Aggregation of Integrins and RhoA Activation Are Required for Thy-1-induced Morphological Changes in Astrocytes*

Ana María Avalos‡, William T. Arthur§, Pascal Schneider¶, Andrew F. G. Quest‖, Keith Burridge§, and Lisette Leyton‡**

From the Programs of ‡Morphology and ‖Cell and Molecular Biology, FONDAP Center for Molecular Studies of the Cell, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Independencia 1027-A, Santiago, Chile, §Department of Cell and Developmental Biology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, and ¶Institute of Biochemistry, University of Lausanne, CH-1066 Epalinges, Switzerland

Thy-1, a cell adhesion molecule abundantly expressed in mammalian neurons, binds to a β_3-containing integrin on astrocytes and thereby stimulates the assembly of focal adhesions and stress fibers. Such events lead to morphological changes in astrocytes that resemble those occurring upon injury in the brain. Extracellular matrix proteins, typical integrin ligands, bind to integrins and promote receptor clustering as well as signal transduction events that involve small G proteins and cytoskeletal changes. Here we investigated the possibility that the cell surface protein Thy-1, when interacting with a β_3-containing integrin on astrocytes, could trigger signaling events similar to those generated by extracellular matrix proteins. DI-TNC$_1$ astrocytes were stimulated with Thy-1-Fc immobilized on beads, and increased RhoA activity was confirmed using an affinity precipitation assay. The effect of various inhibitors on the cellular response was also studied. The presence of Y-27632, an inhibitor of Rho kinase (p160ROCK), a key downstream effector of RhoA, significantly reduced focal adhesion and stress fiber formation induced by Thy-1. Similar effects were obtained when astrocytes were treated with C3 transferase, an inhibitor of RhoA. Alternatively, astrocytes were transfected with an expression vector encoding fusion proteins of enhanced green fluorescent protein with either the Rho-binding domain of Rhotekin, which blocks RhoA function, or the dominant-negative N19RhoA mutant. In both cases, Thy-1-induced focal adhesion formation was inhibited. Furthermore, we observed that RhoA activity after stimulation with soluble Thy-1-Fc molecule was augmented upon further cross-linking using protein A-Sepharose beads. The same was shown by cross-linking β_3-containing integrin with anti-β_3 antibodies. Together, these results indicate that Thy-1-mediated astrocyte stimulation depended on β_3 integrin clustering and the resulting increase in RhoA activity.

Focal adhesions (FAs)[1] mediate strong adhesion to the substrate and anchor actin stress fibers (SFs) through a supramolecular complex that consists of many different cytoskeletal as well as signaling molecules (reviewed in Refs. 1 and 2). The unique features of FAs suggest that they are employed as mechanosensors to inform cells about the immediate environment. As such, FAs are implicated in transmembrane cross-talk between extracellular matrix (ECM) proteins and intracellular signal transduction pathways via integrin receptors (reviewed in Refs. 3 and 4).

The interaction between ECM components and integrins leads to reorganization of the cytoskeleton and regulation of cell migration. Such events have been most extensively studied in fibroblasts (reviewed in Ref. 5) and linked there to activation of small GTPases belonging to the Rho family of monomeric 20–30-kDa GTP-binding proteins. Like other members of the Ras superfamily, Rho acts as a molecular switch, cycling between an active GTP-bound state and an inactive, GDP-bound state (reviewed in Refs. 6 and 7). The best-characterized members of this family are Rho, Rac, and Cdc42, all of which influence cell morphology and migration. In fibroblasts, Rho is required for the formation and maintenance of FAs and also for the formation of contractile actin-myosin filaments to form SFs (8).

Lysophosphatidic acid (LPA) and other soluble serum factors, such as sphingosine-1-phosphate, activate Rho as well as induce FA and SF formation in fibroblasts via G protein-coupled receptors (8). In astrocytes, the major type of glial cell in the brain, LPA also activates Rho and, consequently, the formation of FAs and SFs (9).

In the central nervous system, astrocytes have a stellate shape. When exposed to blood factors upon brain injury, they convert to a fibroblast-like morphology and undergo massive proliferation and migration to the site of injury to produce the glial scar. The latter protects the brain from secondary lesions but constitutes a major impediment to neuronal regeneration (10). Crushed or cut nerve fibers in the central nervous system often react with spontaneous but unsuccessful regenerative sprouting, a process that is linked to the presence of proteins that inhibit neurite outgrowth (11–13).

We previously demonstrated that Thy-1, an abundant neuronal surface protein, binds to a β_3-containing integrin receptor on astrocytes and triggers the assembly of FAs and SFs,

[1] The abbreviations used are: FA, focal adhesion; ECM, extracellular matrix; LPA, lysophosphatidic acid; SF, stress fiber; EGFP, enhanced green fluorescent protein; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; RBD, Rho-binding domain.

thereby promoting cell adhesion (14). Therefore, Thy-1-mediated interaction between neurons and astrocytes is expected to represent another means of neuron-glia communication. Indeed, Thy-1-dependent adhesion of neurons to astrocytes has been reported to inhibit neurite outgrowth (13, 15, 16). Our data have also implicated Thy-1 in the regulation of astrocyte morphology and adhesion through integrin β_3-dependent activation of FA and SF formation. In doing so, neuronal Thy-1 appears to be involved in bidirectional signaling between astrocytes and neurons (14).

Because most work has focused on evaluating the effects of Thy-1 in neurons (13, 16), little is known about the events triggered by Thy-1 in astrocytes required for the formation of FAs and SFs. More recent studies from our laboratory using Y-27632, an inhibitor of the Rho effector p160ROCK, suggested that Thy-1-dependent FA and SF formation requires RhoA activation (17).

