Cell adhesion and integrin binding to recombinant human fibrillin-1

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Abstract **Fibrillin-1 is a major constituent of tissue microfibrils that occur in most connective tissues, either in close association with or independent of elastin. To test possible cell-adhesive functions of this protein, we used recombinant human fibrillin-1 polypeptides produced in a mammalian expression system in cell attachment and solid-phase integrin binding assays. Fibrillin-1 polypeptides containing the single RGD sequence located in the fourth 8-cysteine domain, mediated distinct cell adhesion of a variety of cell lines and bound to purified integrin αVβ3. Integrins αIIbβ3, α5β1, α2β1 and α1β1 did not interact with any of the recombinant fibrillin-1 peptides. Our results indicate a novel role for fibrillin-1 in cellular interactions mediated via an RGD motif that is appropriately exposed for recognition by integrin αVβ3.**

Key words: Fibrillin-1; Integrin; RGD; Cell adhesion

1. Introduction

Fibrillins are a family of large extracellular glycoproteins, which are integral components of the 10–12 nm diameter microfibrils found throughout the extracellular space, in elastic and non-elastic tissues [1,2]. So far two members of this family, fibrillin-1 [3–6] and fibrillin-2 [4,7], have been described as products of different genes. These proteins share a common organization of domains consisting mostly of calcium-binding EGF-like repeats and novel motifs containing 8 cysteines, which are also found in the family of TGF-β binding proteins [3,5,8]. Also common to both proteins is the presence of RGD sequences suggesting possible interactions with integrin adhesion receptors [9].

In situ, microfibrils are often found in close proximity to cells. They appear to connect endothelial cells to elastic fibers in the subendothelial matrix of the mouse aorta [10] and of lymphatic capillaries [11,12]. In the mouse aortic media the elastic laminae are linked to smooth muscle cells by bundles of microfibrils [13]. Furthermore in rat bone marrow, microfibrils appear to connect endothelial cells of the sinusoidal wall to adventitial reticular cells [14] and in skin, microfibrils often extend from the elastic fibers in the dermis to the basement membrane of the dermal-epidermal junction closely approximating the epidermal cell layer [15,16]. Recently Kielty et al. [17] reported cell attachment of smooth muscle cells to isolated microfibrils. Thus evidence for cellular interactions with microfibrils is accumulating, however it remains unclear which microfibrillar component is responsible for this activity.

As the purification of fibrillin from cell culture medium necessitates the use of buffers containing denaturing agents and cannot separate fibrillin-1 and fibrillin-2 [18], we chose to use recombinant human fibrillin-1 polypeptides for a study of possible cell-adhesive functions. These human fibrillin-1 peptides were produced in a mammalian expression system and purified under non-denaturing conditions [19]. Extensive characterization indicated correct folding [19]. Using an experimental approach already applied to characterize cell-adhesive and integrin-binding functions of fibulins [20], laminin [21] and collagen VI [22], we demonstrate the ability of cells to adhere to fibrillin-1 via an RGD motif located in the fourth 8-cysteine domain. In addition we identify integrin αVβ3 as a major cellular receptor for this site.

2. Materials and methods

2.1. Peptides and protein ligands

Human plasma fibronectin (Behringwerke, Marburg) and fibrinogen (Calbiochem, Bad Soden) were purchased. Vitronectin was purified by heparin chromatography [23]. Synthetic fibrillin RGD peptides were produced in our own facilities and kindly provided by Dr. L. Moroder. Synthetic GRGDS was obtained from Bachem, Heidelberg.

2.2. Recombinant production of human fibrillin-1 fragments

The construction of expression vectors from human fibrillin-1 cDNA, the transfection of human fibrosarcoma HT1080 or embryonic kidney 293 cells and the purification and functional characterization of the recombinant proteins have been described in detail elsewhere [19]. Recombinant fibrillin-1 peptides were purified from culture supernatants under non-denaturing conditions and were considered >95% homogeneous based on their appearance in SDS polyacrylamide gel electrophoresis. The correct structure was verified by Edman degradation, glycosylation analysis, rotary shadowing, analysis of cysteines, and by functional characterization, including Ca^{2+} binding and binding of monoclonal antibodies [19].

