

Integrins in Point Contacts Mediate Cell Spreading: Factors That Regulate Integrin Accumulation in Point Contacts vs. Focal Contacts

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Abstract. We have studied the function and distribution of the $\alpha_1\beta_1$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$ heterodimers on type-1 astrocytes with antibodies specific for integrin subunits (α_1 , α_5 , α_6 , and β_1). The $\alpha_1\beta_1$ heterodimer mediates adhesion to laminin and collagen, the $\alpha_5\beta_1$ to fibronectin in an RGD-dependent manner. The $\alpha_5\beta_1$ integrin is found in focal contacts in long-term cultures of well-spread astrocytes colocalizing with vinculin and the termini of actin stress fibers. $\alpha_1\beta_1$ heterodimers can occasionally be found as small aggregates within focal contacts but they do not accumulate there. Instead, $\alpha_1\beta_1$ integrins are found in punctate deposits called point contacts which are distributed over the upper and the lower cell surfaces whether laminin, collagen, fibronectin or polylysine is used as a substratum. Unlike focal contacts, point contacts contain clathrin but rarely codistribute with actin or vinculin.

Two observations indicate that these point contacts are functional. First, mAb 3A3, directed against the rat α_1 subunit, inhibits the attachment of astrocytes to laminin and collagen. Second, during the spreading of astrocytes, a band of point contacts forms around the cell perimeter at a time when no focal contacts are visible. While $\alpha_1\beta_1$ integrins are found only in point contacts in astrocytes, the $\alpha_6\beta_1$ integrin, another laminin receptor, is localized within focal contacts. Moreover, $\alpha_1\beta_1$ heterodimers accumulate in focal contacts in fibroblasts. Thus, the α subunit contributes, independent of its ligand, to functional integrin heterodimer accumulation in focal contacts or in point contacts. This accumulation varies among different cell types with apparently identical heterodimers as well as with the motile state (spreading vs. flattened) of the same cells.

CELLS in culture form specialized contacts among themselves and with their extracellular matrix (ECM)¹ that are often identical to those found in vivo (Geiger et al., 1985; Burridge et al., 1988). Integrins, the major family of ECM receptors (Hynes et al., 1987; Hemler, M. E., 1990; Ruoslahti, E., 1991), are well known to concentrate within attachment sites called focal contacts (Chen et al., 1985; Damsky et al., 1985; and for review see Burridge et al., 1988). Integrins in focal contacts bind to extracellular ligands such as laminin, collagen or fibronectin within the ECM as well as the cytoskeletal proteins vinculin, talin and α -actinin (Horwitz et al., 1986; Tapley et al., 1989; Otey et al., 1990) via their cytoplasmic tails. Much attention has focused on focal contacts because of their characteristic and well-ordered structure. However, several other types of substratum contacts have been reported including: podosomes, dot contacts, and point contacts (Tarone et al., 1985; Nermut et al., 1991; Streeter and Rees, 1987). Podosomes are described as large aggregates (200–400 nm) containing actin (Tarone et al., 1985). Point contacts and dot contacts are

small (90–200 nm) punctate regions of the cell closely apposed (15 nm) to the substratum (Streeter and Rees 1987; Nermut et al., 1991). We have been drawn to the study of these nonfocal contacts by our interest in neural cells in which focal contacts tend to be sparse or nonexistent (Wilson, P., N. Tawil, and S. Carbonetto. 1991. International Brain Research Organization Abstracts. 3:59).

Here we show that well-spread astroglia in culture form both focal contacts and point contacts. These cells express multiple integrins of the β_1 subclass including the $\alpha_1\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$ heterodimers. While $\alpha_5\beta_1$ and $\alpha_6\beta_1$ heterodimers accumulate within focal contacts, the $\alpha_1\beta_1$ integrins are concentrated in punctate arrays, essentially identical to point contacts described by Nermut et al. (1991). $\alpha_1\beta_1$ heterodimers can be found within focal contacts but their occurrence there is no greater than elsewhere on the cell surface. Since $\alpha_5\beta_1$, $\alpha_1\beta_1$, and $\alpha_6\beta_1$ integrins share the same β subunit we conclude that the α subunit is involved in regulation of integrin accumulation within the two different types of cell–substratum contacts. Localization of the $\alpha_1\beta_1$ heterodimer is ligand independent and found in point contacts on astrocytes regardless of the substratum (collagen, lami-

1. *Abbreviations used in this paper:* ECM, extracellular matrix; NEM, *N*-ethyl maleimide.

nin, or fibronectin). In contrast, the $\alpha_6\beta_1$ integrin, also a laminin receptor, is found within focal contacts on the same cells. The early stages of cell attachment and spreading are mediated exclusively by integrins in point contacts. These and other data directly implicate integrins in point contacts in this motile phase of these otherwise sessile astrocytes. Finally, $\alpha_1\beta_1$ integrins in well-spread rat fibroblasts, unlike well-spread astrocytes, were localized to focal contacts. Even though an α subunit may be involved in the localization of integrins to two types of cell-substratum contacts within the same cell, there are cell-specific mechanisms which regulate the interaction of the same heterodimer with intracellular proteins targeting its accumulation in point contacts or focal contacts.

Materials and Methods

Cells and Cell Culture

Cell cultures highly enriched in type 1 astrocytes (>95%) were prepared according to methods described previously (McCarthy and De Vellis, 1980; Noble et al., 1984). Briefly, day 1 postnatal rat cerebral cortices were treated with trypsin (0.25% in Minimal Essential Medium (MEM; Gibco Laboratories, Grand City, NY) for 30 min at 37°C, following trituration cells were seeded into poly-L-lysine-coated culture flasks. After 10 d in culture, flasks were shaken overnight in DME (Gibco Laboratories). The suspended cells were discarded and the attached cells were removed by trypsin (0.25%), seeded into poly-L-lysine-coated flasks (5 μ g/ml), treated with 0.01 mM cytosine arabinoside for 1 wk, and then left in CNS medium which consists of DME medium with 10% FCS. Primary cultures of heart fibroblasts were prepared by simply excising heart tissue from newborn rats, trypsinizing it, and plating the cells on polylysine-coated dishes.

Antibodies

The mAb 3A3 is of the IgG₁ subclass and is directed against the rat α_1 subunit (Turner et al., 1989). Anti- β_1 antiserum was made to a purified β_1 subunit (Tawil et al., 1990). Anti- α_5 and anti- α_6 antisera (a generous gift from Dr. Erkki Ruoslahti, La Jolla Cancer Research Institute, San Diego, CA) were made to peptides whose sequences were derived from the cytoplasmic domain of the α_5 or α_6 subunits. mAbs to vinculin- and rhodamine-conjugated phalloidin were purchased (Sigma Chemical Co., St. Louis, MO).

Immunocytochemistry

Type 1 astrocytes or heart fibroblasts were harvested from confluent cultures after treatment with 5 mM EDTA in Hanks' solution without Ca^{2+} or Mg^{2+} (Gibco Laboratories) for 10 min at 37°C. The cells were centrifuged at 1,000 rpm for 10 min, resuspended in DME with 10% FCS, and seeded at 37°C on polylysine-, laminin-, or fibronectin-coated glass coverslips. 2–12 h later, cells were rinsed with PBS, and fixed with 3% paraformaldehyde in 100 mM phosphate buffer, pH 7.2, for 10 min. When required, cells were permeabilized with cold 0.1% NP-40 or 0.5% Triton X-100 in PBS for 10 min. After rinsing with PBS, cells were incubated with primary antibodies in 10% FCS in DME (Gibco Laboratories) for 1 h at 37°C. The cells were then rinsed several times with PBS and incubated for another hour with rhodamine or FITC-conjugated goat anti-rabbit or anti-mouse antisera (Bio/Can Scientific Inc., West Grove, PA) in 10% FCS in DME. The coverslips were washed with PBS and mounted onto glass slides in a solution of 0.1 M Tris and 70% glycerol at pH 7.0 or in Immunofluor mounting medium (ICN Radiochemicals, Irvine, CA). The slides were examined with a Zeiss epifluorescence microscope (Carl Zeiss, Oberkochen, Germany). Experiments in which astrocytes were sheared were carried out essentially as described by Avnur and Geiger (1981). Briefly, cells on coverslips were rinsed in buffer A (50 mM MES, 4-morpholin-ethanesulfonic acid, 5 mM MgCl_2 , 3 mM EGTA, pH 6.0), and then incubated in buffer B (buffer A plus 1 mM ZnCl_2) for ~2 min at room temperature. The cells were sheared from the coverslips with several brisk streams of PBS (pH 7.2) from a Pasteur pipette. This procedure removed all of the cell except for those regions of the ventral surface in close contact with the substratum. These

preparations were fixed in 3% paraformaldehyde and labeled as described above.