In the present study, the role of Rho activation in the formation of FAs and SFs in Thy-1-stimulated astrocytes was characterized. A variety of Rho inhibitors were employed, and changes in Rho activity as well as in the formation of FAs and SFs upon Thy-1 stimulation of astrocytes were studied. Our results indicate that Thy-1 binding to astrocytes stimulated RhoA activity. Furthermore, negative regulators of Rho and one of its effectors, p160ROCK, inhibited the formation of FAs and SFs triggered by Thy-1. Finally, as has been reported for ECM proteins, the efficiency of Thy-1-mediated RhoA stimulation depended on multivalent engagement of integrins. In summary, Thy-1 interaction with an astrocytic β_3-containing integrin promotes FA and SF formation via RhoA activation by a process that involves integrin receptor clustering.

EXPERIMENTAL PROCEDURES

Cells, Recombinant Proteins, and Reagents—The rat astrocytic cell line DI-TNC$_1$, which displays many of the characteristic properties of neonatal astrocytes (18), was used in these studies. These cells were maintained in RPMI 1640 medium (Invitrogen) containing 10% serum (fetal bovine serum; HyClone), 0.1 mM 2-mercaptoethanol (Invitrogen), and 1% of the antibiotics penicillin and streptomycin (Invitrogen).

Reagents used were His-tagged C3 transferase (Cytoskeleton), Y-27632 (Calbiochem), and LipofectAMINE Plus (Invitrogen). The recombinant proteins Thy-1-Fc and TRAIL-R2-Fc were obtained as reported previously (14) and used either alone or coupled to protein A-Sepharose beads (Sigma). GST-Rho-binding domain (RBD) fusion proteins for the affinity precipitation assay were obtained as described previously (19). Enhanced green fluorescent protein (EGFP), EGFP-RBD, and EGFP-N19RhoA DNAs were purified using a Qiagen plasmid purification kit.

Immunofluorescence reagents were rhodamine-conjugated phalloidin (Sigma) and the antibodies mouse anti-paxillin monoclonal antibody (mAb) (Transduction Laboratories), rabbit anti-N-cadherin (Santa Cruz Biotechnology), and goat anti-mouse fluorescein isothiocyanate and goat anti-mouse Cy3 polyclonal antibodies (Jackson Immuno-Research). Other antibodies used were anti-β_3 integrin mAb (clone F11; Pharmingen), mouse anti-RhoA mAb (Cytoskeleton and Santa Cruz Biotechnology), and horseradish peroxidase-conjugated goat anti-mouse polyclonal antibody (Bio-Rad). Peroxidase reactivity was visualized using Super Signal West Pico Chemiluminescent Substrate (Pierce). All other reagents used for immunofluorescence, affinity precipitation assays, and Western blots were from Sigma.

RhoA Activity Assay (Affinity Precipitation)—Astrocytes were grown in 6-cm plates, and RhoA activity was measured using the affinity precipitation assay as described previously (20). Cells were serum-starved for 16 h and subsequently stimulated with Thy-1-Fc- or TRAIL-R2-Fc-protein A-Sepharose beads for 5 and 15 min. TRAIL-R2-Fc, a fusion protein prepared and obtained in the same way as Thy-1-Fc, was used as a negative control in these experiments (14). Starved cells and treatment with anti-N-cadherin-protein A-Sepharose beads were also used as negative controls, whereas LPA- or serum-stimulated cells (2 min) represented positive controls. In other experiments, cells were stimulated with soluble Thy-1-Fc protein only or anti-β_3 integrin antibody (15 min) and further cross-linked by addition of protein A-Sepharose beads (15 min) or treated with Thy-1-Fc-protein A-Sepharose

beads (15 min). After stimulation, cells were washed twice with 20 mM HEPES, pH 7.5, containing 0.15 N NaCl and lysed with buffer (50 mM Tris, pH 7.6) containing 0.15 N NaCl; 0.1% SDS; 0.5% sodium deoxycholate; 1% Triton X-100; 0.5 mM MgCl$_2$; 10 μg/ml each of aprotinin, leupeptin, and benzamidine; and 1 mM sodium orthovanadate. Lysates were cleared at $13,000 \times g$ for 4 min, and the supernatant was used for the Rho activity assay as described previously (20). Proteins on the beads were recovered by boiling the samples in Laemmli buffer and separated by SDS-PAGE on a 15% gel. Proteins were then transferred to a polyvinylidene difluoride membrane and probed with anti-RhoA mAbs. BCA reagent (Pierce) was used to determine the protein concentration of the whole cell lysates that were also used in immunoblots for RhoA as input controls. Densitometric analysis was performed using the UN-SCAN-IT gel automated digitalizing system (Silk Scientific Corp.).

Indirect Immunofluorescence—The day before treatment with the different RhoA modulators, astrocytes were grown on sterile coverslips in 24-well plates in complete RPMI 1640 medium. Astrocytes were loaded with C3 transferase for 1 h at 37 °C, as described previously (21). Briefly, 2 μg of C3 transferase, mixed with 2 μl of LipofectAMINE and 20 μl of serum-free medium, were incubated for 15 min before addition to the astrocytes in 400 μl of serum-free medium. Astrocytes were also treated with Y-27632 at different concentrations in serum-free medium for 30 min at 37 °C, as described previously (22), or transiently transfected with either EGFP-, EGFP-RBD-, or EGFP-N19RhoA-containing plasmids using LipofectAMINE Plus reagent (Invitrogen), following the manufacturer's instructions.

