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Glial tumor cell adhesion is mediated by binding of the FNIII domain of receptor protein tyrosine phosphatase β (RPTPβ) to tenascin C

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The extracellular domain of receptor protein tyrosine phosphatase β (RPTPβ) is composed of several domains which mediate its interactions with distinct ligands present on the surface of either neurons or glial cells. Here, we demonstrate that the fibronectin type III domain (FNIII) of RPTPβ binds to glial tumor-derived cell lines and primary astrocytes. We used affinity purification to isolate several proteins that specifically bind to the FNIII domain of RPTPβ. One of these, a 240 kDa protein that was purified from U118MG glioblastoma cell, was identified as tenascin C based on the amino acid sequence of several tryptic peptides. The interaction of RPTPβ with tenascin C was found to mediate cell adhesion. Adhesion and spreading of SF763T astrocytoma cells expressing RPTPβ on tenascin C was specifically abolished by the addition of a soluble fragment containing the FNIII domain of the receptor. RPTPβ-dependent cell adhesion was mediated by binding to the alternatively spliced FNIII repeats A1,2,4 (TnfnA1,2,4) of tenascin C. Furthermore, COS cells expressing RPTPβ adhere to TnfnA1,2,4, while the parental cells did not. These results demonstrate that the FNIII domain of RPTPβ binds to tenascin C and suggest that RPTPβ present on glial tumor cells is a primary adhesion receptor system to the extracellular matrix. *Oncogene* (2001) **20,** 609–618.

Keywords: tyrosine phosphatase; extracellular matrix; cell adhesion molecule

Introduction

The interactions between tumor cells and the extracellular matrix is important for neoplastic transformation and cell invasion. Protein tyrosine phosphorylation regulates various signal transduction pathways underlying fundamental cellular processes such as cell motility, growth and differentiation (Angers-Loustau *et al*., 1999; Neel and Tonks, 1997; Stoker and Dutta, 1998). The extracellular domain of most of the known receptor-like protein tyrosine phosphatases (RPTPs) shares structural similarities with cell adhesion molecules suggesting that they play a role in cell-cell and cell-matrix communication by directly coupling cell recognition events to signal transduction pathways within the cell (Brady-Kalnay and Tonks, 1995; Schaapveld *et al*., 1997; Zondag and Moolenaar, 1997).

Receptor protein tyrosine phosphatase β (RPTPβ, also known as RPTPζ) contains in its extracellular domain a region with sequence homology to the enzyme carbonic anhydrase (CAH), followed by a fibronectin type III repeat (FNIII) and by a long unique sequence termed the spacer domain (Krueger and Saito, 1992; Levy *et al*., 1993). Three different isoforms of RPTPβ are generated as a result of alternative splicing: a short and a long receptor form that differ by the absence or presence of a stretch of 860 amino acid residues in the spacer domain, and a third secreted form composed of the extracellular domain of the long receptor known also as phosphacan, 6B4 and DSD-1 proteoglycans (Barnea *et al*., 1994; Garwood *et al*., 1999; Levy *et al*., 1993; Maeda *et al*., 1994). Both the long receptor form and phosphacan are found in rat brain as chondroitin sulfate proteoglycans, whereas the short receptor form (dvRPTPβ) is lacking glycosaminoglycan side chains (Sakurai *et al*., 1996). RPTPβ is predominantly expressed in the nervous system where it is mainly found in glial precursors, radial glia and astrocytes (Canoll *et al*., 1993), as well as in certain neurons (Snyder *et al*., 1996). The expression of the different forms of RPTPβ is regulated during development of the glial lineage. Early in development, high levels of the receptor forms are found in proliferating precursor cells at the subventricular zones. As development progresses and cells mature, these receptor forms are replaced by the secreted non-phosphatase form (Canoll *et al*., 1996; Sakurai *et al*., 1996). The short receptor form of RPTPβ is found in certain astrocytomas and glioblastomas and may represent transformed early proliferating glial precursors (Krueger and Saito, 1992; Sakurai *et al*., 1996). RPTPβ is also found in human cutaneous melanomas which developmentally originate from the neural crest (Goldmann *et al*., 2000).

The different forms of RPTPβ bind to multiple ligands (Margolis and Margolis, 1997; Peles *et al*., 1998; Oohira *et al*., 2000). It has been previously shown that phosphacan interacts directly with the neuronal recognition molecules N-CAM, Ng-CAM (Milev *et al*., 1994) and TAG-1 (Milev *et al*., 1996), with the extracellular matrix molecules tenascin C and

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tenascin R (Barnea *et al*., 1994; Grumet *et al*., 1994; Milev *et al*., 1995; Xiao *et al*., 1997), and with several heparin-binding growth factors, including HB-GAM (Maeda *et al*., 1996) amphoterin (Milev *et al*., 1998a) and bFGF (Milev *et al*., 1998b). In contrast, the non-proteoglycan short receptor form was found to interact with the GPI-linked molecule contactin (Peles *et al*., 1995) and with lower affinity with Ng-CAM, Nr-CAM and N-CAM (Sakurai *et al*., 1997). The multifunctional interaction of phosphacan and RPTPβ with different ligands involves different domains of the receptor and, at least for phosphacan, depends on the presence of chondroitin sulfate or is mediated by N-linked oligosaccharides (Peles *et al*., 1998).

Tenascin C is a multifunctional protein that has been implicated in glial cell adhesion and migration (Giese *et al*., 1996; Zagzag *et al*., 1995). Although the direct interaction between phosphacan and tenascin C suggests a novel mechanism for matrix-cell signaling and raises the possibility that RPTPβ may modulate cell adhesion, it is presently unknown whether, like phosphacan, the short receptor form also binds to extracellular matrix proteins such as tenascin C. This is of particular interest as the short receptor form is found in early migratory glial precursors and in tumor cells (Canoll *et al*., 1996). We have previously found that while the CAH domain of RPTPβ binds to contactin present in neurons, the FNIII domain of this phosphatase may bind a distinct ligand present on the surface of glial cells (Peles *et al*., 1995). Here we extend these observations and identify this glial ligand as tenascin C. The interactions between RPTPβ and tenascin C mediate the adhesion of glial tumor cells and involve the FNIII domains of both molecules.