Inhibition of Cell Attachment

Cell attachment assays were carried out in 96-well plates using methods similar to those reported previously (Turner et al., 1989). The wells were coated overnight with 5 μ g/ml of laminin, collagen, fibronectin, or polylysine in PBS in 4°C. After rinsing with PBS three times, wells were blocked with 20 mg/ml BSA in PBS for 3 h. Type 1 astrocytes were radiolabeled with Trans- ^{35}S methionine in CNS medium (ICN Radiochemicals) for 24 h. Radiolabeled astrocytes were harvested with 5 mM EDTA and rinsed twice with PBS containing 1 mM CaCl_2 , and 1 mM MgCl_2 to remove unincorporated ^{35}S methionine, cells were then resuspended at 4×10^6 cells per ml in PBS with CaCl_2 and MgCl_2 . Antibodies (Fab fragments of anti- β_1 antisera, Fab fragment of anti-rabbit antiserum, or mAb 3A3) or peptides (RGD or RGE) were incubated with the resuspended cells for 10 min and then equal aliquots of cells were added to each well and allowed to attach for 90 min at 37°C. Unattached cells were removed by washing the wells three times with PBS. Attached cells were extracted with 1% NP-40 for 10 min and the extracts transferred to scintillation vials for counting in beta counter. Assays were performed in duplicate and repeated in at least two separate experiments. The percentage of attached cells calculated as follows:

$$\frac{\text{CPM of radiolabeled cells in wells containing antibodies}}{\text{CPM of radiolabeled cells in wells without antibodies}} \times 100\%$$

Immunoprecipitation

Type 1 astrocytes were surface labeled with ^{125}I iodine as described by Tomaselli et al. (1987). In brief, confluent cultures of cells were washed

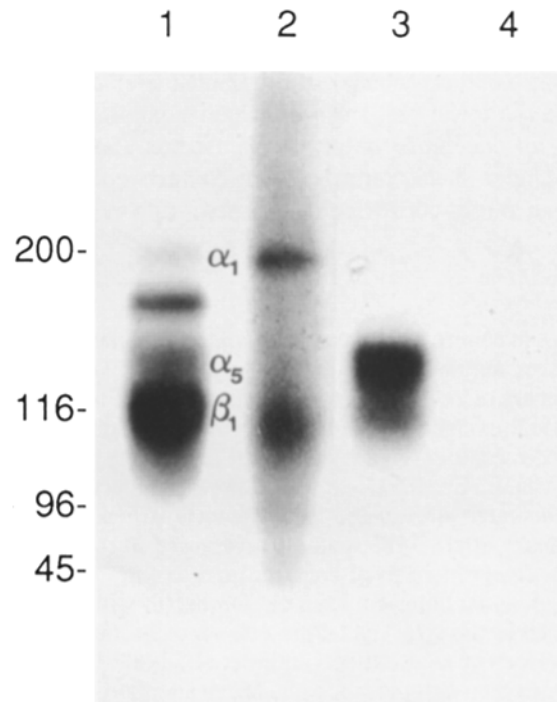


Figure 1. Type 1 astrocytes express $\alpha_1\beta_1$ and $\alpha_5\beta_1$ integrins. Type 1 astrocytes were surface labeled with ^{125}I iodine and extracted with 1% NP-40 detergent. The extract was used for immunoprecipitation with anti- β_1 , anti- α_5 antisera, and mAb 3A3. Samples were analyzed on 6% SDS-PAGE gels under reducing conditions and the radiolabeled bands were visualized with autoradiography as described in Materials and Methods. (Lane 1) Anti- β_1 antiserum; (lane 2) mAb 3A3; (lane 3) anti- α_5 antiserum; (lane 4) normal rabbit serum. Molecular mass markers are indicated on the left.

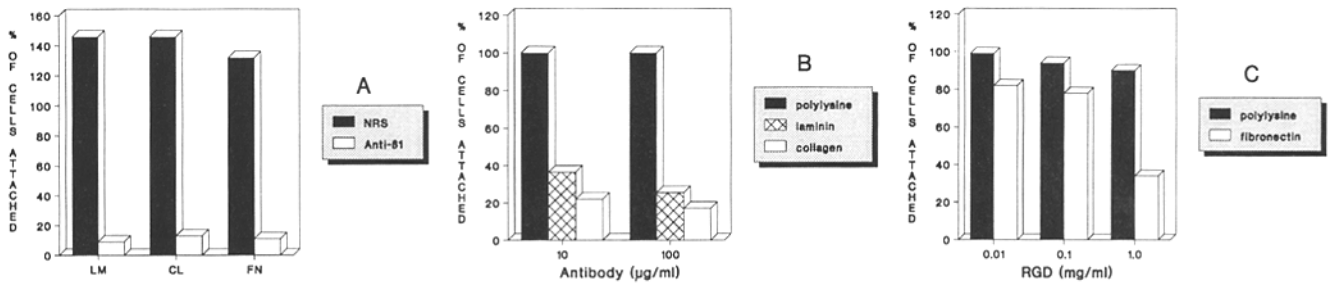


Figure 2. (A) Inhibition by anti- β_1 antisera of type 1 astrocyte adhesion to laminin, collagen and fibronectin. [35 S]-methionine-labeled astrocytes were allowed to attach to microwells coated with laminin, collagen, fibronectin, or polylysine in the presence of different antibodies as described in Materials and Methods. After 90 min, wells were rinsed to remove the nonadherent cells. The adherent cells were extracted with 1% NP-40 and transferred to scintillation vials and their cpm were determined with a beta counter. Data are expressed as a percentage of cells attached to the different substrata in the absence of antibody (see Methods). Each bar represents the mean of two experiments done in duplicate. In each experiment, the standard error was <5%. Fab fragments of anti- β_1 antisera (500–600 μ g/ml) inhibited the attachment of type 1 astrocytes to laminin (LM), collagen (CL), and fibronectin (FN) by >90%. Fab fragments of normal rabbit serum had no inhibitory effect in this adhesion assay. (B) The effect of mAb 3A3 on the attachment of type-1 astrocytes to laminin (LM), collagen (CL), and polylysine (PL). [35 S]methionine-labeled astrocytes were allowed to adhere to coated substrata in the presence of two different concentrations of mAb 3A3. The experiment was carried as explained in A. Data are expressed as the percent of attachment in the absence of an antibody. JG22, a mAb against the chick β_1 subunit had no effect on the attachment of astrocytes to the above substrata (data not shown). Each bar represents the mean of two experiments with duplicate wells in each experiment. The average standard error is <3%. (C) The effect of RGD peptide on the attachment of type 1 astrocytes to fibronectin or polylysine. [35 S]methionine-labeled astrocytes were allowed to adhere to coated wells in the presence of different concentrations of RGD or control (RGE) peptide for 90 min. Cell attachment was quantified as in A. Data are expressed as a percentage of attachment in the presence of RGE which had no effect on attachment. Each bar represents two separate experiments with duplicate determinations in each. The average standard error is <3%.

