Tenascin Mediates Cell Attachment through an RGD-dependent Receptor

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Abstract. Tenascin is an extracellular matrix glycoprotein expressed in association with mesenchymal-epithelial interactions during development and in the neovasculature and stroma of undifferentiated tumors. This selective expression of tenascin indicates a specific role in cell matrix interactions. We now show that tenascin can support the adhesion of a variety of cell types, including various human tumor cells, normal fibroblasts, and endothelial cells, all of which can attach to a substrate coated with tenascin. Detailed studies on the mechanism of the tenascin-promoted cell attachment were carried out with the human glioma cell line U251MG. The attachment of these cells and others to tenascin were inhibited specifically by peptides containing the RGD cell attachment signal. Affinity chromatography procedures similar to those that have been used to isolate other adhesion receptors yielded a heterodimeric cell surface protein which bound to a tenascin affinity matrix in an RGD-dependent fashion. One of the subunits of this putative tenascin receptor comigrates with the β subunit of the fibronectin receptor in SDS-PAGE and cross reacts with antibodies prepared against the fibronectin receptor in immunoblotting. These results identify the tenascin receptor as a member of the fibronectin receptor family within the integrin superfamily of receptors. The cell attachment response on tenascin is distinctly different from that seen on fibronectin, suggesting that cell adhesion and motility may be modulated at those sites where tenascin is expressed in the extracellular matrix.

The extracellular matrix is a complex assembly of molecules that interact with one another as well as with cells to effect a wide range of cellular and tissue functions. The extracellular matrix molecules include fibronectin, laminin, interstitial and basement membrane collagens, and proteoglycans. A number of these molecules have been shown to have important functional properties including the promotion of cell adhesion and spreading, cell motility, directed cell migration, cellular differentiation, and proliferation (Cardarelli and Pierschbacher, 1986; Couchman et al., 1982; Edgar et al., 1984; Ekbloem, 1984; Gospodarowicz et al., 1980; Greenberg and Hay, 1986; Lacovara et al., 1984; Manthorpe et al., 1983; Rovasio et al., 1983; Ruoslahti and Pierschbacher, 1987). More recently, it has been found that a number of extracellular matrix components interact with cells through specific cell surface receptors (Giancotti et al., 1985; Horwitz et al., 1985; Pytel et al., 1985a,b; Pytel et al., 1986; Takada et al., 1989; Takuma et al., 1986; Tomasselli et al., 1987). These receptors belong to an integrin superfamily of proteins and many of them recognize the tripeptide sequence Arg-Gly-Asp (RGD) in their extracellular ligands (Hynes, 1987; Pierschbacher and Ruoslahti, 1984a; Ruoslahti and Pierschbacher, 1987). While a number of extracellular matrix molecules have been well-characterized, new molecules are likely to be found that play specific roles in cell matrix interactions.

One such novel extracellular matrix molecule is the glial-mesenchymal extracellular matrix glycoprotein tenascin (Bourdon et al., 1983; Chiquet-Ehrismann et al., 1986). This glycoprotein has been described as GEMEM (Bourdon et al., 1983) cytotactin (Grun et al., 1985), hexabrachion protein (Erickson and Taylor, 1987), and myotendinous antigen (Chiquet and Pambrough, 1984). Human tenascin is a 250-kD glycoprotein that is secreted as a high molecular mass (>105 kD) disulfide-bonded oligomer (Bourdon et al., 1983; Bourdon et al., 1985). In rotary shadowing images tenascin appears as a hexamerlike structure (Erickson and Taylor, 1987; Vaughn et al., 1987). This structurally unusual matrix molecule is further distinguished by its highly selective oncodevelopmental expression.

Tenascin is expressed in a variety of solid tumors, but is largely absent in normal adult tissues (Bourdon et al., 1983; Mackie et al., 1987; McComb et al., 1987). In human gliomas, it is expressed around the tumor neovasculature, and in fibrosarcomas within the stroma. Developmentally, tenascin is selectively expressed in condensing mesenchyme during the initial stages of organogenesis of mammary gland, toothbud, and kidney (Auferheide et al., 1987; Chiquet-Ehrismann et al., 1986). In each of these organs, epithelial-mesenchymal interactions are of key importance in normal organ development. Temporally restricted expression of tenascin is also seen in the developing nervous system (Gru-
met et al., 1985; Crossin et al., 1986). The selective on-codevelopmental expression of tenascin within the extracellular matrix makes it likely that this molecule plays a specific role in cell–matrix interactions and that such interactions are mediated by cell surface receptors.

