Thy-1 binds to integrin β_3 on astrocytes and triggers formation of focal contact sites

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Background: Thy-1 is an abundant neuronal glycoprotein in mammals. Despite such prevalence, Thy-1 function remains largely obscure in the absence of a defined ligand. Astrocytes, ubiquitous cells of the brain, express a putative Thy-1 ligand that prevents neurite outgrowth. In this paper, a ligand molecule for Thy-1 was identified, and the consequences of Thy-1 binding for astrocyte function were investigated.

Results: Thy-1 has been implicated in cell adhesion and, indeed, all known Thy-1 sequences were found to contain an integrin binding, RGD-like sequence. Thy-1 interaction with β3 integrin on astrocytes was demonstrated in an adhesion assay using a thymoma line (EL-4) expressing high levels of Thy-1. EL-4 cells bound to astrocytes five times more readily than EL-4^{-f}, control cells lacking Thy-1. Binding was blocked by either anti-Thy-1 or anti-β3 antibodies, by RGD-related peptides, or by soluble Thy-1-Fc chimeras. However, neither RGE/RLE peptides nor Thy-1(RLE)-Fc fusion protein inhibited the interaction. Immobilized Thy-1-Fc, but not Thy-1(RLE)-Fc fusion protein supported the attachment and spreading of astrocytes in a Mn^{2+}-dependent manner. Binding to Thy-1-Fc was inhibited by RGD peptides. Moreover, vitronectin, fibrinogen, denatured collagen (dcollagen), and a kistrin-derived peptide, but not fibronectin, also mediated Mn^{2+}-dependent adhesion, suggesting the involvement of β3 integrin. The addition of Thy-1 to matrix-bound astrocytes induced recruitment of paxillin, vinculin, and focal adhesion kinase (FAK) to focal contacts and increased tyrosine phosphorylation of proteins such as $p130^{Cas}$ and FAK. Furthermore, astrocyte binding to immobilized Thy-1-Fc alone was sufficient to promote focal adhesion formation and phosphorylation on tyrosine.

Conclusions: Thy-1 binds to β3 integrin and triggers tyrosine phosphorylation of focal adhesion proteins in astrocytes, thereby promoting focal adhesion formation, cell attachment, and spreading.

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Background

Thy-1 is a glycosyl phosphatidylinositol (GPI)-anchored glycoprotein of the immunoglobulin superfamily (IgSF) expressed in various cell types, particularly those of the T cell lineage and the neuronal system. In neurons, Thy-1 expression is developmentally regulated, whereby both initial appearance and ultimate distribution are controlled to ensure that Thy-1 is excluded from regions of axonal growth [1]. Thy-1 expression is preferentially initiated toward the end of axon extension [2], consistent with the idea that it might participate in stabilizing existing neuronal connections and inhibiting future neurite outgrowth [1, 3].

Interestingly, Thy-1-deficient mice breed and behave normally, despite the fact that this protein is highly expressed in the adult brain. These mice show an impairment in long-term potentiation, which does not appear to affect spatial learning [4]. While this suggests a more restricted role for Thy-1 than might have been anticipated, it is important to note that similar modest effects have been reported for other molecules involved in neural adhesion, such as L1 and N-CAM (reviewed in [5]). Thus, although having been described initially over 30 years ago and representing one of the most abundant surface glycoproteins in neurons of the mammalian central nervous system (CNS), the physiological role of Thy-1 remains to be defined.

Thy-1 appears to be involved in cell adhesion and activation. For instance, Thy-1 promotes the adhesion of thymocytes to thymic epithelia [6], the adhesion of CTL clones to L cells [7], T cell activation [8, 9], and the adhesion of a Thy-1-transfected lymphoma to astrocytes [10]. Furthermore, an astrocytic binding site for neuronal Thy-1 was described, whereby the interactions between Thy-1

and the putative ligand modulate neurite outgrowth [11, 12]. The identification of a Thy-1 ligand in the neuronal system was considered more relevant to human physiology, since Thy-1 is expressed on both human and murine neurons, but not on human T cells. Taking these points into consideration, astrocytes represented the most promising cellular system in which to search for the elusive Thy-1 ligand.

Like Thy-1, L1 is another member of the IgSF known to modulate neurite outgrowth [13]. L1 is a large multidomain glycoprotein that mediates cell-cell adhesion by homophilic [14], as well as heterophilic, interactions. In the latter case, interactions with several extracellular matrix (ECM) components and membrane proteins like laminin and $\alpha_V\beta_3$ integrin have been proposed [15, 16]. Most importantly, interactions between L1 and $\alpha_V\beta_3$ integrin on neuronal cells are known to modulate neurite outgrowth [17].

Integrins often recognize short peptide segments containing an RGD motif, whereby the essential nature of the aspartic acid residue in this context was first identified in the central integrin binding domain of fibronectin. RGD peptides or related motifs are now known to be present in many cell-surface molecules, like L1, that interact with integrins [17]. Interestingly, the alignment of the Thy-1 sequences from human, mouse, and rat [18] led to the identification of a single RLD motif in a highly conserved sequence element. Since RLD is a binding motif for integrins $\alpha_V\beta_3$ and $\alpha_M\beta_2$ [19], the hypothesis that Thy-1 might indeed function as a heterophilic ligand for members of the integrin family was tested. In this context, $\alpha_V\beta_3$ integrin appeared to be the more likely candidate, since this molecule is present on the surface of astrocytes [20], while the expression of $\alpha_M\beta_2$ is restricted to leukocytes [21].

Engagement and clustering of integrin receptors directly initiate a variety of signal transduction events, including an increase in tyrosine phosphorylation of a subset of proteins (see below), activation of serine-threonine kinases, and alterations in cellular phospholipid and calcium levels (reviewed in [22]). These events are associated with the formation of focal adhesions, specialized sites of adhesion formed by many cells in culture that are known to be important in cell spreading and motility [23]. Focal adhesions contain a variety of structural (e.g., talin, vinculin, and α-actinin), signaling (focal adhesion kinase [FAK] and Src-family kinases), and adaptor molecules (including paxillin, tensin, and $p130^{Cas}$) and represent the intracellular sites in which tyrosine phosphorylation levels are highest [24].

In this study, we provide the first step toward unraveling the function of Thy-1 by showing that a β_3 integrin in astrocytes, most likely expressed as $\alpha_V\beta_3$, is a receptor for Thy-1. Furthermore, Thy-1 binding to astrocytes induced cell-signaling events specifically linked to focal adhesion formation, promoting astrocyte attachment and spreading. Hence, as for other IgSF molecules [25], Thy-1 interaction with β_3 integrin may elicit bidirectional signaling between neurons and astrocytes ([11] and this paper).

Table 1

Binding of EL-4 cells to astrocytes.

