Biochemical and Biophysical Research Communications xxx (2018) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Ephrin-B3 binds both cell-associated and secreted proteoglycans

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ARTICLE INFO

Article history:
Received 31 May 2018
Accepted 25 June 2018
Available online xxx

Keywords:
Ephrin-B3
Proteoglycans
CD44
Affinity
Protein complex

ABSTRACT

The ephrin family of membrane proteins binds Eph tyrosine kinase receptors. We have previously shown that ephrin-B3 also binds to heparan sulfate proteoglycans (HSPGs). We now show that ephrin-B3 can bind both secretory and cell associated PGs, such as agrin, collagen XVIII, Perlecan, and CD44, and indicate that such interaction with cell associated PGs involves a complex including 20 and 45 kDa proteins. Ephrin-B3 binding to HEK-293T cells is blocked by a secretory variant of CD44 (v3-v10), while over-expression of membrane associated CD44 increased ephrin-B3 binding. In addition, ephrin-B3 precipitated CD44 expressed by the oral squamous carcinoma cell line H376. Moreover, ephrin-B3 binding affinities to heparin and CD44 in solution was strong.

In conclusion, we have identified secretory and cell associated PGs with high ability to bind ephrin-B3 and suggest that ephrin-B3 can bind to a protein complex organized by a membrane associated PG.

1. Introduction

The ephrin family comprises eight membrane-bound proteins named after their counterpart Eph receptors [1]. Eph receptors and ephrins are widely expressed in body tissues, and their interactions and resulting bi-directional signaling mechanisms [2] affect a variety of cellular functions; morphogenesis, positioning, motility, adhesion, and survival [3–8], making these interactions vital in tissue patterning as illustrated in neural and vascular development [9–11]. Ephrins are subdivided according to sequence similarity and plasma membrane linkage mode, via a glycosylphosphatidylinositol anchor (ephrin-As) or transmembrane domain (ephrin-Bs) [12]. Generally, ephrin-A ligands bind EphA and ephrin-B ligands EphB receptors [13].

In addition to binding Eph receptors, ephrin-B ligands have been shown to bind to the secreted glycoprotein Reelin, involved in guidance of neurons. Reelin also associates with VLDLR and ApoER2 in neurons, and clustering of ephrin-Bs leads to recruitment and phosphorylation of Dab1 which is required for Reelin signaling [14]. Ephrin-B3 is also entry receptor for Henipa virus [15–17], which also binds to cell surface heparan sulfate (HS). Both binding modes are blocked by heparin [18]. Ephrin-B3 binds several EphA receptors, but not all EphB receptors [19–21].

Previously, we have shown that ephrin-B3 binds HSPGs on HEK-293T, HeLa, and CHO cells, where heparin blocks binding to HEK-293T cells independently of Eph receptors, and a heparin/HS-binding domain in ephrin-B3 was identified outside of the Eph-receptors binding domain. . The two positively charged residues, Arg178 and Lys179, in the ephrin-B3's juxtamembrane region is important for heparin/HS binding. Changing the corresponding amino acids in the non-heparin binding ephrin-B1 to positively charged residues gave heparin binding [22]. Ephrin-A3 also binds HS [23], where Lys176 corresponds to Lys179 in ephrin-B3. Ephrin-B3 binding to lymphocytes and lymphoma cell lines may also depend on other residues near the transmembrane domain, in particular Arg188 which is less affected by heparin [22], suggesting several mechanisms for ephrin-B3 binding to cells. Functional studies revealed that ephrin-B3 binding to cells induces signaling, influencing both cell rounding and spreading [22].

Here we extend previous observations and show that ephrin-B3 can bind both cell associated and secreted proteoglycans. In particular, we observe high affinity of CD44 to ephrin-B3. Moreover, ephrin-B3 binding to cells may involve additional cell associated proteins.

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https://doi.org/10.1016/j.bbrc.2018.06.140
0006-291X/

2. Material and methods

2.1. Fusion proteins and antibodies

Control and ephrin proteins fused to mouse IgG2b Fc domain (including hinge) were produced as described [22,24,25]. We also purchased ephrin-B3 with human Fc (R & D technology). CD44 v3-v10-Fc cDNA was kindly provided by Dr. Ivan Stamenkovic [26] and recombinant protein was produced as for ephrin-Fc proteins. A general anti-CD44 antibody was used for Western blotting (R & D technology) and anti-CD44V3 (R & D technology) for flow cytometry. An anti-mouse-HRP conjugated antibody (Dako) was used for Western blot detection.

2.2. Cells and incubations

Daudi, HeLa, HEK-293T, and MDCK-1 cells, the rat salivary gland cell lines SM10 and A253 and the human oral squamous carcinoma cell line H376 were used in this study. Cells were incubated in RPMI 1640 medium (HeLa, Daudi) or high-glucose DMEM both with L-glutamine (HEK-293T, MDCK-1, A253, HeLa (PAA Laboratories)), 10% (v/v) heat-inactivated FBS (PromoCell), and penicillin/streptomycin (PAA Laboratories) at 37 °C in humidified atmosphere with 5% CO_2. H376 was cultured in DMEM F12 (Lonza), 10% FBS, 0.5 μg/ml sodium hydrocortisone succinate (Sigma-Aldrich) and penicillin/streptomycin. HEK-293T cells were transfected using polyethylenimin (Polysciences) [27]. Expression vectors with cDNAs encoding Agrin (membrane bound, accession no. NM_198576) and Perlecan were from Origene (US), while Collagen XVIII cDNA was from Source BioScience (UK). Secretory agrin cDNA was a kind gift from Professor Trond Lømo [28]. The expression clones of syndecans and glypicans were described previously [22].

