CELL STRUCTURE AND FUNCTION 16: 135–139 (1991)

Expression of Chicken Integrin β_1 Subunit in Rat PC12 Cells

Yokichi Hayashi[1,2], Alfred Reszka[2], Taisen Iguchi[3], Barb Reinish[2], Takuji Kawashima[1], and Alan F. Horwitz[2]

[1]Biochemical Research Institute, Morinaga Milk Ind. Co. LTD, 5-1-83 Higashihara, Zama, Kanagawa 228, Japan, [2]Department of Cell and Structural Biology, University of Illinois, Urbana, IL 61801, USA, and [3]Department of Biology, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama 236, Japan

Key words: Integrin β_1 subunit/Chimeric receptor/PC12 cells/Cell spreading

ABSTRACT. **We transfected rat pheochromocytoma (PC12) cells with a cDNA encoding chicken integrin β_1 subunit. The chicken integrin β_1 subunit produced in stable transfectants associated with two major α subunits of rat integrins to form interspecific chimeric receptors. These receptors mediated cell spreading and initial neurite outgrowth on laminin as did corresponding endogenous integrins, although they were slightly less effective in inducing cell adhesion to laminin. These results indicate that chicken integrin β_1 may functionally substitute for β_1 subunit of rat integrins in PC12 cells. Apparently, the structure of the integrin β_1 subunit is highly conserved in the evolution of these species.**

Nerve growth factor (NGF)-induced differentiation of PC12 cells is widely accepted as a relevant model of neuronal development (7). The cells stop dividing, commence neurite outgrowth and become excitable after NGF treatment, as do most neurons. To cause these changes in neuronlike cells such as PC12, NGF must bind to specific cell surface receptors which interact with more than one transducing molecule to modulate intracellular levels of several second messengers such as cAMP, Ca^{2+} and phosphatidylinositol (2, 4, 9).

Recent evidence has shown that neurite outgrowth from PC12 cells cultured with astrocytes is mediated by the interaction between extracellular matrix components (collagen or laminin) and their receptor integrins or homohelic binding molecules such as N-cadherins (13). The neuritic response of PC12 cells on laminin is completely inhibited by antibodies to integrins (15). It seems possible that NGF-NGF receptor complexes might somehow activate the integrin systems by stimulating de novo synthesis of integrins and/or by modifying the coupling efficiencies of integrins.

To study the effect of exogenous integrin on cell adhesion to laminin, we introduced a cDNA encoding chicken β_1 subunit into rat PC12 cells and examined the response of these cells to NGF as well as laminin. We report here that chicken-rat chimeric integrins are also functional in mediating cell spreading and initial neurite outgrowth on laminin.

MATERIALS AND METHODS

Cell culture. PC12 cells were grown as monolayers in DME medium (GIBCO) supplemented with 10% calf serum (GIBCO), 5% horse serum (GIBCO), 1 mg/ml of glutamine, 100 units/ml of penicillin and 100 μg/ml of streptomycin (Sigma). Cultures were incubated at 37°C in 95% air/5% CO_2 and fed every other day. Culture dishes were previously coated with laminin (10 μg/ml) at 4°C overnight; this was followed by washing with PBS and incubation with PBS containing 2% BSA for 2 h at 37°C.

Transfection. A cDNA encoding the chicken integrin β_1 subunit was excised with EcoRI from pGEM1-integrin (12), blunt-ended, and cloned into the Sma I site of the pMAMneo vector (Toyobo, Tokyo). PC12 cells were transfected with this plasmid (pMN-INT) by electroporation according to the Bio-Rad instruction manual and cultured in the presence of G418 (0.8 mg/ml). Since the pMAMneo vector contains two promoters in line, one of which is inducible with dexamethasone, expression of chicken integrin in cultured cells was carried out in the presence of dexamethasone (2 μM). The positive clones were selected with immunostaining for chicken integrin.

Immunostaining. Two days after culture in the presence or absence of dexamethasone, transfectants or mock-transfectants were fixed with 3.7% formaldehyde for 15 min and permeabilized with 0.4% Triton X-100 for 5 min at room temperature. The cultures were incubated with 0.15 M glycine in phosphate-buffered saline (PBS) for 10 min, 5% goat serum in PBS for 1 h, and antibodies to chicken or mouse integrin at a 1:100 dilution for 30 min at room temperature as described previously (6). Cells were then treated with an affinity-purified fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Cappel) for 30 min, and observed under an Axioplan fluorescence microscope (Nikon).

