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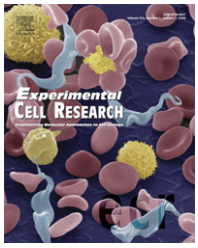

Research Article

The widely expressed extracellular matrix protein SMOC-2 promotes keratinocyte attachment and migration

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ABSTRACT

SMOC-2 is a recently discovered member of the BM-40/SPARC/osteonectin family of extracellular multidomain proteins of so far unknown function. While we have shown earlier that the homologous protein SMOC-1 is associated with basement membranes, in this study we demonstrate that, in the mouse, SMOC-2 could be detected in a large number of non-basement membrane localizations, often showing a diffuse tissue distribution. A more distinct expression pattern was seen in skin where SMOC-2 is mainly present in the basal layers of the epidermis. Functionally, recombinant SMOC-2 stimulated attachment of primary epidermal cells as well as several epidermal-derived cell lines but had no effect on the attachment of non-epidermal cells. Inhibition experiments using blocking antibodies against individual integrin subunits allowed the identification of $\alpha v\beta 6$ and $\alpha v\beta 1$ integrins as important cellular receptors for SMOC-2. Cell attachment as well as the formation of focal adhesions could be attributed to the extracellular calcium-binding domain. The calcium-binding domain also stimulated migration, but not proliferation of keratinocyte-like HaCaT cells. We conclude that SMOC-2, like other members of the BM40/SPARC family, acts as a regulator of cell–matrix interactions.

Introduction

SMOC (secreted *mo*dular *c*alcium-binding protein)-1 and -2 are paralogs sharing a common domain organization [1,2]. They belong to the BM-40/SPARC/osteonectin family of secreted proteins, which all contain an extracellular calcium-binding (EC) domain and a follistatin-like (FS) domain. BM-40/SPARC (also known as osteonectin) is the best studied member of this family and was originally isolated from bone [3], but subsequently found in a variety of other tissues [4–6]. In addition to the EC and the FS domain BM-40/SPARC contains an acidic N-terminal domain. In the EC domain two EF-hands are present that bind one calcium ion each with high affinity and cooperativity and are thought to be calcium saturated under physiological conditions [7]. Calcium binding enhances the affinity for extracellular ligands, such as collagens [8–10].

BM-40/SPARC belongs to the so called matricellular proteins defined as secreted macromolecules that regulate cell–matrix interactions, but not by themselves contribute to extracellular matrix structures [11]. BM-40/SPARC interacts

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Abbreviations: BSA, bovine serum albumin; EC, extracellular calcium binding; FS, follistatin-like; MMP, matrix metalloproteinase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TY, thyroglobulin-like

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with platelet-derived growth factor [12] and vascular endothelial growth factor [13]. *In vitro* assays showed that BM-40/SPARC influences cytokine activities [14], inhibits cell adhesion [15], interrupts the cell cycle [16], regulates cell differentiation [17] and activates matrix metalloproteinases [18]. The mechanisms underlying these activities are not known in detail. Nevertheless, several reports implicate that at least some effects, including those on extracellular matrix organization [19] and cellular survival and invasion [20], are associated with the activation of intracellular signalling cascades involving the non-receptor tyrosine kinases focal adhesion kinase (FAK) and integrin-linked kinase (ILK). Further, hevin-1, a member of the same protein family, was recently shown to inhibit adhesion and migration of fibroblasts in a Rac-1 dependent manner [21].

The nature of the cellular receptors mediating these effects has not been fully explored, but BM-40/SPARC was shown to bind to β3 integrins [22] and to induce an increased migration of tumor cells into bone by the activation of αvβ3 and αvβ5 integrins [23].

BM-40/SPARC is expressed already during embryonic development, but also in adult animals where it is present mainly in tissues undergoing repair or remodelling due to physiological processes, wound healing or disease. Deletion of the murine BM-40/SPARC gene does not alter embryogenesis, but BM-40/SPARC-deficient mice develop cataracts with an abnormal cell surface-basement membrane interface [24,25], a severe osteopenia [26] and display defects in wound healing [27,28].