In this study, we show that tenascin has RGD-dependent cell adhesion activity and describe an integrin-type cell surface receptor that binds to tenascin with the same RGD-dependent specificity as the cells.

Materials and Methods

Cell Culture

Tumor cell lines and normal fibroblasts were cultured in DME supplemented with 10% FBS, glutamine, penicillin, and streptomycin. Cultures were maintained at 37°C in 7% CO2. Human umbilical vein endothelial cells were cultured in DME, supplemented with 20% FBS, heparin, and endothelial cell growth factors (Collaborative Research, Lexington, MA). Adherent cell lines were passaged by treatment with 100 μg/ml trypsin/0.02% EDTA in PBS.

Cell Attachment Assay

Cells for the cell attachment assay were detached using 0.02% EDTA in PBS, pH 7.4, washed in DME containing 2 mg/ml BSA and plated at 2 × 10^4 cells per well in 96-well flat bottom microtiter plates (Titertek; Flow Laboratories, McLean, VA). Wells were previously coated overnight with dilutions of cell attachment proteins in PBS. Plates were washed and then incubated for 30 min with a solution containing the DMEM-BSA medium to block nonspecific binding sites before their use in cell attachment assays. Peptides added to the cell attachment assays were dissolved in DME. The peptides were not cytotoxic at the concentration used as determined by trypan blue exclusion by cells in the assays. Assays were carried out at 37°C in a CO2 incubator for 90 min. Nonadherent cells were removed by washing with PBS and adherent cells fixed with 0.5% toluidine blue. Adherent cells were counted directly or their numbers determined by lysing cells with 1% SDS and measuring dye absorbance at 600 nm in a Multiscan plate reader (Flow Laboratories).

Purification of Tenascin

Tenascin was purified from the spent culture media of U251MG human glioma cells by affinity chromatography on an 81C6 antitenascin monoclonal antibody (Bourdon et al., 1983) coupled to Sepharose 4B. The spent culture media was first concentrated by tangential flow filtration over PLMK300 filters (Millipore Corp., Bedford, MA). A Sepharose 4B column was used to remove debris and aggregated protein before application of the sample to the monoclonal antibody affinity column. Nonbound proteins were washed from the antibody-Sepharose column with 0.5 M NaCl, 1 M urea, 10 mM sodium phosphate, pH 7.4, and tenascin eluted with 0.5 M NaCl, 4 M urea, 10 mM sodium phosphate, pH 7.4. Protein elution was monitored at 280 nm. Purity of the tenascin preparations was monitored by SDS-PAGE on 7% acrylamide gels followed by Coomassie Blue or silver staining. The receptors were eluted with 1 mg/ml GRGDSP peptide. Fractions were analyzed by SDS-PAGE and autoradiography on XAR5 x-ray film with an enhancer screen.

Immunoblot and Immunoprecipitation Analyses

Receptors isolated as described above from unlabeled cells were concentrated by precipitation in acetone and separated by SDS-PAGE on 7.5% acrylamide gel. The separated proteins were then electroblotted onto a nitrocellulose membrane. Blotted protein bands were visualized by staining the membrane with ponceau S stain and destained in PBS. The blots were incubated in PBS containing 1% BSA for 1–2 h to block nonspecific protein binding sites and subsequently incubated with primary antibodies in PBS–1% BSA overnight at 4°C. After the incubation, the blots were washed and incubated with either HRP-conjugated goat anti-mouse IgG or goat anti-rabbit IgG antibodies (Bio-Rad Laboratories, Richmond, CA) in PBS–1% BSA for 1 h. Bound antibodies were visualized by addition of diaminobenzidine tetrahydrochloride in PBS 0.01% H2O2 solution to washed blots.

Immunoprecipitation was performed by incubating 125I-labeled samples with antireceptor antisera overnight at 4°C, followed by recovery of the bound label with protein A-Sepharose. Bound material was analyzed by SDS-PAGE followed by fluorography as described above.

