Binding of Discoidin Domain Receptor 2 to Collagen I: An Atomic Force Microscopy Investigation

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Received January 29, 2002

ABSTRACT: Collagens have recently been identified as ligands for discoidin domain receptors (DDR1 and DDR2), generating an interest in studying the properties of binding of DDR to its ligand. We are interested in the interaction of DDR2 with collagen I because of its potential role in liver fibrosis. Our in vitro binding assay utilizes DDR2−Fc fusion proteins, which can be clustered (multimerized) by use of antibodies to form DDR2 complexes. Binding of DDR2 complexes to collagen I coated on plastic plates was established by a microplate-based assay using Eu^{3+}-labeled proteins and time-resolved fluorometry. Clustering of the DDR2−Fc with antibody was found to be requisite for binding to collagen in vitro. Using atomic force microscopy (AFM) in an aqueous environment, we characterized the surface topographies of DDR2 complexes and collagen I, and investigated binding of this receptor−ligand pair. We were able to image and identify binding of DDR2 complexes onto individual molecules of triple-helical collagen and provide insight into the number and locations of binding sites on collagen I. In most cases, a single receptor complex bound to a single collagen molecule and there were preferred DDR2 binding sites on the collagen I triple helix. These data were validated by rotary-replication transmission electron microscopy (TEM) of glycerol-sprayed samples.

Receptor tyrosine kinases (RTKs)[1] are a class of transmembrane proteins that relay extracellular information to intracellular regions. Ligand binding to the extracellular domain of RTKs induces receptor dimerization and kinase activation (*1*). Discoidin domain receptors 1 and 2 (DDR1 and DDR2, respectively) are 125 and 130 kDa glycosylated proteins belonging to the family of RTK (*2*, *3*). They are expressed in several mammalian tissues and overexpressed in human malignancies. It is being established that DDRs play a role in regulation of collagen (*4*), cell proliferation (*5*), wound healing (*6*), and mammary gland development (*7*). DDRs and alterations in collagen biosynthesis and function may also be involved in the pathology of fibrosis (*8*).

Collagen was recently identified as a ligand for DDR1 and DDR2 (*9*, *10*). Collagens are extracellular matrix proteins characterized by Gly-X-Y repeats in their polypeptide chains, where X and Y are frequently prolines and hydroxyprolines. The native triple-helical structure of collagen, formed by assembly of three such polypeptide chains, is essential for DDR activation, and the binding epitopes are located in the Gly-X-Y repeat regions of collagen (*9*, *10*). Activation of DDRs by collagen is direct, and tyrosine phosphorylation of the receptors is stimulated by soluble fibrillar collagen and immobilized collagen. While collagens I−III and V activate both DDR1 and DDR2, collagen IV activates only DDR1.

Binding of DDRs to collagen is under active investigation and only partly understood. Examining the interaction of DDR2 with collagen I is of special interest to us because of its potential role in regulation of collagen I in liver fibrosis (*8*). Our objective is to understand where and how DDR2 binds to this ligand and if there are preferred sites of binding on the collagen I triple helix. We are interested in knowing what distinguishing features of collagen (arising from hydroxylations, glycosylations, and/or overlapping molecules) are necessary for functional DDR binding. Binding affinity and the slow kinetics of this receptor−ligand interaction need to be explored.

Our earlier attempts to identify DDR binding sites on collagen molecules using synthetic triple-helical collagen-like peptide, renatured individual chains of collagen, or renatured CNBr fragments of collagen were not successful (unpublished observation). In this work, we used atomic force microscopy (AFM) to investigate binding of DDR2 to collagen I. AFM is a relatively new technique (*11*) used to visualize biomolecules at nanoscale resolution (*12*, *13*). AFM produces a topographic image of a sample by scanning the surface with a sharp, nanometer scale probe (tip) attached to a flexible cantilever. AFM has been used previously to map heparin-binding sites on fibronectin molecules (*14*), human factor IX (*15*), and laminin (*16*) to collagen IV on

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[1] Abbreviations: DDR, discoidin domain receptor; AFM, atomic force microscopy; TEM, transmission electron microscopy; RTK, receptor tyrosine kinase; IgG, immunoglobulin; TBS, Tris-buffered saline.

10.1021/bi020087w CCC: $22.00
Published on Web 08/23/2002

dried samples. This high-resolution microscopy technique also permits imaging in a fluid environment, therefore avoiding artifacts due to drying and/or coating of samples, and has the advantage of maintaining biological molecules, and their interactions, in near-physiological conditions.

We have established in vitro binding of DDR2 to its ligand, native, triple-helical collagen I, by use of DDR2−Fc fusion proteins (*9*). This fusion protein is a chimera of the DDR2 extracellular domain and the constant domain (Fc) of human immunoglobulin IgG1. The chimeric molecule is frequently termed a receptor body. The DDR2−Fc chimera can be multimerized (henceforth termed "clustered") with antibodies to form DDR2 complexes. Using a microplate-based assay and time-resolved fluorometry, we have demonstrated that clustering of the receptor body is essential for its binding in vitro. We have characterized surface topologies of DDR2 complexes and collagen I, and investigated binding of this receptor−ligand pair by AFM in an aqueous environment. We imaged the binding of DDR2 complexes onto individual molecules of triple-helical collagen I, and provided insight into the number and locations of preferred binding sites. Our results have demonstrated that in most cases a single receptor complex binds to a single collagen molecule, and there are at least two preferred sites for binding of DDR2 on collagen I. These data were validated by rotary-replication transmission electron microscopy (TEM) of glycerol-sprayed samples.