Immunoprecipitation. To label cells metabolically with ^{35}S-methionine, cells were transferred to methionine-free synthetic medium (N2) supplemented with 2 μM dexamethasone, ^{35}S-methionine (1100 Ci/mmol, Dupont) and the antibiotics, and then incubated in 5% CO_2/air at 37°C for 12 h. The cells were washed three times in PBS, collected mechanically in PBS containing protease inhibitor mixture (2 mM PMSF, 10 μg/ml leupeptin, 0.1 μg/ml pepstatin, and 0.1 mM *o*-phenanthroline), and then solubilized in 0.5% NP-40 suspended in PBS. The labeled integrins were immunoprecipitated from the NP-40 extract with antibodies to integrins as described previously (3). Immunoprecipitated proteins were separated on SDS-PAGE, and autoradiography procedures were carried out (6).

Cell attachment assay. Cells were cultured for 2 days in the presence of 100 ng/ml of NGF. In some experiments, cells were cultured in the presence of dexamethasone as well as NGF. The NGF-primed cells were collected in a mild condition as described previously (6), and resuspended in DME medium containing 2% BSA and various amounts of rabbit antibodies to chicken or mouse integrin (3, 6). These anti-chicken and anti-mouse antibodies specifically recognized the β_1 subunit of their own integrins without cross-reacting between the two species, and blocked adhesion of fibroblasts from chicken and mouse to matrix proteins, respectively (6). Aliquots of the above cell suspension (10^4 cells) were dispensed to laminin-coated multiwells (96 wells). The numbers of spreading cells and round cells were counted separately in each well 1 h after the incubation at 37°C in 5% CO_2/air, and ratios of spreading cells to the total cells were expressed as percentages.

RESULTS

PC12 cells transfected with pMN-INT were cultured in the presence of G418 for a few weeks until clones were visible. Thirty clones were isolated at random, and an aliquot of the cell suspension of each clone was replated and treated with dexamethasone. We obtained several positive clones for chicken integrin, one (PC12W19-I) of which was selected for further characterization. This clone produced only a minimal amount of chicken integrins when cultured without dexametha-