Cells	Relative binding[a]
Primary mouse astrocytes	5.1 ± 1.6
Rat astrocytic cell line (DI TNC_1)	4.9 ± 1.0

[a] Ratio of the number of EL-4 and EL-4^{-f} cells bound (mean value of three experiments).

Results

Identification of a Thy-1 ligand in murine astrocytes

Several cells have been identified as potential carriers of Thy-1 ligand(s) including thymic epithelial cells [26], murine fibroblasts [7], and astrocytes [10]. The latter represents the most favorable cellular system to identify such a molecule since, in the brain, astrocytes form an intimately associated network with neurons, which express high levels of Thy-1. Thus, a cell adhesion assay was developed that allowed the identification and characterization of the interaction between astrocytes and Thy-1-expressing cell lines.

In this assay, the well-characterized murine thymoma cell line EL-4 and the Thy-1 loss mutant, EL-4^{-f}, were employed. Adhesion of such cells to either primary mouse astrocytes or a rat astrocytic cell line (DI TNC_1) was studied. This cell line was chosen because it displays many properties that are characteristic of neonatal astrocytes, including the expression of similar cell surface and ECM adhesion molecules and the ability to promote neurite outgrowth [27]. Given the limited amount of primary astrocytes that can be obtained and the inherent variability between preparations, the immortalized astrocytic cell line was used in most of the experiments.

Wild-type EL-4 cells and the Thy-1 loss mutant line, EL-4^{-f}, were labeled with two different fluorescent probes and incubated simultaneously with astrocytes. EL-4 cells expressing Thy-1 on their surface bound to astrocytes 4–5 times more readily than the EL-4^{-f} cells, regardless of whether primary astrocytes or the rat astrocytic cell line were employed in this assay (Table 1). When EL-4 cells were preincubated with anti-Thy-1 mAb, adhesion to

Table 2

Inhibition by antibodies of EL-4 cell binding to astrocytes.

Antibody	Relative binding[a] to mouse primary astrocytes	Relative binding to DI TNC_1 cells
anti-Thy-1 (V8)	4.5 ± 1.3	5.1 ± 1.1
anti-CD3 (KT3)	1.2 ± 0.3	1.2 ± 0.2
anti-TSA-1 (MTS-35)	1.1 ± 0.2	1.1 ± 0.1

[a] Ratio of astrocyte-bound nontreated EL-4 to antibody-treated EL-4 cells (mean value of three experiments).

astrocytes decreased 4- to 5-fold (Table 2). No such effect was observed for cells that were pretreated with mAbs against CD3ϵ or thymic-shared antigen-1 (Table 2), two molecules expressed on the surface of EL-4 cells [9]. These experiments indicated that a binding site for Thy-1 existed on the surface of astrocytes and that Thy-1 was critically involved in mediating binding between astrocytes and EL-4 cells. In addition, mouse Thy-1 bound to both the mouse (primary astrocytes) and rat (DI TNC_1 cells) astrocytic ligand equally well. Therefore, subsequent experiments were performed using only the rat astrocytic cell line.

Inhibition of EL-4 cell binding to astrocytes by Thy-1-Fc fusion proteins, RGD-like peptides, and anti-integrin mAbs

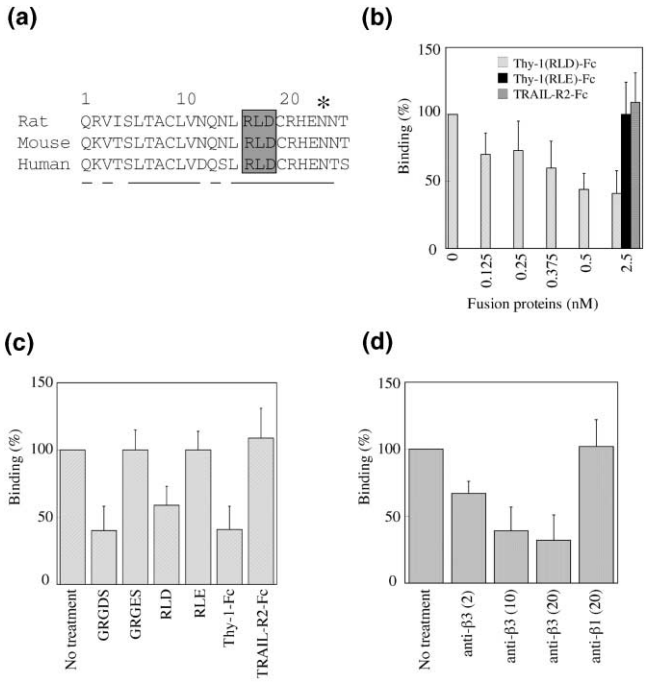
Analysis of the amino acid sequence of human, mouse, and rat Thy-1 revealed the presence of a conserved RLD motif (Figure 1a), an RGD-like peptide that is recognized by some integrins, including $\alpha_V\beta_3$ and $\alpha_M\beta_2$ [19]. To corroborate the above results in an independent manner and to test whether the Thy-1 RLD motif mediated association between EL-4 cells and astrocytes, the effect of soluble chimeric Thy-1-Fc protein, the mutated Thy-1(RLE)-Fc fusion protein, TRAIL-R2-Fc as a control, as well as several RGD-like peptides was examined in the cell-cell adhesion assay.

Astrocyte pretreatment with Thy-1-Fc inhibited, in a dose-dependent manner, up to 70% of EL-4 cell binding, whereas no effect was observed upon preincubation with the same concentration of Thy-1(RLE)-Fc recombinant protein or TRAIL-R2-Fc (Figure 1b). These data confirmed the existence of a specific Thy-1 binding component on the surface of astrocytes and implicated the RLD sequence of Thy-1 as an essential element in the interaction with the astrocytic ligand.

Preincubation of astrocytes with the peptide GRGDS (1 mg/ml) containing the integrin binding motif inhibited the adhesion of EL-4 cells by 60% (average value), while the control peptide GRGES had no effect at the same concentration (Figure 1c). Likewise, the RLD tripeptide (1 mg/ml) reduced cell adhesion by 40%, whereas an RLE peptide had no effect at the same concentration. Interestingly, inhibition observed with GRGDS was comparable to that observed with the recombinant Thy-1-Fc molecule, suggesting that a similar, if not identical, interaction was being blocked by either RGD-like peptides or Thy-1-Fc fusion proteins.

