Archives of Biochemistry and Biophysics 509 (2011) 147–156

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

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

sFRP-1 binds via its netrin-related motif to the N-module of thrombospondin-1 and blocks thrombospondin-1 stimulation of MDA-MB-231 breast carcinoma cell adhesion and migration

Gema Martin-Manso [a], Maria J. Calzada [a,1], Yoshiro Chuman [b,2], John M. Sipes [a], Charles P. Xavier [b], Vladimir Wolf [b,3], Svetlana A. Kuznetsova [a,4], Jeffrey S. Rubin [b], David D. Roberts [a,*]

[a] Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA
[b] Laboratory of Cellular and Molecular Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

ARTICLE INFO

Article history:
Received 1 December 2010
and in revised form 3 March 2011
Available online 12 March 2011

Keywords:
Secreted frizzled-related proteins
Netrin-binding proteins
Thrombospondins
Breast carcinoma

ABSTRACT

Secreted frizzled-related protein (sFRP)-1 is a Wnt antagonist that inhibits breast carcinoma cell motility, whereas the secreted glycoprotein thrombospondin-1 stimulates adhesion and motility of the same cells. We examined whether thrombospondin-1 and sFRP-1 interact directly or indirectly to modulate cell behavior. Thrombospondin-1 bound sFRP-1 with an apparent K_d = 48 nM and the related sFRP-2 with a K_d = 95 nM. Thrombospondin-1 did not bind to the more distantly related sFRP-3. The association of thrombospondin-1 and sFRP-1 is primarily mediated by the amino-terminal N-module of thrombospondin-1 and the netrin domain of sFRP-1. sFRP-1 inhibited α3β1 integrin-mediated adhesion of MDA-MB-231 breast carcinoma cells to a surface coated with thrombospondin-1 or recombinant N-module, but not adhesion of the cells on immobilized fibronectin or type I collagen. sFRP-1 also inhibited thrombospondin-1-mediated migration of MDA-MB-231 and MDA-MB-468 breast carcinoma cells. Although sFRP-2 binds similarly to thrombospondin-1, it did not inhibit thrombospondin-1-stimulated adhesion. Thus, sFRP-1 binds to thrombospondin-1 and antagonizes stimulatory effects of thrombospondin-1 on breast carcinoma cell adhesion and motility. These results demonstrate that sFRP-1 can modulate breast cancer cell responses by interacting with thrombospondin-1 in addition to its known effects on Wnt signaling.

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Introduction

Thrombospondin-1 (TSP1)[5] was initially described as a 450 kDa trimeric glycoprotein released from the α-granules of platelets [1]. In addition to modulating platelet activation [2,3], TSP1 was the first identified endogenous angiogenesis inhibitor [4,5]. Consistent with this activity, TSP1 expression was shown to inhibit tumor growth and metastasis [6]. Moreover, TSP1 expression is frequently lost during malignant transformation due to regulation of its expression by oncogenes, tumor suppressor genes, and hypermethylation [7–9]. However, TSP1 can become highly expressed by stromal fibroblasts and endothelial cells within tumors during tumor progression [10], which results in elevated levels of circulating TSP1 in some cancers [11] that may also inhibit tumor growth [12,13]. In addition to modulating angiogenesis, tumor-associated and circulating TSP1 can also modulate tumor blood flow [14], anti-tumor innate immunity [15], and radiotherapy responses [16,17].

The diverse and sometimes conflicting activities of TSP1 in the context of tumor progression may be rationalized in part by the complexity of TSP1 interactions with other proteins. Each subunit of TSP1 is composed of several domains with multiple ligand binding specificities [18]. TSP1 interacts with cell surface integrin and non-integrin receptors [19–22], heparan sulfate proteoglycans (HSPG) [23], growth factors [24], and other bioactive molecules [25–27].