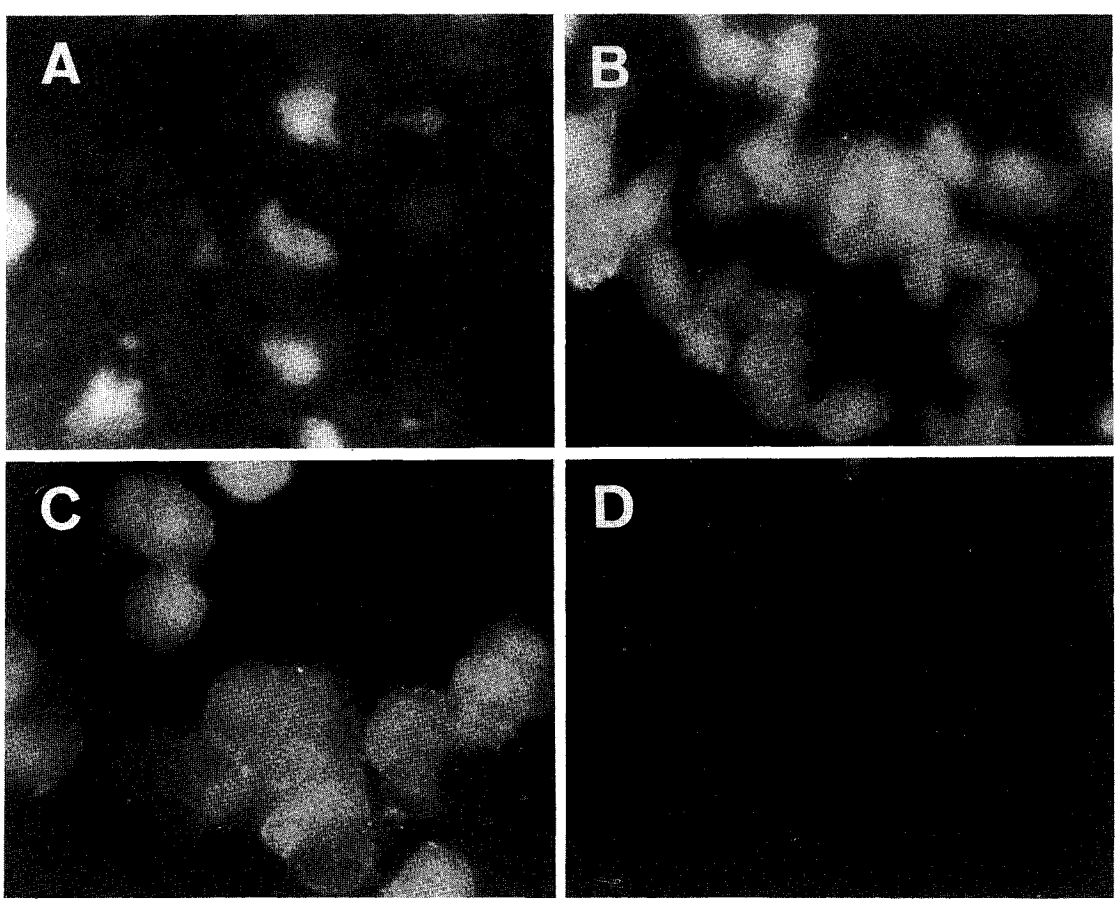

Fig. 1. Immunostaining of chicken integrin β_1 subunit expressed in rat PC12 cells. Transfected (A, B) and mock-transfected cells (C, D) were cultured for 2 days in the absence (A) or presence (B, C, D) of dexamethasone. The cells were immunostained with anti-chicken integrin β_1 antibody (A, B, D) or anti-mouse integrin antibody (C) using FITC conjugated anti-rabbit antibody.

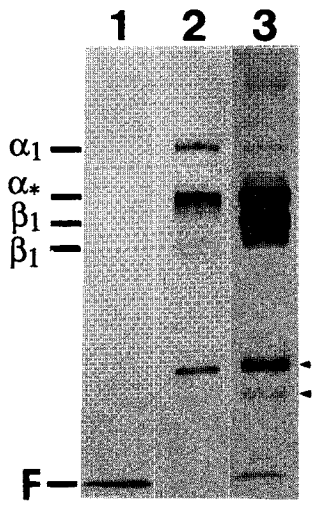

Fig. 2. Immunoprecipitation of chicken integrin β_1 subunit from transfected PC12 cells. Cells were metabolically labeled with ^{35}S-methionine, and integrins were immunoprecipitated with antibodies to chicken integrin (lanes 1, 2) and to mouse integrin (lane 3). The sample in lane 1 was prepared from uninduced transfectant cells, and samples in lanes 2 and 3 were from induced transfectant cells and induced mock-transfectant cells, respectively. α_1, rat α_1 subunit; α^*, rat α subunit; β_1 (larger β_1), rat β_1 subunit; β_1 (smaller β_1), chicken β_1 subunit; F, front line of electrophoresis.

sone, showing a positive reaction to chicken integrin β_1 subunit in a small number of cells (Fig. 1A). In contrast, the addition of dexamethasone induced chicken integrin expression in all cells of the clone; the molecules spread through the cytoplasm as well as the cell surface (Fig. 1B). These results indicated that chicken integrin cDNA from the plasmid was incorporated into chromosomal DNA of the recipient rat PC12 cells and expressed stably in an inducible fashion.

Immunoprecipitation was carried out to determine whether the synthesized integrin could be associated with the α subunits of rat integrins. As shown in Fig. 2 (lane 1), uninduced transfectants did not synthesize a detectable amount of chicken integrin. In contrast, dexamethasone-treated transfectants expressed three distinct protein bands which were similar, if not identical, to the protein bands immunoprecipitated with anti-mouse integrin antibody from mock transfectants (Fig. 2, lanes 2 and 3). As PC12 cells are known to express two major integrins (13), $\alpha_1\beta_1$ and another α subunit associated with β_1 ($\alpha^*\beta_1$), this result suggests that both α subunits of these integrins associated with the chicken integrin β_1 subunit as well as the rat integrin β_1 subunit. There were a few bands in the range smaller than 50 kDa as detected by autoradiography (Fig. 2); there probably represent proteolytic fragments of integrins which still contain epitopes recognizable by the antibodies. The amount of immunoprecipitated chimeric receptor was *ca.* 30–40% of that of rat integrin when the ratio was estimated by the amount of α subunit. The chicken β_1 band was somewhat faint compared to the bands coprecipitated, showing slow labeling with ^{35}S-methionine.