Taking into consideration that $\alpha_M\beta_2$ integrin expression is restricted to leukocytes [21], the possibility that $\alpha_V\beta_3$ might be a ligand for Thy-1 was investigated. The presence of β_1- and β_3-containing integrins on the surface of astrocytes [20] was confirmed by flow cytometry using

Figure 1



Inhibition of EL-4 cell binding to astrocytes. **(a)** Partial sequences of rat, mouse, and human Thy-1, corresponding to amino acids 1–25 in the respective mature proteins, are shown. Underlined amino acid residues are identical for all three species and include the RGD-like sequence present in Thy-1 (box). **(b)** Astrocytes were incubated with the indicated concentrations of recombinant fusion proteins before the addition of equal numbers of EL-4 and EL-4^{-f} cells. Thy-1-Fc inhibited binding in a concentration-dependent manner, while no such effect was observed with the mutated Thy-1(RLE)-Fc fusion protein or TRAIL-R2-Fc used as a control. **(c)** Inhibition of EL-4 cells binding to astrocytes is shown after astrocytes were pretreated with different RGD-related peptides at 1 mg/ml or recombinant fusion proteins at 2.5 nM. **(d)** EL-4 cell-astrocyte adhesion was also analyzed in the presence of either increasing concentrations of anti-rat β_3 or anti-rat β_1 integrin mAb (up to 20 μg/ml). Binding values for each incubation condition are equivalent to the number of EL-4 cells bound after pretreatment and are expressed as a percentage of the bound cells observed in nontreated controls. The error bars indicate the standard deviation resulting from at least four independent experiments. In each case, at least 500 cells were counted per condition.

specific anti-rat integrin antibodies (data not shown). The effect of these antibodies was then tested in the cell-cell adhesion assay. Preincubation of astrocytes with the anti-β_3 integrin mAb inhibited, in a concentration-dependent manner, ~70% of EL-4 binding, while no such effect was observed with the mAb against β_1 integrin, even at the highest concentration tested (Figure 1d). Here, it is important to note that both of the anti-integrin Abs employed reportedly block cell adhesion to their respective ECM substrates [28, 29]. Taken together, these data implicated a β_3-containing integrin, probably $\alpha_V\beta_3$, in mediating adhesion between Thy-1$^+$ EL-4 cells and astrocytes.

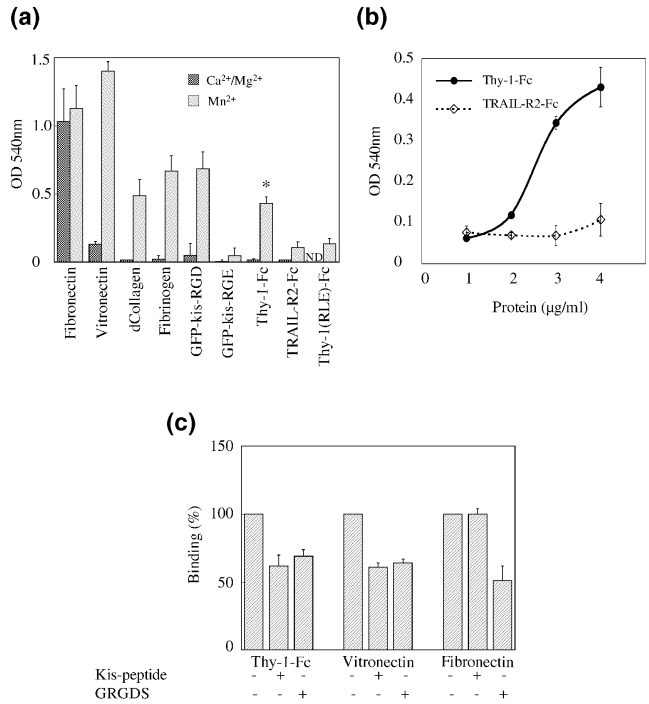
Adhesion of astrocytes to different ECM proteins and to Fc-chimeric molecules

The β_3 subfamily of heterodimeric integrin receptors includes $\alpha_{IIb}\beta_3$, which is specifically expressed in megakariocytes and platelets, and $\alpha_V\beta_3$ integrin, which is more widely distributed [21]. Functionally, these integrins differ in their ligand specificity and sensitivity to divalent cations, including Mn^{2+} [30–32]. Thus, to investigate further whether $\alpha_V\beta_3$ was involved in the adhesion of astrocytes to Thy-1, the ability of astrocytes to adhere to specific integrin ligands and the sensitivity of this interaction to Mn^{2+} were assessed using a cell-matrix adhesion assay.

Several ECM proteins showing differential $\alpha_5\beta_1$- and $\alpha_V\beta_3$-dependent adhesion were used in this assay. A protein consisting of 13 amino acids of the integrin binding loop (**CRIP**RGD**MPDDR**C**) present in the snake venom disintegrin kistrin, fused to the C-terminal end of a his-tagged green fluorescent protein (GFP) and expressed in bacteria (GFP-kis -RGD), was also used in these experiments. Kistrin was chosen because, in contrast to most disintegrins that are potent inhibitors of adhesive ligands to integrin receptors of the β_1 and β_3 families, it binds preferentially to β_3-containing integrins [33–35]. In addition, the 13 amino acid kistrin loop alone was chemically synthesized (Kis-peptide) for use as a soluble inhibitor of matrix adhesion. The specificity of kistrin-derived proteins and peptides for $\alpha_V\beta_3$ was tested and confirmed in preliminary experiments (O. Dormond, L.L., C.R., and P.S., unpublished data). Thus, to further characterize the astrocytic Thy-1 binding molecule, the Kis-peptide was used to block astrocyte adhesion to ECM proteins or Thy-1-Fc fusion proteins.

Binding to plates coated with vitronectin, denatured collagen (dcollagen), fibrinogen, GFP-kis-RGD ($\alpha_V\beta_3$-dependent adhesion), or fibronectin ($\alpha_5\beta_1$- and $\alpha_V\beta_3$-dependent adhesion) [36] was studied using astrocytes in serum-free medium containing either Ca^{2+}/Mg^{2+} or Mn^{2+}. Recombinant Thy-1-Fc and Thy-1(RLE)-Fc proteins, as well as recombinant GFP-kis-RGD and GFP-kis-RGE proteins, were compared with ECM proteins in this assay. Astrocytes attached to and spread on vitronectin, dcollagen, fibrinogen, and GFP-kis-RGD only in the presence of Mn^{2+}, while they attached to fibronectin under both conditions. As expected, no binding to GFP-kis-RGE was detected under the conditions tested (Figure 2a). Thy-1-Fc also permitted the adhesion of astrocytes in the presence of Mn^{2+}, to an extent comparable with dcollagen, fibrinogen, and the GFP-kis-RGD. However, mutation of a single amino acid at position 18 (D → E) in the Thy-1 protein sequence abolished astrocyte adhesion even in the presence of Mn^{2+}. It is important to note that, in competition experiments using EL-4 cells, both Thy-1(RLD)-Fc and Thy-1(RLE)-Fc fusion proteins, but not TRAIL-R2-Fc, were equally effective in preventing bind-

