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# Purification and Characterization of Mammalian Integrins Expressed by a Rat Neuronal Cell Line (PC12): Evidence That They Function as $\alpha/\beta$ Heterodimeric Receptors for Laminin and Type IV Collagen

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**Abstract.** Cells of the rat neuronal line, PC12, adhere well to substrates coated with laminin and type IV collagen, but attach poorly to fibronectin. Adhesion and neurite extension in response to these extracellular matrix proteins are inhibited by Fab fragments of an antiserum (anti-ECMR) that recognizes PC12 cell surface integrin subunits of  $M_r$  120,000, 140,000, and 180,000 (Tomaselli, K. J., C. H. Damsky, and L. F. Reichardt. 1987. *J. Cell Biol.* 105:2347–2358). Here we extend our study of integrin structure and function in PC12 cells using integrin subunit-specific antibodies prepared against synthetic peptides corresponding to the cytoplasmic domains of the human integrin  $\beta_1$  and the fibronectin receptor  $\alpha$  ( $\alpha_{FN}$ ) subunits. Anti-integrin  $\beta_1$  immunoprecipitated a 120-kD  $\beta_1$  subunit and two noncovalently associated integrin  $\alpha$  subunits of 140 and 180 kD from detergent extracts of surface-labeled PC12 cells. Immunodepletion studies using anti-integrin  $\beta_1$  demonstrated that these two putative  $\alpha/\beta$  heterodimers are identical to those recognized by the adhesion-perturbing ECMR antiserum. Anti- $\alpha_{FN}$  immunoprecipitated fibronectin receptor heterodimers in

human and rat fibroblastic cells, but not in PC12 cells. Thus, low levels of expression of the integrin  $\alpha_{FN}$  subunit can explain the poor attachment of PC12 cells to FN. The PC12 cell integrins were purified using a combination of lectin and ECMR antibody affinity chromatography. The purified integrins: (a) completely neutralize the ability of the anti-ECMR serum to inhibit PC12 cell adhesion to laminin and collagen IV; (b) have hydrodynamic properties that are very similar to those of previously characterized integrin  $\alpha/\beta$  heterodimeric receptors for ECM proteins; and (c) can be incorporated into phosphatidylcholine vesicles that then bind specifically to substrates coated with laminin or collagen IV but not fibronectin. Thus, the ligand-binding specificity of the liposomes containing the purified PC12 integrins closely parallels the substrate-binding preference of intact PC12 cells. These results demonstrate that mammalian integrins purified from a neuronal cell line can, when incorporated into lipid vesicles, function as receptors for laminin and type IV collagen.

**I**NTERACTIONS with extracellular matrix (ECM)<sup>1</sup> components are likely to regulate neuronal differentiation during the development of the nervous system. Several well-characterized constituents of the ECM affect neuronal adhesion, process outgrowth, or survival in vitro. The basement membrane glycoprotein, laminin (LN), stimulates neuronal adhesion (Hall et al., 1987), process outgrowth (Rogers et al., 1983; Manthorpe et al., 1983; Lander et al., 1983, 1985), and survival (Edgar et al., 1984; Calof and Reichardt, 1984, 1985) of both central and peripheral neurons. Two other ECM components, fibronectin (FN) and type IV

collagen (Col IV), also stimulate neuronal adhesion and, for some neurons, process outgrowth (cf. Akers et al., 1981; Rogers et al., 1983, 1985; Hall et al., 1987; Carbonetto et al., 1983). Studies of ECM protein expression in nervous tissue suggest that ECM proteins are likely to influence axon growth both during development and in response to injury in vivo. In particular, LN immunoreactivity has been demonstrated in regions of the developing central and peripheral nervous systems where axonal pathways are established (cf. Rogers et al., 1986; Cohen et al., 1987; Letourneau et al., 1988; Adler et al., 1985; Liesi, 1985). More recently, antibodies to a putative LN-proteoglycan complex have been shown to transiently inhibit sympathetic nerve regeneration and/or differentiation in the iris after chemical sympathectomy (Sandrock and Matthew, 1987).