with PBS three times, harvested with 5 mM EDTA in HBSS without Ca^{2+} and Mg^{2+} , resuspended in PBS, and labeled with 0.5 mCi of [125 I]iodine. Cells were then washed to remove unincorporated iodine and then extracted with 1% NP-40 in PBS for 40 min at 4°C in the presence of 1 mM of the following protease inhibitors: PMSF, *N*-ethyl maleimide (NEM), and soybean trypsin inhibitor. The extract was then centrifuged at 100,000 *g* for 35 min at 4°C, and the pellet was discarded. Equal amounts of radioactivity from the supernatant (2×10^7 cpm) were added to PBS solution containing 50 μ g/ml of antibodies. After incubation with gentle shaking for 3–4 h at 4°C, 100 μ l of 50% (vol/vol) protein-A Sepharose stored in 1% NP-40 (Sigma Chemical Co.) was added to each mixture and shaken overnight at 4°C. Since mAb 3A3 is an IgG₁ and does not bind well to protein-A Sepharose, an anti-mouse IgG₁ antibody was added to the mixture for 1–2 h, and then the protein-A Sepharose beads were added as before. The next day, the beads were washed five times with 1% NP-40 in PBS to remove the unbound proteins. The bound proteins were eluted from the beads by boiling for 5 min in SDS-PAGE sample buffer without β -mercaptoethanol and then samples were electrophoresed on 6% SDS-polyacrylamide gels, stained, dried, and autoradiographed.

Results

Astrocytes Express $\alpha_1\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_1$ Integrins

In a previous study, we have shown that astrocytes express integrins from the β_1 subclass (Tawil et al., 1990). To further identify these integrins, type 1 astrocytes were surface labeled with [125 I]iodine and then extracted with 1% NP-40 detergent. Anti- β_1 antisera immunoprecipitated four proteins from these astrocyte extracts. The four bands were of 200, 170, 150, and 115 kD under nonreducing conditions on SDS-polyacrylamide gels. The lowest band migrates as a β_1 subunit (Fig. 1, lane 1) in agreement with other studies (Turner et al., 1989; Tawil et al., 1990). The other three bands were characterized further by immunoprecipitation with antibodies specific for integrin α subunits. mAb 3A3 recognizes the α_1 subunit (Turner et al., 1989; Tawil et al., 1990) and immunoprecipitated a band of 200 kD along with a band at 115 kD representing the α_1 and the β_1 subunits,

respectively (Fig. 1, lane 2). Antisera to the α_5 subunit immunoprecipitated two bands at 150 and 115 kD (Fig. 1, lane 3) while immunoprecipitation with anti- α_6 antisera immunoprecipitated bands at 210, 140, and 115 kD representing $\alpha_6\beta_1$ and $\alpha_6\beta_4$ integrins (Tawil, N., P. Wilson, and S. Carbonetto, manuscript in preparation). The 170-kD protein immunoprecipitated with anti- β_1 antisera has not been identified and may represent a novel α subunit. Thus type 1 astrocytes express integrins from the β_1 subclass including previously identified heterodimers $\alpha_1\beta_1$, $\alpha_3\beta_1$, and $\alpha_6\beta_1$.

$\alpha_1\beta_1$ Integrin Mediates the Attachment of Astrocytes to Laminin and Collagen, $\alpha_3\beta_1$ Integrin Mediates Attachment to Fibronectin

To determine which integrins astrocytes use in their binding to ECM molecules, we used a cell attachment assay in which either anti-integrin antibodies or ARG-GLY-ASP (RGD) synthetic peptides were assayed for inhibition of astrocyte attachment to different ECM substrata. Fab fragments of anti- β_1 antisera inhibited almost completely the attachment of astrocytes to laminin, collagen and to fibronectin (Fig. 2 A) suggesting that astrocytes attach to these ECM molecules mainly through members of the β_1 integrin family. mAb 3A3 inhibited the attachment of astrocytes to laminin and collagen by 75 and 85%, respectively, (Fig. 2 B) indicating that the $\alpha_1\beta_1$ integrin is the main laminin and collagen receptor on astrocytes. Anti- α_5 antisera used for immunoprecipitation studies are directed at a synthetic peptide derived from the cytoplasmic tail of the α_5 subunit and therefore would not be expected to inhibit cell attachment. However, the $\alpha_3\beta_1$ integrin binds to the RGD sequence of fibronectin (Ruoslahti and Pierschbacher, 1986) and soluble RGD inhibited by 65% the attachment of astrocytes to fibronectin (Fig. 2 C). RGE peptide, a control peptide, had no effect on the adhesion of astrocytes to fibronectin (Fig. 2 C). These