EXPERIMENTAL PROCEDURES

Materials. Our in vitro binding assay makes use of receptor body fusion proteins as described previously (*9*). Briefly, the extracellular domain of mouse DDR2 (*3*) was fused with a constant domain (Fc) of human immunoglobulin (IgG1) and overexpressed in COS cells (*9*). The fusion protein, DDR2−Fc, was then incubated with anti-human IgG antibody. This resulted in clustering of the receptor bodies to form DDR2 complexes. Fusion proteins of TrkB, a receptor for the brain-derived growth factor (*17*), and Tie2, a receptor for angiopoietin (*18*), were used in place of DDR2 as negative controls.

Antibodies to the human IgG Fc region were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Eu^{3+}-labeled antibodies to the human IgG Fc region and Eu^{3+}-labeled protein G were from PerkinElmer Wallace and Wallac Oy (EG&G), respectively. Trimeric molecules of bovine dermal vitrogen collagen I were purchased from Collagen Corp. (Faber Place, CA); this collagen was pepsin-digested and consisted of 3−5% collagen III and a certain amount of cross-linked species as well as smaller proteolytic fragments.

Microplate Assay. The microplate-based assay was performed on 96-well ELISA plates (Nunc-Immuno Plate MaxiSorp Surface). Collagen I (10 μg/mL) was dissolved in 50 mM acetic acid (pH 3.5) and immobilized on ELISA plates by aliquoting 100 μL in each well, sealing the plates, and leaving them overnight at room temperature. Tris-buffered saline (TBS) comprising 25 mM Tris and 150 mM NaCl (pH 7.5) with 1% BSA and 0.1% Tween-20 was used for all other proteins. Blocking solution consisted of TBS with 5% BSA and 0.1% Tween-20. Wells were blocked (250 μL/well) for at least 2 h to prevent nonspecific binding. Washing was carried out using TBS (4 × 250 μL/well). Enhancement solution was from Perkin-Elmer Wallace, and 200 μL/well was used.

To establish specific binding of DDR2 to collagen I, the DDR2−Fc and Tie2−Fc chimera at various concentrations (0.5−5.0 μg/mL) were clustered with Eu^{3+}-labeled antibodies [5 μg/mL, 1:1 (v/v)] for 20 min. DDR2 and Tie2 complexes thus formed were incubated (100 μL/well) over immobilized collagen on separate plates for 1 h. Wells were then washed, and enhancement solution was added. The amount of bound receptor was determined by measuring the signal from Eu^{3+}-labeled antibodies present in receptor complexes, using dissociation-enhanced time-resolved fluorometry (*19*).

To verify that clustering of the DDR2−Fc chimera is essential for its binding to collagen, the DDR2−Fc chimera at various concentrations (0.5−10.0 μg/mL) was clustered with unlabeled antibodies [50 μg/mL, 1:1 (v/v)] for 20 min. DDR2 complexes thus formed, and native, unclustered DDR2−Fc were incubated over immobilized collagen on separate plates for 1 h. The plates were then washed and incubated with Eu^{3+}-labeled protein G (100 μL/well), which binds to the Fc tail in the DDR2−Fc chimera. After another wash, enhancement solution was added and the amount of bound receptor was determined by measuring the signal from Eu^{3+}-labeled protein G by time-resolved fluorometry.

Atomic Force Microscopy. Freshly cleaved mica (Ruby muscovite) was used as a substrate for all AFM preparations. The DDR2−Fc chimera (150 ng/mL) was clustered with anti-IgG antibody [150 ng/mL, 1:1 (v/v)] in TBS (pH 7.5) for 2−3 h. DDR2 complexes did not adhere to mica at pH 7.5; therefore, adherence was promoted by treating mica with a 100 mM $MgCl_2$ solution (30−40 min). Collagen I was dissolved in 50 mM acetic acid (pH 3.2), a pH below its isoelectric point, thus making the protein positively charged and promoting adherence to mica. An aliquot of collagen (0.5−1.0 μg/mL) was deposited on mica, allowed to set for 5−7 min, and washed. For binding experiments, an aliquot of DDR2 complexes [in TBS (pH 7.5)] was applied to collagen already immobilized on mica and incubated overnight in a moist Petri dish at 4 °C. The TrkB complex was used in place of the DDR2 complex as a negative control. Care was taken to prevent drying of proteins at any stage. All samples were washed (using a spray bottle) and imaged in filtered distilled water.

A Nanoscope III instrument (Digital Instruments, Santa Barbara, CA) was used in tapping mode for imaging. All samples were imaged in filtered, distilled water using a fluid cell. Oxide-sharpened SiN cantilevers (Digital Instruments) with a nominal spring constant of 0.32 N/m were driven at a resonance frequency of 9 kHz. The E scanner type was used; the scanning rate was 2 Hz with 512 lines per scan direction. Images were recorded in height and amplitude modes. Most images were recorded with a scan size of 1 μm × 1 μm, though occasionally 2 μm × 2 μm scans were also recorded.