Neuronal responses to ECM proteins must depend on the

1. *Abbreviations used in this paper:* anti-ECMR, anti-extracellular matrix receptor serum; Col IV, collagen IV; ECM, extracellular matrix; FN, fibronectin; LN, laminin; NRK, normal rat kidney; octylglucoside, *n*-octyl- $\beta$ -D-glucopyranoside; VLA, very late antigen; WGA, wheat germ agglutinin.

function of specific receptors on the neuronal plasma membrane. Cell surface receptors for several adhesive ECM proteins, including LN, FN, Col IV, vitronectin, fibrinogen, and von Willebrand factor, have recently been identified on a variety of adherent cells (Horwitz et al., 1985; Buck et al., 1986; Akiyama et al., 1986; Gardner and Hynes, 1985; Santoro, 1986; Pytela et al., 1985a, b; 1986; Plow et al., 1985). A common structural feature of these receptors is the noncovalent association of one of several homologous  $\alpha$  subunits with a common  $\beta$  subunit to form  $\alpha/\beta$  receptor heterodimers with distinct ligand-binding specificities. Protein and cDNA sequencing of these receptor  $\alpha$  and  $\beta$  subunits has established the existence of two multigenic families (corresponding to  $\alpha$  and  $\beta$ ) whose members have been called integrins (Tamkun et al., 1986; Takada et al., 1987b; Argraves et al., 1987a, b; Kishimoto et al., 1987; Suzuki et al., 1986, 1987; Corbi et al., 1987; Poncz et al., 1987; reviewed in Hynes, 1987, and Ruoslahti and Pierschbacher, 1987). At present, three integrin  $\alpha/\beta$  heterodimer subfamilies, distinguished by three distinct but homologous  $\beta$  subunits, have been characterized (Hynes, 1987). At least five  $\alpha/\beta$  heterodimers comprise the integrin  $\beta_1$  subfamily: on human cells, these correspond to the five "VLA" protein heterodimers (VLA 1-5) first described on T cells as very late activation antigens (Hemler et al., 1985, 1987a; Takada et al. 1987b). One of the VLA heterodimers (VLA 5) appears to correspond to the integrin  $\beta_1/\alpha_{FN}$  FN receptor heterodimer initially purified from human cells (Pytela et al., 1985a; Takada et al., 1987a; see Hynes, 1987 for integrin nomenclature). A similar FN-binding heterodimer has been characterized in rodent cells (Brown and Juliano, 1986; Patel and Lodish, 1986).  $\beta_1$ -class integrins that bind to FN and LN have also been purified from avian cells using an adhesion-perturbing monoclonal antibody (Horwitz et al., 1985; Buck et al., 1986). These avian integrins are also likely to be heterodimeric in structure (Horwitz et al., 1985). Integrin  $\beta_1$  receptors are expressed on the surfaces of avian neurons and function in neuronal adhesion and process outgrowth in response to LN, FN, and Col IV (Bozyczko and Horwitz, 1986; Tomaselli et al., 1986; Hall et al., 1987; Cohen et al., 1987). Integrin  $\beta_1$  antibodies have also been shown to diminish but not prevent central and peripheral neuronal process outgrowth on glial and muscle cell surfaces (Bixby et al., 1987, 1988; Tomaselli et al., 1988; Neugebauer et al., 1988).

The molecular characterization of neuronal receptors for ECM proteins is hampered by the difficulty in obtaining pure neuronal cultures in large quantities for biochemical studies. We have attempted to circumvent some of these difficulties by studying the interactions of a rat pheochromocytoma cell line, PC12, with ECM proteins. PC12 cells display many properties of differentiated sympathetic neurons after exposure to nerve growth factor, including process outgrowth, electrical excitability, and synapse formation (Tischler and Greene, 1975; Schubert et al., 1977). Previous studies have shown that PC12 cells adhere efficiently to and extend neurites on LN and Col IV, but attach poorly to FN (Tomaselli et al., 1987; Turner et al., 1987). PC12 cell adhesion and process outgrowth on these ECM proteins is inhibited by antibodies (anti-ECMR) that immunoprecipitate three PC12 cell surface glycoproteins of 120, 140, and 180 kD. The 120-

kD protein, which cross reacts with antibodies to the avian integrin  $\beta_1$  subunit, is noncovalently associated with both the 140- and 180-kD glycoproteins (Tomaselli et al., 1987). In the present report, we have purified and further studied the physical properties and ligand-binding specificities of these PC12 cell integrins. Our results indicate that these integrins are organized as  $\alpha/\beta$  heterodimers that function as receptors for LN and Col IV on the surfaces of PC12 cells.