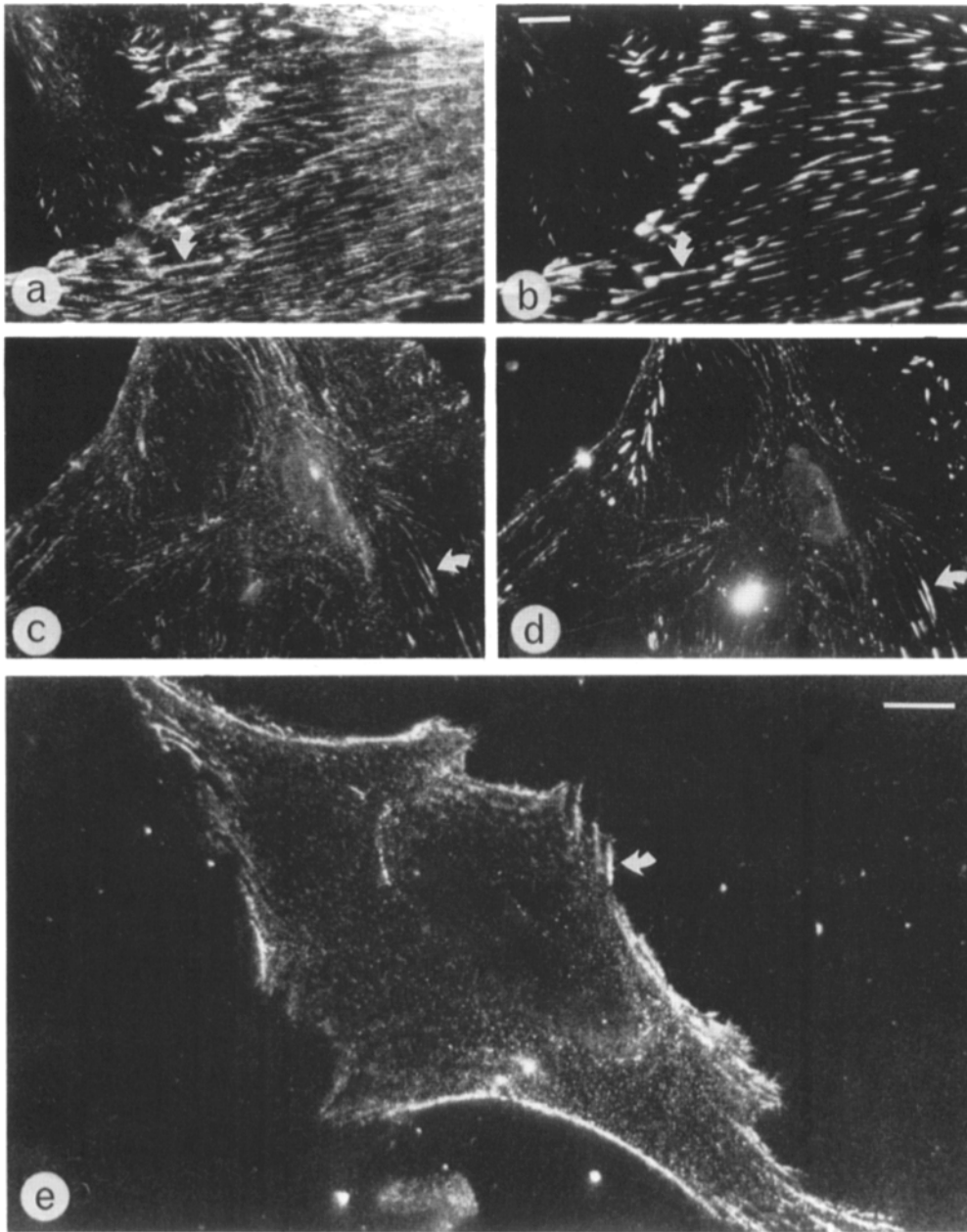


Figure 3. Immunocytochemical studies of astrocytes with anti- β_1 , anti- α_5 antisera, and anti-vinculin antibodies. Type 1 astrocytes were allowed to attach and spread for a minimum of 24 h on polylysine, laminin, or fibronectin overnight. The cells were then fixed and permeabilized and allowed to react with primary antibodies for 1 h at 37°C and then with FITC-conjugated anti-rabbit antisera for another hour. (A) Labeling with anti- β_1 antisera or (C) anti- α_5 antisera detected with fluorescein conjugated anti-rabbit antisera; (B and D) the same cells double labeled with anti-vinculin antibodies detected with rhodamine anti-mouse antibodies. (E) Labeling with anti- β_1 antibodies gave two patterns, focal contacts (arrow), and point contacts. Many point contacts are visible over the cell surface. Bar, 10 μ m.

data suggest that while the $\alpha_1\beta_1$ integrin mediates the attachment of astrocytes mainly to laminin and collagen, the $\alpha_5\beta_1$ integrin mediates their attachment to fibronectin.

$\alpha_5\beta_1$ and $\alpha_6\beta_1$ Integrins Are Localized to Focal Contacts while $\alpha_1\beta_1$ Integrins Are Concentrated in Point Contacts

Immunocytochemical studies were performed to determine the distribution of $\alpha_1\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$ integrins on type-1 astrocytes on different substrata. In fixed and permeabilized astrocytes, anti- β_1 antisera prominently labeled linear arrays of integrins (Fig. 3 a, arrow) on the lower cell surface which can be readily identified as focal contacts by virtue of their arrow codistribution with vinculin (Fig. 3 b, arrow). Labeling with anti- α_5 antisera (Fig. 3 c, arrow) was similarly localized to focal contacts codistributing with vinculin (Fig. 3 d, arrow) as was labeling with anti- α_6 antisera

(Fig. 4 d). Essentially identical results were seen in long-term cultures 1–3 d after seeding on laminin-, fibronectin-, or polylysine-coated substrata. In addition to focal contacts, anti- β_1 antisera labeled many small aggregates of integrins over the cell surface (Fig. 3 e). Interestingly, mAb 3A3-labeled well-spread astrocytes only in this punctate pattern and not in focal contacts (Fig. 4 a) regardless of the substratum (polylysine, collagen, or laminin). The same pattern of labeling was seen on live cells at 37°C (data not shown). To reduce antibody-induced redistribution and endocytosis of membrane proteins at this temperature, the labeling was also performed at 4°C, again with similar results. The punctate appearance seen with mAb 3A3 is distinct from that seen following labeling with fluorescent wheat germ agglutinin which labeled astrocytes diffusely (Fig. 4 b) suggesting that these punctate deposits are not in foldings of the plasma membrane but are aggregates of $\alpha_1\beta_1$ integrins in an otherwise uniform distribution of cell-surface glycoproteins.

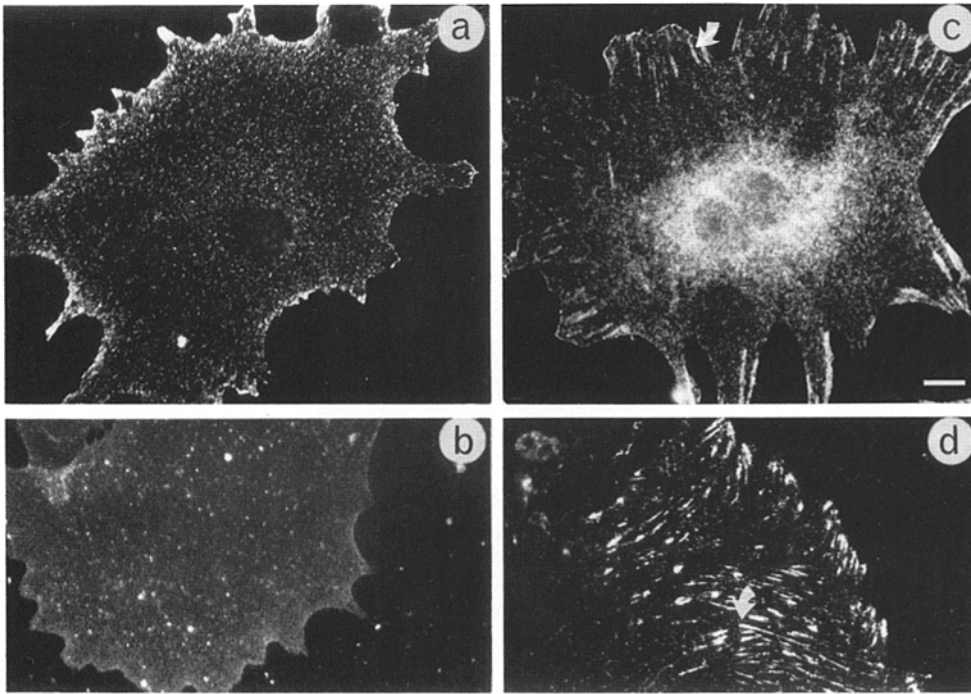


Figure 4. Immunocytochemical studies of α_1 , α_6 integrins on astrocytes and fibroblasts cultured on laminin or collagen substrata. (a) Labeling of an astrocyte on laminin with mAb 3A3 shows a punctate pattern with concentrations at the cell periphery. (b) An astrocyte labeled with wheat germ agglutinin has a much more diffuse uniform labeling. Many of the intensely bright spots are aggregates of lectin that can be seen over the culture dish as well as the cell. (c) Rat fibroblasts labeled with mAb 3A3 also shows prominent focal contacts (arrow). (d) Astrocytes seeded on laminin-coated coverslips and labeled with anti- α_6 antisera, have prominent focal contacts (arrow). Bar, 10 μ m.

Carter et al. (1991) have suggested that functionally inhibitory antibodies to integrins may be competitively excluded from binding to integrins within focal contacts by their ligands. Several data suggest that the failure of mAb 3A3 to label focal contacts is not the result of a restricted access to its antigen on the lower cell surface or receptor occupancy by ligand. First, as mentioned above, antisera to the β_1 subunit readily labeled focal contacts in astrocytes under identical conditions (Fig. 3 a). Similar labeling of focal contacts was seen with antisera to the α_6 subunit (Fig. 4 d); α_6 like $\alpha_1\beta_1$ heterodimers recognize laminin (Sonnenberg et al., 1988). Second, when astrocytes were labeled under conditions in which mAb 3A3 detaches cells, those remaining on the substratum and in the process of being detached, contained no visible focal contacts (data not shown). Third, mAb 3A3 clearly labels focal contacts in rat heart fibroblasts that are attached and spread on collagen (Fig. 4 c; Gullberg et al., 1990). Thus, similar to Wayner and co-workers (1991), we find that functionally inhibitory mAbs are not always competitively excluded from focal contacts, but may identify functional receptors in nonfocal contacts.