Antibodies

Monoclonal antibody 81C6 is an antitenascin antibody previously described (Bourdon et al., 1983). Polyclonal antisera against tenascin were prepared by immunization of rabbits with purified human tenascin. The antisera were absorbed with fibronectin-Sepharose and bovine plasma protein-Sepharose, and their IgG fraction was isolated on protein A-Sepharose. Polyclonal rabbit antibodies to vitronectin receptor were affinity purified on vitronectin receptor-Sepharose (Suzuki et al., 1987). Polyclonal rabbit antibodies to fibronectin receptor β subunit were affinity purified as described (Argoves et al., 1987).

Synthetic Peptides

The peptide GRGDSP derived from the fibronectin cell attachment site and control peptide GROeSP were synthesized on a peptide synthesizer (Applied Biosystems Inc., Foster City, CA) using solid phase chemistry. Peptides were purified by ion exchange HPLC and lyophilized. Peptides were resuspended in appropriate buffered solutions for cell attachment assays or elution of tenascin receptors.

Results

Isolation and Characterization of Tenascin

Human tenascin was isolated from spent culture media of U251MG human glioma cells by 81C6 monoclonal antibody affinity chromatography. The purified tenascin migrated as a single prominent band at ~250 kD in SDS-PAGE under reducing conditions (Fig. 1 A). Unreduced tenascin behaved as a high molecular mass (>105 kD) disulfide bonded oligomer both in SDS-PAGE and HPLC TSK 400 size chromatography (not shown). In electron microscopic images obtained after rotary shadowing, tenascin appeared as a hexameric oligomer (Fig. 1 B). Polyclonal antisera to chicken tenascin (Chiquet and Fambrough, 1984) immunoprecipitated the same 250-kD tenascin polypeptide from U251MG spent culture medium as the 81C6 antibody (not shown). These results identified the isolated protein as highly purified tenascin.

Cell Attachment Activity of Tenascin

Cell attachment to tenascin was examined in an in vitro cell attachment assay. Cells adhering to tenascin included a variety of tumor cell lines of glial (U251MG, Rugli), epithelial (A431), endodermal (PFHR-9), and mesenchymal (MG63, HT1080) origin as well as fibroblasts, and human umbilical
Figure 1. Analysis of purified human tenascin by SDS-PAGE and electron microscopy. (A) Tenascin (20 μg) isolated from the spent culture media of U251MG cells by affinity chromatography on monoclonal antibody 81C6-Sepharose was characterized by SDS-PAGE on a 7% acrylamide gel under reducing conditions. Protein was stained with Coomassie Blue. (B) A rotary shadowed image of one tenascin molecule is shown.

Figure 2. Cell attachment to purified tenascin. The attachment of U251MG cells to tenascin (TN), fibronectin (FN), and bovine serum albumin (BSA) are shown. Microtiter wells were coated with 5 μg/ml protein in PBS. Cells were seeded into the coated wells at a density of 2 x 10^4 cells/200 μl in DME-BSA and incubated at 37°C for 90 min. Bar, 100 μm.

Inhibition of Cell Attachment to Tenascin by RGD Peptides

The peptide GRGDSP inhibited the cell attachment of the U251MG cells to tenascin in a dose-dependent manner (Fig. 5), while the control peptide GRGESP (Pierschbacher and Ruoslahti, 1984b) had no effect, even at high concentrations (10 mg/ml, not shown).

Inhibition of cell attachment to tenascin by the GRGDSP peptide occurred at concentrations of 30 and 150 times lower than were needed for comparable inhibition of cell attachment on vitronectin or fibronectin (Fig. 5). The concentra-
Comparison of cell attachment activity of tenascin and fibronectin. The attachment of U251MG cells to microtiter wells coated with tenascin (○) or fibronectin (□) at various concentrations was assayed. Cells at 2 × 10⁴/200 μl in DME-BSA were seeded into the wells and allowed to adhere. The cell attachment activity is plotted relative to maximum cell attachment to fibronectin.

Figure 4. Inhibition of tenascin-mediated cell attachment by antibodies. Cell attachment to fibronectin (FN), tenascin (TN), and vitronectin (VN) was assayed in the presence or absence of polyclonal antifibronectin antibodies and polyclonal antitenascin antibodies. Wells were coated with 5 μg/ml adhesion protein. Antibody concentrations per well were 20 μg/ml antifibronectin antibody or 200 μg/ml antitenascin antibody. Values represent relative cell attachment ± SD. Controls represent maximum cell attachment on each adhesion protein in the absence of added antibody. □, control; ○, anti-TN; △, anti–FN.