## Materials and Methods

### Materials

LN and Col IV were purified from Engelbreth-Holm-Swarm sarcoma tumors using published methods (Kleinman et al., 1982; Timpl et al., 1982) and were the generous gifts of Drs. J. L. Bixby, A. D. Lander, and J. Winter. Human plasma FN was from Collaborative Research Inc. (Waltham, MA). Cyanogen bromide-activated Sepharose CL-4B, protein A-Sepharose CL-4B, and Sephacryl S-300 were purchased from Pharmacia (Piscataway, NJ). Affigel 10 was from Bio-Rad Laboratories (Richmond, CA). Wheat germ agglutinin (WGA), *N*-acetyl-D-glucosamine, and *n*-octyl- $\beta$ -D-glucopyranoside (octylglucoside) were from Calbiochem-Behring Corp. (La Jolla, CA). Aqualon and [ $^3$ H]phosphatidylcholine, (1- $\alpha$ -dipalmitoyl [2-palmitoyl-9,10- $^3$ H(N)] were from New England Nuclear (Boston, MA). Na $^{125}$ I was purchased from Amersham Corp. (Arlington Heights, IL). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

### Cell Culture

Rat pheochromocytoma (PC12) cells (Tischler and Greene, 1975) were grown in monolayer culture in DME with 4.5 g/liter of glucose (DME H-21; University of California, San Francisco, Cell Culture Facility) supplemented with 10% heat-inactivated horse serum, 5% newborn calf serum, 2 mM glutamine, and 100 U/ml of penicillin/streptomycin. Cells were passaged with Ca $^{++}$ /Mg $^{++}$ -free PBS (15 mM Na $_2$ HPO $_4$ , 1.5 mM KH $_2$ PO $_4$ , 0.14 M NaCl, 2 mM KCl) containing 5 mM EDTA. Human placental fibroblasts were isolated as described in Fischer et al. (1984). Normal rat kidney (NRK) and human placental fibroblastic cells were grown in DME H21 supplemented with 10% FCS, 2 mM glutamine, and 100 U/ml of penicillin/streptomycin.

### Antibodies

The anti-ECMR serum was made in goats to a preparation of 120-160-kD cell-substrate adhesion-related integral membrane glycoproteins purified from baby hamster kidney fibroblastic cells as described in Knudsen et al. (1981). IgG was prepared from the ECMR antiserum by sodium sulfate precipitation as described in Wylie et al. (1979). ECMR IgG used in the preparation of ECMR IgG-Sepharose CL-4B for affinity chromatography was further purified on protein A-Sepharose CL-4B before coupling to Sepharose CL-4B. Fab fragments of anti-ECMR IgG were prepared by digestion with papain as described in Parham (1986). Rabbit anti-LN serum was the generous gift of Dr. J. Winter. Col IV antibodies were purchased from Dr. H. Furthmayr, Yale University, New Haven, CT. Polyclonal antisera to integrin  $\alpha$  and  $\beta$  subunit peptides were prepared as described below.

### Preparation of Antisera to Synthetic Integrin Peptides

A 24-amino acid peptide consisting of an NH $_2$ -terminal cysteine linked to the last 23 COOH-terminal amino acids of the cytoplasmic domain of the human and chicken integrin  $\beta_1$  subunits (DTGENPIYKSAVTTVVNPKYEGK; Tamkun et al., 1986; Argraves et al., 1987b) was synthesized by Dr. Chris Turk at the Howard Hughes Medical Institute, University of California, San Francisco. A second peptide of 19 amino acids consisting of an NH $_2$ -terminal cysteine coupled to the last 18 COOH-terminal amino acids of the cytoplasmic domain of the human fibronectin receptor  $\alpha$  subunit (YGTAMEKAQLKPPATSDA; Argraves et al., 1987b; henceforth referred to as the integrin  $\alpha_{FN}$  subunit as described in Hynes, 1987) was also synthesized. The peptides were synthesized on a peptide synthesizer (model 430A; Applied Biosystems, Inc., Foster City, CA) and cleaved from the resin and deprotected using liquid hydrogen fluoride. Purification of the peptides by