Results

The FNIII domain of RPTPβ mediates its binding to glial cells

The extracellular domain of RPTPβ is composed of several domains that mediate its interactions with distinct ligands that are present in neuronal and non-neuronal cells (Peles *et al*., 1998). To further identify such ligands, we produced soluble proteins that contained different subdomains of RPTPβ fused to human IgG (Figure 1a). These recombinant proteins were used in binding experiments using glioblastoma (U251MG, U373MG, C6) and astrocytoma (SF763T, U118MG) cell lines. As depicted in Figure 1b, all constructs containing the FNIII domain of RPTPβ bound well to these cell lines. No binding was detected with the carbonic anhydrase domain (βC), the spacer region (βS), or when an Fc-fusion protein containing the carbonic anhydrase and the FNIII domains of the related phosphatase RPTPγ was used (Figure 1b and data not shown). Similar results were obtained using cultured primary astrocytes (Figure 2a). Staining of GFAP-positive astrocytes with βF-Fc revealed a punctate staining pattern on the surface of the cells (Figure 2b). In agreement with our binding analysis, no staining of astrocytes was detected with βC-Fc. In contrast, βC-Fc intensely labeled few oligodendrocytes that were occasionally present in the cultures. Given that contactin is expressed by oligodendrocytes (Koch *et al*., 1997), it is likely that this staining represents the interaction between the carbonic anhydrase domain of RPTPβ and contactin in these cells (Peles *et al*., 1995). Previous *in situ* hybridization and immunohistochemistry data suggested that RPTPβ is expressed by astrocytes (Canoll *et al*., 1996). As depicted in Figure 2b, we confirmed the presence of the receptor form of RPTPβ in our cultures by staining with anti-RPTPβ specific antibody, as well as with a contactin-Fc fusion protein. Altogether, these analyses revealed the presence of a specific ligand for the FNIII domain of RPTPβ on the surface of various glial-derived cell lines and primary cultured astrocytes.

Identification of a glial ligand for RPTPβ

In order to isolate a ligand for the FNIII domain of RPTPβ from glial cells, membrane proteins were prepared from ^{35}S-methionine-labeled cell lines and incubated with βF-Fc or βC-Fc as a control (Figure 3a). Several distinct sets of proteins specifically bound to the βF-Fc affinity matrix from the different cell lines. In SF763T and C6 cells, βF-Fc precipitated four proteins with the apparent molecular weight of 160, 170, 200 and 210 kDa, while two proteins of 220 and 240 kDa were detected from U138MG and U118MG cells. These proteins were not detected using βC-Fc, indicating that they interact specifically with the FNIII domain of RPTPβ. Furthermore, βF-Fc precipitated these proteins only from glial-derived, but not from several neuroblastoma (neuro 2A and H4) or neuroendocrine (GH3) cell lines. In contrast, βC-Fc precipitated a 140 and a 190 kDa proteins from GH3 that were identified as contactin and Caspr respectively (Peles *et al*., 1995, 1997). We next used βF-Fc to purify the 240 kDa protein from U118MG cells (Figure 3b). The 240 kDa protein was excised from the gel, digested with trypsin and the resulting peptides were separated by HPLC (data not shown). Selected peptides were analysed by automated Edman degradation. The sequence of one of the peptides (PVLSAEASTGETPNLGEVV) was identical to positions 1151–1169 of human tenascin C (accession #P24821). All other sequenced peptides were also derived from human tenascin C (data not shown). Furthermore, we have confirmed the identity of both the 220 and 240 kDa proteins isolated by βF-Fc from U118MG cells as tenascin C by immunoblot analysis (Figure 3c). In conclusion, the FNIII domain of RPTPβ binds to several distinct ligands present in glial cells, two of which were identified as isoforms of the extracellular matrix protein tenascin C. The identity of the other proteins is yet to be determined.

RPTPβ is expressed in glial tumor cell lines

The identification of tenascin C as a glial ligand for RPTPβ raises the possibility that it may modulate the