Although the $\alpha_1\beta_1$ integrins were in punctate arrays, it was not clear whether all of these arrays participated in attachment of cells to their substrata. To examine more closely sites of adhesion, therefore, we employed a technique primarily used to study cell-substratum adhesions. It involves shearing well-spread cells, from the coverslips to leave behind portions of their ventral surfaces tightly adherent to the substratum (Avnur and Geiger, 1981). Labeling these sheared cells with both anti- β_1 antisera and mAb 3A3 showed that the areas which remain adherent to the substratum had abundant focal contacts as well as many punctate deposits distributed over the lower cell surface. In several instances, punctate deposits containing the $\alpha_1\beta_1$ integrins could be seen within vinculin-containing focal contacts (Fig. 5), although they rarely accumulated to a density greater than that seen on the rest of the cell or formed continuous

linear arrays like $\alpha_6\beta_1$ and $\alpha_5\beta_1$ heterodimers (Fig. 4 d). After dual-labeling studies with mAb 3A3 (Fig. 6 a) and rhodamine-phalloidin (Fig. 6 b) or with anti- β_1 antisera (Fig. 6 c) and vinculin (Fig. 6 d), a small subset of the point contacts were found to contain actin or vinculin. Many point contacts, however, colocalized with clathrin as shown by dual labeling with mAb 3A3 (Fig. 6 e) and anti-clathrin antisera (Fig. 6 f).

In the studies of Nermut et al. (1991) integrins of the β_1 subclass were found within point contacts in transformed cells. To further confirm these observations, we examined the expression of integrins in a Schwannoma cell line. We found that β_1 integrins were found mainly in point contacts; no focal contacts were seen on these cells (Fig. 7, a and b). Immunoprecipitation studies showed that these cells have three to five different β_1 heterodimers (Fig. 8). Thus, several integrins with β_1 subunits, and not only the $\alpha_1\beta_1$, may be localized to point contacts.

We conclude that multiple β_1 integrins are distributed in focal contacts as well as in point contacts. While the $\alpha_5\beta_1$ and $\alpha_6\beta_1$ integrins on astrocytes form focal contacts colocalizing with vinculin and the end of actin cables, the $\alpha_1\beta_1$ heterodimer fails to accumulate there and is instead found in point contacts. Some point contacts are seen within the focal contacts but they don't accumulate there. Only a small subset of them colocalize with vinculin or actin, the majority with clathrin.

Integrins in Point Contacts Are Important in the Attachment and Spreading of Astrocytes

Immunocytochemical studies shown above, in addition to the cell attachment data, suggest that $\alpha_1\beta_1$ integrins concentrated in point contacts aid in the attachment of astrocytes to laminin and collagen. However, the presence of some aggregates of $\alpha_1\beta_1$ integrins in focal contacts raises the question of whether this small population are the only functional heterodimers. This seems not to be the case since astrocytes

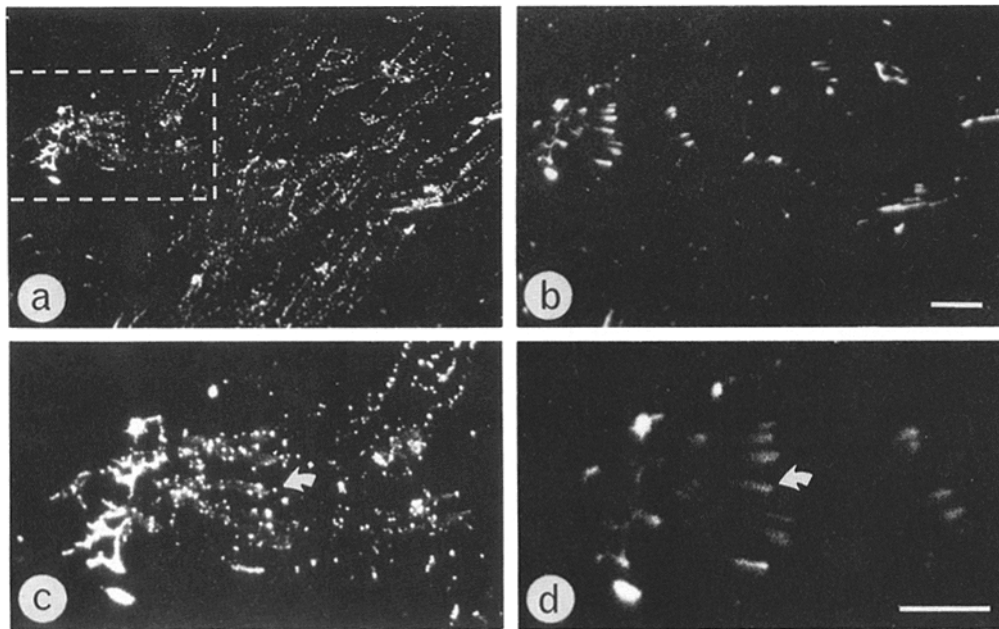


Figure 5. $\alpha_1\beta_1$ aggregates are found in focal contacts but do not accumulate there. Astrocytes were allowed to adhere and spread for at least 48 h on polylysine, laminin, or fibronectin coverslips and then were sheared (Materials and Methods) to remove most of the cell. Some cytoskeletal proteins and parts of the membrane firmly adherent to the substratum remained attached. *a* Shows labeling with mAb 3A3 and *b* shows labeling with phalloidin revealing the distribution of microfilaments. An inset (in *A*) is enlarged in *c* and the same area from *b* is enlarged in *d*. Punctate deposits of $\alpha_1\beta_1$ integrins can be found (*c*, arrow) colocalizing with phalloidin in focal contacts (*d*, arrow). Bar, 10 μm .

in the process of attaching and spreading lack focal contacts. This can be seen in Fig. 9 where point contacts containing $\alpha_1\beta_1$ integrins are distributed in a band surrounding the periphery of the spreading cells. A similar distribution is also seen with anti- β_1 antisera (Fig. 9, *b-d*) or anti- α_1 antisera (data not shown). The same obtains in fibroblasts where the $\alpha_1\beta_1$ heterodimer is found in focal contacts in well-spread cells (Fig. 9 *f*) but is in point contacts before they flatten (Fig. 9 *e*). In this early stage of cell spreading several integrins are found in point contacts and at a later stage, some of these heterodimers are found in focal contacts. These data also suggest that focal contacts are not necessary for the spreading of astrocytes or fibroblasts which is mediated by integrins in point contacts. Note also, that in the early stages of cell spreading (Fig. 9 *a*) there is a diffuse distribution of integrins in the middle of the cell. This diffuse distribution diminishes at later stages of spreading when more point contacts are seen (Fig. 9 *b*), and it likely represents a pool of integrins that move into point contacts as the cell continues to spread. This reorganization into point contacts may contribute to adhesion of motile cells or motile regions of cells which became stabilized by subsequent formation of focal contacts.

Discussion

The experiments described here were aimed at characterizing point contacts structurally and functionally as well as describing factors that regulate integrin localization within point or focal contacts. That the $\alpha_1\beta_1$ heterodimer is found constitutively in point contacts on astrocytes has led us to observations that these contacts function in cell-substratum adhesion especially during flattening of these normally sessile cells. In addition, we have found that localization of integrins in point vs. focal contacts varies with the α subunit although this can be strongly influenced by the cell type in which apparently identical heterodimers are expressed.

Integrins in Point Contacts Mediate Spreading of Astrocytes

In time-lapse studies of spreading astrocytes the periphery is the most active portion of the cell (Wilson, P., and S. Carbonetto, unpublished observations) and is surrounded by a ring of lamellipodia evidently responsible for the flattening of these otherwise sessile cells. As astrocytes attach and spread onto culture substrata coated with laminin, collagen or fibronectin, small aggregates of integrins (Fig. 9) form a band around the periphery of the cell. The integrins in these bands have several features of cell-substratum adhesions called point contacts (discussed below). mAb 3A3 labels the integrin $\alpha_1\beta_1$ which is localized exclusively in point contacts (Fig. 4 *a*) and serves as a marker for them. Since we have shown that mAb 3A3 inhibits the adhesion of astrocytes (Tawil et al., 1990; Fig. 2 *b*) to laminin and collagen, it follows that this heterodimer in point contacts is functional. In addition, antisera to the β_1 subunit and the RGD peptide inhibit attachment of astrocytes (Fig. 2 *a* and *b*) suggesting that point contacts function in the early stages of astrocyte flattening.