Transmission Electron Microscopy. TBS at pH 7.5 was used for all proteins. DDR2 (60 μg/mL) was incubated with anti-IgG antibody [60 μg/mL, 1:1 (v/v)] to form DDR2 complexes. Samples for characterization of DDR2 complexes and collagen I were made by spraying glycerol (50%) solutions of the proteins on freshly cleaved mica with an atomizer (Uli Aebi, Basel, Switzerland). For binding studies,

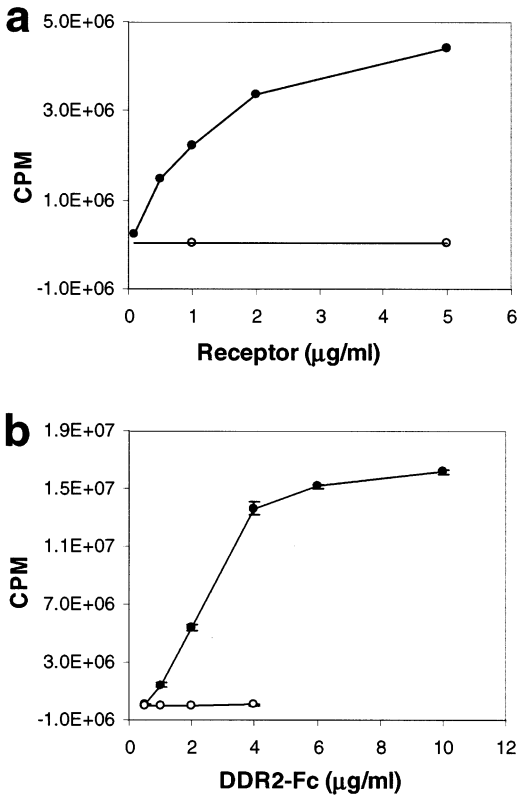

FIGURE 1: Time-resolved fluorometry of DDR2 complexes binding to collagen I immobilized on 96-well microplates. Eu^{3+}-labeled proteins were used to detect receptor binding. The amount of bound receptor was measured as the Eu^{3+} signal released [in counts per minute (CPM)] vs receptor concentration. (a) Eu^{3+}-labeled anti-Fc antibody was used to cluster (multimerize) DDR2−Fc and Tie2−Fc. Receptor complexes thus formed were incubated over immobilized collagen. The Eu^{3+} signal increased with receptor concentration for DDR2 complexes (●), while Tie2 complexes (○) showed no detectable signal. (b) The DDR2−Fc clustered with unlabeled antibody and the native, unclustered DDR2−Fc were incubated over collagen. Eu^{3+}-labeled protein G (which binds to Fc tail) was then added, and the amount of released Eu^{3+} was counted. A dramatic increase in the Eu^{3+} signal with receptor concentration was observed from the microplate containing DDR2 complexes (●) as compared to that of the native, unclustered DDR2−Fc (○).

collagen I (100 μg/mL) was mixed with an equal volume of DDR2 complexes and incubated overnight at 4 °C. Glycerol was then added to the ligand/receptor mixture to give a final glycerol concentration of 50%. The mixture was sprayed onto freshly cleaved mica. Preparations were dried under vacuum (10^{-5} Pa) and rotary shadowed with Pt/C at 6 °C in a JEOL RFD 9010 CR freeze fracture instrument. Replicas were separated from mica in water, washed, mounted on grids, and examined by TEM (JEM 1200 EX) at an accelerating voltage of 80 kV. Micrographs were recorded at a magnification of 100 or 120 K, and the negatives were scanned (AGFA Duoscan) at 600 dpi as tif files.

Data Analysis. Lengths of collagen molecules were measured by manual tracing on AFM (amplitude) images, using the software WSxM (Nanotec Electronica). The position of bound DDR2 complexes was measured from each end; the experimental error in length per position measurement was ±3 nm. Nanoscope III software was used to flatten and convert height mode images to tif files and to ascertain the height profile along a line across the image to estimate particle sizes. Adobe Photoshop was used for illustrations. Length measurements and image analysis on TEM data were performed using ImageJ (NIH) on tif files scanned from TEM negatives. The number of measurements (n) made to estimate the size of biomolecules was 20.

RESULTS

Binding of DDR2 complexes to immobilized collagen I was established by a microplate-based assay using time-resolved fluorometry and Eu^{3+}-labeled proteins. The DDR2−Fc and another fusion protein, Tie2−Fc, were clustered with Eu^{3+}-labeled antibodies. DDR2 and Tie2 complexes thus formed were incubated over collagen immobilized on separate plates. As shown in Figure 1a, the amount of Eu^{3+} signal increased with increasing DDR2 complex concentrations, while the sample with Tie2 complexes showed no detectable Eu^{3+} signal. This result established that DDR2 complexes bind to immobilized collagen I, and Tie2 complexes do not.

Antibody-mediated clustering of the DDR2−Fc complex was found to be requisite for binding to collagen in vitro (Figure 1b). Native DDR2−Fc and DDR2 complexes formed with unlabeled antibodies were incubated over immobilized collagen on two separate microplates. Eu^{3+}-labeled protein G (which binds to the Fc tail) was then incubated on these plates. A dramatic increase in the Eu^{3+} signal released as a function of receptor concentration was observed from the plate containing DDR2 complexes as compared to that for the native, unclustered DDR2−Fc. This signifies that Eu^{3+}-labeled protein G was binding to DDR2 complexes already bound to collagen while the unclustered DDR2−Fc was hardly present over collagen to enable binding of protein G.

The size and geometry of DDR2 complexes and collagen I were established by AFM and TEM. In AFM images, topography was mapped to color using a linear gray scale from 0 to 5 nm from black to white on height images. DDR2 (Figure 2a) and TrkB (data not shown) complexes appeared to be globular particles, 20−35 nm in width and 3−5 nm in height, as determined by the height profile along a line (inset, Figure 2a) in AFM images. The TEM results of rotary shadowed DDR2 complexes are shown in Figure 2b; the lateral diameter was determined to be 5−10 nm, inclusive of a Pt coating with a thickness of ≈1−2 nm. In preparations containing collagen I, randomly oriented filamentous structures characteristic of collagen I molecules (*20*) were easily distinguished in AFM (Figure 2c) and TEM images (data not shown). Some molecules were occasionally overlapping each other. Collagen filaments were clean, and the preparations were free of particulate matter. Diameters of these filaments, obtained by lateral width measurements on AFM images, were 8−10 nm, and the height was 0.5−1.2 nm (inset, Figure 2c). The width of collagen filaments estimated from TEM images of rotary shadowed samples was 2−4 nm, inclusive of a Pt coating. The length of pepsin-digested collagen was heterogeneous and ranged from 30 to 300 nm for both AFM and TEM data.