RGD-dependent Tenascin Receptor

A receptor for tenascin was isolated from octylglucoside extracts of surface-labeled cells by affinity chromatography on a tenascin-Sepharose column. After the column was washed with octylthioglucoside, it was eluted with peptides, first GRGESP and then GRGDSP. As has been found to be the case with other RGD-directed receptors (Pytela et al., 1985a,b), the GRGESP peptide at 1 mg/ml eluted from the tenascin column a small proportion of protein, but the bulk of the bound protein eluted with the GRGDSP peptide. SDS-PAGE revealed specifically eluted 145- and 125-kD polypeptides (Fig. 6A). The eluted material also contained a minor band at ~200 kD, the identity of which was not studied further because it was not observed in all receptor preparations. Upon reduction of disulfide bonds, the GRGDSP-eluted material gave only a minor band at 130 kD, presumably because the main polypeptides comigrate after reduction (Fig. 6B).

The decrease of the size of the larger (α) subunit on reduction indicated the α subunit may be composed of a heavy and light chain as is seen for the fibronectin receptor α subunit. However, the light chain of the tenascin receptor α subunit could not be readily identified in the reduced receptor preparations, perhaps due to a paucity of radiolabeling sites in this particular chain. The polypeptide composition is similar to that of the integrin-type adhesion receptors (Hynes, 1987; Ruoslahti and Pierschbacher, 1987). In particular the tenascin receptor α subunit had the same chain mobility as the fibronectin receptor α subunit, suggesting that the tenascin receptor was a member of the fibronectin receptor subfamily of integrins. This assumption was confirmed by immunological analysis as shown below.

The tenascin receptor was compared with the vitronectin receptor and fibronectin receptor isolated from U251MG cell extracts. The previously characterized vitronectin receptor (Pytela et al., 1985b) was obtained when the U251MG cell extract was fractionated on a GRGDSP-Sepharose affinity matrix (not shown). The tenascin receptor did not bind detectably to a GRGDSP-K-Sepharose column. Moreover, the tenascin receptor could be isolated on tenascin-Sepharose even after the U251MG extract was first passed over GRGDSP-K-Sepharose. Similar experiments with the cell-binding 120-kD fragment of fibronectin (Pytela et al., 1985a) showed that the U251MG cells also have a distinct fibronectin receptor (Fig. 7) and that no detectable tenascin receptor bound to this affinity matrix.

Receptor immunoprecipitation and immunoblotting were used to confirm the heterodimer composition of the receptor.
and to identify the β subunit of the tenasin receptor. The tenasin receptor α and β subunits were both immunoprecipitated with antifibronectin receptor β subunit polyclonal antibodies, suggesting an immunologic identity between the β subunits of these receptors (Fig. 7, left). Receptor immunoblots with an antifibronectin receptor β subunit monoclonal antibody confirmed the immunological similarity between the tenasin receptor and fibronectin receptor β subunits (Fig. 7). Based on these results it appears that the two receptors share the same β subunit. The α subunit of the tenasin receptor was not reactive with an antibody against the fibronectin receptor α subunit and neither subunit of the tenasin receptor bound anti-vitronectin receptor antibodies.

Discussion

In this paper we have shown by using cell attachment assays that tenasin can interact with cells in an RGD-dependent manner and have isolated and partially characterized a receptor that binds to tenasin with a similar specificity as the cells.

Cell attachment assays showed that human tenasin insolubilized on plastic interacts with a number of tumor and normal cell types supporting their attachment. Perhaps as significant was the finding that at least several cell lines and lymphoid cells did not attach or attached poorly to tenasin. This may indicate that these cells either lack cell receptors or the receptor interaction is not detectable by cell attachment.

Titration curves comparing the attachment of cells to tenasin and fibronectin in a serum-free medium showed that similar numbers of cells attached to the two proteins. Inhibition of cell attachment to tenasin by tenasin-specific antibodies but not by antifibronectin antibodies, indicated a specific interaction of tenasin with the cells. Among the cell lines tested, the U251MG glioma cells attached to tenasin particularly well, and these cells were chosen for detailed studies on the cell–tenasin interaction.

