Matrix Biology 30 (2011) 225–233

Contents lists available at ScienceDirect

Matrix Biology

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

SMOC1 is a tenascin-C interacting protein over-expressed in brain tumors

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ARTICLE INFO

Article history:
Received 22 November 2010
Received in revised form 4 February 2011
Accepted 10 February 2011

Keywords:
Tenascin-C
SPARC-related modular calcium-binding protein 1
Brain cancer
Biomarker

ABSTRACT

Tenascin-C is an extracellular matrix protein over-expressed in a large variety of cancers. In the present study, we aimed at identifying new interactors of tenascin-C by purifying secreted proteins on a tenascin-C affinity column. Analysis of eluates by mass spectrometry revealed phosphoglycerate kinase 1, clusterin, fibronectin, SPARC-related modular calcium-binding protein 1 (SMOC1) and nidogen-2 as potential interactors of tenascin-C. The interaction between tenascin-C and SMOC1 was confirmed by co-immunoprecipitation and further analyzed by Surface Plasmon Resonance Spectroscopy, which revealed an apparent dissociation constant (K_D) value of 2.59 10^{-9}M. Further analyses showed that this binding is reduced in the presence of EDTA. To investigate whether SMOC1 itself could be over-expressed in the context of tumorigenesis, we analyzed data of two independent RNA profiling studies and found that mRNA levels of SMOC1 are significantly increased in oligodendrogliomas compared to control brain samples. In support of these data, western blot analysis of protein extracts from 12 oligodendrogliomas, 4 astrocytomas and 13 glioblastomas revealed elevated levels compared to healthy brain extract. Interestingly, cell migration experiments revealed that SMOC1 can counteract the chemo-attractive effect of tenascin-C on U87 glioma cells. The present study thus identified SMOC1 as a new cancer-associated protein capable of interacting with tenascin-C *in vitro*.

1. Introduction

Tenascins are large multimeric extracellular matrix proteins found in many tissues. The tenascin family comprises 4 members: tenascin-C, -X, -R and -W with distinct expression patterns (for review, see Brellier et al., 2009). Tenascin-C, the best described member of the family, is known to be implicated in both modeling and re-modeling of tissues: it is expressed during embryogenesis, reduced in the adult under normal conditions but re-expressed in various pathological situations such as asthma, fibrosis, inflammation, infection, and cancer. In the context of these pathologies, the presence of tenascin-C does not only modulate the structural and mechanical features of the matrix, but also affects cellular behavior through activation of signaling pathways. Several ligands of tenascin-C have been described during the last decades (reviewed in Orend and Chiquet-Ehrismann, 2006). Most of the identified tenascin-C ligands are also extracellular proteins, such as fibronectin (Chung et al., 1995), perlecan (Chung and Erickson, 1997), lectican (Day et al., 2004) and heparin (Fischer et al., 1995). Others are cell surface transmembrane proteins such as a wide set of integrins (α2β1, α7β1, α8β1, α9β1, αvβ3 and αvβ6) as well as epidermal growth factor receptor (Swindle et al., 2001) and annexin II (Chung and Erickson, 1994). Furthermore, a recent study identified tenascin-C as an activator of toll-like receptor 4 (Midwood et al., 2009).

In the present study, we aimed at identifying new ligands of tenascin-C. For this purpose, we pulled-down proteins present in the culture medium of HEK293 cells with tenascin-C coupled Sepharose 4B resin. Among the candidates identified by mass spectrometry, SPARC-related modular calcium-binding protein 1 (SMOC1) caught our attention since other proteins of the SPARC family have been shown to contribute to the tumorigenesis process (for review, see Arnold and Brekken, 2009; Sullivan and Sage, 2004). We thus characterized, by different approaches, the binding between tenascin-C

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doi:10.1016/j.matbio.2011.02.001

and SMOC1 and investigated whether SMOC1 is over-expressed in tumors, similarly to tenascin-C.

2. Results

2.1. Identification of tenascin-C associated proteins

To identify new interactors of tenascin-C (TNC), we performed pull-down experiments with full-length tenascin-C protein immobilized on a CNBr-activated Sepharose 4B. Affinity chromatography was performed using concentrated serum-free conditioned medium of HEK293 cells. Potential tenascin-C interactors were eluted with 8 M urea and identified by mass spectrometry analysis. Two control columns (as described in Section 4) were run in parallel. A total of 102 proteins were identified with at least two peptides each (Supplemental Table 1). A false discovery rate of 0.4% was calculated using Scaffold. After elimination of all proteins with no known extracellular localization and proteins that were found in similar amounts in the control column eluates, we were left with 5 potential tenascin-C ligands for further analysis: phosphoglycerate kinase 1 (PGK1), clusterin, fibronectin, SPARC-related modular calcium-binding protein 1 (SMOC1) and nidogen-2, as presented in Table 1. Identification of fibronectin among these candidates validated our method since fibronectin is a known binding partner of tenascin-C. Table 2 indicates for each candidate the MASCOT Score and the spectral count after control-, FN- and TNC-columns. Two common background proteins, alpha-enolase (ENOA) and peptidyl-prolyl cis-trans isomerase A (PPIA) are also listed. The second to last column of Table 2 shows the fold enrichment of each candidate from the tenascin-C column compared to the control column. This factor was calculated normalizing the enrichment factor of spectra identified for each protein with the ones obtained for the two background proteins.