Figure 2



Astrocyte adhesion to different ECM and recombinant Fc proteins. Astrocytes were seeded into plates coated with the the indicated proteins. Cell attachment to the plate was determined 2 hr after adding the cells. **(a)** Astrocytes attached to fibronectin both in the presence of Ca^{2+}/Mg^{2+} or Mn^{2+}, while binding to vitronectin, dcollagen, fibrinogen, GFP-kis-RGD, and Thy-1-Fc was only detected in the presence of Mn^{2+}. The mutated Thy-1(RLE)-Fc fusion protein and the GFP-kis-RGE, in contrast, did not support astrocyte adhesion in the presence of Mn^{2+}. Nonparametric Mann-Whitney analysis was used to compare Thy-1-Fc in the presence of Mn^{2+} relative to the control (TRAIL-R2-Fc). *$p < 0.002$ was calculated using average values from seven independent experiments. ND = not determined. **(b)** Adhesion of astrocytes to Thy-1-Fc increased in a concentration-dependent manner, while no such effect was seen for TRAIL-R2-Fc. **(c)** Astrocytes were incubated with the indicated peptides before adding them to the plates coated with Thy-1-Fc, vitronectin, or fibronectin as indicated. Thy-1-Fc- and vitronectin-mediated astrocyte adhesion was inhibited by both soluble Kis-peptide and GRGDS peptide, while fibronectin-mediated adhesion was reduced only by GRGDS.

ing of different anti-Thy-1 mAbs to EL-4 cells (see Supplementary material available with this article online). This observation indicates that the single amino acid mutation in Thy-1(RLE)-Fc did not lead to dramatic changes in the conformation of the mutated fusion protein. Additionally, adhesion to Thy-1-Fc was significantly higher than for TRAIL-R2-Fc (Figure 2a) and was enhanced by increasing amounts of Thy-1-Fc on the plate, whereas TRAIL-R2-Fc failed to produce such an effect (Figure 2b).

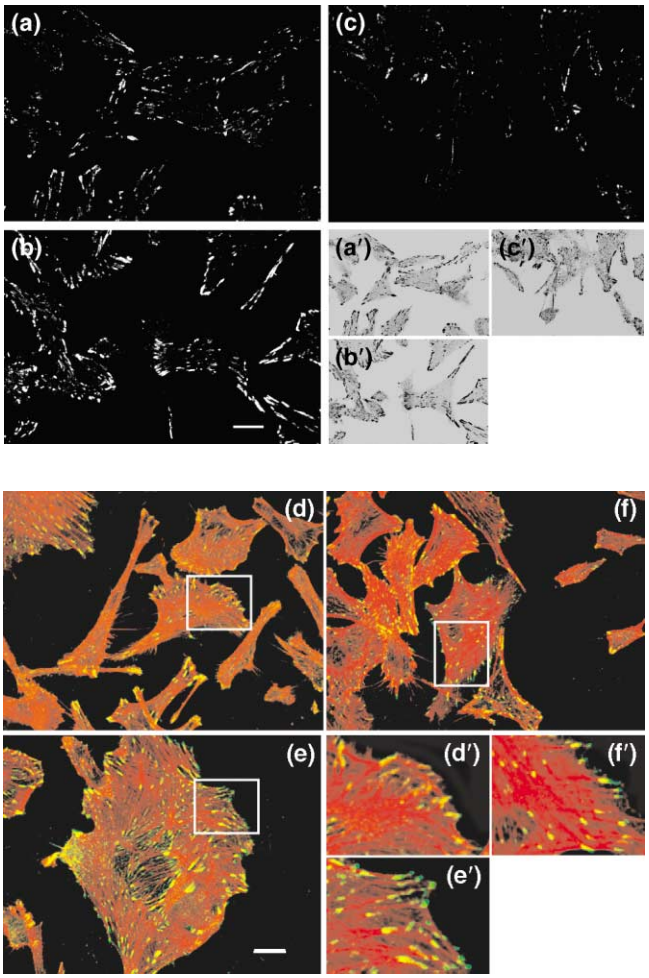
Competition assays using either RGD peptides (1 mg/ml) or the soluble Kis-peptide (0.25 mg/ml) to treat astrocytes prior to adding them to Thy-1-Fc-, vitronectin-, or fibronectin-coated plates showed that, while GRGDS inhibited adhesion of astrocytes to all three proteins by 40%–50%, the Kis-peptide only did so for vitronectin and Thy-1-Fc, but was ineffective in blocking adhesion to fibronectin (Figure 2c). Thus, astrocytes attached to and spread on ECM proteins, as well as on the purified, recombinant Thy-1-Fc protein. Binding to Thy-1 was inhibited by RGD peptides. Taken together, the data strongly support a role for Thy-1 in promoting astrocyte adhesion and spreading by engaging β_3 integrin, most likely as $\alpha_V\beta_3$ integrin, on the surface of astrocytes.

Thy-1-induced tyrosine phosphorylation of focal adhesion proteins in astrocytes

Integrin signaling has been linked to phosphorylation on tyrosine of proteins present in focal adhesions. Thus, as an initial step to define the cellular responses initiated upon integrin ligation by Thy-1, signaling events associated with integrin activation in cultured astrocytes were studied by indirect immunofluorescence using anti-phosphotyrosine mAbs.

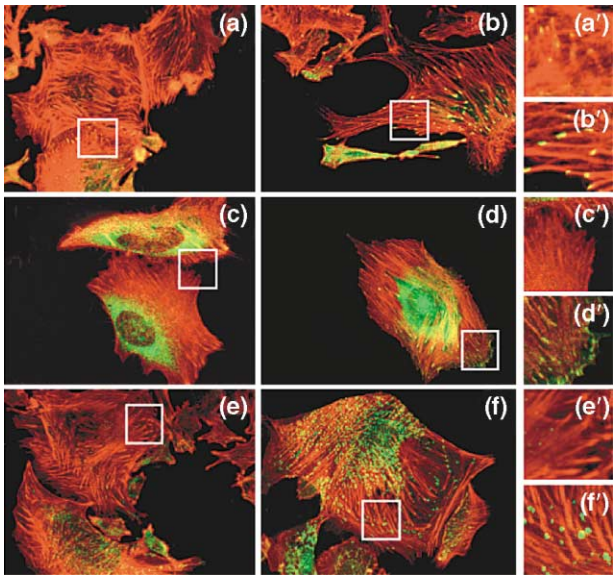
In a first approach, DI TNC_1 astrocytes were incubated with either EL-4 or EL-4^{-f} cells. Astrocytes on coverslips displayed low levels of tyrosine phosphorylation (Figure 3a). Such levels were particularly enhanced in the focal contacts upon incubation with EL-4 cells (Figure 3b), while astrocytes incubated with EL-4^{-f} cells displayed only background levels of phosphorylation (Figure 3c). To confirm that this effect was triggered by Thy-1 molecules and not by other proteins on the surface of EL-4 cells, astrocytes were incubated with Protein A-coated beads conjugated with either Thy-1-Fc or TRAIL-R2-Fc recombinant proteins. Levels of phosphotyrosine (Figure 3d) were increased only upon incubation with Thy-1-Fc (Figure 3e), but not when using TRAIL-R2-Fc-coupled beads (Figure 3f). Green fluorescence was compared in boxes showing similar surface areas of cells after treatment. Phosphotyrosine-containing dots increased in number, as well as intensity, indicating that focal complexes had been formed (compare [e] with [d] and [f], as well as [e′] with [d′] and [f′] in Figure 3). Quantification of the number of green pixels per unit area (Figure 3d′–f′) revealed an increase of at least 2-fold in Thy-1-stimulated cells (Figure 3e′). Higher levels of cell spreading were

