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; Ekblom, 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., 1985; Pytel et al., 1985a; Pytel et al., 1985b; Pytel et al., 1986; Takada et al., 1989; Takmunk et al., 1986; Tomaselli 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-Ehriismann et al., 1986). This glycoprotein has been described as GEMM (Bourdon et al., 1983) cytotactin (Grumet 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 (>10 kD) disulfide-bonded oligomer (Bourdon et al., 1983; Bourdon et al., 1985). In rotary shadowing images tenascin appears as a hexameric structure (Erickson and Taylor, 1987; Vaughn et al., 1987). This structurally unusual matrix molecule is further distinguished by its highly selective oncdevelopmental 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-Ehriismann 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-
The selective on-codevelopmental expression of tenasin 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 tenasin has RGD-dependent cell adhesion activity and describe an integrin-type cell surface receptor that binds to tenasin with the same RGD-dependent specificity as the cells.

Materials and Methods

Cell Culture

Tumor cell lines and normal fibroblasts were cultured in DMEM 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 DMEM, supplemented with 20% FBS, heparin, and endothelial cell growth factors (Collaborative Research, Lexington, MA). Adherent cell lines were passaged with 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 DMEM containing 2 mg/ml BSA and plated at 2 × 10⁴ 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 non-specific binding sites before their use in cell attachment assays. Peptides added to the cell attachment assays were dissolved in DMEM. 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 3% paraformaldehyde and stained with 0.5% toluidine blue. Adherent cells were either 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 Tenasin

Tenasin was purified from the spent culture media of U251MG human glioma cells by affinity chromatography on an 8IC6 anti-tenasin 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 tenasin 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 tenasin preparations was monitored by SDS-PAGE analysis on 7% acrylamide gels followed by Coomassie Blue or silver staining, by HPLC chromatography on a TSK-400 column (7.5 x 60 mm), and by ELISA. Fibronectin and vitronectin were purified from human plasma 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).

Results

Isolation and Characterization of Tenasin

Human tenasin was isolated from spent culture media of U251MG human glioma cells by 8IC6 monoclonal antibody affinity chromatography. The purified tenasin migrated as a single prominent band at ~250 kD in SDS-PAGE under reducing conditions (Fig. 1A). Unreduced tenasin behaved as a high molecular mass (>10⁶ kD) disulfide bonded oligomer both in SDS-PAGE and HPLC TSK 400 sizing chromatography (not shown). These results identify the isolated protein as highly purified tenasin.
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 8IC6-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.

Human M21 melanoma cells, F9 mouse embryonic carcinoma cells and cells of lymphoid origin (EL4, WR.1, thymocytes), and monocytic U937 cells adhered poorly or not at all to tenascin. Results for the attachment of U251MG cells to tenascin are shown in Figs. 2 and 3. The morphology of cells adhering to tenascin was distinctly different from the morphology of cells adhering to fibronectin or vitronectin. The U251MG cells generally assumed a more polar, elongated morphology on tenascin with larger numbers of cellular extensions and less extensive spreading than they did on fibronectin (Fig. 2) or on vitronectin (not shown). Cells remained attached to tenascin-coated wells for periods of up to at least 24 h. Cells cultured for 24 h on fibronectin or vitronectin appeared indistinguishable from cells adhering to tenascin. Despite the reduced cell spreading observed for cells adhering to tenascin, the level of cell attachment to tenascin was found to be similar to the level of attachment to fibronectin. As shown for the U251MG cells in Fig. 3, the cell attachment titration curves for tenasin and fibronectin closely paralleled one another with maximum cell attachment (75–85% of cells added) on either tenascin or fibronectin occurring at a coating concentration of 3 μg/ml protein and higher. The similarity of the attachment efficiencies of the two proteins indicated that the attachment of cells to tenascin was not due to contamination of tenascin by fibronectin. This was further supported by the finding that there was no detectable fibronectin in tenascin samples as tested by ELISA. Moreover, antitenascin antibodies blocked cell attachment to tenascin but not to fibronectin (Fig. 4), whereas antifibronectin antibodies inhibited cell attachment to fibronectin, but not to tenascin. Neither type of antibody inhibited the attachment of cells to vitronectin.

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 GRGES (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).
Figure 3. 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 $2 \times 10^4$/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.

The decrease of the size of the larger (α) subunit on reduction indicated that 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 β subunit had the same chain mobility as the fibronectin receptor β 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-Sepharose column. Moreover, the tenascin receptor could be isolated on tenascin-Sepharose even after the U251MG extract was first passed over GRGDSP-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.

