The International Journal of Biochemistry & Cell Biology 57 (2014) 45–53

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

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

The NTR domain of procollagen C-proteinase enhancer-1 (PCPE-1) mediates PCPE-1 binding to syndecans-1, -2 and -4 as well as fibronectin

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ARTICLE INFO

Article history:
Received 2 July 2014
Received in revised form
14 September 2014
Accepted 25 September 2014
Available online 5 October 2014

Keywords:
Procollagen processing
Procollagen C-proteinase enhancer-1
Heparin binding
Syndecans
Fibronectin

ABSTRACT

Procollagen C-proteinase enhancer 1 (PCPE-1) is an extracellular matrix glycoprotein that can stimulate procollagen processing by procollagen C-proteinases (PCPs) such as bone morphogenetic protein-1 (BMP-1). PCPE-1 consists of two CUB domains that bind to the procollagen C-propeptide and are responsible for enhancing activity and a netrin-like (NTR) domain that binds to BMP-1 as well as heparin and heparan sulfate. The NTR domain also mediates binding of PCPE-1 to cells, an interaction inhibited by heparin, thus suggesting involvement of cell membrane heparan-sulfate proteoglycans (HSPGs). Using pull-down experiments and an ELISA type binding assay we show here that PCPE-1 binds to three cell membrane HSPGs, syndecans-1, -2 and -4. We also demonstrate that this binding is mediated by the NTR domain and depends on the glycosaminoglycan chains of the syndecans. Using co-immunoprecipitation and an ELISA type binding assay we show that PCPE-1 can also bind fibronectin (an established binding partner of BMP-1), another interaction involving the NTR domain. Consistently, fibronectin inhibits cell attachment to PCPE-1 although it does not affect PCPE-1 enhancing activity. PCPE-1 is not an adhesive protein since cell attachment to PCPE-1 is not associated with cell spreading and/or actin filaments formation. The results suggest that PCPE-1 binding to syndecans and/or fibronectin may control collagen fibril assembly on the cell surface. Further characterization of these interactions may pave the way for future design of new means to modulate collagen deposition in pathological conditions such as fibrosis.

1. Introduction

Tolloid proteinases play important regulatory roles in extracellular matrix (ECM) assembly, tissue morphogenesis and developmental processes, achieved by proteolytic processing of matrix macromolecules such as procollagens, lysyl oxidases, proteoglycans, growth factors and related proteins (Ge and Greenspan, 2006; Hopkins et al., 2007; Muir and Greenspan, 2011). In mammals, the tolloid family of proteases includes bone morphogenetic protein-1 (BMP-1) and mammalian tolloid (mTld) (splice variants of the *BMP-1* gene) and mammalian tolloid-like 1 and 2 (mTll-1 and -2). BMP-1 and mTld are also known as procollagen C-proteinases (PCPs) because both are responsible for C-terminal processing of fibrillar procollagens (Kessler et al., 1996; Li et al., 1996).

The activity of the PCPs on fibrillar procollagens is increased by procollagen C-proteinase enhancer proteins 1 and 2 (PCPE-1 and 2) (Kessler and Adar, 1989; Takahara et al., 1994; Steiglitz et al., 2002) and to a lesser extent, by fibronectin (Huang et al., 2009). Enhancement of BMP-1 activity by PCPE-1 seems to be restricted to fibrillar procollagens because PCPE-1 does not affect the activity of BMP-1 on other tolloid substrates (Moali et al., 2005; von Marschall and Fisher, 2010). PCPE-1 however diminishes C-terminal processing of procollagen III by meprins (Kronenberg et al., 2010), BMP-1-related proteases possessing both PNP and PCP activity (Broder et al., 2013). The normal expression pattern of PCPE-1 matches that of collagen type I (Takahara et al., 1994;

Abbreviations: BMP-1, bone morphogenetic protein 1; CEPC, H^3-tryptophan-labeled chick embryo procollagen type I; CUB, Complement C1r/C1s, Uegf, BMP-1; C1, C-propeptide of the proα1(I) procollagen chain; DAPI, 4′,6-diamidino-2-phenylindole; ECM, extracellular matrix; FITC, fluorescein isothiocyanate; GAG, glycosaminoglycan; HSPGs, heparan sulfate proteoglycans; MFs, 3T3 mouse fibroblasts; mTld, mammalian tolloid; PCP, procollagen C-proteinase; PCPE-1, procollagen C-proteinase enhancer 1; PNP, procollagen N-proteinase; RIPA, radioimmunoprecipitation assay; TIMP, tissue inhibitor of matrix metalloproteases.

