Journal of Structural Biology 183 (2013) 495–500

Contents lists available at SciVerse ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Oligomerization of DDR1 ECD affects receptor–ligand binding

David Yeung [a,1], David Chmielewski [b,1], Cosmin Mihai [b], Gunjan Agarwal [a,b,*]

[a] Biomedical Engineering Department, 270 Bevis Hall, 1080 Carmack Road, The Ohio State University Columbus, OH 43210, USA
[b] Davis Heart and Lung Research Institute, 473 West 12th Ave., Columbus, OH 43210, USA

ARTICLE INFO

Article history:
Received 5 January 2013
Received in revised form 15 May 2013
Accepted 20 June 2013
Available online 28 June 2013

Keywords:
Collagen
Discoidin domain receptor
Extracellular domain
Oligomerization
Atomic force microscopy

ABSTRACT

Discoidin domain receptor 1 (DDR1) is a widely expressed receptor tyrosine kinase (RTK) which regulates cell differentiation, proliferation and migration and remodeling of the extracellular matrix. Collagen(s) are the only known ligand for DDR1. We have previously reported that collagen stimulation leads to oligomerization of the full length receptor. In this study we investigated the effect of oligomerization of the DDR1 extracellular domain (ECD) pre and post ligand binding. Solid phase binding assays showed that oligomers of recombinant DDR1-Fc bound more strongly to collagen compared to dimeric DDR1-Fc alone. In addition, DDR1-Fc itself could oligomerize upon in-vitro binding to collagen when examined using atomic force microscopy. Inhibition of dynamin mediated receptor endocytosis could prevent ligand induced endocytosis of DDR1b–YFP in live cells. However inhibition of receptor endocytosis did not affect DDR1 oligomerization. In summary our results demonstrate that DDR1 ECD plays a crucial role in receptor oligomerization which mediates high-affinity interactions with its ligand.

1. Introduction

Discoidin domain receptors (DDRs) are widely expressed receptor tyrosine kinases (RTKs) that bind to and get activated by collagen(s), the major component of the extracellular matrix (Vogel et al., 1997; Shrivastava et al., 1997). There are two known members of the DDR family; namely, DDR1 and DDR2, both distinguished from other RTKs by the presence of an extracellular discoidin (DS) domain and an unusually long juxtamembrane (JM) region. DDRs have an unusually slow activation process compared to other RTKs requiring longer stimulation to achieve full scale ligand-induced tyrosine phosphorylation, the reasons behind which are not completely understood (Vogel et al., 1997; Shrivastava et al., 1997).

In vitro work by us and others revealed that high affinity interaction with collagen requires dimerization and/or pre-oligomerization of DDR1 (Agarwal et al., 2007; Abdulhussein et al., 2008; Leitinger, 2003). It has also been reported that a significant percentage of the DDR1 population forms ligand independent dimers on the cell-surface (Abdulhussein et al., 2008; Noordeen et al., 2006; Mihai et al., 2009). Using fluorescence microscopy and live cell imaging, we had also shown that ligand binding results in receptor oligomerization and endocytosis (Mihai et al., 2009). The specific sites in DDR1 responsible for receptor dimerization have been described to be the leucine zipper motif in the transmembrane domain (Noordeen et al., 2006) and the cysteine residues in the JM region of the DDR1 extracellular domain (ECD) (Abdulhussein et al., 2008). While it is speculated that regions in DDR1 ECD may contribute to receptor dimerization (Carafoli et al., 2012), the role of the ECD in receptor oligomerization is not completely understood.

In this study we investigated how oligomerization of DDR1 ECD pre- and post-binding to collagen impacts its ligand-binding ability. Solid phase binding assays were used to compare how pre-oligomerization of recombinant DDR1 ECD (dimeric DDR1-Fc) impacts its collagen binding ability. Atomic force microscopy (AFM) was used to ascertain oligomerization of DDR1-Fc post ligand binding. A YFP tagged full length DDR1b was used to examine receptor oligomerization on the cell surface. Our results generate new insights into how oligomerization of the DDR1 ECD is crucial for this ligand–receptor interaction.