preparative HPLC was carried out with a Vydac C18 column (Vydac, Hesperia, CA), 1 × 25 cm, using a gradient of 5–65% acetonitrile in water plus 0.1% trifluoroacetic acid. Peptide identity was verified by gas phase sequencing. Each peptide was coupled via the NH<sub>2</sub>-terminal cysteine residue to Keyhole Limpet Hemocyanin (KLH) using the water-soluble hetero-bifunctional cross-linking reagent *m*-maleimidobenzoyl sulphosuccinimide ester according to manufacturer's instructions (Pierce Chemical Co., Rockford, IL). Rabbit antisera to the peptide–KLH conjugates were raised in New Zealand White rabbits by the Berkeley Antibody Co. (Richmond, CA). Antisera that reacted with unconjugated peptides by ELISA were screened on human placental and NRK fibroblastic cells by antigen blotting. The antiserum to the peptide derived from the integrin β<sub>1</sub> subunit cytoplasmic domain used in this study recognizes a single 110-kD integrin β<sub>1</sub> subunit in antigen blots of both human and rat fibroblastic cell proteins. The antiserum to the peptide derived from the integrin α<sub>FN</sub> subunit cytoplasmic domain used in this study recognizes a 160-kD integrin α<sub>FN</sub> subunit in antigen blots of both human and rat fibroblastic cell proteins.

### Cell Surface Iodination and Immunoprecipitation

PC12 cells, NRK cells, and human placental fibroblasts were labeled by lactoperoxidase-catalyzed iodination as described in Tomaselli et al. (1987). About 3 × 10<sup>7</sup> labeled cells were extracted in 1 ml of PBS plus 1 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1% Triton X-100 as described previously (Tomaselli et al., 1987). Immunoprecipitations were performed on detergent extracts as detailed in Tomaselli et al. (1987) using 10 μl of the appropriate antiserum and 50 μl packed volume of protein A–Sepharose CL-4B. Immunoprecipitates were washed five times in extraction buffer by centrifugation, eluted in SDS gel sample buffer (Laemmli, 1970) without β-mercaptoethanol, and stored at –70°C until use.

For the immunodepletion studies with the integrin β<sub>1</sub>-specific antibodies, a 1-ml aliquot of PC12 cell extracts (10<sup>7</sup> cpm) was sequentially immunoprecipitated four times with 30 μl of anti-integrin β<sub>1</sub> serum, followed by two extractions with protein A–Sepharose alone, and, finally, 20 μl of the anti-ECMR serum. In control experiments, the ECMR antiserum recognized proteins of 120, 140, and 180 kD in PC12 cell extracts that had been extracted five times with a control antiserum.