After the treatments or transfections described previously, astrocytes were either stimulated with Thy-1-Fc-protein A-Sepharose beads for 10 min or left unstimulated. Then, the beads were removed by three gentle washes with phosphate-buffered saline (PBS), and cells were fixed for 15 min with 4% paraformaldehyde in 100 mM PIPES buffer, pH 6.8, containing 0.04 M KOH, 2 mM EGTA, and 2 mM MgCl$_2$. Afterwards, they were washed three times with 150 mM Tris-HCl, pH 7.5, and twice with PBS. Cells were permeabilized with 0.1% Triton X-100 in PBS containing 1 mM sodium orthovanadate and aprotinin, leupeptin, and benzamidine (10 μg/ml each) for 10 min, washed twice with PBS, and then blocked with 1% bovine serum albumin in PBS for 15 min. Cells were then stained with an anti-paxillin mAb, followed by a second antibody coupled to fluorescein isothiocyanate and phalloidin conjugated to rhodamine or a second antibody coupled to Cy3 when cells were transfected with green fluorescent protein constructs. After washing and mounting the coverslips on microscope slides with Mowiol-2.5% 1,4-Diazabicyclo [2.2.2]octane, fluorophores were visualized by confocal microscopy in a Zeiss LSM-410 Axiovert-100 confocal microscope (Unidad de Analisis Celular Integral, Institute of Biomedical Sciences, University of Chile).

The number of FA sites per cell was quantified using the ImageJ software program from the National Institutes of Health public domain. The number of focal adhesions was estimated by selecting structures of determined pixel range above a threshold value.

RESULTS

Tyrosine phosphorylation at focal contacts formed by astrocytes cultured on plates is enhanced by incubation with either Thy-1-Fc coupled to protein A-Sepharose beads or cells containing high levels of Thy-1 on their surface. Thy-1-dependent cell-cell interaction is blocked by RLD-containing peptides and by anti-β_3 integrin antibodies (14). Hence, FA formation observed in astrocytes in culture is enhanced by cell-cell interactions requiring a β_3 integrin, and this can be mimicked by adding Thy-1-coated beads to the cells (14). Because integrins are generally considered receptors for ECM, rather than cell surface proteins, molecular mechanisms employed in integrin-mediated FA and SF formation were investigated in Thy-1-stimulated astrocytes.

We first tested whether Thy-1-induced FA and SF formation required activation of RhoA. To measure the activity of RhoA in astrocytes under various conditions, we used an affinity precipitation assay described previously (23). DI-TNC$_1$ astrocytes were stimulated with either LPA (a known activator of RhoA), Thy-1-Fc-protein A-Sepharose beads (Thy-1-Fc beads), or TRAIL-R2-Fc-protein A-Sepharose beads (TRAIL-R2-Fc beads) as a control. Subsequently, active Rho-GTP from cell lysates
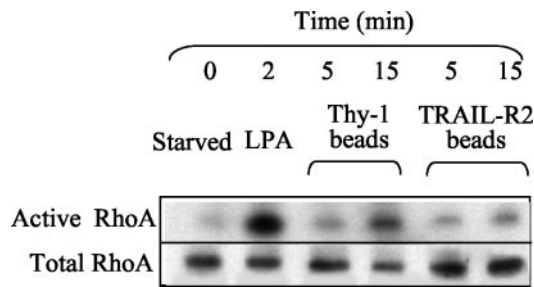
Fig. 1. **Thy-1 binding to astrocytes stimulated RhoA activity.** DI-TNC$_1$ astrocytes were serum-starved and then stimulated with Thy-1-Fc- or TRAIL-R2-Fc-protein A-Sepharose beads for 5–15 min or with 2 μg/ml LPA for 2 min before lysing the cells. Affinity precipitated RhoA (*Active RhoA*) and whole cell lysates (*Total RhoA*) were visualized by immunoblotting with anti-RhoA mAbs.

was precipitated by incubation with the GST-RBD fusion protein coupled to glutathione-agarose beads (20, 23). Similar levels of total Rho protein were observed in all whole cell lysates (Fig. 1, *Total RhoA*), whereas incubation with Thy-1-Fc beads, but not TRAIL-R2-Fc beads, stimulated RhoA activity (*Active RhoA*). Activation was time-dependent because incubation with Thy-1-Fc beads yielded higher levels of active RhoA at 15 min than after 5 min.

To corroborate these results, astrocytes were treated with different modulators of Rho activity. As reported previously, Thy-1-Fc coupled to protein A-Sepharose beads stimulated the formation of FAs and SFs (Fig. 2*A*, *gray bars*; Fig. 2*C*, *top panels*) (14). Controls in these experiments included treatments with either anti-N-cadherin antibodies coupled to protein A-Sepharose beads (anti-N-cadherin beads) or TRAIL-R2-Fc beads. Anti-N-cadherin antibodies bound to the surface of astrocytes, as revealed by immunofluorescence experiments (data not shown), but did not affect FA and SF formation (Fig. 2*A*; Fig.2*C*, *top panels*). TRAIL-R-2-Fc beads do not bind to astrocytes; however, because this fusion protein was prepared in the same manner as Thy-1-Fc, it served as an appropriate control for the Fc-fusion protein and beads used. As expected, based on our previous results, no changes in astrocyte morphology or FA and SF formation were observed in response to treatment with TRAIL-R2-Fc beads (Fig. 2*A*; Fig.2*C*, *top panels*).