The two SMOCs are the most recently discovered and least characterized members of the BM-40/SPARC protein family and display a unique domain composition. Whereas in the other members the EC and the FS domains always appear as a pair, in SMOCs the two domains are separated by two thyroglobulin-like (TY) domains and the SMOC-specific domain. The latter has a high proportion of aromatic amino acids, suggesting the presence of a hydrophobic core region. The TY domains [29], each containing six cysteine residues and the Cys-Trp-Cys-Val motif [30], have been found in proteins involved in either cell–cell and cell–matrix interactions or in growth factor binding [31]. The EC domain includes two well conserved EF-hands and for both SMOCs a reversible calcium-dependent conformational transition was shown by circular dichroism spectroscopy [1,2].

Both SMOC-1 and SMOC-2 have been expressed as recombinant proteins in eukaryotic expression systems and specific antibodies were generated. The expression of mRNA for SMOC-1 and -2 indicates a broad tissue distribution for both proteins [1,2]. By immunostaining at the light and electron microscopic levels SMOC-1 was shown to be present in many basement membranes, an exception being the ovarian follicle where SMOC-1 is found in the zona pellucida and not in the basement membrane [2]. The expression of SMOC-2 has not been systematically studied at the protein level, but human SMOC-2 was described under the name smap2 (smooth *m*uscle *a*ssociated *p*rotein *2*) as being associated with smooth muscle and upregulated during neointima formation [32]. The protein was also found expressed at the extracellular periphery of cultured human umbilical vein endothelial cells and overexpression of SMOC-2 after adenoviral infection of human umbilical vein endothelial cells stimulated DNA synthesis in a manner synergistic with vascular endothelial growth factor and basic fibroblast growth factor [33]. It was proposed that SMOC-2 is an angiogenic factor that potentiates the effect of growth factors. Further, a recent study showed that SMOC-2 contributes to cell cycle progression by maintaining integrin-linked kinase (ILK) activity during the G1 phase [34].

We have now studied the expression of SMOC-2 protein in mouse and show that, in contrast to SMOC-1, its deposition is not limited to basement membranes. Functional analysis using recombinant protein fragments shows that migration, but not proliferation, of cultured keratinocytes is stimulated by the SMOC-2 EC domain, which also mediates cell attachment by binding to cell surface receptors of the integrin family.

Materials and methods

Production of an antiserum against SMOC-2

A guinea pig was immunized with purified recombinant full-length mouse SMOC-2 (Pineda Antikörper Service). The antiserum was affinity-purified on a column of CNBr-activated Sepharose CL6B carrying immobilized recombinant SMOC-2 and did not react with any other known member of the BM-40/SPARC family (BM-40/SPARC, SC1, TSC-36, testican-1, -2, -3, SMOC-1) in dot blots of native proteins bound to nitrocellulose or in SDS-PAGE followed by immunoblotting. Specificity of the affinity-purified antibody was further demonstrated by incubation of the antibody with an excess of recombinant protein prior to use in immunoblots or on tissue sections. Under these conditions, signals, that were present without preincubation, were completely inhibited (see Supplemental Fig. 1).

Indirect immunofluorescence microscopy of mouse tissue sections

Tissues from embryonic or adult C57Bl/6 mice were embedded in Tissue-Tek (Sakura) and 7 μm cryosections were prepared and stored at −20 °C. Sections were air dried for 30 min and fixed with 0.5% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min. After washing with 0.1% Triton X-100 in 0.15 M NaCl, 50 mM Tris–HCl, pH 7.4 (TBS), sections were blocked for 30 min with 5% normal goat serum in Triton/TBS. The antibody against SMOC-2 was diluted in blocking solution and applied to the sections overnight at 4 °C. Detection was done with Cy3-labeled goat anti-guinea pig IgG (Jackson ImmunoResearch) and the slides were examined under a Zeiss Axiophot fluorescence microscope.

SDS-PAGE and immunoblot of cell culture supernatants and skin extracts

Serum-free cell culture supernatants were collected, 1-ml aliquots were precipitated by the addition of 10 μl of 0.1% Triton X-100 and 250 μl 50% trichloroacetic acid in water, and the pellets dissolved in SDS-containing sample buffer. Skin was prepared from the back of newborn mice and incubated in 1 M NaCl to separate the epidermis from the dermis. Both parts were homogenized in 100 mM NaCl, 10 mM Tris–HCl, pH 7.6, containing 1 mM EDTA and protease inhibitors. After centrifugation, pellets were dissolved in SDS-containing sample