Point contacts have been identified previously by interference reflection microscopy, immunocytochemistry, and EM as regions of the cell closely apposed to the substratum (Neyfakh et al., 1983; Vasiliev et al., 1985; Streeter and Rees, 1987; Nermut et al., 1991). Several features of the punctate deposits of $\alpha_1\beta_1$ integrins found on the substratum side of sheared astrocyte preparations make it likely that these are point contacts rather than podosomes (Streeter and Rees, 1987; Nermut et al., 1991). First, punctate deposits of $\alpha_1\beta_1$ integrins have the dimensions (0.2–0.3 μm) of point contacts (Fig. 4 *a*). Second, unlike focal contacts, one class of point contacts are not associated with vinculin or actin (Nermut et al., 1991; Fig. 6) but instead colocalize with clathrin (Fig. 6 *f*). Third, after shearing of cells, the punctate deposits of integrins and point contacts remain (Fig. 5) firmly adherent to the substratum (Nermut et al., 1991). Fourth, both point

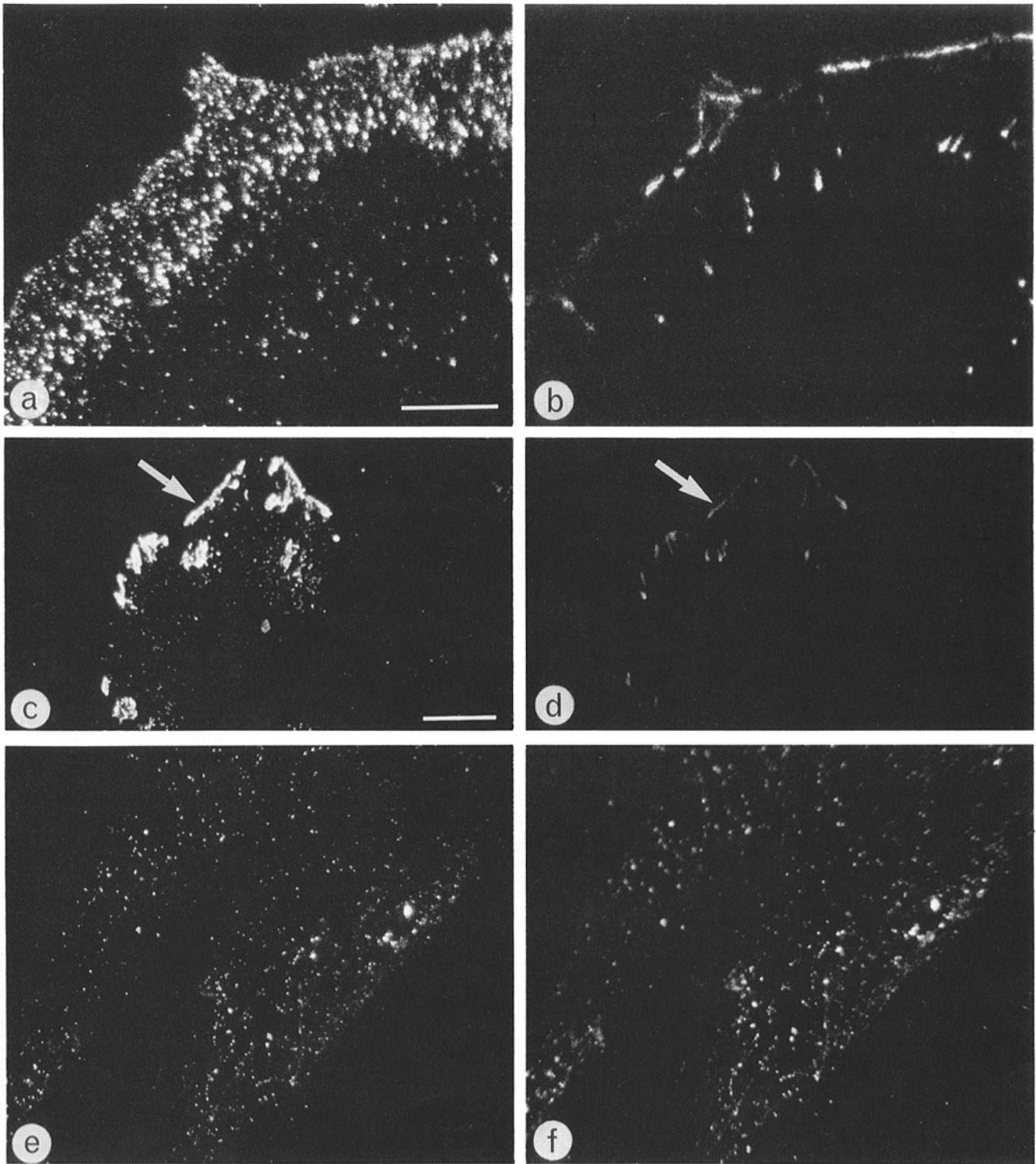


Figure 6. Colocalization of vinculin, actin and clathrin in point contacts. Astrocytes attached to laminin were sheared as explained in Materials and Methods and then labeled as in Fig. 5. Double-labeled astrocytes with mAb 3A3 (a) and phalloidin (b) or with anti- β_1 antisera (c) and vinculin (d). Some punctate contacts colocalize with vinculin (arrows). However, many point contacts, labeled in e with mAb 3A3, colocalize with clathrin f. Bar, 10 μm .

contacts (Neyfakh et al., 1983; Vasiliev, 1985) and the integrin aggregates (Fig. 9) are prominent on the leading edge of spreading cells. Additionally, Nermut et al. (1991), have reported that integrins of the β_1 class found within point contacts are involved in fibroblast motility (Nermut et al.,

1991). Consistent with this point of view, RN22 cells, a Schwannoma cell line that is motile, rarely forms focal contacts and their β_1 integrins are localized within point contacts (Fig. 7). However, the association of $\alpha_1\beta_1$ containing point contacts with cell motility is not absolute for such con-

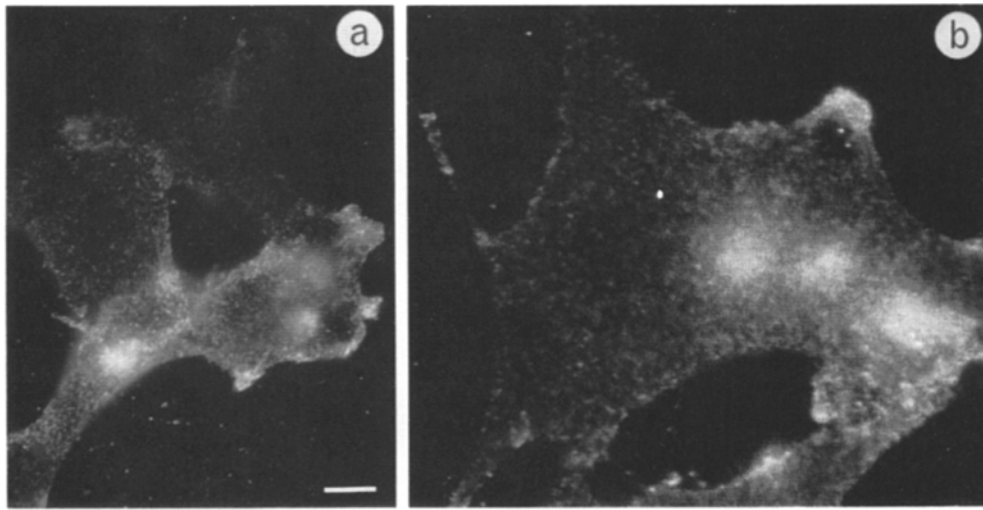


Figure 7. Immunocytochemical studies of RN22 Schwannoma cells. (a and b) RN22 Schwannoma cells were allowed to adhere and spread on laminin and then fixed and labeled with anti- β_1 antisera following the procedure in Fig. 3. No focal contacts were seen, only a punctate distribution of β_1 integrins. Bar, 10 μm .

tacts exist even in stationary astrocytes (Fig. 4 a). Indeed, long-term incubation of well-spread astrocytes with mAb 3A3 detached the cells from laminin and collagen substrata, though it localizes in a punctate pattern over the cell surface (Tawil, N. J., and S. Carbonetto, unpublished data). While we emphasize the dominant contribution of point contacts to cell adhesion during cell spreading, they also appear to contribute, along with focal contacts, to the adhesion of flattened cells.