### Purification of PC12 Cell Integrins

For analytical studies (cf. Figs. 3 A, 5, and 7) ~10<sup>8</sup> PC12 cells were surface labeled by lactoperoxidase-catalyzed iodination as previously described (Tomaselli et al., 1987). Labeled cells were extracted for 30 min on ice in 5 ml of ice-cold PBS containing 1.0 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 200 mM octylglucoside, 1 mM PMSF, 1 mg/ml leupeptin, and 1 mg/ml pepstatin (extraction buffer). Detergent-insoluble material was removed by centrifugation at 12,000 *g* for 20 min at 4°C. For the purification of larger amounts of protein used in functional studies (cf. Figs. 3 B, 4, and 6), ~5 × 10<sup>9</sup> unlabeled PC12 cells were extracted with stirring as described above in ~20 ml of extraction buffer and insoluble material was removed by centrifugation at 12,000 *g* for 30 min at 4°C. This cell extract was then mixed with a 5-ml extract of ~10<sup>8</sup> PC12 cells that had been surface labeled and extracted with octylglucoside as described above. Before continuing with the purification, PC12 cell extracts were mixed with an equal volume of extraction buffer without detergent to yield a final octylglucoside concentration of 100 mM. Extracts were mixed for 5 h at 4°C by end-over-end rotation with 10 ml packed volume of WGA coupled to Affi-gel 10 (coupling of 10 mg of WGA per ml of Affi-gel 10 was achieved following manufacturer's instructions [Bio-Rad Laboratories]). The slurry was loaded into a column (15 × 3 cm) and washed with 30 column vol of extraction buffer containing 50 mM octylglucoside (wash buffer). Bound proteins were eluted with 5% *N*-acetyl-D-glucosamine in wash buffer, and the peak <sup>125</sup>I-containing fractions were pooled and mixed for 2 h at 4°C with 10 ml packed volume of normal goat IgG coupled to Sepharose CL-4B (coupling of 10 mg of goat IgG per ml of cyanogen bromide-activated Sepharose was achieved using manufacturer's instructions). The gel slurry was loaded into a column (15 × 3 cm) and the unbound, flow-through material was collected and mixed end-over-end with 1.5 ml of anti-ECMR IgG coupled to Sepharose CL-4B (4 mg of ECMR IgG/ml of gel) for 3–4 h at 4°C. The anti-ECMR IgG–Sepharose CL-4B was loaded into a column (1 × 5 cm), the flow-through was collected, and the column was washed with 50–100 ml of wash buffer followed by 10 ml of wash buffer containing 0.5 M NaCl at a flow rate of ~1 ml/min. Bound proteins were eluted with 50 mM diethylamine

pH 11.5, 1.0 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 0.1 M NaCl, 50 mM octylglucoside, and 1 mM PMSF, and immediately neutralized with Hepes pH 7.4.

### Neutralization of Anti-ECMR Serum with Purified PC12 Cell Integrins

Immunopurified PC12 cell proteins (~5 μg in 2 ml of elution buffer as judged from silver-stained gels by comparison to known amounts of BSA) were concentrated eightfold by centrifuging at 2,500 *g* for 20 min at 4°C through an Amicon filter with a 30,000-D cutoff (Centricon 30; Amicon Corp., Danvers, MA). Concentrated proteins (in ~250 μl) were mixed with 50 μl of normal goat serum (to help minimize protein loss in subsequent manipulations) and dialyzed for 48 h at 4°C against four 1-liter changes of PBS with 1.0 mM CaCl<sub>2</sub> and 0.5 mM MgCl<sub>2</sub> to remove the octylglucoside. Dialyzed proteins were mixed with the anti-ECMR serum and incubated 30 min at 25°C before testing the ECMR antiserum for its ability to inhibit PC12 cell attachment. As a control, the ECMR IgG-column elution buffer was mixed with 50 μl of normal goat serum, dialyzed, and mixed with the ECMR antiserum. PC12 cell attachment was measured in the presence of the anti-ECMR serum that had been premixed with either purified proteins or control proteins (normal goat serum) using a cell attachment assay described previously (Hall et al., 1987; Tomaselli et al., 1987).

### Reconstitution of Purified PC12 Cell Integrins into Lipid Vesicles

Immunopurified proteins (~20 μg as judged by silver-stained gels) were concentrated to 1 ml using a Centricon 30 (Amicon Corp.) as described in the preceding section. A mixture of unlabeled phosphatidylcholine (100 μg for analytical studies [Fig. 5]; 50 μg for liposome-binding experiments [Fig. 6]) and [<sup>3</sup>H]phosphatidylcholine (10<sup>6</sup> cpm for analytical studies [Fig. 3]; 10<sup>7</sup> cpm for liposome-binding experiments [Fig. 4]) was dried onto a glass tube under a stream of N<sub>2</sub> and then redissolved in either the antibody column elution buffer alone or the antibody column elution buffer containing the purified PC12 cell proteins. Liposomes were formed by detergent dialysis as described in Mimms et al. (1981). Liposomes that were formed by dialyzing for 36 h at 4°C against four 1-liter changes of PBS containing 1 mM CaCl<sub>2</sub> and 0.5 mM MgCl<sub>2</sub> were made 45% in sucrose, overlaid with 2 ml of 30% sucrose, and then 2 ml of 10% sucrose in PBS plus Ca<sup>++</sup> and Mg<sup>++</sup> and centrifuged at 45,000 rpm for 18–24 h in an SW 60 rotor (Beckman Instruments Inc., Palo Alto, CA). The resulting sucrose gradient was fractionated and fractions were analyzed for the presence of [<sup>3</sup>H]phosphatidylcholine by scintillation counting and for the presence of [<sup>125</sup>I]-labeled proteins by gamma counting. <sup>3</sup>H-labeled liposomes were recovered as an opaque band at the top of the gradient.