* Corresponding author. Fax: +1 301 402 0043.
E-mail addresses: mansog@mail.nih.gov (G. Martin-Manso), mcalzada.hlpr@salud.madrid.org (M.J. Calzada), chuman@sci.hokudai.ac.jp (Y. Chuman), sipesj@mail.nih.gov (J.M. Sipes), Charles.Xavier@nih.gov (C.P. Xavier), vladimir.wolf@qiagen.com (V. Wolf), kuznetsova4872@yahoo.co.uk (S.A. Kuznetsova), rubinj@mail.nih.gov (J.S. Rubin), droberts@helix.nih.gov (D.D. Roberts).
[1] Present address: Department of Medicine, Hospital Universitario de la Princesa, Universidad Autónoma de Madrid, Madrid, Spain.
[2] Present address: Department of Chemistry, Hokkaido University, Sapporo 060 0810, Japan.
[3] Present address: Qiagen, Inc., Germantown, MD 20874, USA.
[4] Present address: Department of Immunology, Research Institute for Experimental Medicine RAMS, St.-Petersburg, Russia.
[5] *Abbreviations used*: sFRP, secreted frizzled-related protein; TSP1, thrombospondin-1; HSPG, heparan sulfate proteoglycan; CRD, cysteine-rich domain; NTR, netrin; TIMP, tissue inhibitor of metalloproteinases; pCOLCEs, procollagen C-proteinase enhancer proteins; GST, glutathione-S-transferase; vWC, von Willebrand factor type C; LDL, low-density lipoprotein; EGF, epidermal growth factor.

0003-9861/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.abb.2011.03.004

In the context of breast cancer, TSP1 expression appears to be a marker of aggressiveness and correlates with the microvessel density [28,29] but is not related to p53 status or VEGF expression [30]. Endogenous TSP1 inhibits primary tumor growth and angiogenesis but promotes metastasis to the lung in the Pyt transgenic mouse breast cancer model [31]. TSP1 stimulates breast carcinoma cell adhesion and chemotaxis by engaging $\alpha3\beta1$ integrin [32,33]. TSP1 can also promote human breast cancer cell proliferation in vitro, but the mechanism remains to be elucidated [34,35].

The secreted frizzled-related proteins (sFRPs) comprise a family of 5 proteins that bear homology to frizzleds, the seven-pass transmembrane cell surface receptors for Wnts [36,37]. sFRP-1, 2 and 5 are closely related and form one subgroup, and sFRP-3, and 4 form a second subgroup [37,38]. Constitutive activation of Wnt signaling is common in neoplasia [39]; in particular, autocrine Wnt pathways contribute to the proliferative and metastatic properties of breast cancer cells [40,41]. The sFRPs were first identified as Wnt antagonists, and accordingly they have been viewed as tumor suppressors [42–45]. Consistent with this assessment, four of the five human *SFRP* genes contain dense CpG islands that often are hypermethylated in many cancers, resulting in silencing of their expression [45]. Loss of sFRP-1 expression in breast cancer has been associated with decreased survival [46], and restoration of expression in colorectal and renal cell carcinoma lines attenuated the malignant phenotype [47,48]. Similarly, ectopic sFRP-1 expression in MDA-MB-231 breast cancer cells suppressed tumor growth and metastasis [49]. In contrast to sFRP-1, sFRP-2 has positive effects on breast carcinoma cells and endothelial cells that imply a stimulation of tumor growth [37,50–52].

The sFRPs contain two distinct structural domains: a frizzled-related cysteine-rich domain (CRD) and a netrin (NTR) module [36,37]. The CRD consists of 110–120 amino acid residues including ten invariant cysteines that form a conserved set of five disulfide bonds. The CRDs of frizzleds bind Wnt ligands [53], and initial studies suggested that sFRPs inhibit signaling by binding to Wnts via their CRDs [54,55]. However, additional experiments demonstrated that sFRPs and frizzleds could associate with each other through CRD-CRD interactions, implying there are additional mechanisms of Wnt inhibition [55]. The NTR module is defined by a set of six characteristically spaced cysteines, stretches of hydrophobic and positively charged amino acids and, where three-dimensional structural data are available, two α-helices packed against a five-stranded β-barrel [56,57]. NTR domains are found in the carboxyl (C) -terminus of netrins, laminin-related proteins in the extracellular matrix that control axon guidance. While the function of the NTR module in netrins is unknown, in the tissue inhibitors of metalloproteinases (TIMPs) it mediates binding to their protease targets [56]. The NTR module also is present in type I procollagen C-proteinase enhancer proteins (pCOLCEs), complement proteins C3, C4 and C5 as well as other molecules [56]. The NTR domain of sFRP-1 associates with Wnt proteins and modulates their activities [58,59]. Besides its interaction with Wnts, the NTR domain is responsible for sFRP association with HSPG [58].