Figure 3



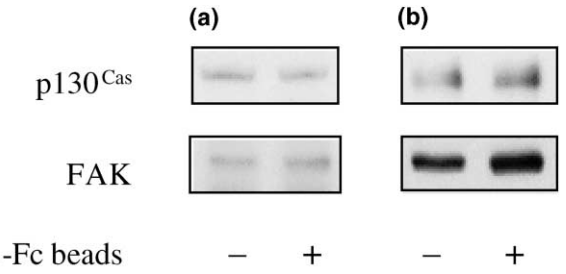
Enhanced tyrosine phosphorylation in astrocytes induced by EL-4 cells or Thy-1-Fc chimeric molecules. Astrocytes were incubated with **(a)** PBS/FCS, **(b)** EL-4 cells, or **(c)** EL-4^{-f} cells for 5min. Then, cells were fixed, permeabilized, and incubated with anti-phosphotyrosine mAb as described. Phosphorylation was increased for astrocytes incubated with Thy-1-containing EL-4 cells (b), while only background levels were observed otherwise (a and c). **(a′–c′)** Respective inverted images of identical fields are shown miniaturized to demonstrate that equivalent cell numbers are present in each picture. Astrocytes were incubated with either **(d)** Protein A beads, **(e)** Thy-1-Fc conjugated to Protein A beads, or **(f)** TRAIL-R2-Fc conjugated to Protein A beads. After 5 min of incubation, cells were treated as before and then incubated with anti-phosphotyrosine mAb. Binding of this mAb was detected with anti-mouse FITC that was added together with rhodamine-conjugated phalloidin to visualize F-actin. (e) Tyrosine phosphorylation was enhanced for astrocytes incubated with Thy-1-Fc-conjugated beads. Similar cell surface areas are shown enlarged to permit comparison of green fluorescence (white-lined boxes in [d–f]). Note that phosphotyrosine staining was more abundant and of higher intensity in **(e′)** Thy-1-treated cells as compared to **(d′ and f′)** controls. The scale bar shown in (e) is equivalent to 20 μm and is valid for (a–f).

Figure 4



Distribution of paxillin, vinculin, and FAK in astrocytes. Distribution of focal adhesion proteins (green) was studied by indirect immunofluorescence (FITC-coupled second antibody) using first antibodies against **(a and b)** paxillin, **(c and d)** vinculin, and **(e and f)** FAK. Fluorescence was viewed either (a, c, and e) before or (b, d, and f) after treating astrocytes attached to coverslips with Thy-1-Fc conjugated to Protein A beads. Rhodamine-conjugated phalloidin (red) was also used to follow the actin fibers. The three focal adhesion proteins studied were recruited to the contact sites upon incubation of astrocytes with Thy-1-containing beads. Enlarged views of comparable regions (white-lined boxes in [a–f]) at the end of the stress fibers are also shown before and after Thy-1 incubation (compare **[a′]** to **[b′]**, **[c′]** to **[d′]**, and **[e′]** to **[f′]** for paxillin, vinculin, and FAK recruitment, respectively).

Figure 5



Binding of Thy-1-Fc to astrocyte-induced tyrosine phosphorylation of the signaling proteins $p130^{Cas}$ and FAK. Astrocytes attached to plates were incubated for 5 min with (+) Thy-1-Fc-containing beads or (−) Protein A beads only. Cells were then harvested directly in lysis buffer. (**b**) Proteins indicated were immunoprecipitated from such extracts as described and probed after transfer to nitrocellulose with anti-phosphotyrosine mAb. (**a**) The same membranes were then stripped and reprobed with the antibodies used for immunoprecipitation, indicated to the left.

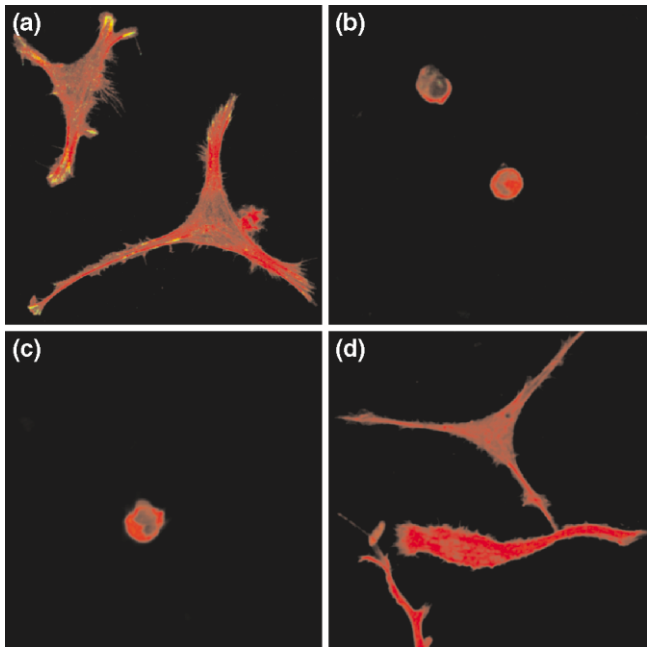
commonly observed in astrocytes treated with Thy-1-Fc. The striking increase in size of the cell shown in Figure 3e is representative of this phenomenon. Concomitant labeling with phalloidin to visualize F-actin showed that enhanced tyrosine phosphorylation occurred predominantly at the ends of actin stress fibers in focal contact sites (Figure 3d–f).