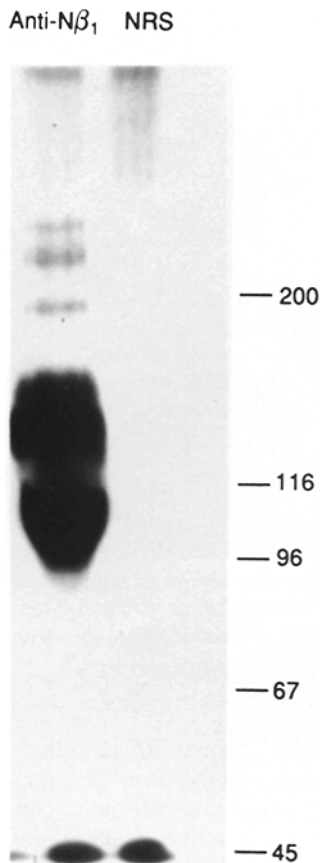


Figure 8. Immunoprecipitation with anti- β_1 antisera from RN22 Schwannoma cell line. [^{125}I]iodine-labeled RN22 Schwannoma cells were extracted and the lysates were used for immunoprecipitation as explained in Fig. 1. (Lane A) Anti- β_1 antisera; (lane B) normal rabbit serum.

Integrin Heterodimers with the Different α Subunits Localize to Point Contacts or Focal Contacts

Integrin localization in focal contacts depends on interaction with cytoskeletal proteins (Burrige et al., 1988). Mutation of the cytoplasmic tail of the β_1 subunit results in failure to form focal contacts and reduced cell-substratum adhesion (Solowska et al., 1989; Hayashi et al., 1990; Marcantonio et al., 1990; Rezka et al., 1992). In addition integrins are recruited into focal contacts as a function of the ligand coated on the substratum (Singer et al., 1988). Since the alpha subunits largely (Hemler, 1990), but not exclusively (Vogel et al., 1990; Smith et al., 1990; Bodary and McLean, 1990), determine ligand specificity they also influence this integrin localization. Our studies support the notion that the α subunit contributes to the localization of integrins on the cell surface by showing that $\alpha_1\beta_1$ and $\alpha_5\beta_1$, both functional on astrocytes, are found in distinct types of cell-substratum contacts on flattened cells. Astrocytes also have an $\alpha_6\beta_1$ integrin which, like the $\alpha_1\beta_1$, recognizes laminin (Sonnenberg et al., 1988). Localization of the $\alpha_6\beta_1$ heterodimer to focal (Fig. 4) contacts on astrocytes where $\alpha_1\beta_1$ is found only in point contacts is apparently not dictated by the ligand alone. Our antisera to the α_6 subunit is not functionally inhibitory and we have not been able to show that the $\alpha_6\beta_1$ receptor is actually interacting with laminin, though its participation in focal contacts is certainly consistent with this. In fact, the $\alpha_1\beta_1$ integrin is found in point contacts regardless of the ligand (laminin, fibronectin, and polylysine) on the substratum. Its function, of course, requires laminin or collagen and the detachment by mAb 3A3 of long-term cultures, (Tawil, N. J., and S. Carbonetto, unpublished results) but not freshly attaching cells (Fig. 2), from polylysine likely reflects the fact that astrocytes synthesize and assemble laminin matrices (Liesi, 1985; David, 1988; Ard and Bunge, 1988). Similarly, the localization of $\alpha_5\beta_1$ in focal contacts on substrata initially coated with polylysine likely reflects the production of fibronectin by these same cells (Price and Hynes, 1985).

The contribution of the cytoplasmic regions of alpha subunits to cell-surface localization has been less well studied than that of the cytoplasmic domains of the β subunits or of the α subunits' extracellular domains. In one study, de-

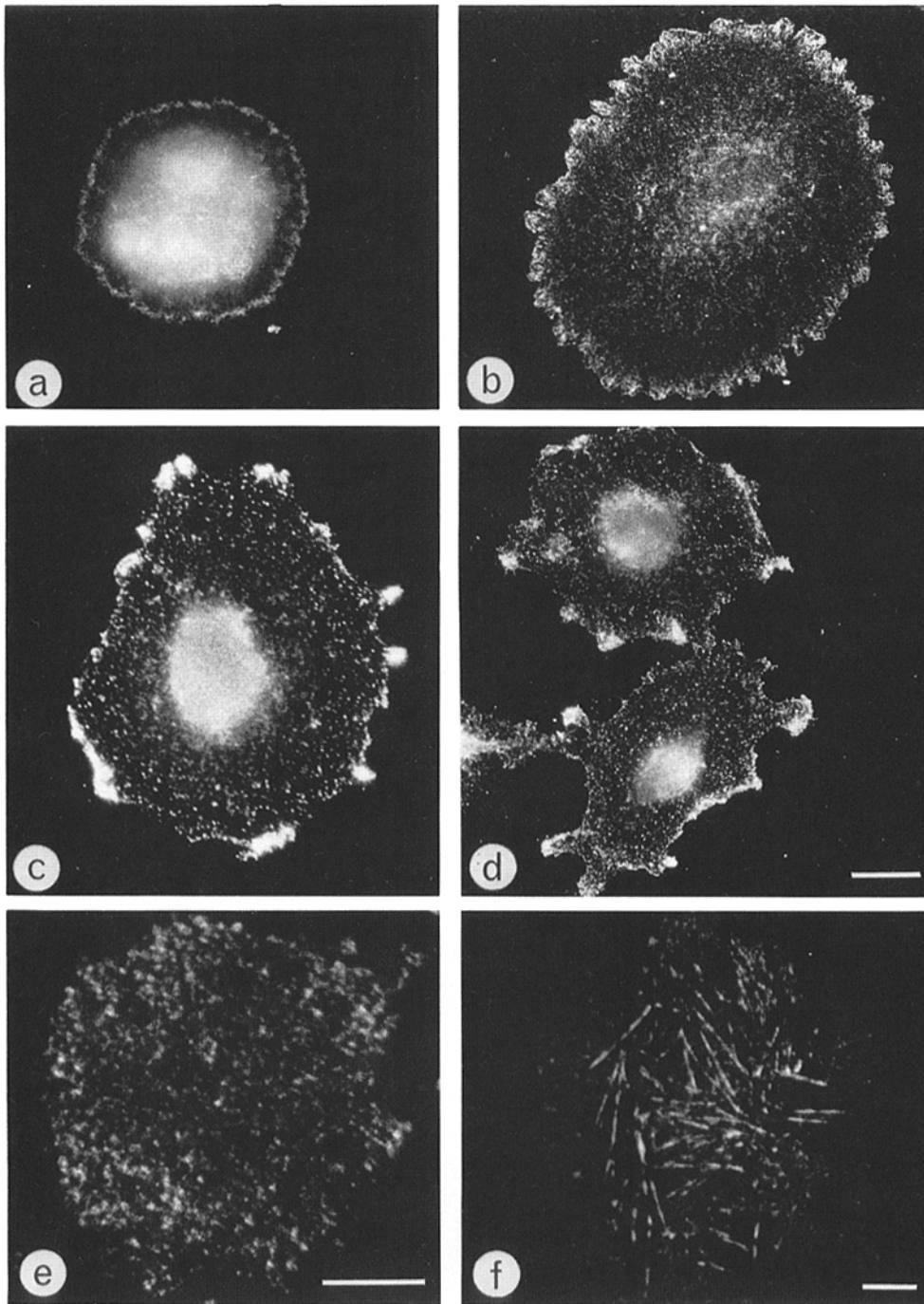


Figure 9. Immunocytochemical localization of integrins on spreading astrocytes and fibroblasts. Astrocytes were allowed to attach and spread on laminin or fibronectin for 2 h, and then fixed and labeled as in Fig. 5 with mAb 3A3 (on laminin), (a) or anti- β_1 antisera (on fibronectin) (b). (c and d) Astrocytes 2–4 h after seeding on fibronectin-coated coverslips and labeled with anti- β_1 antisera. Notice that mAb 3A3, or anti- β_1 antisera show integrins localized only within point contacts as the cells spread; labeling within anti- α_5 antisera gave a similar result (data not shown). (e) Rat fibroblasts spreading on fibronectin and labeled with mAb 3A3 also reveal no focal contacts seen in early stages of spreading as compared with spread fibroblasts (f). Bar, 10 μ m.