For AFM imaging of receptor−ligand binding, unwanted or nonspecifically bound particles on substrate and collagen were minimized by using TBS buffer at pH 7. DDR2 complexes did not adhere to mica under this buffer condition (Figure 3a), and the samples were no different from those of bare mica surfaces incubated with TBS buffer overnight.

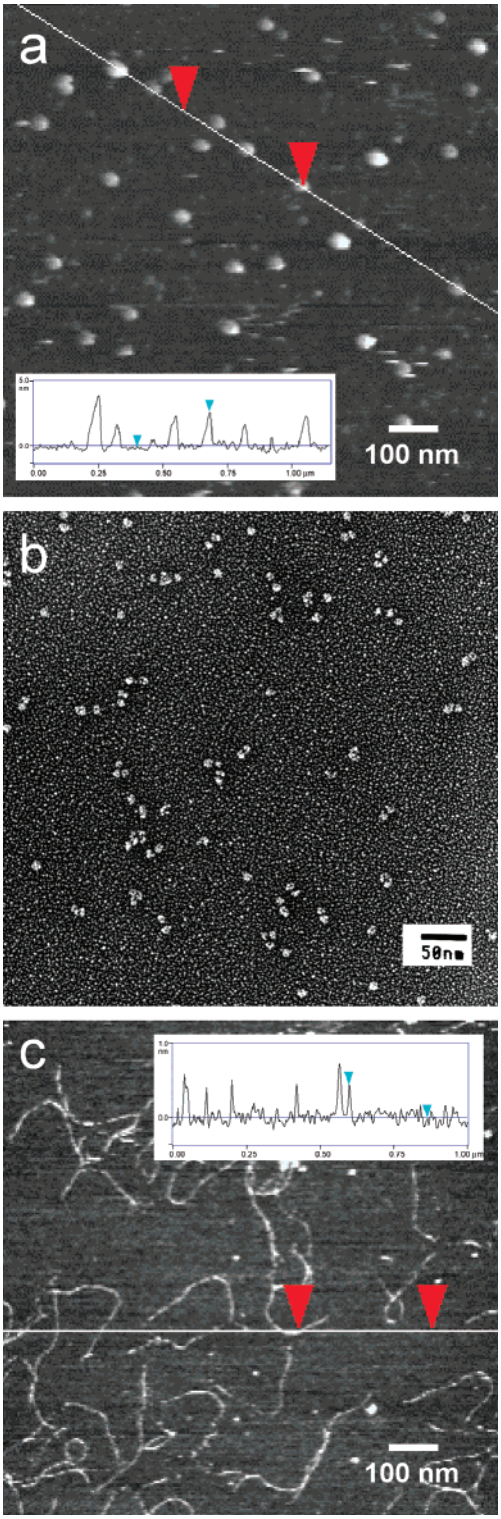

FIGURE 2: Imaging of DDR2 complexes and collagen I. (a) DDR2 complexes were applied to $MgCl_2$-treated mica, washed, and imaged in water by tapping mode AFM (height mode scan of size 1 μm × 1 μm). The inset shows a vertical height profile of DDR2 complexes along a line; particles were 3−5 nm in height. (b) TEM image of rotary shadowed DDR2 complexes sprayed with glycerol on freshly cleaved mica. DDR2 complexes were 5−10 nm thick, inclusive of the Pt coating (~1−2 nm). (c) Collagen I was applied to freshly cleaved mica in 50 mM acetic acid, washed, and imaged by AFM (height mode scan of size 1 μm × 1 μm). Randomly oriented filamentous structures were easily distinguished. Some molecules were occasionally overlapping; the preparation was free of particulate matter comparable to sizes of DDR2 complexes. The inset shows the height of collagen was 0.5−1.0 nm; lengths of collagen were heterogeneous.