The cell spreading assay of this transfectant was performed on laminin-coated dishes (Fig. 3). Cells cultured in the presence of 100 ng/ml of NGF for 2 days were mixed with varying amounts of antibodies to chicken and/or mouse integrins and then plated onto laminin-coated culture wells. Both mock and uninduced transfectant cells spread on laminin within 1 h and started to extend neurites in the absence of any antibodies (data

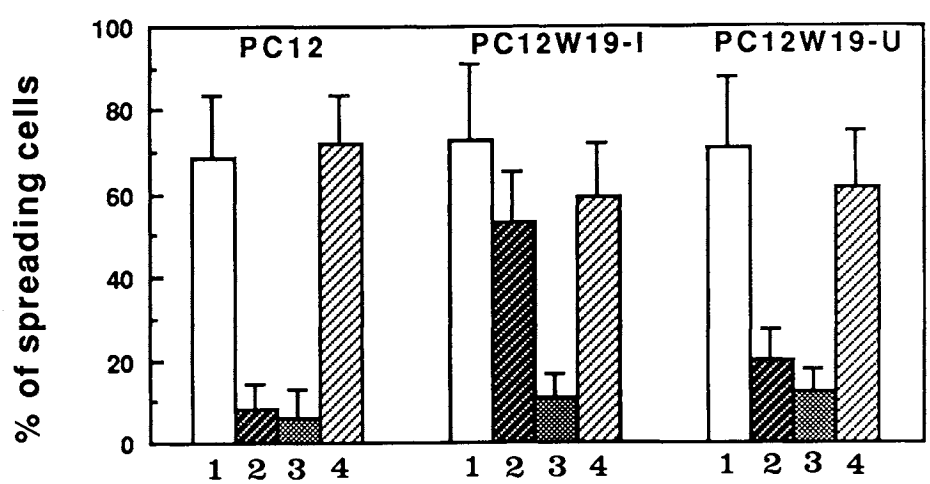

Fig. 3. Inhibition of transfectant cell attachment to laminin by the antibody to mouse and chicken integrins. One h after plating on laminin-coated plates, the percentage of spreading cells among total cells was calculated. Column 1, no antibody addition; column 2, antibody to mouse integrin; column 3, antibodies to chicken and mouse integrins; column 4, antibody to chicken integrin. PC12, PC12W19-I and PC12W19-U are mock transfectants, induced transfectants and uninduced transfectants, respectively.

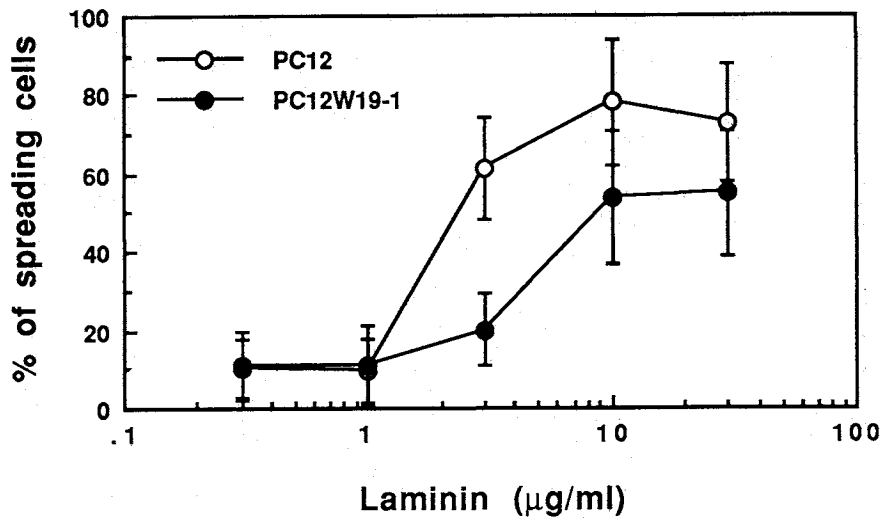

Fig. 4. Cell adhesion to laminin-coated dishes. PC 12 and PC12w19 were cultured for 2 days in the presence of both NGF and dexamethasone, and the cell suspensions were prepared. After mixing with antibody to mouse integrin (at a dilution of 1:100, PC12w19) or preimmune serum (at the same dilution, PC12), the cell suspensions were dispensed to multiplates which were previously coated with various concentrations of laminin. The percentage of spreading cells in each well was calculated as shown in Fig. 3.