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http://dx.doi.org/10.1016/j.biocel.2014.09.023
1357-2725/

Kessler et al., 1990) and its expression increases in fibrosis in parallel to the characteristic increase in collagen expression (Ogata et al., 1997; Shalitin et al., 2003; Kessler-Icekson et al., 2006; Yu et al., 2013; Beaumont et al., 2014). Thus, PCPE-1 seems to function as a specific positive regulator of collagen deposition.

PCPE-1 consists of two N-terminal CUB domains that bind to the procollagen C-propeptide and are responsible for enhancing activity (Kessler and Adar, 1989; Takahara et al., 1994; Hulmes et al., 1997; Ricard-Blum et al., 2002; Vadon-Le Goff et al., 2011; Bourhis et al., 2013) and a C-terminal NTR (netrin-like) domain that binds to heparin and heparan sulfate (Moschcovich et al., 2001; Weiss et al., 2010). This latter interaction induces further enhancement of procollagen processing by BMP-1, an effect referred to as superstimulation (Beckhouche et al., 2010). The NTR domain also interacts with BMP-1 (Beckhouche et al., 2010) and mediates PCPE-1 binding to β2-microglobulin (Morimoto et al., 2008). Additional interaction targets of PCPE-1 in the ECM include laminin 111, collagens VI and IV, thrombospondin-1 and endostatin (Salza et al., 2014).

The NTR domain of PCPE-1 shares homology with TIMP (Bányai and Patthy, 1999) and is preceded by an un-structured linker that is sensitive to proteolysis (Salza et al., 2014; Kronenberg et al., 2009; Williamson et al., 2008). Although naturally occurring NTR fragmens were reported to inhibit the activity of matrix metalloproteinases (MMPs) (Mott et al., 2000), no inhibitory activity was detected by other investigators against a range of metalloproteinases, including MMPs and BMP-1 (Beckhouche et al., 2010).

We have shown that 3T3 mouse fibroblasts (MFs) can attach to PCPE-1 and that cell attachment is inhibited by heparin and the NTR domain (Weiss et al., 2010). This pointed at cell-associated heparan sulfate proteoglycans (HSPGs) as potential PCPE-1 receptors and suggested the cell surface as a physiological site of PCPE-1 action. The main purpose of this study was therefore to identify cell-associated interaction targets of PCPE-1, thereby laying the basis for the design of new means to modulate collagen deposition in pathological conditions.

2. Materials and methods

2.1. Proteins

Recombinant human PCPE-1 (huPCPE-1) and C-terminally Flag-tagged huPCPE-1 were produced in human embryonic kidney cells (Weiss et al., 2010). The NTR and CUB1CUB2 fragments were generated from huPCPE-1 by limited proteolysis and separated by heparin-Sepharose chromatography (Weiss et al., 2010). The laminin 322 LG45 fragment was prepared as previously described (Okamoto et al., 2003). Recombinant mouse syndecan-2 was from R&D.

2.2. Antibodies

Mouse anti-Flag antibody M2, rabbit anti-Flag antibody, rabbit anti-human fibronectin antibody and alkaline phosphatase (APA) and horse radish peroxidase (HRP) conjugated antibodies were from Sigma. Rat monoclonal antibodies against mouse syndecans-1 and -4 were a kind gift of Dr. PW Park. Rabbit antibody H-174 against human syndecan-1 and rabbit antibody to mouse syndecan-2 were from Santa Cruz. A sheep antibody to mouse syndecan-2 and HRP-coupled donkey anti-sheep IgGs were from R&D. Rabbit polyclonal antibody to huPCPE-1 was raised by us and the IgG fraction was prepared from the immune sera using standard protocols. Rabbit antibody (LF41) against the C-propeptide of the proα1(I) chain (Fisher et al., 1995) was a kind gift of Dr. Larry Fisher. Monoclonal antibodies 7A11/1 and 7A11/5 against the CUB2 and NTR domains of huPCPE-1 respectively were developed by us and are available commercially.