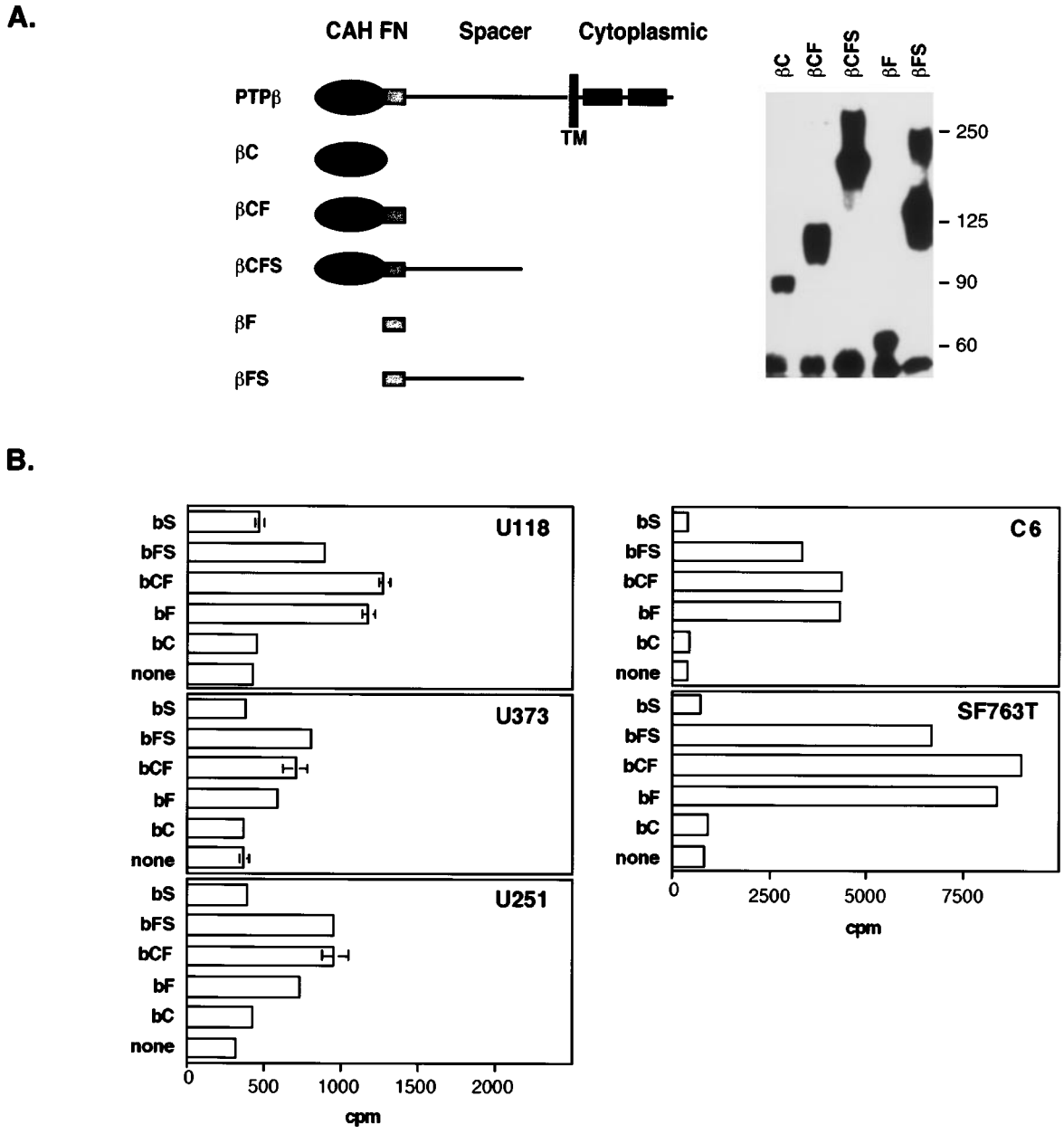

Figure 1 Binding of different subdomains derived from the extracellular region of RPTPβ. (**a**) Schematic presentation and expression of the RPTPβ Fc-fusion proteins. Abbreviations: C - carbonic anhydrase domain, F - fibronectin type III, S - spacer domain. Anti-human Fc immunoblot of the Fc-fusion proteins is shown on the right along with molecular weight markers in kDa. (**b**) Binding of the RPTPβ Fc-fusion proteins to different glial tumor-derived cell lines. Cells were incubated with the indicated Fc-fusion proteins. Bound proteins were detected using ^{125}I-labeled protein A as described in Materials and methods. An average of two sets of experiments done in triplicates is shown

binding of RPTPβ present in these cells with other ligands. We first examined the expression of RPTPβ in the various cell lines. Immunoblot analysis revealed that the short receptor form of RPTPβ was found in U251MG, SF763, SF763T, U373MG, C6 and SF767T (Figure 4a). No expression of RPTPβ transcript or protein was detected in U118MG (Figure 4a and b) or U138MG cells (data not shown), the two cell lines from which tenascin C was precipitated by βF-Fc. We next compared the cell surface expression of RPTPβ in several cell lines using a specific antibody against its extracellular domain with the binding of a soluble contactin-Fc, a known ligand of the carbonic anhydrase domain (Peles *et al*., 1995). As depicted in Figure 4c, strong binding of anti-RPTPβ antibody was detected in SF763T, C6, U251MG and U373MG cells, indicating that RPTPβ is expressed on the surface of these cells. However, in contrast to the antibody binding which was equivalent in all RPTPβ-expressing cells, we observed higher levels of Contactin-Fc binding

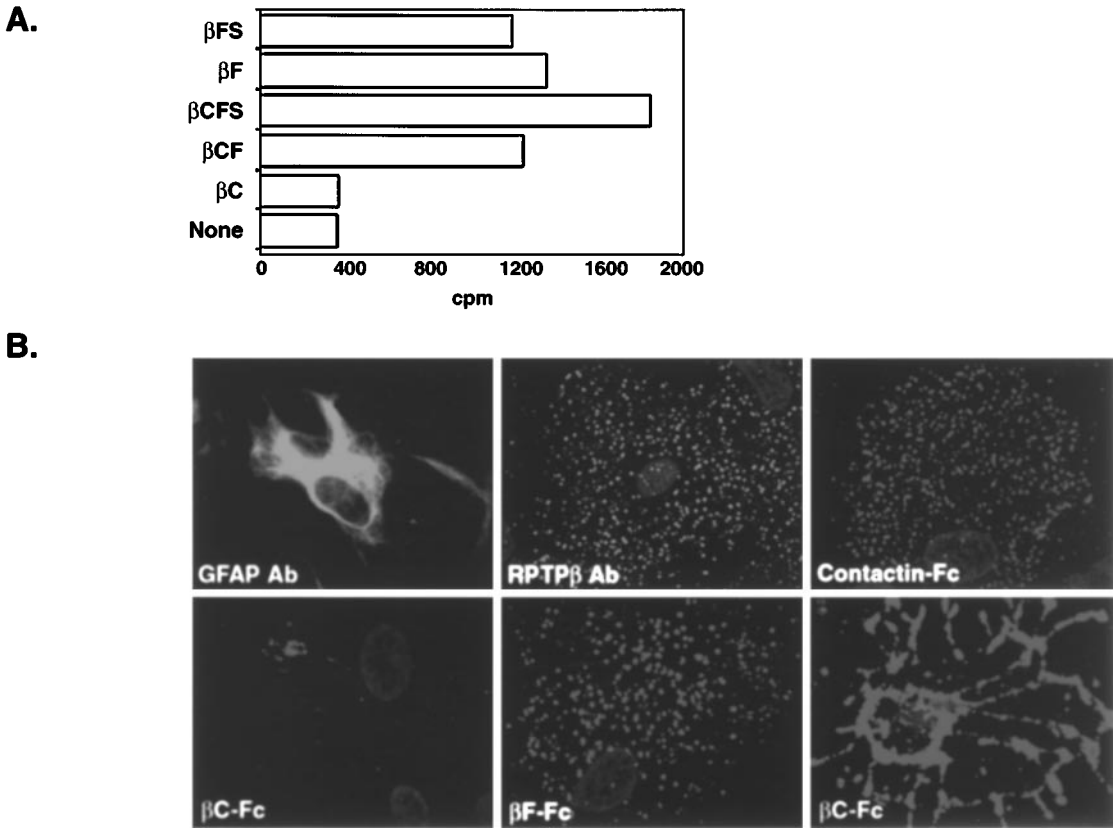

Figure 2 The fibronectin type III domain of RPTPβ binds to cortical astrocytes. (**a**) Binding of the different RPTPβ Fc-fusion proteins to cultured astrocytes. Binding was done as described in the first figure. Note that binding pattern is similar to that obtained using glial-derived cell line. (**b**) Astrocytes were stained with antibodies against GFAP, RPTPβ, or using βC-Fc, βF-Fc and contactin-Fc fusion proteins as indicated. Staining of GFAP-positive astrocytes with anti-RPTPβ revealed a punctate staining. Similar staining pattern was obtained with contactin-Fc fusion protein. The βF-Fc protein binds to astrocytes, whereas the carbonic anhydrase domain (βC-Fc) labeled only few oligodendrocytes that were present in the culture (lower right panel)

to SF763T and C6 cells than to U251MG and U373MG. Given that U251MG and U373MG, but not the SF763T and C6 cell lines we used produce tenascin C (Figure 4d), these results may suggest that the binding of contactin to RPTPβ is modulated by tenascin C binding to RPTPβ in cells which express both proteins.