2. Materials and methods

2.1. Reagents

Fc-tagged ECDs of human DDR1 and TrkB were purchased as recombinant protein from R&D Biochemicals, MN and reconstituted in sterile phosphate buffered saline (PBS) at a stock concentration

Abbreviations: DS, discoidin; DDR1, discoidin domain receptor 1; ECD, extra cellular domain; RTK, receptor tyrosine kinase; AFM, atomic force microscopy; FRET, Förster resonance energy transfer; YFP, yellow fluorescent protein.

* Corresponding author. Address: Davis Heart and Lung Research Institute, 473 West 12th Ave., Columbus, OH 43210, USA. Fax: +1 614 247 7799.
E-mail address: agarwal.60@osu.edu (G. Agarwal).

[1] These authors contributed equally to this work.

1047-8477/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jsb.2013.06.010

of 100 μg/ml. Bovine dermal collagen type I was obtained from Advanced BioMatrix. Mouse monoclonal anti-DDR1 (against ECD) was from R&D Biochemicals, MN. Anti-Fc antibody was from Jackson Immunoresearch, (West Grove, PA). Anti-mouse and anti-goat IgG horseradish–peroxidase-conjugated antibodies were obtained from Santa Cruz Biotech. The DDR1b–YFP construct was generated using a plasmid containing the entire mouse DDR1b sequence (obtained from Regeneron Pharmaceuticals, Tarrytown, NY), as previously described (Mihai et al., 2009). Dynasore, an inhibitor for dynamin mediated endocytosis (Macia et al., 2006) was purchased from Sigma–Aldrich, St. Louis, MO. Glass-bottom culture dishes for live cell microscopy were obtained from MatTek Glassware (Ashland, MA).

2.2. Solid phase binding assays

Collagen was immobilized in 96-well micro-titer plates by incubating the wells with 25 μg/ml of collagen in phosphate buffer saline (PBS), overnight at 37 °C. Thereafter, the plates were washed three times with 200 μl TBS (tris buffered saline) (Bio-Rad, Hercules, CA) containing 0.05% tween (GE Healthcare, Uppsala, Sweden), followed by blocking with 300 μl of 1% bovine serum albumin (Santa Cruz Biotechnology, Santa Cruz, CA) with 0.05% tween overnight at 4 °C. The wells were washed again three times with TBS-tween and incubated with 100 μl of recombinant DDR1 or TrkB ECDs at concentrations ranging from 0 to 10 μg/ml, overnight at 4 °C. To pre-oligomerize DDR1 ECD, the protein was incubated with an equal mass of anti-Fc antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) in PBS at 4 °C overnight as previously described (Agarwal et al., 2007). Collagen-coated wells were incubated with non- or pre-oligomerized proteins containing equal amounts of DDR1 ECD. To detect binding of recombinant proteins to collagen, the plates were washed and thereafter probed with anti-Fc or anti-DDR1 antibodies, followed by washing and incubating with HRP conjugated secondary antibodies. Bound protein was detected by adding 100 μl of 3,3′,5,5′-tetramethylbenzidine, (TMB) to each well for 20 min at room temperature protected from light. The reaction was stopped using 1 N HCl (Sigma Aldrich, St. Louis, MO) solution, and the absorbance of the plates was recorded at 450 nm using a spectrophotometer. All experiments were performed at least three times.

2.3. Atomic force microscopy

Collagen (1 μg/ml) was mixed with recombinant DDR1-Fc (0.2 μg/ml) in ice cold PBS and incubated at 4 °C. As a control DDR1-Fc and collagen alone samples were also incubated in PBS under similar conditions. At specific time points (0, 1, 2, 4 and 24 h), the samples were aliquoted onto chilled and freshly cleaved mica substrates, incubated for 5 min, washed and air dried and subjected to AFM imaging using the Multimode AFM (Digital Instruments, Santa Barbara, CA). AFM imaging was performed in tapping mode in ambient air using NSC15 cantilevers (Micromasch, Estonia) with a nominal spring constant of 40 Nm^{-1}. Both height and amplitude images were recorded at 512 lines per scan direction. Topographic heights of DDR1-Fc in samples with or without collagen were measured from AFM images, by the section analysis feature of the Nanoscope software. At least $n = 50$ particles were analyzed per sample.

