that deletion of loop 1 (ΔS52–57 and ΔS53/54–56A) or loop 3 (Δ105–112 or Δ108–112) largely abolished the binding of the DDR1 discoidin domain to collagen, whereas deletion of loop 2 (Δ68–70) or loop 4 (Δ171–172) led only to a partial reduction.

To further define the contribution of individual loop regions in binding collagen, we generated synthetic cyclic peptides mimicking loops 1–3 (Fig. 7A). We tested the ability of these peptides to compete with the binding of DDR1 (over-expressed in 293 cells) to collagen in an ELISA. Despite using a 1250-fold molar excess of cyclic peptides over collagen, we failed to observe any significant interference of discoidin-collagen interaction using a single peptide or a combination of all three peptides (Fig. 7B). Furthermore, none of the peptides was able to interfere with DDR1 activation in cells transfected with or endogenously expressing DDR1 (data not shown).

**DISCUSSION**

In this study, by mutating a total of 11 individual loop residues to alanine, we showed for the first time that three neighboring, exposed loops in the discoidin domain of DDR1 are critically involved in collagen binding. In addition, we generated and tested six variants with partial deletions within these loops. We identified a deep groove at one end of the DDR1
discoidin domain as centrally involved in the ligand recognition process. This groove is basically formed by residues donated by loops 1 (Ser-52 to Thr-57) and 3 (Arg-105 to Lys-112), which protrude roughly parallel to each other from the bottom of the conserved discoidin $\beta$-barrel (Fig. 3B). It is noteworthy that the interloop cavity possesses the appropriate width to properly accommodate a triple-helical collagen molecule with an average diameter of 12 Å (distance between the C$\alpha$ atoms of Trp-53 and Phe-114: 14 Å). According to our present model, we predict that collagen docks into this groove with its major molecular axis running roughly perpendicular to the plane of the $\beta$-sheets. Given the plasticity evidenced for the equivalent loop region of the factor V discoidin domain (17), it is tempting to speculate that the loops of DDR1 (and DDR2) are flexible in a similar way, being able to undergo some structural rearrangement upon collagen engagement.

Although our data clearly indicated that loops 1 and 3 are essential for DDR1-collagen recognition, synthetic cyclic peptides mimicking these sequences failed to inhibit DDR1-collagen binding in an ELISA (Fig. 7). A number of possibilities could explain this outcome. The peptides used were 11- or 12-mers and should, after cyclization, form unconstrained loops resembling the DDR1 solution structure. Despite this fact, we cannot completely rule out the possibility that they are unable to mimic the fold of the native binding epitope. In light of the large body of evidence supporting the use of peptides for mapping protein-protein binding sites, however, we favor a simpler explanation for the apparent discrepancy between the results obtained with mutated/truncated proteins on the one hand and synthetic DDR1 peptides on the other, i.e., that the configuration of the interloop groove, which cannot be appropriately mimicked by the untethered peptides, is most critical for collagen binding. In other words, only simultaneous contacts with both “walls” of the groove (Fig. 3B, loops 1 and 3) would result in appreciable collagen binding, and this requires the framework provided by the discoidin $\beta$-barrel. Along these lines, a recent study failed to detect binding of peptides derived from the serpin, pigment epithelium-derived factor to type I collagen, although they partially cover the putative ligand-binding site (26).

Our mutagenesis data indicate that the nearby DDR1 residues Arg-105 and Ser-175 (distance between Arg-105 N$\gamma$ and Ser-175 O$\gamma$: 7.5 Å), located at the bottom of the groove, are essential for high-affinity collagen binding. It is conceivable that the side chains of both residues engage in important electrostatic interactions with specific collagen residues within the receptor-ligand complex, which are, in turn, strictly required for subsequent activation of the tyrosine kinase domain of DDR1. Loop 1 contains one threonine and four serine residues, which would facilitate ample hydrogen bond formation to collagen chains (Fig. 3A). In contrast, loop 3 has a hydrophobic tip (A-G-G-L-G) flanked by the basic residues Arg-105, His-106, and Lys-112. These three side chains might potentially form salt-bridges to acidic residues within the collagen helix; however, only point mutation of Arg-105, but not of His-106 or Lys-112, eliminated DDR1 function in our activation and binding assays (Figs. 5 and 6). Therefore, and although the high-affinity binding sequence within the collagen triple-helix is unknown for DDR1 (and DDR2), our data suggest that at least one acidic collagen residue is involved in the binding process. It is interesting to note that collagen binding by the integrin I-domain involves salt-bridge formation as well. In this case, however, the arginine is not part of the receptor but is located within the collagen recognition sequence GFOGER (O = hydroxyproline) (27). The binding groove in DDR1 identified here may have evolved analogously not only to the integrin I-domain but also to the collagen-binding trench in the adhesin protein from Staphylococcus aureus (28, 29).