Integrin engagement results in receptor clustering. The latter triggers the recruitment and phosphorylation of focal adhesion proteins such as FAK, $p130^{Cas}$, paxillin and vinculin, and the recruitment of actin binding proteins and actin stress fibers. Thus, increased tyrosine phosphorylation observed at the tips of stress fibers would be expected to parallel a recruitment of focal adhesion proteins to the same region. To explore this possibility, indirect immunofluorescence using antibodies directed against focal adhesion proteins was performed. Astrocytes were analyzed before and after treatment with Thy-1-Fc beads, and, indeed, increased fluorescence was observed at the tip of stress fibers for all focal adhesion proteins studied. Thus, paxillin (Figure 4a,b), vinculin (Figure 4c,d), and FAK (Figure 4e,f) were recruited to focal contacts upon astrocyte treatment with recombinant Thy-1-Fc protein (Figure 4b,d, and f).

To conclusively demonstrate that tyrosine phosphorylation was enhanced in focal contacts, $p130^{Cas}$ and FAK were immunoprecipitated before and after treating cultured astrocytes with Thy-1-Fc-coupled beads and tested for tyrosine phosphorylation content by immunoblotting (Figure 5). In this experiment, Thy-1-Fc-coated beads rapidly increased tyrosine phosphorylation in astrocytes 2-fold (assessed by scanning densitometry) for both aforementioned proteins (Figure 5b), while no such effect was observed with TRAIL-R2-Fc (data not shown).

At this point, it was not clear whether β_3 ligation by Thy-1 alone was sufficient to induce increased tyrosine phosphorylation at focal adhesion sites or whether this effect required integrins already engaged at the ventral surface of the cell. To answer this question, astrocytes were added to Thy-1-Fc-coated coverslips in the absence of serum. In indirect immunofluorescence experiments, tyrosine phosphorylation was only observed at the end of the stress fibers when astrocytes were plated on Thy-1-coated coverslips (Figure 6a), but not with mutated Thy-1(RLE)-Fc protein or BSA (Figure 6b,c, respectively). In the absence of any protein (nontreated coverslips), astrocytes started adhering, but no tyrosine phosphorylation was detected (Figure 6d). These results demonstrated that neither preexisting integrin engagement nor ECM proteins secreted by astrocytes themselves during the assay accounted for the effect observed in Figure 6. Thus, Thy-1 interaction with its receptor was sufficient to induce astrocyte adhesion and signaling events leading to focal contact formation.

Figure 6



Binding of astrocytes to Thy-1-Fc-coated coverslips promoted focal contact formation and induced tyrosine phosphorylation. Astrocytes were plated on coverslips coated with **(a)** Thy-1-Fc, **(b)** Thy-1(RLE)-Fc, **(c)** BSA, or **(d)** coverslips that were not treated, and they were then analyzed by indirect immunofluorescence using anti-phosphotyrosine antibodies as described for Figure 3d–f. Thy-1, on its own, promoted the drastic changes in morphology of the astrocytes and the signaling events expected for focal adhesion formation. Although some astrocyte adhesion was observed in (d) nontreated coverslips, tyrosine phosphorylation and focal contacts were only detected in (a) Thy-1-coated coverslips.

In summary, these results support a model in which neuronal Thy-1 binds to β_3 integrin, most likely as $\alpha_V\beta_3$ integrin, on astrocytes, enhances tyrosine phosphorylation of focal adhesion proteins, and recruits such proteins into mature focal complexes, thereby promoting the assembly of focal contacts and the spreading of astrocytes.

Discussion

Thy-1, a member of the IgSF, is a protein that is highly conserved throughout evolution and is expressed at high levels in neurons where it constitutes 5%–7% of total surface protein [37]. Such abundance suggests an important role for Thy-1 in the nervous system; however, unraveling the function of Thy-1 has been hampered by the absence of any known molecular counterpart. In this paper, we provide the first insights at a molecular level into Thy-1 function in the CNS by identifying a receptor/ligand molecule on astrocytes. The results presented here show that: β_3 integrin on astrocytes is a ligand for Thy-1; Thy-1-β_3 integrin interaction triggers rapid tyrosine phosphorylation of focal adhesion proteins in astrocytes, thereby promoting focal adhesion formation, cell attachment, and spreading; and the β_3 integrin involved is likely to be $\alpha_V\beta_3$.

β_3 integrin is a ligand for Thy-1

Experiments using the cell adhesion assay described here revealed that EL-4 cell binding to astrocytes was mediated by interactions between Thy-1 and the integrin β_3. Inhibition achieved using recombinant Thy-1-Fc or the RGD peptides was 60% (Figure 1b,c), whereas antibodies against either β_3 integrin or Thy-1 were capable of blocking 70%–90%, respectively, of the EL-4-astrocyte binding (Figure 1d, Tables 1 and 2). These results indicate that, although Thy-1 and β_3 integrin play an important role in mediating the binding observed between those cells, the possibility that other proteins are involved cannot be excluded.

Thy-1, a member of the IgSF, has been shown to be an adhesion molecule [6]. A number of neuronal glycoproteins from the same family can modulate neurite outgrowth by either homophilic or heterophilic interactions with molecules in other cells. For example, L1 participates in heterophilic interactions with other members of the IgSF, components of the ECM, and integrins [15, 17, 38, 39]. The presence of a single RGD motif in human L1 and of two such domains in the murine and rat L1 homologs led to the discovery of RGD interactions with various integrin family members. The evidence specifically favoring $\alpha_V\beta_3$ as a Thy-1 ligand/receptor was essentially 4-fold: Thy-1 contains an RLD instead of an RGD motif in its sequence; a single mutation of RLD → RLE in the Thy-1 molecule renders the protein unable to inhibit EL-4-astrocyte interactions and to support astrocyte adhesion and spreading; astrocytes express the RLD binding integrin $\alpha_V\beta_3$, but not $\alpha_M\beta_2$; and astrocyte adhesion to Thy-1 requires Mn^{2+}, but not Ca^{2+}/Mg^{2+}. Thus, although these results, in conjunction with the inhibition experiments using anti-β_3 integrin mAbs and RGD-containing peptides, strongly favor $\alpha_V\beta_3$ as the Thy-1 ligand/receptor, a role for other integrins or for a yet to be characterized α chain in the nervous system cannot be excluded. Moreover, incomplete inhibition observed using RGD peptides, Thy-1-Fc, or anti-β3 integrin antibodies in the cell-cell adhesion assay (Figure 1) and in the kistrin competition experiments (Figure 2) indicated that adhesion of astrocytes to Thy-1 may additionally be mediated by a non-RGD motif in the molecule, as has been described for other IgSF members, like L1 [40]. Two questions still remain: why did anti-Thy-1-antibodies inhibit astrocyte/EL-4 interaction almost completely (Table 2), and why was Thy-1(RLE)-Fc so "inactive?" The answer to the first question may be linked to Thy-1 abundance on the surface of EL-4 cells [1]. A possible answer to the second question is that RLD binding to its ligand/receptor might be necessary to yield a conformation/orientation of the

protein required for the second interaction to occur. Further investigation is required to address this issue.