Upon binding to GTP, Rho interacts with and activates different downstream effectors (reviewed in Refs. 24 and 25). Among these effectors, p160ROCK (also known as Rho kinase) is involved in Rho-induced formation of FAs and actin SFs (26). To investigate the role of p160ROCK in Thy-1-stimulated formation of FAs and SFs in astrocytes, the specific inhibitor Y-27632 was employed (22, 27). Our previous results with this compound indicate that 50 μM Y-27632 decreases the formation of FAs and SFs induced by Thy-1 (17). However, because Y-27632 is reported to be specific for p160ROCK at 10 μM (22), the effect of lower concentrations of this inhibitor was tested. In cells treated with Y-27632, morphological changes were apparent at 5 μM when compared with nontreated cells (Fig. 2*B*). Because basal FAs were reduced over 50% with 10 μM Y-27632, and this value was not significantly different from the results obtained with 50 μM Y-27632, the former concentration was used to assess the effect of this p160ROCK inhibitor on Thy-1-stimulated FAs and SFs. In general, only a few FAs and SFs, as well as small focal complexes, were visible at 10 μM Y-27632. The remaining actin bundles and adhesion contacts were mainly associated with the lateral cell border (Fig. 2*C*, *bottom panels*). Morphological changes produced by Y-27632 were not reversed by treatment with Thy-1-Fc beads (Fig. 2*A*, *black bars*), indicating that FA and SF formation induced by Thy-1 depended on at least one Rho effector, p160ROCK.

To test a different Rho activity modulator, astrocytes were loaded with the exoenzyme C3 transferase that specifically inactivates Rho-dependent signaling pathways by selective Rho-ADP-ribosylation. C3 transferase was introduced into cells using liposomes containing polycationic and neutral lipids, as has been reported for NIH-3T3 fibroblasts (21) and HeLa cells (28) (see Experimental Procedures). Treatment with C3 transferase led to the disassembly of basal levels of FAs and SFs in both nonstimulated (Fig. 3, *top right panels*) and TRAIL-R2-Fc bead-treated cells (data not shown). This effect was not reversed upon Thy-1 stimulation (Fig. 3, *bottom right panels*). Therefore, RhoA inhibition by C3 transferase prevented Thy-1-induced FA and SF formation in astrocytes.

As an alternative strategy to block RhoA activity, astrocytes were transiently transfected with a vector expressing the RBD of Rhotekin as an EGFP fusion protein. This RBD binds to active RhoA, thereby sequestering RhoA and preventing its interaction with endogenous effectors. In astrocytes expressing EGFP-RBD, a diminished number of FAs were apparent, and these structures failed to develop in response to Thy-1 (Fig. 4, *right panels*). No effect was detected when expressing EGFP alone (Fig. 4, *left panels*). Thus, these results corroborate the notion that FA formation induced by Thy-1 in astrocytes is RhoA-dependent.

To further demonstrate that Thy-1-stimulated formation of FAs depends on active RhoA, astrocytes were transiently transfected with a vector expressing an EGFP-tagged dominant-negative mutant of Rho (N19RhoA). This mutant binds to guanine nucleotide exchange factors, thereby preventing the activation of endogenous Rho (29). EGFP alone did not affect the formation of FAs in nonstimulated cells (Fig. 4, *NS*) or Thy-1-Fc-stimulated cells (Fig. 5, *top panels*; Fig. 4, *left panels*). In astrocytes expressing EGFP-N19RhoA, decreased basal levels of FAs were detected (data not shown). This phenotype was not altered upon Thy-1 stimulation (see the *outlined area* in the *bottom panels*), whereas untransfected adjacent cells still responded to Thy-1-Fc beads (Fig. 5, *bottom panels*). Taken together, these results demonstrate that Rho activation is required for Thy-1-induced FA formation in astrocytes.

Signal transduction pathways triggered by integrin ligation are known to involve clustering of the receptors, which permits their association with cytoplasmic proteins that connect them to the cytoskeleton (reviewed in Ref. 30). Because cell surface Thy-1 induces its effects in astrocytes engaging a β_3 integrin (14), the hypothesis that Thy-1 acted as a multivalent ligand was tested. Astrocytes were stimulated with serum or soluble Thy-1-Fc (sThy-1), and the latter was subsequently cross-linked by the addition of protein A-Sepharose beads. In astrocytes treated only with sThy-1, a small but significant stimulation of RhoA activity was observed (Fig. 6*A*). However, upon addition of either Thy-1-Fc previously bound to the beads (*Thy-1-beads*) or protein A-Sepharose beads in the presence of Thy-1-Fc molecules already bound to the cells (*sThy-1 + beads*), RhoA activity was significantly enhanced by 5- and 7-fold, respectively, as determined by densitometric analysis (Fig. 6*A*). As a negative control, anti-N-cadherin antibodies were also used. N-cadherin belongs to a family of Ca^{2+}-dependent adhesion molecules and does not activate RhoA (31, 32). Bound antibodies were cross-linked with protein A-Sepharose beads, and, as expected, RhoA activity did not increase (1.01 $\pm$ 0.094, average $\pm$ S.E.; $n = 5$).