In a previous mutagenesis approach of the DDR1 extracellular domain, we designed mutants based on the sequence homology between disease-linked point mutants in retinoschisin and the DDR1 discoidin sequence (13). These mutations were localized within the conserved strands of the $\beta$-barrel and affected the overall fold discoidin domain rather than the collagen-binding epitope. The molecular model presented here allowed us to precisely target residues in which the side chains are neither buried in the protein core nor involved in electrostatic interactions with other side chain/main chain atoms. These residues could thus be safely mutated to alanine without compromising protein folding. Similar considerations apply to the design of variants with partial deletions of loop stretches. The success of this approach is illustrated by the observation of similar levels of wild-type and mutant proteins properly positioned in plasma membrane of transfected cells (Fig. 5E).

Additional work by others has stressed the importance of the loop region in proteins with discoidin domains other than DDR1. In a recent mutagenesis approach of the DDR2-DiscD, sequences homologous to loops 1–3 of DDR1 were identified to be important for collagen-binding; however, loop 4, which is highly conserved between both DDR, was not detected in this screen (30). It is possible that residues located in loop 4 are critically involved in the more promiscuous collagen-binding preference of DDR1 compared with DDR2. Similar observations have been made in other structurally related domains. In both factor V and factor VIII, the C-terminal discoidin domain has been found to bind phospholipids within the plasma membrane of platelets (reviewed in Ref. 19). In addition, recent results indicated that the more N-terminal discoidin domain of factor V (C1) contributes to the membrane association process as well (31). The C-terminal discoidin domain of factor VIII also provides a critical binding site for von Willebrand factor, a carrier protein. It is noteworthy that several hemophilia mutations are located within the loop region of factor VIII, lending further support to its functional significance during protein-protein and protein-phospholipid interactions (31). Mapping of the factor V/VIII loop regions by site-directed mutagenesis showed that hydrophobic residues located within loops 1 and 3 are critically important for membrane-binding as well as von Willebrand factor-binding (31–33). The third currently available crystal structure of a discoidin domain, the structure of Npn-1 revealed a conserved $\beta$-barrel decorated with four major and two minor loops (16). Two groups of loops within Npn-1 form an interloop cleft region that is believed to harbor the binding site for semaphorins and VEGF-165, the two known ligands of Npn-1. It is interesting that an 18-amino acid, somewhat poorly defined cell adhesion motif in Npn-1 also maps to the third loop within the discoidin domain (34). A molecular model of the discoidin domain in retinoschisin has recently been made available as well (35).

Our data show that the extracellular domain of DDR1 has a 10-fold higher binding affinity to collagen than the discoidin domain alone. Furthermore, we confirm previous findings showing that a 150 nM concentration of DDR2-DiscD is sufficient for maximal collagen-binding (30). However, our recombinant DDR-collagen ELISA data supersede the work by Letinger (2003) in that we were able to produce a complete set of recombinant extracellular DDR proteins and we used a single antibody to detect binding of recombinant proteins containing a uniform N-terminal affinity tag. In addition, the DDR2 discoidin domain construct previously published included additional sequences derived from the stalk region (residues 185–200 and 369–397). In contrast, our discoidin domain constructs, which
were delineated by the N- and C-terminal cysteines, allowed us to eliminate the possibility that residues outside of the discoidin domain contribute to the collagen interaction measured.

Taken together, we showed that the discoidin domain of DDR1 is sufficient for collagen binding; however the extracellular stalk region of DDR1 plays an important role in enhancing this interaction. We propose that the stalk region may serve as a scaffold for the discoidin domain, allowing it to interact with its ligands in a more stable conformation in vivo, and may contribute to the much lower concentration required for the extracellular domain of DDR1 to achieve maximal collagen binding. On the other hand, the stalk region could contribute as an additional binding epitope for collagen. The results of our epitope mapping in conjunction with the three-dimensional modeling presented here will allow further investigation of the collagen-binding surface on DDR1 and DDR2 with the ultimate goal of developing reagents that antagonize the receptor/ligand complex and therefore have a therapeutic potential in diseases such as tissue fibrosis or cancer.

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