2.4. Fluorescence microscopy

HEK 293 cells were seeded on glass bottom culture dishes and transiently transfected with DDR1–YFP construct using Fugene 6 transfection reagent (Roche, Boston, MA). After 24 h of transfection the cells were incubated with 80 μM Dynasore for 30 min prior to stimulation with collagen type 1 (10 μg/ml) for 30 min at 37 °C. At the end of the stimulation interval, the cells were membrane stained using CellMask Deep Red (Molecular Probes, Eugene, OR) at 37 °C for 5 min. Following membrane staining the cells were fixed in 2% formalin buffered in PBS for 10 min and imaged in PBS. Similar samples were prepared using cells not treated with dynasore, and with cells not stimulated with collagen. Imaging was done using a Zeiss LSM 510 confocal microscope with a 63× water immersion objective. YFP was imaged using the 488 nm line of an Argon laser and the membrane stain was imaged using a 633 nm HeNe laser. For each channel a z stack of 1 μm optical slices was acquired in 1 μm steps, covering the entire z range of the cell monolayer.

3. Results

DDR1-Fc dimers and antibody induced DDR1-Fc oligomers have previously been characterized by us using atomic force microscopy (AFM) and SDS PAGE (Agarwal et al., 2007). DDR1-Fc appears as 91 and 197 kD in reducing and non-reducing conditions respectively on SDS PAGE. The anti-Fc mediated oligomers are approximately 600 kD as ascertained using size exclusion chromatography (Mihai et al., 2006). AFM analysis on samples in a fluid environment revealed that DDR1-Fc dimers and antibody mediated oligomers have an average topographic height of 3.24(±0.7) and 4.02(±0.8) nm, respectively.

3.1. Pre-oligomerization of DDR1 ECD increases its collagen binding affinity

To confirm the ability of dimeric DDR1-Fc to bind to collagen, DDR1-Fc or TrkB-Fc were incubated over immobilized collagen in solid-phase binding assays and the bound protein detected using anti-Fc antibodies. As shown in Fig. 1A, DDR1-Fc specifically bound to collagen while the control protein TrkB-Fc showed no significant binding. To examine if pre-oligomerization of DDR1 ECD impacts its ability to bind to collagen, similar assays were performed by incubating DDR1-Fc before and after pre-oligomerization to collagen coated wells and the bound protein detected using anti-DDR1 antibodies. The DDR1-Fc dimers and antibody-induced oligomers of DDR1-Fc used in our samples had identical concentrations of DDR1 ECD. Our solid-phase binding assays showed a rapid saturation of the binding signal for oligomerized DDR1-Fc as compared to the dimeric samples (Fig. 1B). The IC50 for binding of dimeric and oligomeric DDR1-Fc to immobilized collagen type 1 was determined to be 553 ± 179 ng/ml (7.85 ± 2.54 nM) and 176 ± 44.9 ng/ml (2.5 ± 0.64 nM) respectively using the approach of Orosz and Ovádi (2002).

3.2. Collagen induces oligomerization of DDR1–ECD post ligand binding

To examine the oligomeric state of DDR1 ECD upon collagen binding, we performed single-molecule studies using AFM. DDR1-Fc dimers were incubated with collagen in solution and thereafter immobilized on a mica surface for AFM studies. For comparison, DDR1-Fc and collagen alone samples were also imaged using AFM. Dimeric DDR1-Fc imaged as a globular protein with topographic height ranging from 1 to 4 nm, with an average height of 2.23 ± 0.6 nm. Incubation of DDR1-Fc alone for up to 4 h in solution did not change the size distribution or average height (2.14 ± 0.72) of particles (Fig. 2). Upon collagen binding a significant alteration in the morphology of DDR1-Fc was observed in a time-dependent manner. Very few binding events were observed in AFM images when DDR1-Fc and collagen samples were