According to our previous results, Thy-1 elicits responses in astrocytes by engaging a β_3-containing integrin (14). To further demonstrate that in astrocytes Thy-1 activates RhoA by clustering integrin β_3 receptors, astrocytes were treated with anti-β_3 antibodies and subsequently cross-linked by the addition
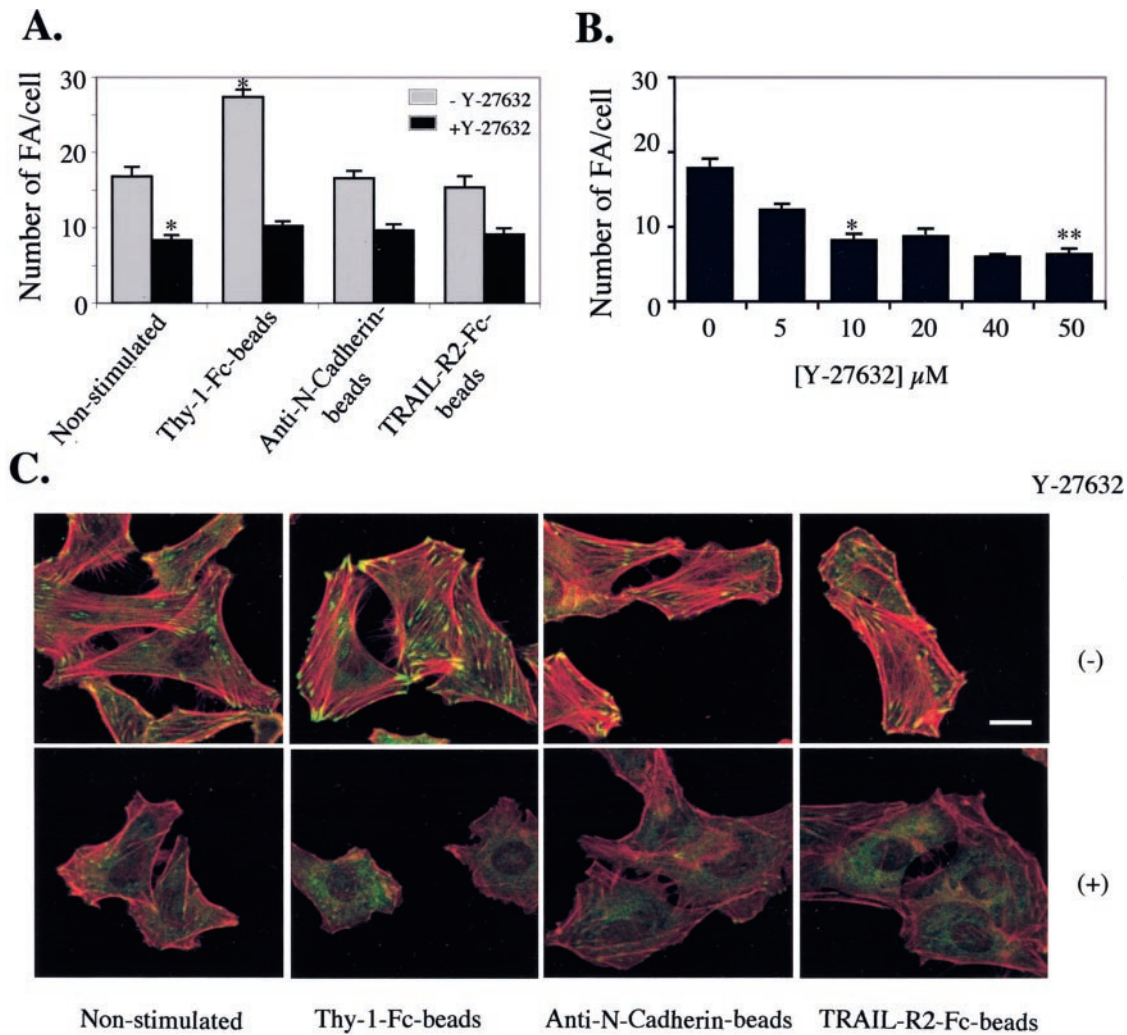
FIG. 2. **Effect of Y-27632 on focal adhesion and stress fiber formation in astrocytes.** DI-TNC$_1$ astrocytes were treated without or with Y-27632 (10 μM, *A* and *C*; 5–50 μM, *B*) for 30 min in serum-free RPMI 1640 medium. Cells were then left without further treatment (*Non-stimulated*, *A* and *C*; 0–50 μM, *B*) or stimulated with Thy-1-Fc-, anti-N-cadherin-, or TRAIL-R2-Fc-protein A-Sepharose beads (*A* and *C*). After the different treatments, cells were washed, fixed, and permeabilized. Focal adhesions were stained with an anti-paxillin mAb followed by fluorescein isothiocyanate-labeled anti-mouse IgG. Rhodamine-conjugated phalloidin was used to visualize filamentous actin by confocal microscopy. The number of focal adhesions per cell was determined for at least 50 cells per experimental condition using the ImageJ program. Data shown are averages ± S.E. of one duplicate experiment. Values shown are representative of three independent experiments. Nonparametric Mann-Whitney analysis was used to compare each experimental condition with non-stimulated cells. A *single asterisk* indicates a significant difference compared with nonstimulated cells in the absence of Y-27632 ($p < 0.001$; *A* and *B*); a *double asterisk* indicates no significant difference compared with cells treated with 10 μM Y-27632 (*B*). *C*, representative photographs of cells treated as indicated in *A* are shown. *Bar* = 25 μm.

of protein A-Sepharose beads. Little effect was obtained when anti-β_3 antibodies (Fig. 6*B*, α-β_3) were used alone. However, if antibodies were cross-linked with protein A-Sepharose beads (α-β_3 + *beads*), activation of Rho protein was apparent (Fig. 6*B*).