2.3. Cell attachment assays

Adhesion of established cell lines to ligand-coated plastic wells for 30–60 min followed by a colorimetric detection of bound cells was performed according to a previously published protocol [24]. In inhibition experiments, cells were incubated for 10 min at 37°C with peptide inhibitors or blocking monoclonal antibodies [22] prior to adding to the coated wells.

2.4. Solid phase binding assays with purified integrins

The purification of human integrins α1β1, α2β1, α5β1, αVβ3, and αIIbβ3, as well as the protocol for solid phase binding assays with purified integrins is outlined in detail [21]. Binding was performed in 0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM $MnCl_2$, 1 mM $MgCl_2$, and 0.1 mM $CaCl_2$, 0.02% Tween 20. Bound ligand was detected using polyclonal rabbit antibodies followed by peroxidase-labeled goat anti-rabbit IgG [21]. Fibrillin-1 fragments rF6 and rF6trunc were detected with a rabbit antiserum raised against rF6trunc. For rF11, monoclonal antibodies 201 and 26 [19] were used and the extent of binding was monitored by parallel binding experiments using immobilized fibulin-2 as a positive control [25]. In inhibition experiments, immobilized integrins were preincubated with inhibitory peptides for 10 min prior to addition of the soluble ligand.

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3. Results

The domain structure of the recombinant human fibrillin-1 peptides relevant to this study is depicted in Fig. 1. Together they span the whole fibrillin-1 molecule. rF2, rF6trunc and rF6 are extensions from a central EGF-like motif adjacent to the fourth 8-cysteine domain, which contains the RGD motif. The small fragment rF2 (30 kDa) extends to the EGF-like repeat C-terminal to the fourth 8-cysteine domain. The large protein rF6 (195 kDa) includes the complete C-terminal half of fibrillin-1 and a truncated product rF6trunc (115 kDa) extends to the sixth 8-cysteine domain. Only the large N-terminal fragment rF11 does not include the RGD motif.

These polypeptides were immobilized on plastic and used in cell attachment assays (Fig. 2, Table 1). Distinct adhesion and spreading to fragments rF6 and rF6trunc was observed, although the overall extent of attachment compared to reference ligands vitronectin (Fig. 2) or fibronectin (Table 1) varied considerably with the cell line. The large N-terminal fragment rF11 (Table 1) as well as the short polypeptide rF2 (data not shown) were inactive in these assays. However, rF2 was a highly active soluble inhibitor of A375 cell adhesion to rF6trunc (Table 2) indicating its inactivation by immobilization onto plastic. The synthetic peptides GRGDS and IRPRGDNGD representing the authentic fibrillin-1 sequence also totally inhibited A375 cell adhesion to rF6trunc (Table 2). Complete inhibition by GRGDS at less than 30 μM was also observed for the attachment of A375, 251MG and A431 cells on rF6. RGES as a control peptide (1 mM) was not inhibitory. Furthermore 251MG cell adhesion to rF6 could be blocked by at least 50% by adding inhibitory monoclonal antibodies specific for the human β3-integrin subunit (C17) (data not shown).

In order to identify possible cellular receptors for fibrillin-1 we performed solid phase binding assays with purified integrins. Integrins αIIbβ3 (Fig. 3A), α5β1, α2β1 and α1β1 (data not shown) did not interact with any fibrillin-1 polypeptide. In contrast, integrin αVβ3 (Fig. 3B) bound specifically to fragment rF6 and rF6trunc, but not to rF11, with either integrin (Fig. 3B) or fibrillin-1 polypeptide (not shown) used as the immobilized ligand. The specificity of this interaction is confirmed by its sensitivity to the presence of 10 mM EDTA (not shown), and also by the inhibition with synthetic GRGDS and IRPRGDNGD peptides or with recombinant fragment