Uni- versus bidirectional signaling between neurons and astrocytes

If Thy-1 triggers astrocyte "activation" by engaging integrin receptors, then the question arises whether Thy-1 is a receptor or a ligand? Signaling events initiated by integrin clustering may ultimately lead to the release of factors from astrocytes that prevent neurite outgrowth. In this case, Thy-1 would be acting as a ligand, and the consequence of Thy-1-ligand interaction for neurons would be indirect. This possibility is not unlikely, since it is known that astrocytes direct neurite outgrowth in the developing brain with the help of newly synthesized cell surface and ECM molecules [41]. Furthermore, astrocytes were recently shown to respond to stimuli by increasing intracellular calcium concentrations and releasing glutamate, which could then activate neighboring neurons [42].

Alternatively, signaling could be bidirectional in that the interaction between Thy-1 and β_3 integrin generates a signal in both neurons and astrocytes. In neurons, Thy-1 occupancy would stop axonal growth by an unknown mechanism, and in astrocytes, integrin clustering would induce tyrosine phosphorylation of focal adhesion proteins. In this case, Thy-1 and β_3 represent either receptors or ligands, depending on the cell being studied. Such bidirectional signaling between neurons and astrocytes, involving molecules other than Thy-1, has already been reported [43]. In the specific case of Thy-1, antibody-mediated cross-linking on the surface of PC12 cells generates an intracellular calcium flux [44]. Moreover, Thy-1 localization in microdomains of the plasma membrane is essential for its ability to modulate neurite outgrowth, also indicating that the Thy-1 GPI anchor is important in neuronal signaling [12]. If Thy-1-mediated effects in neurons were only caused indirectly by astrocyte-derived factors, the presence of Thy-1 in microdomains should be irrelevant, and signaling events should not be observed in neurons upon Thy-1 cross-linking. Thus, bidirectional signaling triggered in neurons by Thy-1 and in astrocytes by β_3 is likely to occur.

Thy-1-β_3 integrin interaction leads to rapid tyrosine phosphorylation of proteins involved in focal adhesion formation in astrocytes

Focal contacts form at attachment sites between cells and the substratum. Several proteins are found at contact sites, including talin, vinculin, $p130^{Cas}$, paxillin, α-actinin, and FAK [23]. Some of these proteins are substrates of nonreceptor tyrosine kinases and are tyrosine phosphorylated upon focal adhesion formation. Thy-1-β_3 interaction promoted focal adhesion assembly of already adherent astrocytes (Figures 3 and 4). Interestingly, cells in suspension in the absence of serum also adhered to immobilized Thy-1-Fc (Figure 6). In particular, adhesion to Thy-1 alone was sufficient to promote spreading as well as de novo formation of focal adhesion contacts. Focal adhesion assembly has been shown to be dependent on the low molecular weight GTPase RhoA [45], and in more recent work, binding via integrins activated RhoA [46, 47], raising the possibility that Thy-1 binding to β_3 may also stimulate RhoA activity. Future experiments will explore this possibility.

In addition, astrocyte adhesion to Thy-1 was comparable to adhesion on vitronectin, fibrinogen, dcollagen, and GFP-kis-RGD in that it was Mn^{2+}-dependent. However, astrocyte binding to Thy-1 was considerably lower than that obtained with the $\alpha_V\beta_3$ ligand, vitronectin. This might reflect a lower affinity of the Thy-1-Fc fusion protein than its cellular counterpart, or, alternatively, it may reflect that additional molecules are required for effective cell attachment. Nevertheless, the data described here indicate that Thy-1 functions as a classical integrin ligand and promotes the formation of focal complexes in astrocytes.

Functional consequences of Thy-1- β_3 integrin interaction in the brain

A wide range of molecules is known to promote nerve cell growth, including the CAMs, NCAM, N-cadherin, and the glycoprotein L1. Like Thy-1, all are members of the IgSF; yet, in contrast to other members of this family, Thy-1 inhibits neurite outgrowth. How may this difference be explained? NCAM, N-cadherin, and L1 are quite broadly expressed and appear to play a role in the development of specific projections in the nervous system. In contrast, Thy-1 expression is restricted to postnatal developmental periods, is observed in axons only after growth has been completed, and is suggested to participate in stabilizing the axonal networks. Thus, Thy-1 differs from other IgSF members in that its expression is very precisely controlled, both spatially and temporally. Because of these restrictions, Thy-1 interaction with its binding partner may have very distinct consequences for neuronal function.

For decades, neurons have been regarded as the only cells involved in the generation and control of brain signaling, while the surrounding glia was assumed to provide only structural and metabolic support to neuronal function. The emerging concept, underscored by findings here, is that astrocytes are active and integrated participants in neurotransmission [43, 48]. Therefore, astrocytes are not static entities but, instead, respond rapidly to their microenvironment and, in doing so, modulate neuronal function. Whether astrocytes actively influence neuronal function by secreting unknown factors upon Thy-1 integrin engagement is another aspect that deserves further investigation.

Conclusions

Thy-1 function remains unknown, largely due to the lack of insight concerning Thy-1-interacting proteins. Here, for the first time, we present evidence that Thy-1 interacts with β_3 integrin on astrocytes. Since Thy-1 and β_3 integrin are expressed on many cell types, a wide variety of possible functions may be linked to Thy-1-β_3 integrin interaction.

Thy-1 has generally been viewed as a receptor molecule. The results presented here show that Thy-1 also functions as a ligand. Thy-1 binding to β3 integrin triggers tyrosine phosphorylation of focal adhesion proteins in astrocytes, thereby promoting focal adhesion formation, cell attachment, and spreading. Thus, Thy-1 modulates neurite outgrowth, not only by triggering a response in the neuronal cells (as described by others [11, 12]), but also by initiating signaling events that promote adhesion of adjacent astrocytes to the underlying surface. These observations argue that Thy-1 functions in a bimodal fashion, as a receptor on neuronal cells and as a ligand for β_3 on astrocytes. Since Thy-1 is implicated in the inhibition of neurite outgrowth, signaling events in astrocytes are likely to play an important role in this process.

Materials and methods

Cells, peptides, and reagents

Primary cultures of cerebral cortical astrocytes from 1- to 2-day old Swiss albino mice [49] and the rat DI TNC_1 astrocytic cell line [27] were kindly donated by Drs. L. Pellerin and P. Magistretti (U. of Lausanne, Switzerland). For details of other cells, RGD peptides, and reagents used, see the Supplementary material.

Antibodies used were anti-Thy-1 mAbs (clone V8) [50]; hamster anti-rat β_1 (clone Ha2/5) and mouse anti-rat β_3 (clone F4) integrin mAbs from Pharmingen; and anti-phosphotyrosine 4G10 from UBI. Other antibodies (anti-vinculin, anti-FAK, anti-$p130^{Cas}$, and anti-paxillin antibodies) were a kind donation of Dr. K. Burridge (U. of North Carolina at Chapel Hill, NC).