Taken together, these results indicate that engagement of a β_3 integrin on astrocytes by Thy-1 triggers Rho activation in a manner that depends on the extent of integrin cross-linking.

DISCUSSION

Thy-1, a highly abundant protein present on the surface of mammalian neurons, was identified as a ligand for a β_3-containing integrin, probably $\alpha_V\beta_3$ (14). Binding of Thy-1 to this integrin present in astrocyte membranes stimulates the assembly of FAs and SFs in astrocytes. High levels of tyrosine phosphorylation of FA proteins and recruitment of proteins that form this large supramolecular complex have also been reported upon Thy-1-stimulation of astrocytes (14).

Typical ligands of integrins are proteins of the ECM. Cell-matrix contacts are specialized sites of the cell surface where integrins link the ECM to the cytoskeleton. Plating cells on

fibronectin or other ECM proteins that engage integrins activates a variety of signaling pathways that result in cell spreading, membrane ruffling, and eventually FA and SF formation. These events require coordinated activation of the small G proteins Rac and Cdc42 (33), as well as Rho (34).

Because molecular mechanisms governing signaling by cell-cell interaction need not be the same as those involved in cell-ECM interaction, we investigated signaling pathways triggered by Thy-1-dependent engagement of integrins on the surface of astrocytes. The results obtained indicated that Thy-1 promoted activation of the small G protein RhoA (Fig. 1) most efficiently when presented as a multivalent ligand (Fig. 6). The role of RhoA and one of its effectors (p160ROCK) was further substantiated by treating the cells with either C3 transferase or the p160ROCK inhibitor (Y-27632), respectively (Figs. 2 and 3). Inhibition of p160ROCK with Y-27632 also appeared to induce membrane ruffles and focal complexes (Fig. 2*C*, *bottom panels*), which were not seen upon C3 treatment (Fig. 3). A similar phenomenon has been reported in fibroblasts to be associated with a different pathway emanating from active Rho
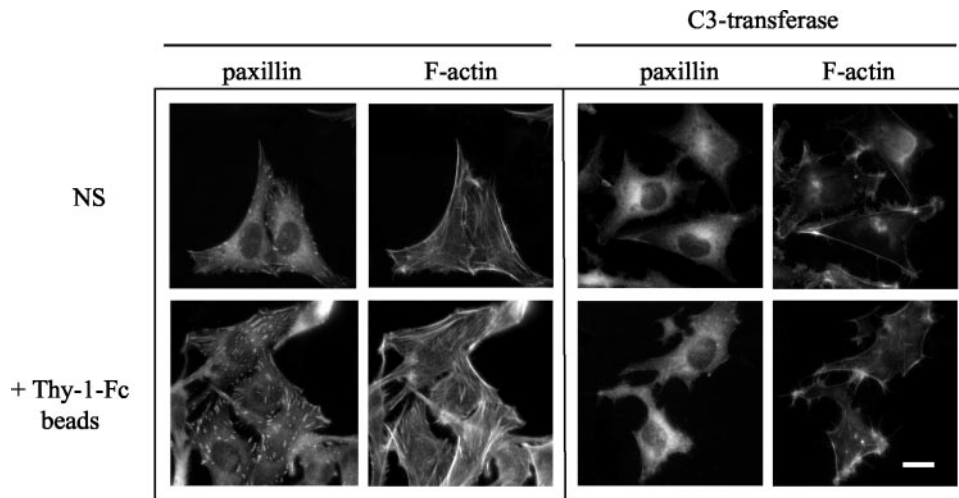
FIG. 3. **C3 transferase blocked Thy-1-stimulated focal adhesion and stress fiber formation in astrocytes.** DI-TNC$_1$ astrocytes were cultured in serum-free RPMI 1640 medium and left in the absence of C3 transferase (*left panels*) or loaded with C3 transferase (*right panels*) for 1 h. Cells were then stimulated for 10 min with Thy-1-Fc bound to protein A-Sepharose beads (+ *Thy-1-Fc beads*) or left unstimulated as a control (*NS*). The cells were then washed, fixed, and permeabilized. Focal adhesions and stress fibers were stained as indicated in the Fig. 2 legend. These structures were visualized by confocal microscopy. Photographs shown are representative of three separate experiments. *Bar* = 25 μm.
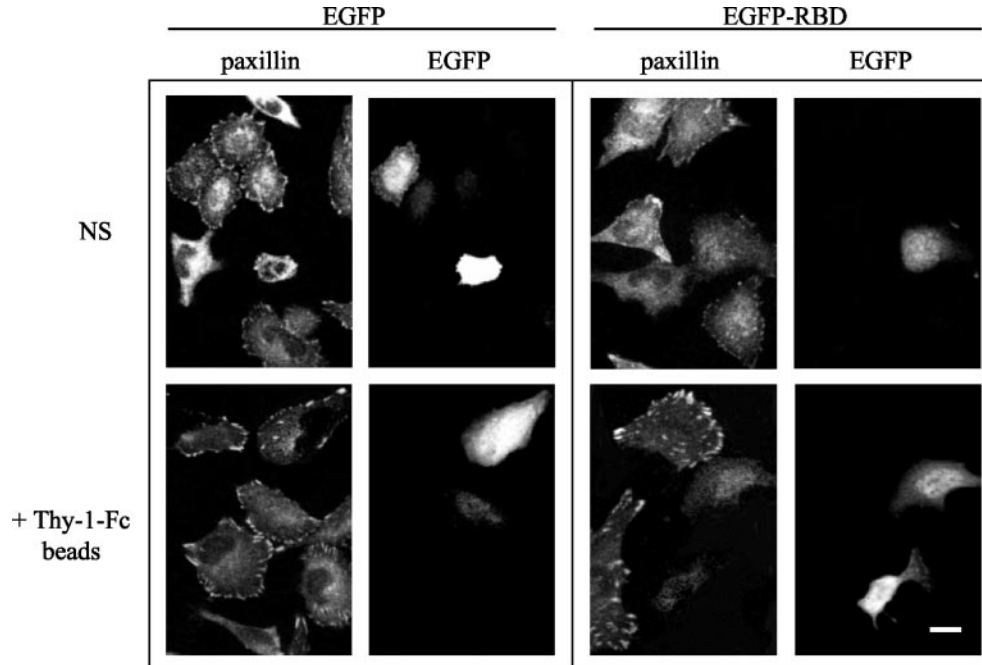