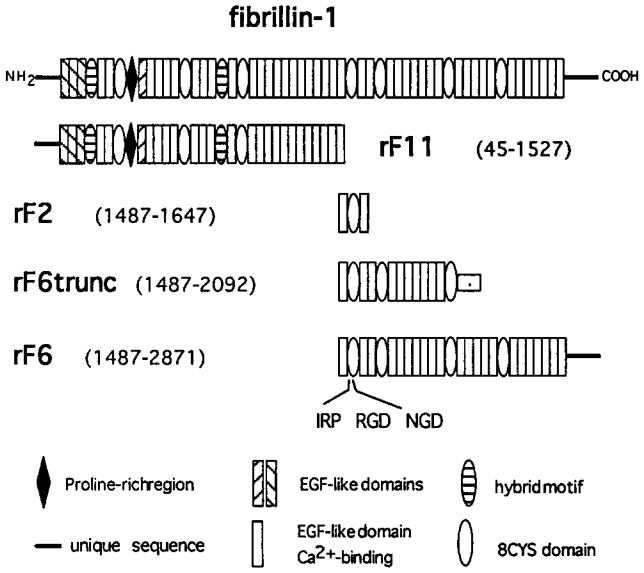

Fig. 1. Domain structures of fibrillin-1 and the recombinant fibrillin-1 polypeptides. The location of the RGD sequence is indicated. Numbers in brackets refer to the numbering of amino acids according to [6]. Due to an unexpected recombination event, fragment rF6trunc was found to contain a C-terminal 40-amino acid extension derived from the major capsid protein VP1 of the SV40 virus. Verification of DNA sequences also revealed a point mutation in the DNA of fragment rF2 leading to an isoleucine to threonine change at position 1538 [19].

rF2 (Table 3). We also compared the inhibitory potential of RGD-containing nonapeptides representing sequences of human fibrillin-1 (Fb-1) and fibrillin-2 (Fb-2/1 and Fb-2/2) in binding assays with three purified RGD-dependent integrins αVβ3, αIIbβ3 and α5β1 and their ligands (Table 3). This revealed about 20-fold reduced inhibitory activities of the three nonapeptides compared to GRGDS with all three integrins.

4. Discussion

In this study, we have identified specific cell-adhesive activity of three human recombinant fibrillin-1 polypeptides rF6, rF6trunc and rF2, all of which include the fourth 8-cysteine domain. This result, together with the potent and complete inhibition of adhesion with RGD peptides, locates the pri-

Table 1
Cell adhesion to recombinant human fibrillin-1 polypeptides

Cell line		rF11	rF6	rF6trunc
251MG	astrocytoma	<5	91	113
RN22	schwannoma	<5	74	53
A375	melanoma	<5	69	39
HBL100	mammary epithelium	<5	67	38
A431	epidermoid	<5	67	25
OVCAR-4	ovarian carcinoma	<5	38	60
Scl I	epithelial carcinoma	<5	23	23
HT1080	fibrosarcoma	<5	12	5
Rugli	glioblastoma	<5	<5	<5
T47D	mammary epithelium	<5	<5	<5
Fibroblasts	adult skin	<5	74	57

Adhesion is expressed as % adhesion compared to fibronectin at 40 μg/ml coating concentration. Cells were of human origin with the exception of RN22 and Rugli (rat).

Table 2
Inhibition of A375 cell adhesion to vitronectin and rF6trunc by synthetic and recombinant RGD peptides

Soluble inhibitor	IC_{50} (μM)	
	Vitronectin (2 μg/ml)	rF6trunc (10 μg/ml)
GRGDS	25	5.6
IRPRGDNGD	>200	120
rF2	10	3.7

Numbers in brackets indicate substrate concentrations used for coating of plastic plates.

mary site for fibrillin-1 cell binding to the single RGD sequence in this domain. This RGD site is located in the middle of a 14-amino acid stretch flanked by two cysteines. Since all 8 cysteines in the 8-cysteine domain are disulfide bonded [19], the RGD tripeptide is predicted to form the tip of a small loop structure. Available sequence data suggest that this loop is conserved between species within fibrillin-1, and a similar RGD motif is present at the same position of fibrillin-2. The latter contains an additional RGD site in a different position of the third 8-cysteine domain [7], which is similarly located at the predicted tip of a small loop.