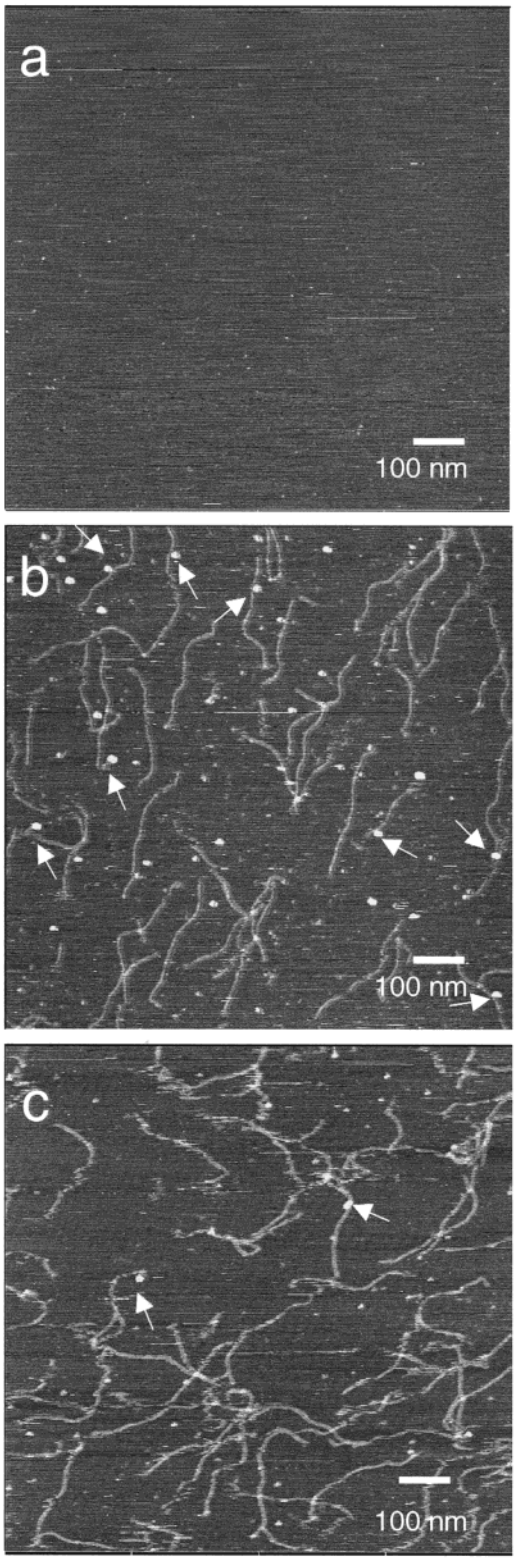

FIGURE 3: Results of a series of AFM experiments illustrating that binding of DDR2 complexes to collagen I is specific. (a) DDR2 complexes in TBS buffer (pH 7.5) incubated overnight on freshly cleaved mica. DDR2 complexes did not adhere to mica under these buffer conditions. (b) DDR2 complexes in TBS (pH 7.5) incubated over collagen immobilized on mica. Globular particles on collagen filaments represent binding of DDR2 complexes (indicated with arrows); few unbound complexes were present. (c) TrkB complexes incubated over collagen as described for panel b. Collagen filaments appeared to be clean and similar to native preparations, with only a few receptor complexes present in each image (indicated with arrows). The scan size was 1 μm × 1 μm for all images.

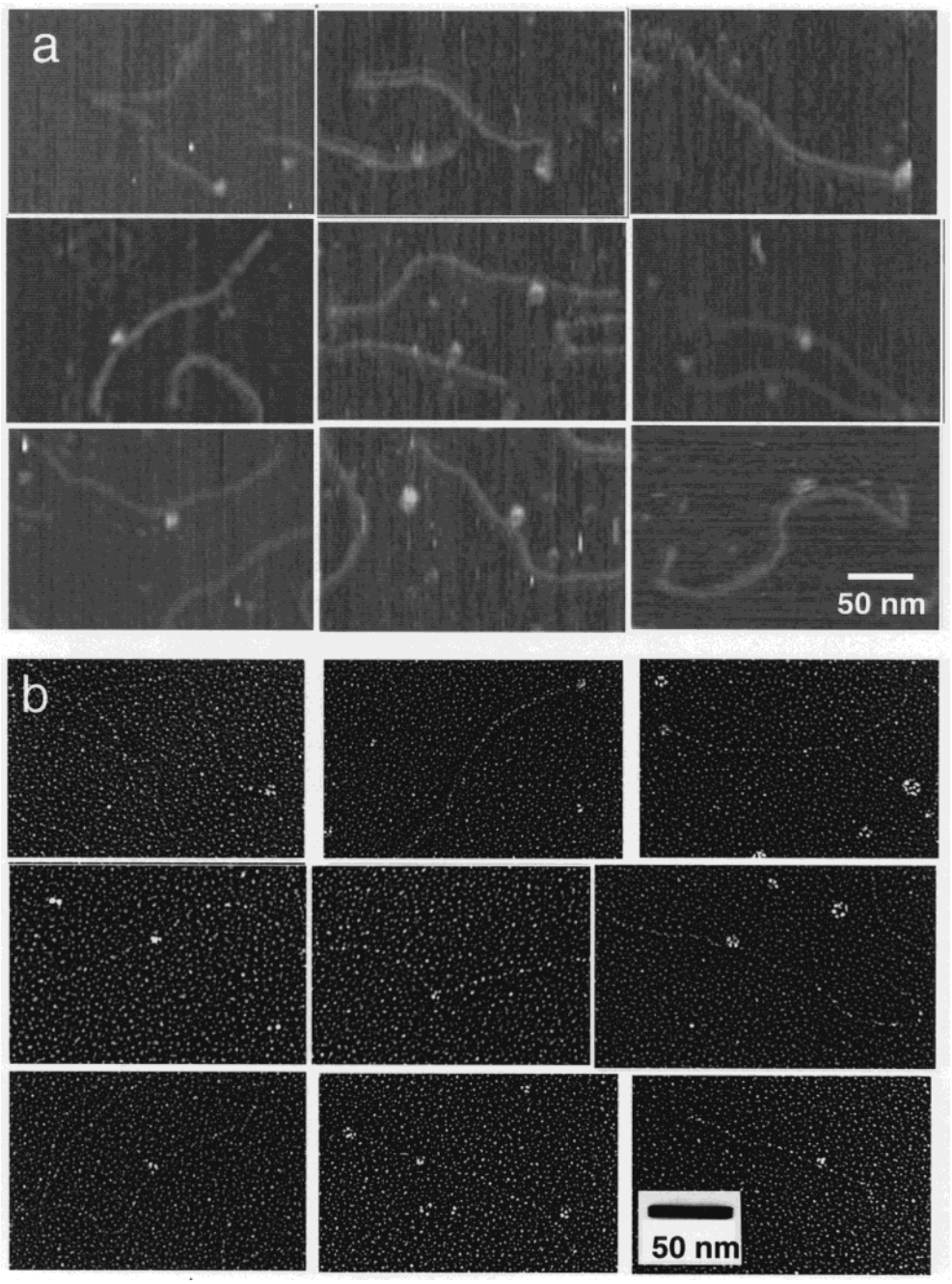

FIGURE 4: Selected regions from AFM and TEM images showing that DDR2 complexes bind at various positions along the collagen I molecule. (a) DDR2 complexes incubated over collagen preattached to mica and imaged by AFM. (b) TEM images of DDR2 complexes and collagen I incubated in solution and sprayed with glycerol on freshly cleaved mica. Top, middle, and bottom rows show binding around 0, 65 ± 8, and 105 ± 8 nm, respectively, from an end of collagen.