RPTPβ mediates adhesion of SF763T cells to tenascin C

Tenascin C is an extracellular matrix component that is implicated in cell adhesion and migration (Faissner, 1997; Jones and Jones, 2000). To determine the functional consequence of the binding of RPTPβ to tenascin C, we have performed a series of adhesion assays. As shown in Figure 5, SF763T cells adhere and spread on polystyrene dishes precoated with either laminin or tenascin C. Adhesion of SF763T cells to tenascin C occurred within less than a minute of plating and did not require calcium (data not shown). No adhesion was observed on an uncoated surface (Figure 6), or when BSA was used as a substrate (data not shown). Cell adhesion and spreading on tenascin C, but not on laminin, was completely inhibited by the addition of a soluble Fc-fusion protein containing the carbonic anhydrase and the FNIII domains of RPTPβ. In contrast, an Fc-fusion protein containing the carbonic anhydrase and the FNIII domains of the highly related RPTPγ, as well as a fusion protein containing the CAH domain of RPTPβ, had no effect on cell adhesion (Figure 6 and data not shown). Furthermore, the effect of the FNIII domain of RPTPβ on adhesion to tenascin C was specific to cells that express this receptor. For example, although U118MG cells that do not express RPTPβ adhere to tenascin C, this adhesion was not effected by the addition of soluble RPTPβ fusion proteins (data not shown). These results demonstrate that RPTPβ mediates adhesion of SF763T cells to tenascin C.

SF763T adhesion involves two distinct domains of tenascin C

In order to examine which domain of tenascin C is involved in RPTPβ-dependent cell adhesion, we have used various recombinant domains of the protein as a substrate. As shown in Figure 6, SF763T cells adhere and spread on FNIII domains 1-3 (TNfn1-3) and FNIII domains A1,A2,A4 (TnfnA1,2,4). No adhesion of SF763T cells was detected when the EGF,

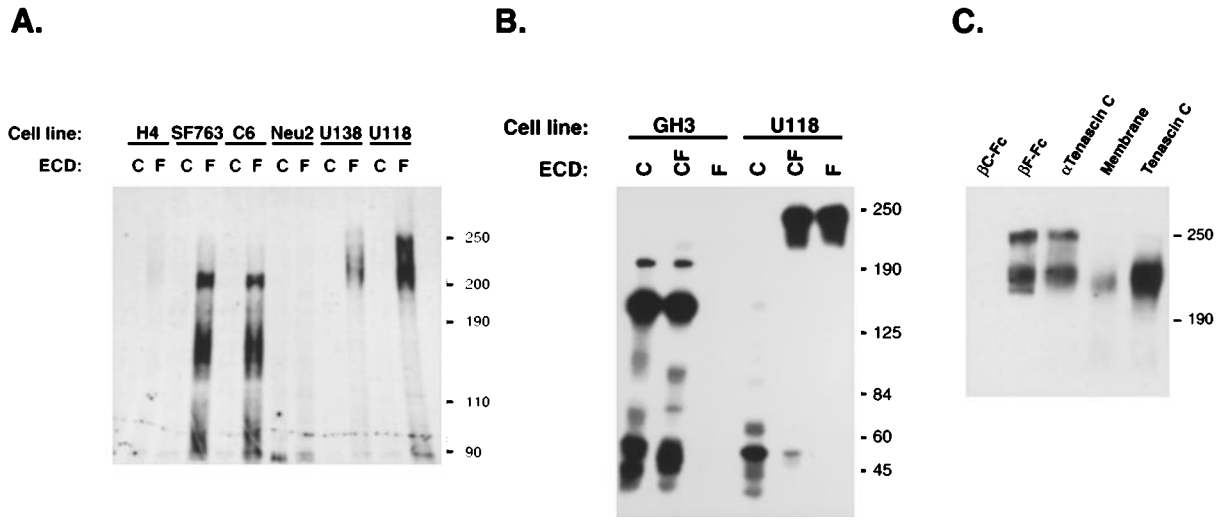

Figure 3 Receptor mediated ligand precipitation (RMLP) of membrane proteins from glial cell lines using the extracellular domain of RPTPβ. (**a,b**) Membrane protein lysates from ^{36}S-methionine-labeled H4 neuroglioma, SF763T, C6, U138MG and U118MG glioblastomas, and Neuro 2A neuroblastoma cells were subjected to precipitation with βC-Fc (C) or βF-Fc (F) as indicated at the top (ECD). The protein complexes were washed as described in Materials and methods and resolved on SDS–PAGE. Autoradiogram of the gel is shown along with the location of molecular weight markers in kDa. The FNIII domain precipitated different sets of proteins from glial but not neuro-derived cell lines. The 140 and 190 kDa proteins precipitated by βC-Fc GH3 cells represent contactin and caspr complex. The 220–240 kDa protein from U118MG membranes (shown in **b**) was used for protein sequencing. (**c**) Identification of the 220 and 240 kDa proteins precipitated with βF-Fc as tenascin C isoforms. Solubilized membrane proteins from U118MG cells were subjected to precipitation with βC-Fc, βF-Fc, or an antibody to tenascin C (αTenascin C) as indicated at the top of each lane, followed by immunoblot with an antibody against tenascin C. Membrane lysate (membrane) and purified tenascin C (Tenascin C) were used as control

fibrinogen, or several other FNIII domains of tenascin C were used as a substrate. We next examined whether adhesion of SF763T cells to Tnfn1-3 or TnfnA1,2,4 could be inhibited by the addition of a soluble βCF-Fc. As shown in Figure 7, βCF-Fc completely abolished adhesion and cell spreading on tenascin C and on TnfnA1,2,4, but not when Tnfn1-3 was used as a substrate.