In solid-phase binding assays with purified integrins we identified integrin αVβ3 as a potent binding partner for fibrillin-1 fragment rF6, whereas two other RGD-binding integrins (αIIbβ3, α5β1) and two collagen/laminin receptors (α1β1, α2β1) were inactive. These data are in agreement with independent experiments showing that transfection of Chinese hamster ovary (CHO) cells with cDNAs for the human αV- and β3-integrin chains confers on them the ability to bind to the rF6 fragment (Martin Pfaff and Joe Loftus, unpublished results). αVβ3 expression in an active form is therefore sufficient to allow cells to adhere to fibrillin-1. However, it is possible that other integrins also recognize the single RGD motif in fibrillin-1. This would explain our failure to get complete inhibition of 251MG cell adhesion to rF6 with inhibitory anti-β3-integrin antibodies. The integrin-binding properties of fibrillin-1 resemble those of another extracellular matrix protein, osteopontin. It is recognized by integrins αVβ3 and also αVβ5 and αVβ1 [26], but not by integrin αIIbβ3 [27]. Yet most other RGD proteins such as fibronectin, vitronectin or the laminin-P1 fragment bind to both αIIbβ3 and αVβ3 [21], and some of them, like mouse fibulin-2, bind distinctly better to αIIbβ3 than to αVβ3 [20].

In the inhibition experiments fragment rF2 was a more potent inhibitor of cell adhesion than the IRPRGDNGD peptide, whereas both the peptide and the recombinant protein were similarly active in the solid phase binding assay with integrin αVβ3 (Tables 2 and 3). This might be explained by the different experimental conditions in both assays leading to different states of activation of integrins and to a preferential stabilization of the larger recombinant protein bound to the integrin in the plasma membrane.

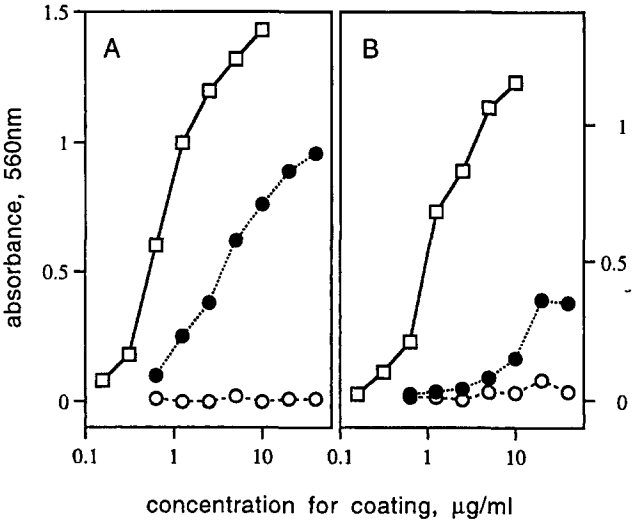

Fig. 2. Cell attachment of A375 melanoma (A) and HT1080 fibrosarcoma (B) cells to vitronectin (open squares), rF6trunc (closed circles) and rF11 (open circles).

Interestingly, the three RGD nonapeptides representing the three RGD sites of fibrillin-1 and fibrillin-2 inhibit integrin αVβ3 as well as αIIbβ3 and α5β1 in solid phase binding assays, although with about 20-fold reduced activity compared to GRGDS (Table 3). This implies that the structural information conferring specificity for integrin αVβ3 is present in the recombinant fibrillin-1 proteins but not in the nonapeptides. Therefore no conclusions on integrin-specific binding activities of fibrillin-2 can be extrapolated from experiments with short linear peptides.

Some insight into the molecular criteria that determine binding specificities of RGD ligands to integrins αVβ3, αIIbβ3 and α5β1 can be obtained by a comparison of the inhibitory activity of soluble GRGDS peptide with the concentrations of soluble ligands in solid phase assays (Table 3; see also [28]). Whereas inhibitory GRGDS concentrations for integrin αVβ3 are within the range of the vitronectin concen-

Table 3
Inhibition of binding to immobilized integrins with GRGDS, RGD nonapeptides corresponding to authentic sequences of fibrillin-1 and -2 and recombinant fragment rF2