When DDR2 complexes [in TBS (pH 7.5)] were incubated over collagen immobilized on mica, 5−10 particles bound to collagen molecules could be seen in each 1 μm × 1 μm scan (Figure 3b). These data were indicative of the receptor binding to its ligand. A few unbound complexes were present, which could have arisen from DDR2 complexes binding to very small strands of collagen and/or weakly bound complexes dissociating from collagen due to physical forces exerted by the AFM tip on repeated scanning. To verify that binding events are from DDR2 complexes and not due to the clustering antibody or the native DDR2−Fc, we incubated these proteins over immobilized collagen overnight, but did not see particles on collagen or mica (data not shown). When TrkB complexes were incubated over immobilized collagen, few (zero to two) complexes were present on the substrate or collagen (Figure 3c) in each image, and we could not determine a binding pattern from these data. These results established that DDR2 complexes bind to collagen I.

In more than 95% of binding events, a single DDR2 complex was observed binding to a single collagen molecule. Selected regions from AFM images show that DDR2 complexes bind at various positions along the collagen molecule (Figure 4a). Rotary shadowed samples of the DDR2 complex and collagen I, incubated in solution and glycerol sprayed on freshly cleaved mica, were examined by TEM; representative regions showing DDR2 complexes bound to collagen I are illustrated in Figure 4b. In data obtained from

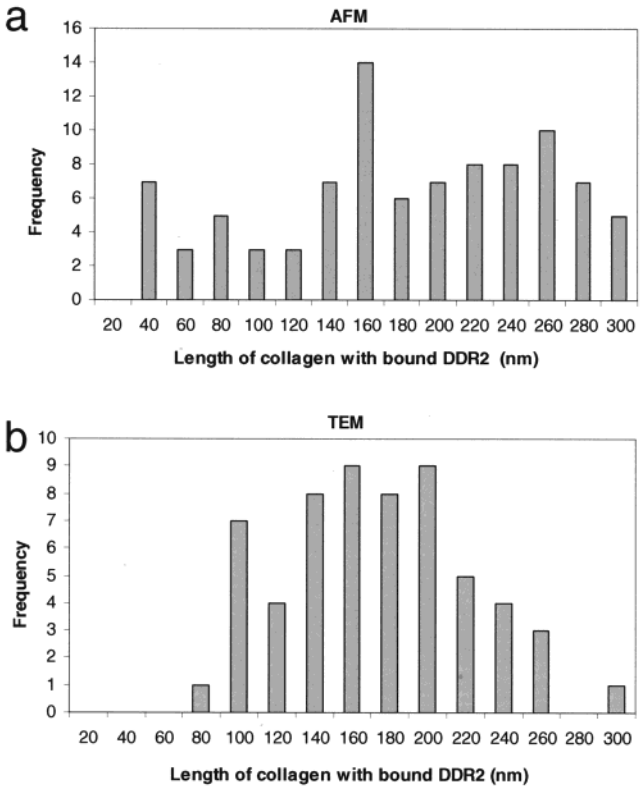

FIGURE 5: Frequency distribution of the length of collagen I-containing bound DDR2 complexes obtained using (a) AFM and (b) TEM. Most of the collagen fragments are >140 nm in length (half-length of intact collagen). Length bins are 10 nm.

both AFM and TEM, globular DDR2 complexes can be seen bound to collagen ends or to the triple-helical contour of collagen.

Quantitative analysis of our data proved to be challenging because of the inherent length heterogeneity of collagen I containing the bound DDR2 complex and a lack of morphological landmarks on collagen I that might indicate some characteristic feature, such as a terminus and/or specific site. The length of receptor-bound collagen I fragments ranged from 30 to 300 nm, with the majority of the fragments being >140 nm (the half-length of intact collagen molecule; *20*) in length (Figure 5). We measured the position of bound DDR2 complexes relative to the two free ends of collagen fibers ($n = 110$ for AFM and 60 for TEM). Each bound complex was therefore associated with two distances (d_1 and d_2) from the ends of collagen. Panels a and b of Figure 6 show a plot of d_1 versus d_2 for each bound DDR2 complex in AFM and TEM experiments, respectively. It can be seen that there are certain rows and columns with a higher density of points in these plots. A sum of all points along each 10 nm wide row (and column) was made, thus binning the data into 10 nm bins along d_1 (rows) and d_2 (columns). For each 10 nm bin, the numbers of points in its corresponding row and column were added to give the total number of bound DDR2 complexes having at least one distance (d_1 or d_2) in the bin. Few preferred regions of binding emerged from these data: at ends (0 nm), between 30 and 40 nm, between 60 and 70 nm, and between 100 and 110 nm. We assume that collagen fragments have been cleaved at just one point (*21*−*23*; see the Discussion). Therefore, one distance, d_1 or d_2, should reflect the true binding site from a native terminus of collagen. One distance (d_1 or d_2) was selected for each bound DDR2 complex, depending on which of these distances was in the peak regions (weighted in the order 60−70, 100−110, 30−40, and 0 nm, based on their frequency values and the binding sites observed for nearly intact collagen molecules; see the Discussion). A distribution of the selected binding distance for AFM and TEM data is shown in panels c and d of Figure 6, respectively. Preferred binding positions were noted around 35 ± 8, 65 ± 8, and 105 ± 8 nm from an end for both AFM and TEM data with an additional peak at 0 nm for AFM data.