not shown). In contrast, cell attachment to the laminin-coated dishes was blocked by the addition of anti-mouse integrin antibody (Fig. 3); the cells adhered weakly to the substratum from which they were easily detached by gentle agitation. On the other hand, transfectants induced by dexamethasone spread on laminin even in the presence of one of either antibodies (Fig. 3). However, the addition of both antibodies inhibited the cell adhesion to the substratum. These results imply that dexamethasone induces a production of chicken integrin in rat PC12 cells, resulting in the formation of functional chimeric integrins. If the chimeric integrins were neither formed nor functional, the cells would not spread on laminin in the presence of mouse antibody. The chimeric integrins in transfectant PC12 cells, therefore, take part in cell attachment to laminin.

The sensitivities of mock-transfected and transfected cells to NGF were almost the same at the concentrations examined (1, 10, and 100 ng/ml, data not shown). Both types of cells were also equally reactive to laminin in terms of cell spreading and initial neuritic response when no anti-integrin antibody was added. However, this does not necessarily indicate that both chimeric and endogenous integrins have comparable affinity to laminin. To clarify this point, we compared the laminin-mediated cell adhesion by chimeric integrins with that of rat endogenous integrins. As shown in Fig. 4, the laminin concentrations that gave rise to the half-maximal cell adhesion were 1.8 μg/ml for rat integrins and 4.3 μg/ml for chimeric integrins, showing only a slight difference in effectiveness between rat and chimeric integrins to promote cell adhesion to laminin.

DISCUSSION

In the present study, we showed that the chicken integrin β_1 subunit is synthesized as a functional molecule in a foreign rat PC12 cell, forming chimeric integrins with rat integrin α_1 and α^* subunits, which are capable of mediating cell adhesion to laminin.

The chicken integrin β_1 subunit expressed in mouse NIH 3T3 cells also assembles with mouse integrin α_3 as well as α_5 subunits, which results in the formation of functional receptors (6, 10). Such integrins were found to have intact binding domains; one of them is present in the extracellular region of integrin molecules, and the other is in the intracellular region and binds to vinculin through talin (6). Thus, chicken β_1 subunit may substitute for the β_1 subunit of not only rat but also the integrins of other species. Most of such chimeric integrins formed in foreign cells are functional (5, 8, 10, 11). Therefore, it is assumed that the integrin domains responsible for binding other protein(s) are highly conserved through evolution.

The antibody to mouse integrin, which recognizes the β subunit of mouse integrins, blocked the function of rat integrins, but not that of the chimeric integrins, showing a condition in which only chimeric integrins are able to work. Using this condition, we showed here that these chimeric integrins mediated initial cell spreading on laminin as well as collagen (data not shown). However, we could not examine the efficiency of chimeric integrins in performing their function during long-term culture in comparison to that of the endo-

genous integrins, since newly synthesized integrins quickly overcame the blockage of integrin by antibody during cultivation.

The sensitivity of the transfectant to NGF was almost the same as that of the original cells. This may suggest that expression of chicken integrin β_1 subunit did not affect NGF-mediated systems which were somehow linked to integrin systems to induce neurite outgrowth from PC12 cells.

If intensities of α subunits in the autoradiography reflect the amount of de novo synthesized integrins, the ratios of chimeric integrins to rat domestic ones in transfected cells could be estimated and were actually 4–10 (for $\alpha_1\beta_1$) and 0.2–0.4 (for $\alpha^*\beta_1$). This raises two questions. The first one is what the reason for such variations in the ratios is. The amount of each synthesized β subunit can affect the total amount of each integrin, which may account for the different values among the transfectants examined. The second one is why the ratios of the two major integrins (chimeric $\alpha_1\beta_1$/rat $\alpha_1\beta_1$ and chimeric $\alpha^*\beta_1$/rat $\alpha^*\beta_1$) are reversed in transfected cells. The affinities of chicken and rat β subunits toward each α subunit seem to be different. Alternatively, the competition between chicken and rat β subunits to assemble with α subunits appeared to be reversed in the case of the two major α subunits. These questions, however, still remain to be clarified.