We next determined whether expression of RPTPβ in transfected cells will enable cell adhesion to the TnfnA1,2,4 domains of tenascin C. As depicted in Figure 8, COS-7 cells adhere to tenascin C, as well as to its first three FNIII repeats (Tnfn1-3), but not to its TnfnA1,2,4 domains. In contrast, COS-7 cells expressing the short receptor form of RPTPβ, also adhere and spread when the TnfnA1,2,4 domains of tenascin C were used as a substrate. Furthermore, similar to SF763T cells, the adhesion of RPTPβ-transfected cells to the TnfnA1,2,4, but not to Tnfn1-3 could be blocked by the addition of a soluble βF-Fc fusion protein (data not shown). Altogether, these results demonstrate that the short receptor form of RPTPβ functions as a cell adhesion receptor for tenascin C.

Discussion

Recent studies have suggested that receptor protein tyrosine phosphatases play a role in cell–cell and cell–matrix interactions. RPTPμ, RPTPκ and RPTPδ have been shown to mediate homophilic binding (Brady-Kalnay *et al*., 1993; Sap *et al*., 1994; Wang and Bixby, 1999); PTP-LAR interacts with the nidogen-laminin complex (O'Grady *et al*., 1998) and galectin was recently identified as a ligand for CD45 (Walzel *et al*., 1999). RPTPs were also found to interact with cell adhesion molecules present on the same plane of the plasma membrane to create multi-protein complexes (Fashena and Zinn, 1997; Zeng *et al*., 1999) and to regulate cell adhesion (Shenoi *et al*., 1999). Finally, studies demonstrating that several RPTPs interact with cytoskeletal proteins (Brady-Kalnay *et al*., 1995; Serra-Pages *et al*., 1995, 1998) further support the possible role of these receptors as mediators of cell–matrix interactions. We have previously shown that various domains found in the short receptor form of RPTPβ differentially mediate the binding of this receptor to distinct ligands found in neurons and glial cells (Peles *et al*., 1995). The carbonic anhydrase-like domain of RPTPβ interacts with the neuronal GPI-linked cell adhesion molecule contactin, leading to cell adhesion and neurite outgrowth of contactin-expressing cells. In the present study, we have identified the glial ligand that binds the FNIII domain of RPTPβ as tenascin C. We further show that glial-derived tumor cells expressing this receptor adhere and spread on tenascin C, suggesting that RPTPβ function as an adhesion molecule.

Does the binding of tenascin C to RPTPβ modulate the interaction of this receptor with other ligands? Tenascin C is a multi-domain protein, which interacts with several adhesion systems including members of the Ig-superfamily neuronal adhesion molecules (Milev *et al*., 1996; Zisch *et al*., 1992), integrins (Giese *et al*.,