The construction of the recombinant Fc molecules, their purification, and their characterization are provided as Supplementary material.

Cell-cell adhesion assay

Murine primary astrocytes or a rat astrocytic cell line (DI TNC_1) were allowed to interact with EL-4 cells (Thy-1-bearing thymoma). As a control for specificity of the interaction, a Thy-1-negative mutant of the EL-4 cell line (EL-4^{-f}) was used. This cell line is deficient in GPI anchor synthesis and, therefore, does not express any GPI-anchored proteins on its surface [51]. Thy-1^+ cells were labeled with CellTracker CMFDA green, and Thy-1^- cells were labeled with CellTracker CMTMR red following the manufacturer's instructions (Molecular Probes). Once labeled, cells (5×10^5 of each kind) were added to adherent astrocytes in a 24-well plate. After 20 min at 37°C, cells were gently washed with PBS. Bound cells were fixed with 4% paraformaldehyde and counted using confocal microscopy and Adobe PhotoShop 5.0 software (see Supplementary material).

In experiments testing the effect of anti-Thy-1 Abs, EL-4 cells were first labeled with the two different CellTrackers, and, then, those labeled with CMTMR (red) were incubated with different Abs for 30 min at 4°C (see Table 2). The experiments then continued as specified previously, comparing binding of nontreated (green) and Ab-treated (red) cells to astrocytes after 20 min at 37°C. When purified Fc-fusion proteins or RGD-like peptides were tested, the astrocytes were preincubated for 10 min at 37°C with the fusion proteins or peptides. Then, both EL-4 cells (labeled with CMFDA, green) and EL-4^{-f} cells (labeled with CMTMR, red) were added together to the treated astrocytes, and binding was assessed. For each experimental condition, at least 500 cells were counted. Relative binding was calculated as the ratio between the Thy-1^+ cells and the Thy-1^- cells present in each well. For all experiments, each condition shown was performed in duplicate or triplicate. To calculate a standard deviation, data values of every experiment were standardized to their own positive control (100%), defined as cell binding observed in the absence of any treatment.

Cell-matrix adhesion assay

Fc molecules, ECM proteins, or recombinant GFP-kis-RGD(E) (1–4 μg/well) were added to coat the surface of each well in a 96-well plate. Proteins were left overnight at 4°C and removed before blocking remaining sites with 2% BSA in PBS for 3 hr at 37°C. The plate was then washed with PBS prior to the addition of astrocytes. Astrocytes, washed and resuspended in serum-free medium, were first incubated with divalent cations (1 mM $CaCl_2$ + 0.5 mM $MgCl_2$ or 0.1 mM $MnCl_2$) for 10 min at 37°C, then added to the plate at 4×10^4 cells/well and incubated in the protein-coated wells for 2 hr at 37°C. In experiments testing the effect of soluble Kis-peptide or RGD peptides, cells were pretreated for 30 min at 37°C with the peptide and then added to the protein-coated plates. Afterwards, astrocytes were gently washed with PBS, fixed with 4% paraformaldehyde for 1 hr, and stained with 0.5% crystal violet. The absorbance obtained after solubilizing stained cells with 0.1% SDS was then monitored at 540 nm.

Indirect immunofluorescence of astrocytes

Astrocytes were grown on sterile coverslips in 24-well plates. After rinsing with PBS containing 2.5% FCS, astrocytes were incubated with 5×10^5 EL-4, EL-4^{-f} cells, or Fc fusion proteins conjugated to Protein A beads for 5 min at 37°C. After three gentle washes with PBS, the astrocytes were fixed for 10 min with 4% paraformaldehyde in PBS and then permeabilized with 0.1% Triton X-100, prepared in PBS containing 1 mM sodium orthovanadate (PBS/Vn). Alternatively, coverslips were coated with Fc fusion proteins as indicated for the cell-matrix adhesion assay, and after blocking the coverslips with 2% BSA, astrocytes were added in PBS containing 0.1 mM $MnCl_2$ to the coverslips and incubated for 3 hr at 37°C. Cells were then fixed and permeabilized as indicated above. Subsequently, cells were blocked for 15 min in 0.5% gelatin (fish-skin gelatin, Sigma) in PBS/Vn and then incubated with either the mAb anti-phosphotyrosine 4G10, anti-vinculin mAb, anti-FAK polyclonal antibody, or anti-paxillin mAb diluted in blocking buffer. After incubation for 1 hr at 37°C, the cells were washed in PBS/Vn and treated with both the appropriate second antibody conjugated to FITC and phalloidin conjugated to rhodamine to visualize F-actin. The fluorophores were visualized by confocal microscopy, and green fluorescence was quantified by counting the number of green pixels in fields of equal cell surface area using Adobe PhotoShop 5.0 software.

Tyrosine phosphorylation of focal adhesion proteins

Astrocytes were treated with Thy-1-Fc-Protein A beads, TRAIL-R2-Fc-Protein A beads, or Protein A beads only. After 5 min at 37°C, beads were removed by gently washing the wells with PBS/Vn, and the astrocytes were lysed with ice-cold cell lysis buffer as described [52]. The lysates were clarified by centrifugation at 13,000×g. Resulting supernatants were used to immunoprecipitate focal adhesion proteins such as $p130^{Cas}$ and FAK. For immunoprecipitation, Protein A beads were preincubated with 2 μg of each antibody for 1 hr at 4°C, followed by the addition of the cell lysates. Proteins bound to the beads were dissolved in SDS sample buffer containing reducing agent, separated by electrophoresis on 10% polyacrylamide gels [53], and transferred to nitrocellulose. Membranes were blocked with 2% gelatin in wash buffer (50 mM Tris-HCl, 150 mM NaCl, 0.2% Tween 20, and 0.4% gelatin) for 1 hr at room temperature. The nitrocellulose was then incubated for 1 hr with anti-phosphotyrosine mAb, followed by an anti-mouse IgG-HRP antibody.

The peroxidase activity was revealed by enhanced chemiluminescence using ECL. The data obtained was analyzed by scanning densitometry using the software programs DeskScan II 2.3 and NIH Image 1.6. To standardize results, numerical values obtained by scanning densitometry for tyrosine phosphorylated bands were normalized to values obtained for the respective protein bands using specific antibodies.

Supplementary material

Supplementary material containing a more detailed description of cells, peptides and reagents, preparation and characterization of the recombinant proteins, and the cell-cell adhesion assay utilized in this paper is available at http://images.cellpress.com/supmat/supmatin.htm. An additional control demonstrating that the mutation of Thy-1(RLD → RLE)-Fc does not result in dramatic conformational changes in the recombinant protein is also supplied with the online version of this article.

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