### Liposome Attachment Assay

LN, Col IV, FN, and hemoglobin were diluted in Ca<sup>++</sup>/Mg<sup>++</sup>-free PBS to the concentrations indicated in the legend to Fig. 6 and 100 μl were used to coat microtiter wells (0.28 cm<sup>2</sup> surface area; Linbro, Flow Laboratories, Hamden, CT) by incubation overnight at 4°C. Wells were washed twice with PBS and blocked for 2–3 h with PBS plus 10 mg/ml hemoglobin. Wells were rinsed again with PBS before the addition of ~10<sup>5</sup> cpm of <sup>3</sup>H-labeled liposomes suspended in PBS plus 1.0 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, and 2 mg/ml hemoglobin. Liposomes were allowed to attach for 4–5 h at 4°C and then the supernatants were removed and the wells were washed twice with ice-cold PBS containing 1.0 mM CaCl<sub>2</sub> and 0.5 mM MgCl<sub>2</sub>. Bound liposomes were dissolved in 1% SDS (100 μl well), mixed with 3 ml of Aquasol, and counted in a scintillation counter.

### Sucrose Gradient Analysis of Immunopurified PC12 Cell Integrins

Aliquots of immunopurified surface-labeled PC12 cell proteins (500 μl, ~10,000 cpm) were dialyzed into PBS plus 50 mM octylglucoside, 1.0 mM CaCl<sub>2</sub>, and 0.5 mM MgCl<sub>2</sub> and mixed with protein standards exhibiting known sedimentation coefficients (*s*<sub>20,w</sub>). The protein standards were: fumarase, 9.09S; aldolase, 7.35S; and BSA, 4.7S. Proteins were layered onto a 5–20% sucrose gradient (11 ml) in PBS plus 1 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, and 50 mM octylglucoside. The tubes were centrifuged at 40,000 rpm for 20 h at 4°C in an SW 41 Ti rotor (Beckman Instruments, Inc.). Fractions were collected and were assayed for protein content by Amido black

staining (to identify the positions of the sedimentation standards) and for  $^{125}\text{I}$  by gamma counting (to identify the positions of the PC12 cell proteins). The  $s_{20,w}$  for the PC12 cell proteins was determined graphically by plotting the known  $s_{20,w}$  values of the sedimentation standards versus fraction number and interpolating the value for PC12 peak fractions.

### Molecular Sieve Chromatography of Immunopurified PC12 Cell Integrins

An aliquot of immunopurified, surface-labeled PC12 cell proteins ( $\sim 200 \mu\text{l}$ ;  $\sim 10,000$  cpm) was dialyzed into PBS plus 50 mM octylglucoside, 1.0 mM  $\text{CaCl}_2$ , and 0.5 mM  $\text{MgCl}_2$ , and layered onto a column ( $29 \times 0.7$  cm) of Sephacryl S-300 that had been precalibrated with blue dextran ( $V_0$ ), phenol red ( $V_1$ ), and standards of known Stokes radii (thyroglobulin, 85 Å;  $\beta$ -galactosidase, 69 Å; and BSA, 36 Å). The column was run in PBS plus 1 mM  $\text{CaCl}_2$ , 0.5 mM  $\text{MgCl}_2$ , and 50 mM octylglucoside at a flow rate of  $\sim 2$  ml/h and 0.25-ml fractions were collected and assayed for  $^{125}\text{I}$ -containing PC12 cell proteins by gamma counting. The distribution coefficients of the standards were calculated and plotted against known values for their Stokes radii. The value for the Stokes radii of the PC12 cell proteins was then interpolated.