2.3. Cells, culture conditions and preparation of cell lysates

NIH 3T3 mouse fibroblasts (MFs) were grown in high glucose Dulbecco modified Eagle's Medium (DMEM; Biological industries, Bet-Haemek, Israel) containing 10% fetal calf serum, 2 mM glutamine, 100 U/ml, penicillin and 100 μg/ml streptomycin. At confluence, the cells were washed with PBS and lysed with RIPA buffer (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 250 μM phenylmethylsulfonyl fluoride, 1 mM N-ethylmaleimide, 1% Nonidet P-40, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS) on ice for 20 min. After centrifugation, the supernatants were stored at −80 °C until use.

Keratocytes were removed from human corneas according to ethical regulations at the Cell and Tissue Bank (Hôpital Edouard Herriot, Lyon, France) (Torbet et al., 2007). Keratocytes were seeded at 10,000 cells/cm^2 and grown in DMEM/Ham-F12 1:1, 10% new born calf serum, 5 ng/ml basic fibroblast growth factor, and antibiotics (Builles et al., 2006). At confluence, keratocytes were lysed with RIPA buffer as above.

2.4. Cell attachment assay

Attachment of MFs to PCPE-1 was determined as described (Weiss et al., 2010). Cells (6×10^4 cells/100 μl) were allowed to attach to wells pre-coated with (biotinylated) PCPE-1 for 45 min. Attached cells were stained with crystal violet and lysed with acetic acid. Absorbance at 595 nm was determined using an ELISA reader. To examine the effect of fibronectin on cell attachment, the cells were incubated (2 h) with fibronectin (100 and 250 μg/ml) before addition to the wells.

2.5. Pull down experiments

For immunoprecipitation of syndecans-1 and -4, MFs cell lysate samples (2 mg protein) were incubated (2 h, 4 °C) with protein G-Sepharose beads (Sigma) harboring either anti syndecan-1 or anti syndecan-4 antibodies (2.5 μg). Syndecan-2 was immunoprecipitated similarly except that beads were coated with a rabbit antibody against syndecan-2 (7.5 μg) and the cell lysate samples contained 3 mg protein. Beads carrying the syndecans were washed with RIPA buffer followed by incubation with PCPE-1-Flag (2.5 μg; 2 μg in the case of syndecan-2) with or without prior treatment (2 h, 37 °C) with either heparitinase I (Seikagaku Corp; 10 mU/ml) alone or combined with chondroitinase ABC (Sigma, 40 mU/ml). Bound proteins were eluted by heating in Laemmli's SDS-sample buffer and analyzed by immunoblotting with anti-Flag antibodies.

In the reverse experiment, cell lysate samples (2 mg protein) were incubated with PCPE-Flag-coated Protein G-Sepharose beads (via the M2 antibody; 5 and 2.5 μg, respectively). In some instances, heparin (Sigma H3400) and PCPE-1 fragments were added as potential competitors of HSPGs binding. In other instances, the pulled down HSPGs were treated (2 h, 37 °C) with either heparitinase I alone or combined with chondroitinase ABC as above to release the core proteins for identification by immunoblotting, a treatment that was omitted in the case of syndecan-2 because unlike syndecans-1 and -4, glycosylated forms of syndecan-2 are readily detected by immunoblotting with antibodies against the core protein of syndecan-2 (Fig. 2A).

To pull down syndecan-1 from keratocytes lysates, PCPE-Flag (2.5 μg) was incubated with Gamma Bind-G Sepharose beads (Amersham GE Healthcare) harboring the M2 antibody (5 μg). The laminin 322 LG45 fragment was coupled to Sepharose-4B as described (Okamoto et al., 2003). Beads harboring PCPE-Flag and