Screening of a peptide phage display library resulted in the identification of a peptide binding motif for sFRP-1 with micromolar affinity [60]. The core of this motif, DGR, is present in the type 3 calcium-binding repeats of TSP1 [18], suggesting that these proteins might interact. Here we report that sFRP-1 binds to TSP1 with high affinity, although not via the DGR motif. This binding is shared by sFRP-2 but not sFRP-3. The interaction primarily involves the NTR module of sFRP-1 interacting with the N-module of TSP1. sFRP-1 specifically disrupts integrin-mediated cell adhesion of MDA-MB-231 breast carcinoma cells to surfaces coated with TSP1 or its N-module, and blocks TSP1-mediated migration of breast carcinoma cells. These activities suggest that physical and functional interactions of TSP1 with sFRP-1 have pathophysiological relevance for breast cancer progression.

Materials and methods

Reagents

Human TSP1 and fibronectin were purified from platelets and plasma, respectively, obtained from the National Institutes of Health Department of Transfusion Medicine [61,62]. Monomeric and trimeric recombinant regions of TSP1 (Fig. 3) expressed in insect cells and prepared as described [63,64] were provided by Dr. Deane Mosher, University of Wisconsin. Monomeric recombinant N-module containing residues 1 to 250 of mature TSP1 was prepared as previously described [65]. A glutathione-S-transferase (GST) fusion protein expressing the von Willebrand factor type C (vWC) domain of TSP1 (provided by Dr. Jack Lawler, Harvard University, Boston, MA) was prepared as described [66]. Recombinant human sFRP-1 and sFRP-2 were prepared as described [58,67]. Recombinant human sFRP-3 was purchased from R&D Systems (Minneapolis, MN). Type I collagen was purchased from Inamed (Fremont, CA).

Recombinant expression and purification of CRD and NTR domain

cDNAs encoding the CRD and NTR domain along with a small amount of flanking sequence were generated by PCR using full-length human sFRP-1 cDNA as template and the indicated primers for the CRD (5′-CCGCTCGAGAAAAGACGCTTCTACACCAAGCCACCT-3′, 5′-GCTCTAGATCATCACGTCATGGCGATGCAGACGTCCCCCT-3′) and NTR domain (5′-CGGAATTCGTGTGTCCTCCCTGTGACAACGAG-3′, 5′-GCTCTAGATCATCACTTAAACACGGACTGAAAGGTGGGGC-3′). After the fidelity of PCR products was verified by sequence analysis, the cDNAs were digested with XhoI/XbaI (CRD) or EcoRI/XbaI (NTR) and subcloned into the pICZaA expression vector (Invitrogen). Competent *Pichia pastoris* cells were transformed with these constructs using Pichia EasyComp Kit (Invitrogen). Transformed clones were grown in 5 ml BMGY medium overnight at 30 °C, 275 rpm. Cultures were transferred into 500 ml BMGY medium in a 2-liter flask with deep baffles and incubated overnight at 30 °C, 250 rpm. Cells were pelleted by centrifugation and resuspended at 80 g/liter in BMMY medium supplemented with 1% methanol/day for 3–5 days to induce expression of the recombinant protein (cultures maintained at 30 °C, 250 rpm).

All purification steps were performed at 4 °C. CRD culture medium was concentrated 10-fold by ultrafiltration using a YM3 membrane (Millipore), dialyzed against solution A (20 mM Tris–HCl, pH 7.4), filtered through a 0.44 μm membrane and applied to a 5 ml HiTrap Q FF column (GE Healthcare) equilibrated with solution A. After washing the column with 50–100 ml of solution A, protein was eluted with a linear gradient of increasing NaCl (solution B: 20 mM Tris–HCl, pH 7.4, 1 M NaCl). The CRD either bound weakly (eluting with 3–10% solution B) or remained in the flow through. The early eluting fractions containing the CRD and flow through were combined, dialyzed in solution A and loaded on a freshly equilibrated HiTrap Q FF column. The CRD was retained on the resin and eluted with 3–10% solution B. NTR culture medium was filtered through a 0.44 μm membrane and loaded directly on a HiTrap Heparin HP column (GE Healthcare) equilibrated with solution A′ (25 mM phosphate buffer, pH 7.4). After washing the resin with 10–20 column volumes of solution A′, NTR protein was eluted with a NaCl step gradient in fractions containing ~1 M NaCl (solution B′: 25 mM phosphate buffer, pH7.4, 2 M NaCl). Purified protein was visualized by SDS–PAGE, followed by staining with Coomassie blue. The mass and amino-terminal sequence of the CRD and NTR domain were confirmed by MALDI-TOF and Edman degradation, respectively.