DISCUSSION

Specific binding of the receptor DDR2 to collagen I has been established in vitro in our microplate-based assay using a Fc fusion protein. Our results confirm that clustering (multimerization) of the receptor is required for its interaction with collagen in vitro. This is in contrast to typical RTKs where ligand binding leads to receptor dimerization. DDRs form a special case where receptor dimerization (or multimerization) is required prior to ligand binding. Cell-based assays have demonstrated that DDR tyrosine phosphorylation requires autocatalytic activity, and it may be possible that DDRs behave like the ephrin receptors and require formation of multimeric clusters to be fully activated (*24*).

We used AFM to visualize and characterize DDR2 complexes and collagen I under near-physiological conditions. Low-angle, rotary-replication TEM has been used in parallel to verify our data and provide a check on AFM tip artifacts. Widths of nanoscale particles are known to be susceptible to tip "convolution" effects arising from finite widths of AFM tips (*25*, *26*); hence, lateral width does not provide a true measure of the particle dimensions in AFM images. Sometimes particles loosely adhered to the substrate can attach to the AFM tip while imaging (making it broader), and the width of the imaged particles can appear larger. This is illustrated in our experiments where the diameter of DDR2 loosely adhered to mica (Figure 2a) appears to be larger than when they are imaged after being bound to collagen (Figure 3b). The height profile is a more accurate measure of particle size in AFM and has been used to ascertain particle size from our data. DDR2 complexes appeared as small globules of a height consistent with measurements obtained using TEM. Polyclonal anti-IgG antibodies were used for clustering the DDR2−Fc, which likely resulted in aggregates of different sizes. Collagen I was similar in size, shape, and features as has been established previously (*20*). The height of collagen as determined by AFM was slightly lower than its reported value (1.2−1.5 nm) obtained using TEM (*20*). A possible explanation for this is that biological molecules are soft in aqueous media and can be slightly compressed due to forces exerted by the AFM tip. The lateral diameter of collagen obtained by AFM was similar to values reported previously (*27*).

To investigate receptor−ligand binding using AFM in an aqueous environment, selective adherence of biomolecules to the substrate (mica) was achieved utilizing buffer conditions. The ligand was immobilized firmly on mica by using a pH below its isoelectric point, and receptor complexes could then be incubated over immobilized collagen under buffer conditions appropriate for binding. Our AFM protocol resembles the steps followed in the microplate-based assay

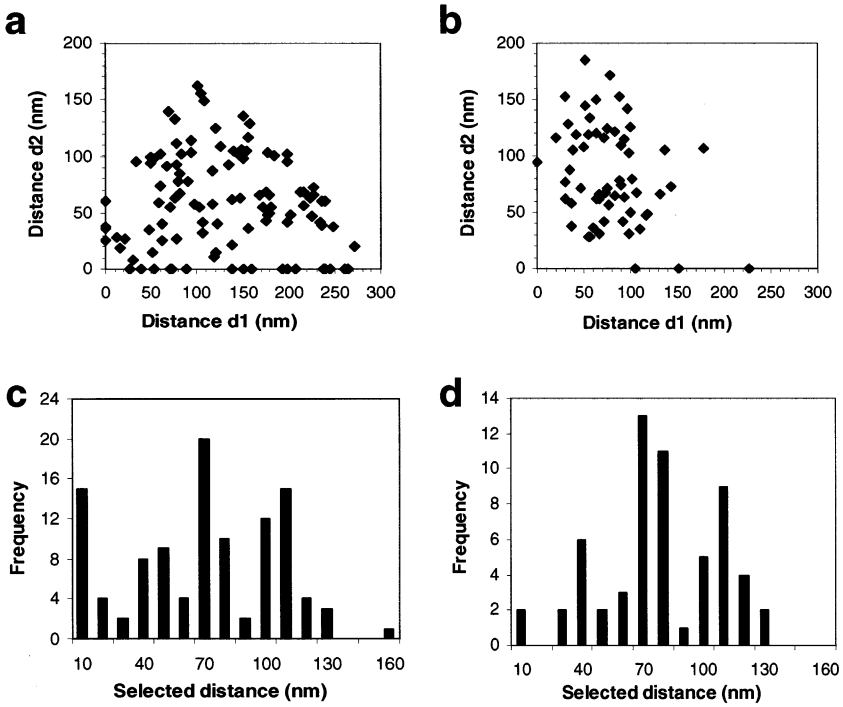

FIGURE 6: Analysis of DDR2 binding sites on collagen I. Distances of bound DDR2 complexes from two ends of collagen molecules plotted relative to each other, i.e., d_1 vs d_2, from (a) AFM ($n = 110$) and (b) TEM data ($n = 60$). One distance, either d_1 or d_2, was selected for each bound DDR2 complex, depending on which of these distances lies in the high-density regions (see the text and the Results) observed in panels a and b. Frequency distribution of selected distance for (c) AFM and (d) TEM data. Preferred binding sites are observed 35 ± 8, 65 ± 8, and 105 ± 8 nm from an end of collagen for both panels c and d, with the peaks at 65 ± 8 and 105 ± 8 nm being the strongest. An additional peak at 0 nm is observed from AFM data.

and helped us in minimizing the presence of nonspecifically bound proteins on mica or collagen. This was especially important to us as DDR2 complexes were small and globular and any spurious particle could easily be confused with the receptor complex. We did not observe a discrepancy in our results when DDR2 complexes and collagen were incubated in solution prior to attaching to mica as shown by our TEM results.