FIG. 4. **RBD expression in astrocytes blocked focal adhesion formation induced by Thy-1.** DI-TNC$_1$ astrocytes were transiently transfected with either EGFP (*left panels*)- or EGFP-RBD (*right panels*)-containing plasmids and left untreated (*NS*) or stimulated with Thy-1-Fc-protein A-Sepharose beads (+ *Thy-1-Fc beads*). Immunofluorescence was performed with mouse anti-paxillin antibodies followed by Cy3-labeled anti-mouse IgG. Focal adhesions in nontransfected and EGFP-transfected cells (green fluorescence) were visualized using confocal microscopy. Photographs shown are representative of at least three separate experiments. *Bar* = 25 μm.

that includes activation of mDia, phosphorylation of p130Cas, and Rac activation (35). Whether this pathway also operates in astrocytes remains to be tested.

On the other hand, sequestration of active Rho by expression of an RBD-containing protein (Fig. 4) or inhibition of RhoA activation by expression of a dominant-negative RhoA protein (Fig. 5) led to disassembly of existing FAs and prevented Thy-1-stimulated FA formation. These results underscore the importance of RhoA activity in both maintenance of FAs in nonstimulated astrocytes and Thy-1-induced FA formation. Furthermore, the morphology of cells expressing these fusion proteins was more strongly altered than in Y-27632-treated cells, where residual actin filaments and adhesion points associated with the cell border were still apparent (Fig. 2C). The

latter observation suggests that the pathway controlled by p160ROCK might not be the only one downstream of Rho important for cytoskeletal organization in astrocytes.

Differential regulation of Rho activity by various integrins has been reported previously (36). Moreover, an initial dip in Rho activity has been observed when fibroblasts are plated on fibronectin (23) or upon treating them in suspension with soluble RGD peptides (34) known to engage $\alpha_5\beta_1$ integrin. Reports also indicate that β_3 and β_1 integrins could lead to different responses, but the effect seems to depend on the cell type (37, 38). However, in all cases, activation of the receptors was achieved using ECM-derived ligands.

In experiments shown here, integrin engagement upon cell-cell interaction was mimicked using adherent astrocytes and
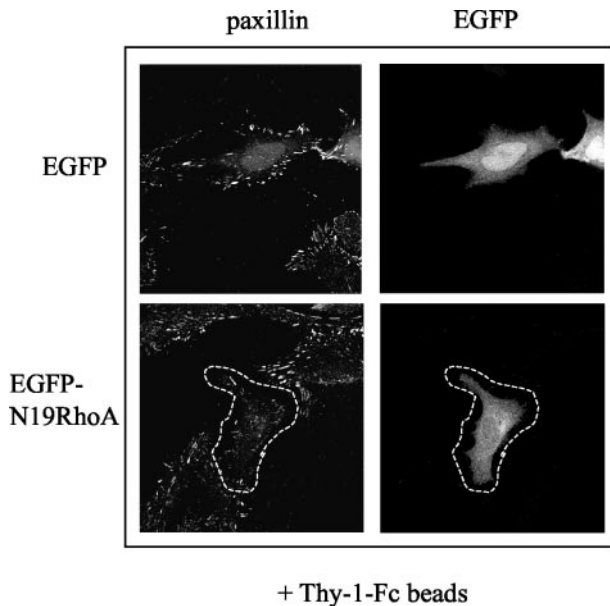
FIG. 5. **The expression of a dominant-negative mutant of RhoA affected the formation of focal adhesions in astrocytes.** DI-TNC$_1$ astrocytes were transiently transfected with either EGFP (*top panels*)- or EGFP-N19Rho (dominant-negative mutant; *bottom panels*)-containing plasmids and then stimulated with Thy-1-Fc beads. Immunofluorescence was performed with mouse anti-paxillin antibodies followed by Cy3-labeled anti-mouse IgG. Focal adhesions in nontransfected and EGFP-transfected cells (green fluorescence) were visualized using confocal microscopy. *Outlined area* in the *bottom panels* defines an EGFP-N19Rho-transfected cell. Images shown are representative of the results observed in a minimum of three separate experiments.

subsequently adding surface-bound Thy-1 ligand. With this approach, Rho activity was stimulated by treatment with soluble Thy-1 and substantially enhanced when the degree of cross-linking was increased (Fig. 6). Addition of soluble Thy-1-Fc fusion protein increased the RhoA activity of treated astrocytes 2–3-fold, indicating that ligand binding and a modest degree of cross-linking (Thy-1-Fc is a dimeric molecule) were sufficient to trigger RhoA activation in adherent cells (Fig. 6). Independent of the manner in which cells were treated, Thy-1 significantly increased RhoA activity when compared with starved cells. Moreover, RhoA activation caused by soluble Thy-1 was significantly enhanced upon subsequent addition of protein A-Sepharose beads, indicating that the extent of RhoA activation depended on the degree of receptor cross-linking. Similar results were obtained when cross-linking anti-β_3 antibodies on astrocytes, whereas no activation was seen when anti-N-cadherin antibodies were cross-linked. Thus, for already adherent astrocytes, the receptor engaged by both soluble Thy-1 and anti-β_3 antibodies activated RhoA when cross-linked on the surface of astrocytes.