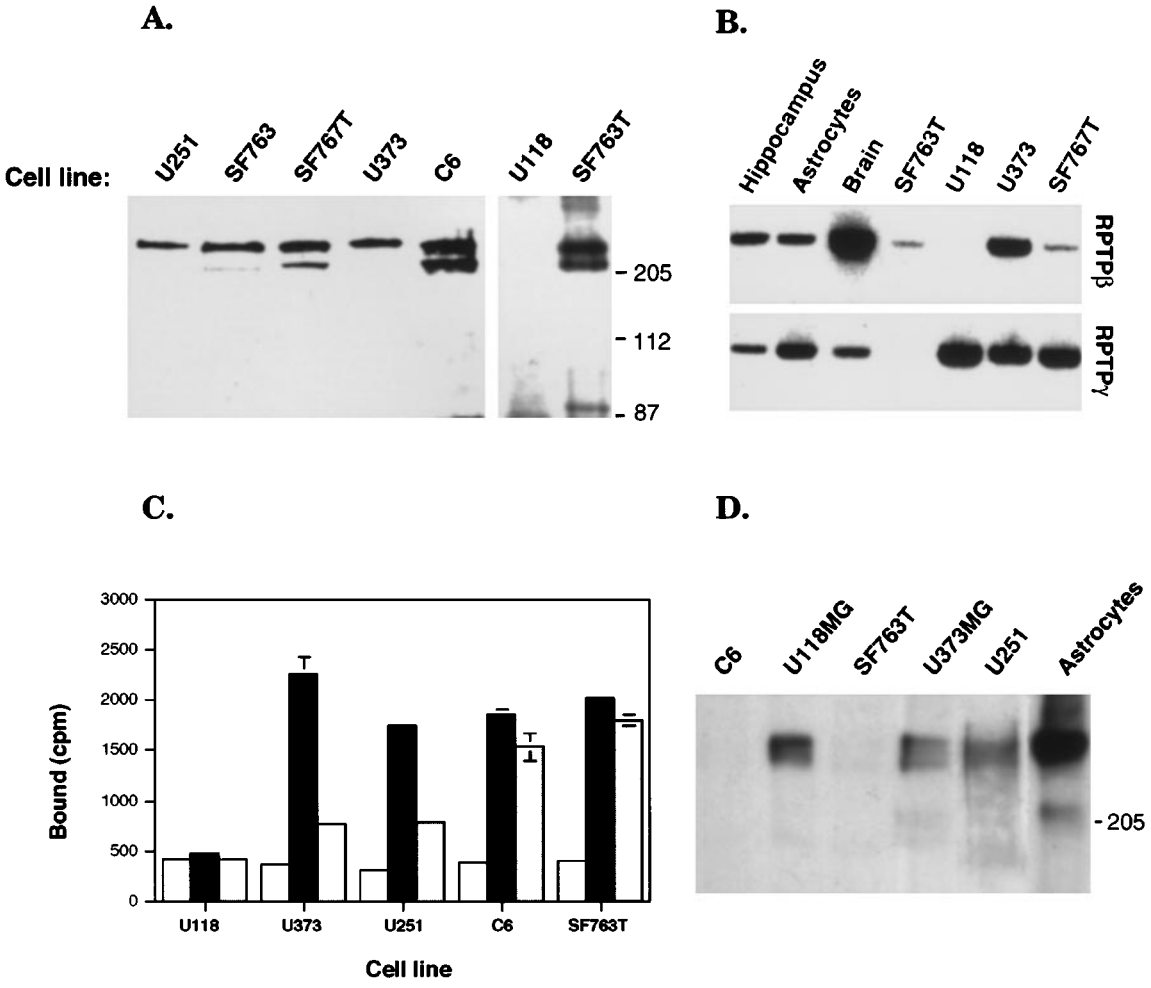

Figure 4 RPTPβ is expressed in human glial tumor-derived cell lines. (**a**) Cell lysates from the various cell lines were subjected to immunoprecipitation and immunoblotting with anti- RPTPβ antibody. The short receptor form of RPTPβ appears as a doublet on the gel. Molecular weight markers are shown in kDa on the left. (**b**) RT–PCR analysis of RPTPβ expression. mRNA from human hippocampus, isolated astrocytes, brain, or several glioblastoma cell lines was extracted and used as a template to amplify RPTPβ or RPTPγ as indicated. (**c**) Binding of antibody directed to the extracellular region of RPTPβ. The different indicated cell lines were incubated with pre-immune serum as control (white bars), antibody against the extracellular region of RPTPβ (black bars), or with human contactin-Fc fusion protein (gray bars). Bound proteins were detected using ^{125}I-labeled anti mouse or protein A as described in Material and methods. An average of two sets of experiments done in triplicates is shown. (**d**) Expression of tenascin C in the various cell lines. Immunoprecipitation and immunoblot analyses were done using antibody against tenascin C as described in (**a**) for RPTPβ

1996; Phillips *et al*., 1998; Prieto *et al*., 1993; Schnapp *et al*., 1995; Yokosaki *et al*., 1998), phosphacan (Barnea *et al*., 1994; Grumet *et al*., 1994; Milev *et al*., 1995, 1997) and the short receptor form of RPTPβ (this study). Both tenascin C and RPTPβ interact with contactin (Peles *et al*., 1995; Revest *et al*., 1999; Zisch *et al*., 1992), suggesting that the binding of each pair (i.e., RPTPβ-contactin, RPTPβ-tenascin and contactin-tenascin) could potentially be regulated by the presence of the third component. Whether such regulation occurs competitively or cooperatively may depend on which specific domain within each protein is directly involved in binding. It has been shown that the first Ig domains in contactin mediate its binding to both tenascin C and phosphacan (Revest *et al*., 1999; Zisch *et al*., 1992). In contrast, contactin, phosphacan and RPTPβ bind to distinct sites in tenascin C. While contactin binds to the fifth and sixth fibronectin repeats of tenascin C (TNfn5-6) (Jones and Jones, 2000), and phosphacan binds to its fibrinogen domain (Milev *et al*., 1997), the short receptor for of RPTPβ interacts with TNfnA1,2,4. We have shown that two distinct sites in RPTPβ, the carbonic anhydrase and its FNIII domains mediate its binding to contactin and tenascin C, respectively (Peles *et al*., 1995; and this study). However, although RPTPβ could potentially bind contactin and tenascin simultaneously, we have observed reduced binding of contactin to cells which express both RPTPβ and tenascin C. This result suggests that the presence of tenascin may negatively regulate the binding of contactin to RPTPβ, and argue against the presence of a stable tertiary complex. This

is further supported by recent findings which have detected contactin-phosphacan and contactin-tenascin C, but not a tertiary complex that contains all of these molecules in rat brain (Revest *et al*., 1999). Taken together, these results suggest that the function of the short receptor form of RPTPβ in cell adhesion is highly regulated by a complex interplay of other cell adhesion molecules and extracellular matrix components such as tenascin C.

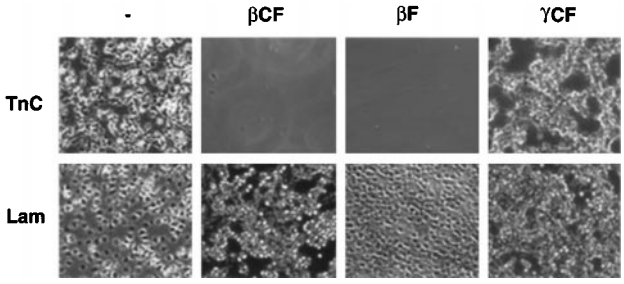

Figure 5 RPTPβ-dependent adhesion of SF763T cells to tenascin C. Polystyrene dish that had been precoated with tenascin C (TnC; upper panels), or laminin (Lam; lower panels) was further incubated with an Fc-fusion containing the carbonic anhydrase and the FNIII domains of RPTPβ (βCF), RPTPγ (γCF), the FNIII domain of RPTPβ (βF), or BSA (−) as indicated. SF763T cells (1×10^5 cells) were placed in the central region of the dish for 1 h and non-adhered cells were removed by gentle washing. The plate was fixed and viewed using inverted microscope. Magnification: × 20

We show that expression of the short receptor form of RPTPβ in COS-7 cells results in adhesion of these cells to the TNfnA1,2,4 domains of tenascin C, indicating that this phosphatase functions as a receptor system for cell adhesion. Previous experiments showed that the third FNIII repeat of tenascin C mediates cell binding through interaction with integrins (Prieto *et al*., 1993; Schnapp *et al*., 1995; Yokosaki *et al*., 1998). These interactions were also reported to be important for astrocytes adhesion and astrocytoma and glioma migration (Giese *et al*., 1996; Phillips *et al*., 1998). Using different domains of tenascin C as a substrate, we have found that adhesion of SF763T cells is mediated by TNfn1-3 and TNfnA1,2,4. Although these two distinct sites are involved in SF763T adhesion, only the adhesion on TnfnA1,2,4 could be inhibited by the FNIII domain of