Very few multiple binding events, i.e., two or more DDR2 complexes bound to a single collagen molecule, were observed, even though the concentration of DDR2 complexes was much greater than number of collagen molecules in all our experiments. This may be due to the existence of only a few intact collagen molecules (having two binding sites available), and/or the weak nature of DDR2 binding as demonstrated by our experiments (long incubation time, only 5−10 binding events per image, and occasional dissociation of the receptor complex bound to collagen by repeated scanning with the AFM tip). Our results also provide a clue that glycosylation of native collagen may be playing a role in the interaction of DDR2 with collagen I, as suggested earlier (*10*). In the glycosylation pattern of collagen I, there is one Glc-Gal-Hyl residue per thousand for α1(I) and α2-(I) chains and one Gal-Hyl residue per thousand for the α2-(I) chain (*28*). Thus, there are possibly only one or two glycosylation sites on a collagen I molecule, which could explain the predominance of single binding events.

Our data analysis assumes that pepsin-digested fragments of trimeric collagen have been cleaved at just one point. Pikkarainen et al. (*21*−*23*) have characterized fractionation of pepsin-digested collagen and concluded that there are points on the tropocollagen molecule that are especially susceptible to pepsin. There is likely more than one unique site of single cleavage for pepsin-digested collagen (*21*−*23*, *29*), which can give rise to the various lengths of collagen fragments. On the basis of the location of pepsin cleavage sites (*22*), the larger fragments, (∼80, 140, 160, and 220 nm in length and others) could arise from a single cleavage, and would have one intact terminus from the native collagen molecule. The smaller fragments are likely to have been cleaved at both ends, and would therefore result in artifactual binding peaks arising from measurements made between arbitrary points along the collagen molecule. Since our DDR2 binding set mostly contains larger collagen fragments, we have used the assumption that the collagen fragments are singly cleaved. Our AFM and TEM data show that binding especially at 65 ± 8 and 105 ± 8 nm from ends occurs mostly on larger fragments. Analysis of nearly intact collagen molecules (260−300 nm in length, $n = 25$) from AFM data showed preferred binding at distances of 65 ± 8 and 105 ± 8 nm from one end of collagen (*30*). These observations support our assumption of single cleavage and suggest that binding at 65 ± 8 and 105 ± 8 nm from one end of collagen may be real and favored sites of binding. We did not observe enough evidence for binding at ends (0 nm) or at 35 ± 8 nm from ends in nearly intact molecules (*30*). Our TEM data do not support preferred binding at ends. In both AFM and TEM data, there are collagen fragments of various lengths having DDR2 complexes bound at ends. Therefore, it is likely that binding at ends may be an artifact arising from more than one cleavage of the collagen molecule. Binding at 35 ± 8 nm from an end may be a weak site of binding or an artifact as indicated by the AFM and TEM data. We did not find any correlation between DDR2 binding site and other

features on collagen-like overlaps or nodes between two collagen, kinks, curls, etc.

To correlate DDR2 binding sites with collagen structure, we searched the databases for a preferred amino acid sequence on the α1 and α2 chains of collagen type 1 around 35 ± 8, 65 ± 8, or 105 ± 8 nm from either the carboxy or amino terminus but were unable to find unique features in these regions. This is not surprising since DDR2 does not bind to individual chains (either α1 or α2) of collagen (*9*, *10*). We are limited by the unavailability of NMR and/or X-ray data on the complete triple-helical structure of collagen. Successful identification of binding sites of various biomolecules such as heparin (*31*), phosphophoryn (*32*), integrins (*33*), and decorin (*34*), reviewed (*35*) on collagen I by high-resolution microscopy, was made possible by other investigators by using either procollagen or individual chains of collagen labeled with antibodies. To complement our understanding of binding of DDR2 to collagen I and identify the structural features of collagen I responsible for the binding site, we need to explore use of uniform length triple-helical collagen and/or collagen with a site-specific identifier with our AFM and TEM protocols. Recombinant collagen (Fibrogen, San Francisco, CA) and procollagen are candidates; however, binding of DDR2 has to be re-established in both cases, as not all commercial sources of collagen are known to activate DDRs (*9*, *10*).

Despite the limitations of our biomolecular systems, our experiments provided us with interesting data on binding of DDR2 to collagen I. We observe four unique features. (1) Receptors have to be multimerized (clustered) to bind to collagen. (2) Binding is weak compared to that of other RTKs. (3) A single DDR2 complex binds to a single collagen I molecule. (4) There are at least two preferred sites of DDR2 binding on the collagen I triple helix.

ACKNOWLEDGMENT

We are grateful to Drs. Lei Yu and Hong You (Cell Biology, University of Cincinnati, Cincinnati, OH) for use of their Nanoscope III atomic force microscope, and for helpful tips throughout this project. We are thankful to Drs. Jarek Sacha and Tom Dufresne for help with image analysis, Paul Correa for analyzing the collagen 1 sequence for our studies, and Babul Borah (Procter & Gamble) for review of our manuscript and many useful suggestions.

SUPPORTING INFORMATION AVAILABLE

Quantitative analysis of binding of DDR2 complexes to collagen I on near full-length collagen (260−300 nm) from AFM data. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI020087W