Tenasin appears to be a member of the RGD family of cell attachment proteins (Ruoslahti and Pierschbacher, 1987), as demonstrated by the specific, dose-dependent inhibition of cell attachment to tenasin by RGD peptides. These results imply the presence of an RGD sequence within the tenasin polypeptide and, indeed at least chicken cytotactin (tenasin) does contain this sequence (Jones et al., 1988).

Previous work on the cellular interactions of tenasin has indicated that tenasin lacks cell attachment–promoting activity (Erickson and Taylor, 1987) or that it can interact with cells promoting attachment, but is an inhibitor of fibronectin-mediated cell attachment (Chiquet-Ehrismann et al., 1988). These results are not necessarily in conflict with ours for the following reasons.

Our results show that the cell attachment–promoting activity to tenasin is distinct from that of fibronectin and vitronectin in that little cell spreading is seen on tenasin. The cell attachment–inhibiting activity observed by others could be an indication that the cell–tenasin interaction, which initially manifests itself as cell attachment, is primarily a signal for another type of response, such as migration or differentiation. In this regard, the cell attachment–promoting activity of tenasin might be considered a manifestation of an interaction between the cell and tenasin rather than an indication of the physiological result of the interaction. In fact, it is an exciting possibility that while some extracellular matrix molecules give to cells signals leading to attachment and spreading others may signal migration or even detachment. In addition to tenasin, such a role has been proposed for thrombospondin (Lahav, 1988), which may also interact with cells in an RGD-dependent manner (Lawler and Hynes, 1986).

We find that the cell–tenasin interaction can be inhibited with an RGD peptide, and others have found that soluble tenasin inhibits the attachment of cells to an RGD peptide substrate (Chiquet-Ehrismann et al., 1988). However, these investigators also found that the soluble RGD peptide was not capable of inhibiting the attachment of the same cells to tenasin. The reasons for this difference are not known, but it may be that cells can also bind to tenasin through a non-RGD–dependent mechanism. Fibronectin and laminin are each known to have more than one integrin-type receptor (Gehlsen et al., 1988; Hemler et al., 1987; Horwitz et al., 1985; Ignatius and Reichardt, 1988; Johansson et al., 1987; Pytela et al., 1985a, 1986; Takada et al., 1988; Wiersma et al., 1988), and other types of molecules, such as proteoglycans, can also mediate cell attachment to fibronectin (LeBaron et al., 1988; Saunders and Bernfield, 1988). If more than one receptor exists for tenasin, differences in the source of tenasin, the methods used in its isolation, the cell type employed in the assays and the details of the assay methodology may favor one receptor over others, explaining the divergent observations by different laboratories. From our results it ap-
pears that one mechanism whereby cells can interact with tenascin is through an RGD-dependent integrin-type receptor.

The tenascin receptor we have isolated has a β subunit that is similar and possibly identical to the fibronectin receptor β subunit. This β subunit is shared by at least six different integrins that together comprise the fibronectin receptor or VLA family within the integrin superfamily (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Hemler et al., 1987; Takada et al., 1988). Like the other integrins, the tenascin receptor appears to be a heterodimer of the β subunit and an α subunit, because the two subunits coprecipitated upon immunoprecipitation with antibodies reactive with the β subunit. The α subunit of the tenascin receptor is clearly distinct from the fibronectin receptor α subunit, but whether it might be identical to one of the many other known integrin α subunits will have to be determined in future studies. It will also be important to determine whether tenasin is the only ligand for the receptor we have identified. The affinity chromatography results presented here suggest that it does not bind to fibronectin and that it has a specificity different from that of the vitronectin receptor. However, the lack of sufficient quantities of the receptor has so far precluded more detailed specificity studies by assays such as the liposome assay used with other receptors (Pytela et al., 1985a,b).

A functional difference between tenascin and fibronectin or vitronectin is suggested by the differences in cell morphology seen as cells attach to these proteins. Since tenascin is selectively expressed in developing organs and in tumors (Aufderheide et al., 1987; Bourdon et al., 1983; Chiquet-Ehrismann et al., 1986; Grumet et al., 1985), the interaction of cells with tenascin may have a special significance in cell differentiation, proliferation, and migration. Our identification of the first receptor for tenascin will facilitate studies on the involvement of tenascin in these phenomena. The sensitivity of this receptor to inhibition by RGD peptides indicates that these peptides can be used to probe its functional role without substantially affecting the functions of other integrins.

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