2.3. Flow cytometry

Cells were suspended in PBS, 1% BSA and incubated with control–Fc or ephrin–Fc fusion proteins (30 μg/ml) for 30 min at 4 °C before staining with goat anti-mouse IgG, Alexa Fluor 488 (Thermo Fisher) or with ephrin-B3 with human Fc tail before staining with phycoerythrin conjugated anti-human IgG (Jackson ImmunoResearch). Cells were analyzed with a FACSort flow cytometer (BD Biosciences), flow data collected with CellQuest 3.3 (BD Biosciences), and data generated using FlowJo 7.2.2 (Tree Star). At least 20,000 cells gated as viable were analyzed per sample.

2.4. ^{35}S-sulfate labelling of cell-surface proteins

Transfected HEK-293T cells were incubated overnight two days after transfection in sulfate-free RPMI 1640 medium with 2% FBS and 0.2 mCi/ml of ^{35}S-sulfate (Hartmann Analytic). Media were removed for analysis, and cells washed in PBS, then lysed in 10 mM Tris/HCl (pH 7.5), 0.5% Nonidet P40, 150 mM NaCl, 0.6 mM PMSF, 3.2 μg/ml aprotinin, and 10 μl/ml phosphatase inhibitor cocktail II (Sigma–Aldrich)] on ice for 30 min before clearing by centrifugation (18,000 g for 5 min at 4 °C). Control–Fc, ephrin-A3–Fc, or ephrin-B3–Fc (1.5 μg) was added to cell lysates, before incubation with agitation for 3 h at 4 °C. Protein G beads (10 μl; Dynal) were added before incubation for another 1 h. The precipitate was washed with PBS followed by SDS-PAGE. Gels were fixed (30 min), treated with Amplify (GE Healthcare Biosciences), dried, followed by Typhoon (9400) scanning.

2.5. Biotin labelling of cell surface proteins

Cells were washed twice in PBS and resuspended in 5 ml bicarbonate buffer (10 mM $NaHCO_3$, 150 mM NaCl, pH 8.5). Cell surface biotinylation was performed by incubating cells with 20 μl reagent (10 mg/ml Biotin-X-NHS; Calbiochem, Darmstadt, Germany) 30 min on ice. Excess biotin was removed by one bicarbonate and two PBS washes. Cells were lysed on ice for 30 min before centrifugation (18,000 g for 5 min at 4 °C).

2.6. Ephrin-B3 protein complex isolation

To precipitate ephrin-B3-binding proteins, ephrin-B3–Fc (0.3 μg) was added to HEK-293T regular lysates or lysates of biotinylated HEK-293T cells and rotated overnight at 4 °C. Protein G–Dynabeads (10 μl) were subsequently added, followed by rotation at 4 °C (1 h) to capture ephrin-B3-Fc protein complexes, followed by two PBS washes. Precipitates were separated in ready-cast SDS-PAGE gels (Bio-Rad Laboratories), transferred to nitrocellulose membranes (Whatman), and incubated for 1 h with primary antibody or streptavidin-HRP, at room temperature (RT), or overnight at 4 °C. Membranes incubated with unconjugated primary antibodies were washed and incubated with HRP-linked secondary antibody (1 h, RT). Antibody binding was detected with ECL Plus Western blotting detection system (GE Healthcare BioSciences).

2.7. MST ligand-binding assay

Microscale thermophoresis (MST) was used to study interactions of ephrin-B1 or ephrin-B3 with heparin, EphB2, or CD44. Ephrin-B1 and ephrin-B3 were labeled with amine-reactive NT-647 dye (Nanotemper Technologies). In brief, 5 μM solutions of each protein were incubated with dye (molar ratio of 1:3) for 30 min (RT) in darkness. Unreacted dye was removed on a desalting/size exclusion column with buffer exchange, using PBS, 0.05% Tween-20. Labeled proteins were cooled until mixing with their ligands. Each ligand was serial diluted 1:1 in PBS, 0.05% Tween-20 before mixing with labeled protein at RT. Samples were left for some minutes before loading into premium coated NT.115 capillaries (Nanotemper). Fluorescence (red) profiles were measured at 25 °C in a Monolith NT.115 instrument. Data collected at 20% (for heparin and CD44) or 40% (for EphB2) LED power, and the fluorescence signals during thermophoresis were monitored for 30 s. Changes in fluorescence (ΔFnorm) due to thermophoresis were recorded as signal difference between 0 and 5 s. Normalized data was plotted as a function of ligand concentration. For experiments with a clear binding curve, the dissociation constants (Kds) were calculated from fitting a standard binding curve to the average of three dilution series (only two for ephrin-B1/EphB2). For the ephrin-B3 and CD44 interaction, the affinity was so strong that only an upper Kd estimate of <1 nM was inferred from the binding curve, as the concentration of labeled protein (1.5 nM) was at the lower end of the instrument sensitivity, but too high compared with the Kd value for a valid curve fitting. Data points and curves were exported to Origin (OriginLab Corporation).

3. Results

3.1. Cell binding of ephrin-B3 and ephrin-B3 mutants

Binding of ephrin-B3 and ephrin-B3-mutants was investigated for HEK-293T, HeLa, SM10, A253, H376, and Daudi by flow cytometry. For most of these cell lines, the 178/179 ephrin-B3 mutant, described in Ref. [22], did not bind efficiently to the cell surface. Particularly low binding was observed to HEK-293T cells (Fig. 1A), as previously shown [22]. The 188/190 mutant [22], bound less efficiently than wild type ephrin-B3, but more efficiently than the 178/179 mutant. One exception was Daudi cells, where the 188/190

Please cite this article in press as: K. Prydz, et al., Ephrin-B3 binds both cell-associated and secreted proteoglycans, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.06.140