Should these and other results now be taken to indicate that cell-ECM interactions involve initial inhibition of RhoA, whereas cell-cell interactions immediately activate RhoA and thereby promote FA formation? The most likely answer is no. The initial inhibition of RhoA seen upon cell-ECM interaction might be due to the fact that rounding of cells upon trypsinization increases Rho activity, possibly to facilitate cell retraction due to changes in cortical actin cytoskeleton (28). Such elevated activity levels need to be reduced to allow cells to adhere to and spread over a substrate. Thus, differential modulation of Rho activity could be due to the cell type used (see above and also see Ref. 39), the integrin engaged, the nature of the ligand, and/or the mode of stimulation. In our experiments, astrocytes were already bound to the plate. Small FAs were apparent, and Rho activity was kept low by starving cells before stimulation.
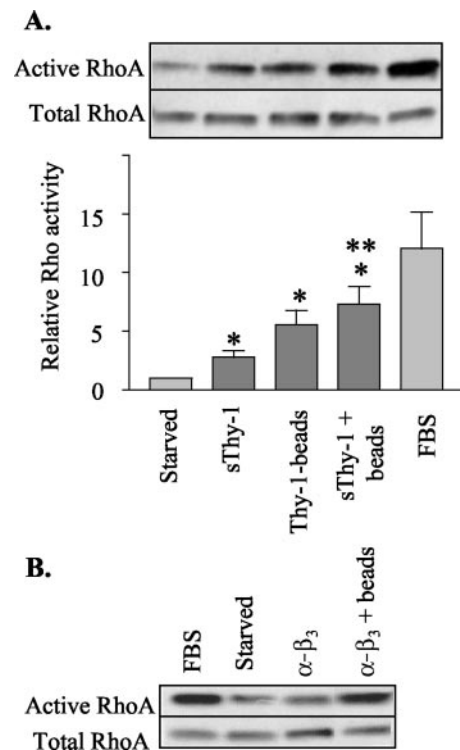

FIG. 6. **Thy-1-stimulated RhoA activity requires aggregation of integrin receptors on astrocytes.** *A*, DI-TNC$_1$ astrocytes were serum-starved and then stimulated with either fetal bovine serum (*FBS*; 2 min), soluble Thy-1-Fc protein (*sThy-1*; 15 min), soluble Thy-1-Fc bound to protein A-Sepharose beads (*Thy-1-beads*; 15 min), or soluble Thy-1 (15 min) followed by the addition of protein A-Sepharose for 15 more minutes (*sThy-1 + beads*). Cells were lysed, and active RhoA levels were determined by affinity precipitation of active Rho and immunoblot analysis. Numerical data shown were obtained by densitometric analysis. Average values ± S.E. calculated from five independent experiments are shown. Nonparametric Mann-Whitney analysis was used to compare each experimental condition (*dark gray bars*) with serum-starved cells or among themselves. A *single asterisk* indicates a significant difference compared with starved cells, and a *double asterisk* indicates a significant difference compared with sThy-1 ($p < 0.01$). *B*, astrocytes were serum-starved before stimulation with fetal bovine serum (*FBS*; 2 min), anti-β_3 integrin antibodies (α-β_3; 15 min), or anti-β_3 antibodies (15 min) followed by the addition of protein A-Sepharose beads (α-β_3 + *beads*; 15 min) before cell lysis and quantification of active RhoA. Affinity precipitated RhoA (*Active RhoA*) and whole cell lysates (*Total RhoA*) were immunoblotted with anti-RhoA mAbs. Results shown are representative of two separate experiments.

Under these conditions, processes such as membrane ruffling and cell spreading are not required. Instead, Thy-1 stimulation increased both the size and number of pre-existing adhesions, which correlates well with the observed activation of RhoA.

FAs and SFs are prominent in many cells grown in culture, including astrocytes, but are rarely seen in cells within the body. They reflect elevation of Rho activity and the resulting tension exerted on a rigid, two-dimensional substratum (1). We have shown here that the induction of FAs and SFs in cultured astrocytes by binding of Thy-1 requires increased Rho activity. In cell culture, increased formation of such structures is associated with elevated contractility. In the brain, increases in astrocytic contractility may represent another form of neuron-astrocyte communication and perhaps contribute to wound healing and scar formation. Because the glial scar constitutes a serious impediment to neuronal regeneration, elevated tension exerted by astrocytes on neighboring neurons may represent one of several mechanisms by which the scar prevents neuronal repair upon brain injury.

In summary, our results indicate that when the cell surface protein Thy-1 engages the astrocytic $\alpha_V\beta_3$ integrin, signaling

cascades that include RhoA and p160ROCK activation are required to induce FA and SF formation in astrocytes. Moreover, we also demonstrate that the degree of Rho activation depended on clustering of integrin β_3 receptors. Additional studies will investigate whether Thy-1-triggered FA and SF formation in astrocytes via Rho-dependent signal transduction pathways is relevant for processes involving neuron-glia communication in the brain.

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