When the expression of chicken β_1 was augmented by dexamethasone, the domestic β_1 subunit did not occupy the α subunit as frequently as in the untreated condition (unpublished data). This implies that the chimeric integrin formation in PC12 cells was as efficient as that of rat integrins, whereas the affinities of chimeric integrins to laminin were shown to be comparable, but slightly lower than those of rat integrins. At present, we do not know which region in integrin subunits is more crucial in decreasing the affinities of the chimeric integrins to laminin. Further experiments are needed to induce mutations in various regions in integrins including laminin binding, cytoplasmic and Ca ion binding domains to access regions responsible for their ligand binding activities.

REFERENCES

1. Akiyama, S.K. and Yamada, K.M. (1987). Biosynthesis and acquisition of biological activity of fibronectin receptor. *J. Biol. Chem.*, **262**: 17536–17642.
2. Alonso, A.P., Malgaroli, A., Vicentini, L.M., and Meldolesi, J. (1986). Early rise in cytosolic Ca^{2+} induced by NGF in PC12 and chromaffin cells. *FEBS Lett.*, **208**: 48–51.
3. Bozyczko, P., Decker, C., Muschler, J., and Horwitz, A.F. (1989). Integrin on developing and adult skeletal muscle. *Exp. Cell Res.*, **183**: 72–91.
4. Contreras, M.L. and Guroff, G. (1987). Calcium-dependent nerve growth factor-stimulated hydrolysis of phosphoinositides in PC12 cells. *J. Neurochem.*, **48**: 1466–1472.
5. Giancotti, F.G. and Ruoslahti, E. (1990). Elevated levels of the $\alpha_5\beta_1$ fibronectin receptor suppress the transformed phenotype of chinese hamster ovary cells. *Cell*, **60**: 849–859.
6. Hayashi, Y., Haimovich, B., Reszka, A., Boettiger, D., and Horwitz, A. (1990). Expression and function of chicken integrin β_1 subunit and its cytoplasmic domain mutants in mouse NIH3T3 cells. *J. Cell. Biol.*, **110**: 175–184.
7. Levi, A., Biocca, S., Catlaneo, A., and Colissano, P. (1988). The mode of action of nerve growth factor in PC12 cells. *Mol. Neurobiol.*, **2**: 201–226.
8. Marcantonio, E.E., Guan, J-L., Trevithick, J.E., and Hynes, R.O. (1990). Mapping of the functional determinants of the integrin β_1 cytoplasmic domain by site-directed mutagenesis. *Cell Regulation*, **1**: 597–604.
9. Schubert, D. and Witlock, C. (1989). Alteration of cellular adhesion by nerve growth factor. *Proc. Natl. Acad. Sci. USA*, **74**: 4055–4058.
10. Solowska, J., Guan, J.L., Marcantonio, E.E., Trevithicle, J.E., Buck, C.A., and Hynes, R.O. (1989). Expression of normal and mutant avian integrin subunits in rodent cells. *J. Cell Biol.*, **109**: 853–861.
11. Symington, B.E. (1990). Fibronectin receptor over expression and loss of transformed phenotype in a stable variant of the K 562 cell line. *Cell Regulation*. **1**: 637–648.
12. Tamkun, J.W., Desimone, D.W., Fonda, D., Patel, R.S., Buck, C., Horwitz, A.F., and Hynes, R.O. (1986). Structure of the cysteine-rich subunit of integrin, the fibronectin receptor. *Cell*, **46**: 271–282.
13. Tomaselli, K.J., Domsky, C.H., and Reichardt, L.F. (1987). Interactions of a neuronal cell line (PC12) with laminin, collagen IV, and fibronectin: identification of integrin related glycoproteins involved in attachment and process outgrowth. *J. Cell. Biol.*, **105**: 2347–2358.
14. Tomaselli, K.J., Neugebauer, K.M., Bixby, J.L., Lilien, J., and Reichadt, L.F. (1988). N-Cadherin and integrins: two receptor systems that mediate neuronal process outgrowth on astrocyte surfaces. *Neuron*, **1**: 33–43.
15. Turner, D.C., Flier, L.A., and Carbonetto, S. (1989). Identification of a cell surface protein involved in PC12 cell substratum adhesion and neurite outgrowth on laminin and collagen. *J. Neurosci.*, **9**: 3287–3296.

(Received for publication, November 27, 1990 and in revised form, January 25, 1991)