letion of the cytoplasmic domain of the α_1 subunit did not alter cell attachment mediated by LFA-1 binding to ICAM-1 in short-term adhesion assays (Hibbs et al., 1991). On the other hand, several recent studies suggest that heterodimers with overlapping ligand specificities have distinct cellular distributions (Elices et al., 1991; Wayner et al., 1991). This is manifest in our studies where both $\alpha_1\beta_1$ and $\alpha_6\beta_1$ recognize laminin but segregate to point vs. focal contacts, respectively. These data suggest indirectly that the α subunit modulates heterodimer interaction with cytoplasmic proteins. The cytoplasmic regions are among the most highly variable regions of the α subunits and offer potential for producing

distinct intracellular signals from heterodimers that may bind the same ligand.

It is also evident from our studies that the interactions between the integrins and cytoskeletal proteins are not dictated merely by the subunit composition of the heterodimers. The $\alpha_5\beta_1$ and other β_1 integrins which are found in focal contacts in flattened astrocytes are only in point contacts as astrocytes spread. Conversely, the $\alpha_1\beta_1$ heterodimer, which is found in point contacts on flattened astrocytes, localizes within focal contacts on rat fibroblasts (Fig. 4 c; Gullberg et al., 1990). Although the subunit composition contributes to the localization of functional integrins within point or fo-

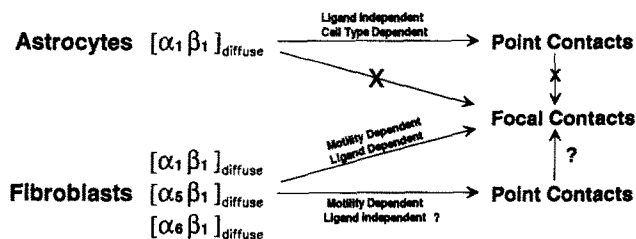


Figure 10. Hypothetical scheme describing the genesis of point and focal contacts on rat astrocytes. Unattached astrocytes have a diffuse distribution of integrins on their surface. During attachment and spreading point contacts of β_1 integrins become apparent over the lamellipodia of the cell. Independent of the ligand, the $\alpha_1\beta_1$ heterodimer remains in point contacts on astrocytes, although in fibroblasts this heterodimer accumulates in focal contacts. The $\alpha_5\beta_1$ and $\alpha_6\beta_1$ integrins also accumulate first in point contacts and later in focal contacts once the cells have flattened. It is unclear whether these are recruited from the pool of diffuse receptors.

cal contacts, this localization may be overridden depending on the cell type and on whether or not the cell is spreading.

Integrin Redistribution during Cell Attachment and Spreading

Fig. 10 illustrates a scheme for the targeting of integrins to point contacts and focal contacts during cell attachment and spreading. During the initial stages of cell attachment, astrocytes or fibroblasts are spherical with a small flattened region at the lower cell surface. At this time, a large pool of β_1 integrins, including the $\alpha_1\beta_1$ heterodimer, are diffusely distributed at the main portion of the cell. As the cell spreads, a diffuse collection of β_1 integrins (Fig. 9 a) is seen over the nucleus but the $\alpha_1\beta_1$ integrin has been recruited into point contacts (Fig. 9 b). Still later, in flattened cells, several β_1 integrins ($\alpha_5\beta_1$, $\alpha_6\beta_1$) are found in focal contacts (Fig. 3, a and c, and 4, c and d), in astrocytes and fibroblasts, though $\alpha_1\beta_1$ heterodimers in astrocytes, unlike fibroblasts, are found only in point contacts (Fig. 4 a).

In principle, the localization of integrins within cell-substratum contacts could involve redistribution of presynthesized receptors at the cell surface as well as selective insertion or degradation of newly synthesized receptors. Receptor metabolism appears unlikely to be a major factor in the localization of receptors in cultured cells which occurs in the 1–2 h that the cells take to flatten; although there is evidence for rapid insertion of integrins in lymphocytes (Isenberg et al., 1987). In rat astrocytes that have been sheared to remove all but the most adherent portion of the lower cell surface, we have observed clusters of $\alpha_1\beta_1$ integrins within regions of focal contacts (Fig. 5) suggesting that these clusters are not constrained from moving into focal contacts. Once there, however, the clusters do not accumulate to form the relatively high density seen with the $\alpha_5\beta_1$, $\alpha_6\beta_1$ heterodimers. In the case of the $\alpha_1\beta_1$ integrin in point contacts, there is little codistribution with vinculin, talin, or actinin (Fig. 6) but colocalization instead with clathrin (Fig. 6). Clathrin is a well-known component of coated pits responsible for endocytosis of cell surface receptors (Goldstein et al., 1985). Bretscher (1989, 1992) has suggested that endocytosis of integrins is intimately linked with cell motility via cycling of receptors in and out of the cell surface. He proposes that the

accumulations of receptors at the periphery of spreading cells (Damsky et al., 1985) results from addition to the surface of unligated receptors. We have found accumulations of integrins in point contacts at the cell periphery, however, we believe that these receptors on spreading astrocytes (Fig. 9) are functioning and hence bound with ligand since antibodies to the $\alpha_1\beta_1$ heterodimer or to the β_1 subunit alone block astrocyte spreading.

That these point contacts codistribute with clathrin is in keeping with Bretscher's suggestion that an endocytic-like mechanism is involved in cell motility. Adhesion of receptors followed by endocytosis, may be important in the transient cell-substratum contacts that participate in motility. Within the cytoplasmic domain of the β subunit are two regions (cyto 2 and 3; Rezka et al., 1992) which contain amino acid sequences similar or identical to the NPXY sequence that is involved in internalization of cell-surface receptors into coated pits (Chen et al., 1990). Rezka et al. (1992) report that mutation of these sequences substantially reduces the localization of integrins in focal contacts; no data are given of their effects on cell adhesion, motility or localization in point contacts. In our studies clathrin colocalizes with the $\alpha_1\beta_1$ integrin in point contacts but not with integrins in focal contacts. Conceivably focal contacts derive from point contacts (Fig. 10) and disruption of the cyto 2 or 3 domains (Reszka et al., 1990) interferes with the formation of clathrin-containing point contacts and hence focal contacts. Possibly related to this hypothesis, Wayner et al. (1991) have shown that the $\alpha_v\beta_3$ integrin but not the $\alpha_v\beta_5$ localizes in focal contacts. The latter has a distribution which is punctate and seems very similar to that of the $\alpha_1\beta_1$ integrin in point contacts. Interestingly the $\alpha_v\beta_5$ heterodimer has an eight amino acid insert near its presumed cyto 3 region that is not found in the β_3 subunit and may alter its cytoplasmic interactions. Taken together these data suggest that the involvement of integrins with clathrin-mediated endocytosis may be a mechanism for regulating cell motility and that this in turn is a function of both β and α subunits. An attractive feature of this proposal is that it has the potential for rapid regulation of adhesion, certainly within the 1–2 h during which cells in culture flatten, and possibly within the minutes required for cell guidance.

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