Figure 4. Inhibition of tenascin-mediated cell attachment by antitenascin 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 of 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.
imactic). (B) Comparison of 125I-labeled tenascin receptor and/3 subunits were both immunopre-
affinity isolation of tenascin receptor. Tenascin receptor was isolated on tenascin-Sepharose from 125I cell surface labeled
Figure 6. Affinity isolation of tenascin receptor. Tenascin receptor was isolated on tenascin-Sepharose from 125I cell surface labeled U251MG cells, solubilized in 50 mM octylglucoside, 1 mM CaCl2, 1 mM MgCl2 (lanes 1, final wash fraction). Fraction volumes were 0.5 ml. Samples of each fraction were subjected to electrophoresis on a 7% SDS-acrylamide gel under reducing conditions and autoradiographed. (B) Comparison of 125I-labeled tenascin receptor subjected to electrophoresis on a 7.5% SDS-acrylamide gel under reducing (1) and nonreducing (2) conditions. Molecular mass markers in kilodaltons are shown.
and to identify the /3 subunit of the tenascin receptor. The tenascin receptor a and /3 subunits were both immunopre-
cipitated with antifibronectin receptor /3 subunit polyclonal antibodies, suggesting an immunologic identity between the /3 subunits of these receptors (Fig. 7, left). Receptor immunobLOTS with an antifibronectin receptor /3 subunit monoclonal antibody confirmed the immunological similarity between the tenascin receptor and fibronectin receptor /3 subunits (Fig. 7). Based on these results it appears that the two receptors share the same /3 subunit. The a subunit of the tenascin receptor was not reactive with an antibody against the fibro-
nectin receptor a subunit and neither subunit of the tenascin receptor bound anti-vitronectin receptor antibodies.

Discussion

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

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

Tenascin 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 tenascin by RGD peptides. These results imply the presence of an RGD sequence within the tenascin polypeptide and, indeed at least chicken cytotactin (tenascin) does contain this sequence (Jones et al., 1988).

Previous work on the cellular interactions of tenascin has indicated that tenascin 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 tenascin is distinct from that of fibronectin and vitronectin in that little cell spreading is seen on tenascin. The cell attachment-inhibiting activity observed by others could be an indication that the cell—tenascin 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 tenascin 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 tenascin, such a role has been proposed for thrombospondin (LaBav, 1988), which may also interact with cells in an RGD-dependent manner (Lawler and Hynes, 1986).

We find that the cell—tenascin interaction can be inhibited with an RGD peptide, and others have found that soluble tenascin 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 tenascin. The reasons for this difference are not known, but it may be that cells can also bind to tenascin 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 (Learon et al., 1988; Saunders and Bernfield, 1988). If more than one receptor exists for tenascin, differences in the source of tenascin, 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-
Figure 7. Immunoprecipitation and immunoblotting analysis of tenascin, fibronectin and vitronectin receptors. Immunoprecipitation (far left): Affinity-purified tenascin receptor (TNR) was immunoprecipitated with polyclonal antibodies to the fibronectin receptor β subunit and analyzed by SDS-PAGE on a 7.5% acrylamide gel under nonreducing conditions. Immunoblots: Affinity-purified tenascin receptor (TNR), fibronectin receptor (FNR), and a vitronectin receptor (VNR) all from the U251MG cells were subjected to electrophoresis as above and electrobotted onto nitrocellulose membranes for immunoblotting. The antibodies used were an antifibronectin receptor β subunit monoclonal antibody 442 (Anti-FNRβ; Cheresh, D., J. O. Gailit, and E. Ruoslahti, unpublished results), a rabbit antibody prepared against the cytoplasmic peptide of the fibronectin receptor α subunit (Anti-FNRα; Argraves, W. S., and E. Ruoslahti, unpublished results), and polyclonal antivitronectin receptor antibodies (Anti-VNR). Approximately equal amounts of receptor protein were blotted as estimated by Ponceau-S staining before immunoblotting. Relative positions of α and β subunits are indicated.

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 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 tenascin 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.

We thank Drs. Scott Argraves, James Gailit, and David Cheresh for receptor antibodies, and Dr. Douglas Fambrough for antisera to chick tenascin. This work was supported by grants CA45586 (to M. A. Bourdon), CA42507, CA28896 (E. Ruoslahti), and Cancer Center Support Grant CA 30199 from the National Cancer Institute.

Received for publication 18 June 1988 and in revised form 8 November 1988.

References