Since relative protein quantification counting spectra is not very accurate especially at low numbers, we further compared these candidates as well as the background proteins by multiple reaction monitoring (MRM), selecting 4 peptides and one transition each. Signal intensities of the different peptides are shown in Supplemental Fig. 1. The last column of Table 2 shows the fold enrichment of each candidate from tenascin-C column compared to the control column, calculated as the mean of enrichment of the analyzed peptides. Again, this factor was then normalized with the ones obtained for the two background proteins. This experiment revealed that SMOC1 was the best candidate since it was 4.5 times enriched in the eluates of the tenascin-C column, which is comparable to the 4.8-time fold enrichment of fibronectin.

2.2. Co-immunoprecipitation of tenascin-C and SMOC1

To further confirm the interaction between SMOC1 and tenascin-C, we performed immunoprecipitation experiments. For this purpose, we over-expressed full-length SMOC1-myc-DDK-his or tenascin-C or both proteins together in COS cells which show undetectable endogenous levels of either protein. On the one hand, we immunoprecipitated SMOC1 with an anti-SMOC1 antibody and showed that tenascin-C is co-immunoprecipitated with SMOC1. Nothing was immunoprecipitated when using an irrelevant anti-HA antibody or the SMOC1 antibody in the absence of SMOC1 transfection (Fig. 1A). On the other hand, we immunoprecipitated tenascin-C with an anti-tenascin-C antibody and showed that SMOC1 can be co-immunoprecipitated (Fig. 1B). In the absence of tenascin-C, over-expression SMOC1 could not be immunoprecipitated with an anti-tenascin-C antibody (Fig. 1B). We concluded from this experiment that tenascin-C and SMOC1 associate, when over-expressed, in the culture medium of mammalian cells.

2.3. Interaction between tenascin-C and SMOC1 by Surface Plasmon Resonance Spectroscopy

In order to firmly establish binding between SMOC1 and tenascin-C, we performed Surface Plasmon Resonance Spectroscopy. Purified his-tagged tenascin-C was coupled to a CM5 chip and SMOC1 was tested as soluble analyte at increasing concentrations. Associations and dissociations of the obtained binding curves were analyzed in a Langmuir 1:1 binding model (Fig. 2 and Supplemental Table 2). The apparent K_D value for SMOC1 as soluble interaction partner for tenascin-C was 2.59 10^{-9} M, which suggests a strong interaction between SMOC1 and tenascin-C. Tenascin-W, another member of the tenascin family which shares with tenascin-C a similar structure and over-expression in many cancers, was also tested in this experiment. Interestingly, tenascin-W did not bind to SMOC1, showing a specificity for tenascin-C as SMOC1 ligand (data not shown).

Since SMOC1 is a calcium-binding protein, we investigated whether the binding between SMOC1 and tenascin-C is calcium-dependant. We repeated the Biacore experiments by supplementing the medium with 5 mM of calcium on the one hand and by chelating calcium by 10 mM of EDTA on the other hand (Fig. 2B and Supplemental Table 2). SMOC1 and tenascin-C still bound in the presence of EDTA but to a

Table 1
Main characteristics of tenascin-C potential ligands.

Abbreviation	Full name (s)	Subcellular localization	Tissue specificity	Function
PGK1	Phosphoglycerate kinase 1	Mainly cytoplasmic, but can sometimes be secreted (Lay et al., 2000)	Tumoral tissues (Lay et al., 2000)	Disulfide reductase, important regulator of the tumoral "angiogenic switch" (Lay et al., 2000)
CLU	Clusterin Apolipoprotein J Ku70-binding protein 1 Complement-associated protein SP-40,40	Extracellular	High in brain, ovary, testis, and liver, lower in heart, spleen, lung, and breast, absent in T lymphocytes (de Silva et al., 1990) and over-expressed in tumors (for review, (Trougakos and Gonos, 2002))	Associated with apoptosis (Zhang et al., 2005), promotes epithelial–mesenchymal transition of breast tumor cells, thereby enhancing metastasis (Lenferink et al., 2009)
FN	Fibronectin	Extracellular	Plasma form secreted by hepatocytes and cellular forms present on cell surfaces, connective tissues, and basement membranes	Promotes adhesion of cells to extracellular materials
SMOC-1	SPARC-related modular calcium-binding protein 1	Extracellular	Widely expressed in many tissues with the strongest signal in the ovary (Vannahme et al., 2002)	
NID2	Nidogen-2	Extracellular	Muscle, heart, placenta, kidney, skin, and testis, weaker expression in liver and brain (Kohfeldt et al., 1998)	Interacts with collagens I and IV and perlecan (Salmivirta et al., 2002) and not essential for basement membrane formation (Schymeinsky et al., 2002)