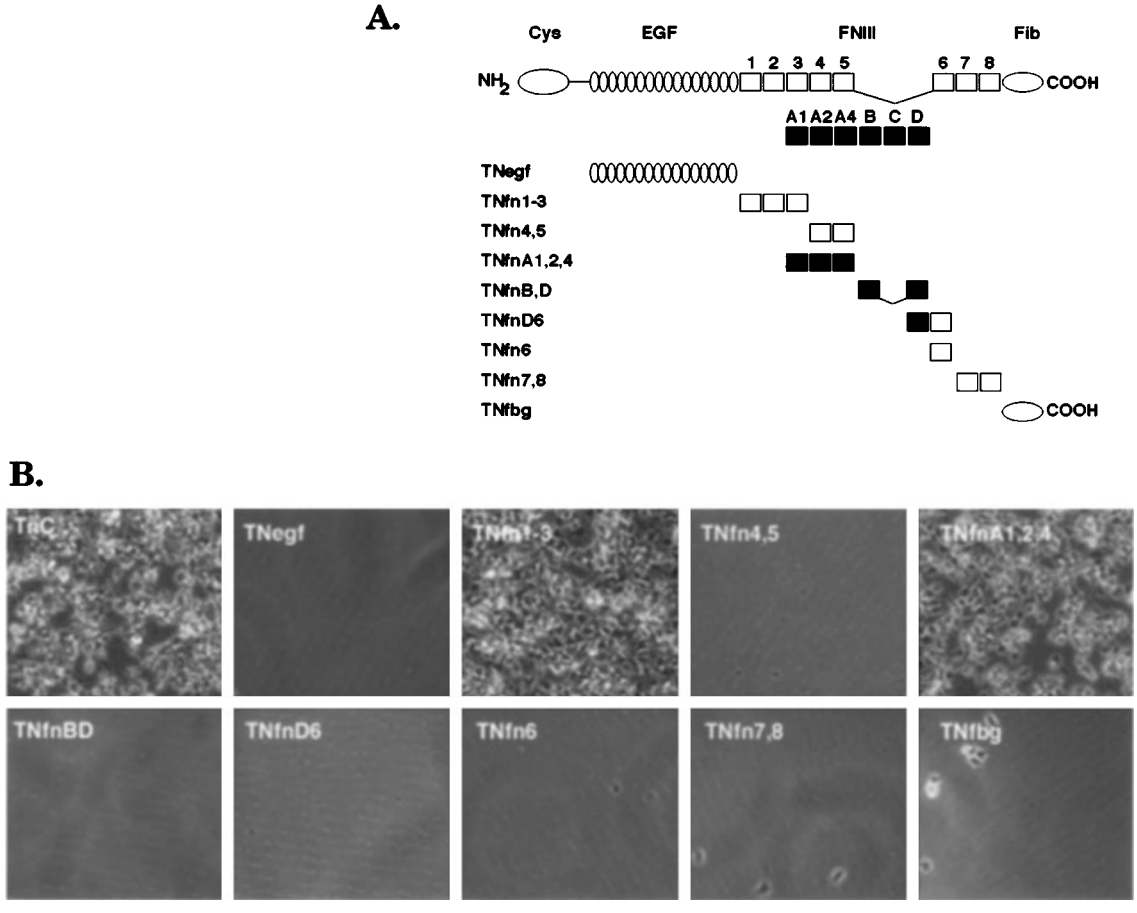

Figure 6 Adhesion of SF763T cells is mediated by two distinct sites in tenascin C. (**a**) Schematic representation of tenascin C and the different recombinant tenascin C fragments used in this study. (**b**) Adhesion of SF763T cells to different tenascin C domains. The indicated fragments (10 μg/ml) were bound to a polystyrene dish and adhesion assay was carried out exactly as described in the legend to Figure 5. Two distinct regions of tenascin C FNIII repeats mediated cell adhesion. While Tnfn1-3 is found in all known tenascin C proteins, TnfnA1,2,4 are alternatively spliced FNIII repeats that are inserted between the fifth and the sixth FNIII repeats only in several isoforms of tenascin C. Magnification: × 20

RPTPβ. Similar specificity was observed using COS-7 cells which express RPTPβ. Thus, it appears that adhesion of these cells is mediated by binding of RPTPβ to TNfnA1,2,4, and by binding of TNfn1-3 to another receptor system, most likely to integrins. Moreover, given that a soluble βFc also inhibits the adhesion of these cells to a full-length tenascin C molecule which contains both the TNfn1-3 and TNfnA1,2,4 domains, it is likely that the interactions of RPTPβ with tenascin C regulates cell adhesion mediated by other receptor systems. An additional receptor may be annexin II which is involved in epithelial and endothelial cell attachment by binding to TnfnA1,2,4 (Chung *et al*., 1996). One possible convergent point between RPTPβ and other adhesion receptor signaling may be via Rho family of small GTPases, which were recently shown to be regulated by tenascin C (Wenk *et al*., 2000). Whether RPTPβ affects Rho activation is yet to be determined.

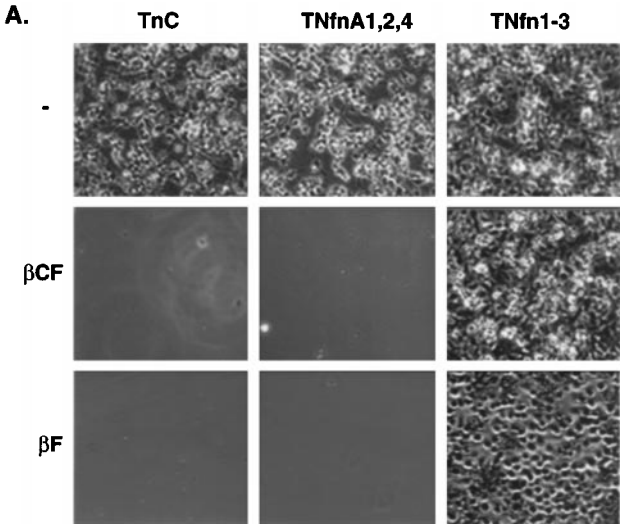

Figure 7 Identification of TnfnA1,2,4 as an RPTPβ-dependent adhesion site for glioblastoma cells. Polystyrene dishes were coated with 10 μg/ml tenascin C (TnC), TnfnA1,2,4, or the Tnfn1-3 fragments as indicated on the top. The prepared substrates were subsequently incubated with BSA control (−, upper panels), or with the Fc-fusion containing the carbonic anhydrase and the FNIII domains of RPTPβ (βCF, middle panels), or the FNIII of RPTPβ alone (βF, lower panels). Following blocking with BSA, SF763T cells were placed in the center of the dish and incubated for one hour. Non-adhered cells were removed by three washes and bound cells were fixed and photographed using a phase-contrast inverted microscope. Magnification: × 20

It has been previously shown that adhesion of glioblastoma cells to tenascin C is modulated by the secretion of phosphacan (Sakurai *et al*., 1996; Milev *et al*., 1997). Thus, a unique feature of this adhesion system is that it is regulated by the presence of the short receptor form of RPTPβ on the cell surface, as well as by the secretion of its proteoglycan form phosphacan. Given the high expression of tenascin C at the edges of glial tumors (Zagzag *et al*., 1995), this may have important implications for the progression of the disease. The extracellular matrix of glial tumors is involved in a variety of cell functions, such as cell attachment, migration and proliferation, which are mediated by complex interactions with cell surface receptors. The modulation of the interactions between RPTPβ and tenascin C would represent one example by which tumor cells remodel their environment by regulating their binding to extracellular matrix proteins.