Integrin/soluble ligand	GRGDS	Fb-1	Fb-2/2	Fb-2/1	rF2
αVβ3 (1.5 μg/ml)					
vitronectin (65 nM)	0.035	0.57	1.7	3.1	0.18
rF6trunc (280 nM)	0.031	0.30	n.d.	n.d.	0.13
αIIbβ3 (3 μg/ml)					
fibrinogen (30 nM)	6.1	92	53	78	n.d.
fibronectin (20 nM)	0.58	9.1	n.d.	n.d.	≫20
α5β1 (0.5 μg/ml)					
fibronectin (20 nM)	4.7	103	85	252	n.d.

n.d.=not determined. Results are expressed as IC_{50} values (μM). Numbers in brackets indicate concentrations used for coating (integrins) or soluble concentrations (ligands). Peptides representing RGD sites of fibrillins were IRPRGDNGD (Fb-1), FGPRGDGSL (Fb-2/2) and FANRGDVLT (Fb-2/1) located in the fourth (Fb-1, Fb-2/2) and third (Fb-2/1) 8-cysteine domains. Data represent mean values of 2–4 independent experiments.

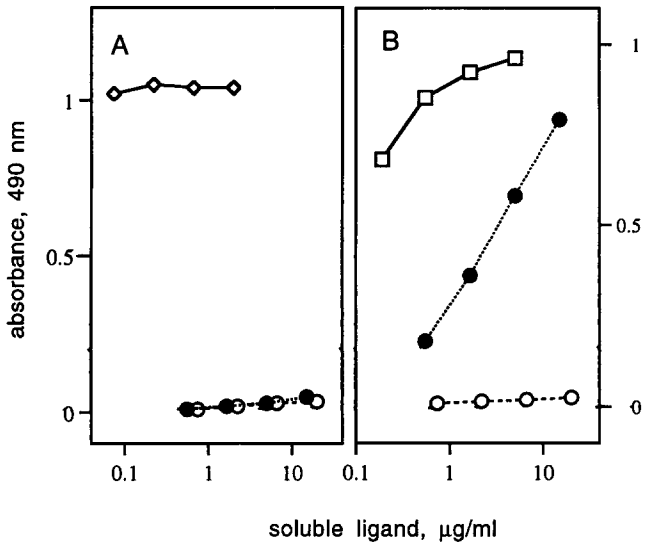

Fig. 3. Binding of fibrinogen (open diamonds), vitronectin (open squares), and recombinant fibrillin-1 peptides rF6 (closed circles) and rF11 (open circles) to immobilized integrins αIIbβ3 (A) and αVβ3 (B).

tration, IC_{50} values for integrins αIIbβ3 and α5β1 are two orders of magnitude higher than the concentrations of their soluble ligands. Therefore, the relative contribution of the RGD sequence alone to the overall binding affinity strongly differs between these integrins, and the simple presence of an appropriately exposed RGD motif might be sufficient to mediate binding of αVβ3. In contrast, recognition by integrins αIIbβ3 and α5β1 seems to require additional binding criteria, as exemplified by the well-defined synergy region in fibronectin [29]. The absence of such additional binding structures in fibrillin-1 might thus explain an insufficient affinity for integrins αIIbβ3 and α5β1.

Although it still remains to be shown that the RGD site of fibrillin-1 is similarly accessible in authentic fibrillin-1 molecules, our results convincingly demonstrate its correct exposure in recombinant fragments of fibrillin-1 for binding to a subset of RGD-dependent integrins. The putative physiological role of fibrillin-cell interactions is presently unclear. They could provide a structural link between the cytoskeleton of cells, microfibrils and elastic fibers. In fact, fibrillin has been identified by immunoelectron microscopy in microfibrils that connect elastic fibers to cytoskeletal structures in endothelial cells [10]. In addition, the assembly of microfibrils and elastic fibers might depend on fibrillin interactions with cells. Elastic fiber assembly typically occurs at infoldings of the cell surface, where microfibrils are seen close to the cell membrane [30]. Specific binding of integrin αVβ3 to fibrillin-1 may also play an important role during angiogenesis. The key role played by αVβ3 expressed on endothelial cells in this process [31], together with the abundance of fibrillin-1 in the vascular wall and in the surrounding connective tissue, underlines this possibility.

5. Note added in proof

During the submission of the manuscript, a report appeared [32] describing cellular interactions mediated by integrin αVβ3 to fibrillin-1 purified from bovine tissues.

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