Materials and methods

Proteins and antibodies

Production and purification of the different Ig-fusion was performed as described previously (Peles *et al*., 1995; Sakurai *et al*., 1997). The following recombinant human Ig-fusion proteins containing different domains of the extracellular regions of dvRPTPβ were used: βC (amino acids from 1–313), βCF (amino acids from 1–415), βF (amino acids from 301–414), βCFS (amino acids from 1–630), βFS (amino

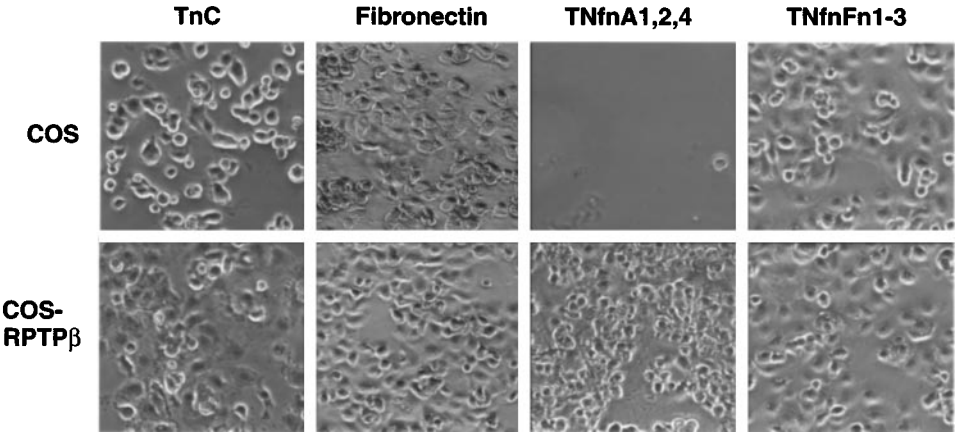

Figure 8 Expression of RPTPβ in COS cells results in cell adhesion to the TnfnA1,2,4 domains of tenascin C. COS-7 cells were transfected with RPTPβ (COS-RPTPβ) or without DNA (COS) were allowed to adhere to dishes precoated with either tenascin C (TnC), fibronectin, TnfnA1,2,4, or TNfn1-3 as indicated. After 1 h, non-adhered cells were removed by gentle washing and bound cells were fixed and viewed using inverted microscope. Magnification: × 20

acids from 301–630), and βS (amino acids from 415–630). The generation of recombinant tenascin C fragments was previously described (Scholze *et al*., 1996). Purified tenascin was a generous gift from Dr Martin Grumet.

Monoclonal antibodies to RPTPβ were purchased from Transduction Laboratories, Cy3 and FITC-conjugated secondary antibodies were from Jackson laboratory, anti GFAP from Sigma and anti-tenascin C antibody (AB1906) from Chemicon. Polyclonal antibody to the extracellular domain of RPTPβ was obtained by immunizing mice with purified βCF-Ig protein and clearing the serum from anti-human Fc antibodies by passing it through a normal human IgG-Sepharose column.

Cell culture

Growth and origin of the different cell lines used in this study were previously described (Peles *et al*., 1995). For culturing rat astrocytes, the cortex of newborn rat pups was dissected and dissociated by incubation with 0.25% trypsin (Gibco) and collagenase (Sigma) for 20 min. Cells were washed several times in DMEM + 10% fetal calf serum and triturated with a pasteur pipette. The cells were plated at 100 000 cells/cm^2 in 75 cm^2 Primaria flasks (Falcon) and grown in DMEM + 10% fetal calf serum. After 8 days, confluent cultures were shaken for 14 h at 37C$^\circ$ to remove oligodendrocyte precursors and the attached cells were trypsinized and replated. For immunofluorescence, cells were grown on glass coverslips precoated with 10 μg/ml polylysine for 3 days before the experiment.

Protein purification and sequencing

Analytical scale purification using the Ig-fusion proteins as affinity reagents was performed from ^{35}S methionine-labeled cells as previously described (Peles *et al*., 1997). In certain cases the complexes were resolved on an SDS gel and subjected to Western blotting with anti-tenascin C antibody. Immunoprecipitation and immunoblotting (Poliak *et al*., 1999) and protein purification were done essentially as described previously (Peles *et al*., 1995).

Ig fusion proteins and antibody binding and immunofluorescence

Binding of Ig-fusion proteins to cultured cell lines was done as described previously (Peles *et al*., 1995). Similar protocol was used for antibody binding experiments but ^{125}I-labeled anti-mouse IgG was used instead of protein-A. For staining experiments, astrocytes grown on coverslips in a 24-well dish were incubated with conditioned media containing the various Ig-fusion proteins for 2 h at ambient temperature. The dish was placed on ice and unbound material was removed by three consecutive washes with cold PBS. Slides were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 30 min and subsequently stained with Cy3-conjugated anti-human Fc antibody as described previously (Poliak *et al*., 1999). Immunofluorescence slides were viewed and analysed using a Deltavision wide-field deconvolution system (Applied Precision) connected to Zeiss fluorescence microscope.

Cell adhesion assays

SF763T cells were removed from the tissue culture plate by washing with PBS containing 10 mM EDTA, washed once with DMEM containing 4% BSA and resuspended in DMEM/F12. 1×10^5 cells in 0.5 ml of binding buffer were placed in the central region of 35 mm polystyrene dish that had been precoated with the various proteins. Substrates for cell adhesion were prepared by incubating 3 μl of protein for 3 h followed by washes and blocking with BSA as previously described (Grumet *et al*., 1994). When double coating was used, Ig-fusion proteins were applied before the blocking step.

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