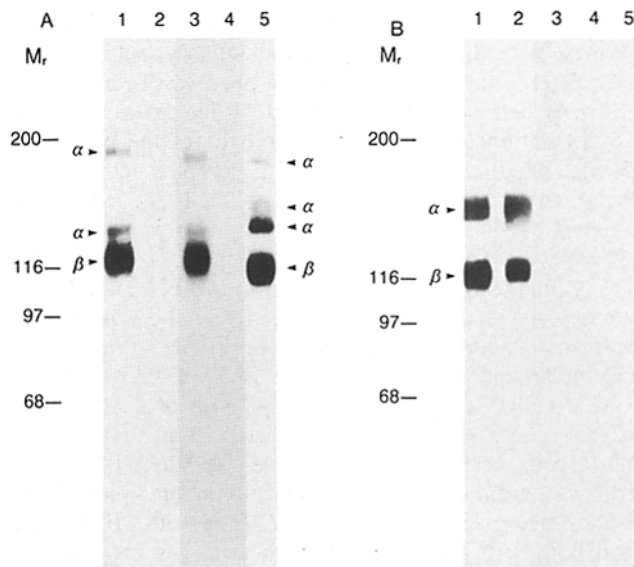
### SDS-PAGE Analysis

Samples were run on polyacrylamide gels according to Laemmli (1970). Gels were stained with either Coomassie Blue or reducing silver (Merrill et al., 1981), dried, and subjected to autoradiography at  $-80^\circ\text{C}$  using Kodak XAR x-ray film and intensifying screens (Dupont Lightning Plus; Newtown, CT). Molecular mass standards used were myosin (200,000 D),  $\beta$ -galactosidase (116,000 D), phosphorylase a (97,000 D), and BSA (68,000 D).

## Results

### Identification of Cell Surface Integrins Expressed by PC12 Cells

PC12 cells attach well to substrates coated with LN and type IV collagen, but attach poorly to FN (Tomaselli et al., 1987; Turner et al., 1987). In a previous study we used an adhesion-perturbing antiserum (anti-ECMR) to identify and initially characterize integrin-related PC12 cell surface glycoproteins of 120, 140, and 180 kD that are involved in attachment and process extension on substrates coated with LN and Col IV (Tomaselli et al., 1987). This study also showed that the 120-kD protein specifically cross reacted with antibodies to the avian integrin  $\beta_1$  subunit and was noncovalently associated with both the 140- and 180-kD proteins. Integrin expression by PC12 cells was studied here using antibodies specific for individual integrin  $\alpha$  and  $\beta$  subunits. A rabbit antiserum raised against a 24 amino acid peptide derived from the COOH-terminal cytoplasmic domains of the human and avian integrin  $\beta_1$  subunits (Tamkun et al., 1986; Argraves et al., 1987b) was used to immunoprecipitate proteins from detergent extracts of surface-labeled PC12 cells. The anti-integrin  $\beta_1$  serum specifically immunoprecipitated three labeled PC12 cell surface proteins of 120, 140, and 180 kD under nonreducing conditions (Fig. 1 A, lane 1). These three glycoproteins comigrate in SDS gels with three glycoproteins that are immunoprecipitated by the adhesion-perturbing anti-ECMR serum (Fig. 1 A, lane 3). When separated under reducing conditions, the mobility of the 120-kD protein is decreased while that of the 140-kD protein is increased, resulting in the comigration of these two proteins at  $\sim 130$  kD. The mobility of the 180-kD protein is not noticeably affected by reduction (see Tomaselli et al., 1987). Since the 120-kD PC12 cell protein is the rat homolog of the avian integrin  $\beta_1$  subunit (Tomaselli et al.,



**Figure 1.** Immunoprecipitation of  $^{125}\text{I}$  surface-labeled cells with integrin  $\beta_1$  and  $\alpha_{\text{FN}}$  peptide antibodies. (A) Immunoprecipitation of PC12 cell extracts with anti-integrin  $\beta_1$  serum (lane 1), preimmune serum (lane 2), anti-ECMR serum (lane 3), or normal goat serum (lane 4). Arrowheads indicate that the 120-kD protein corresponds to the integrin  $\beta_1$  subunit and the 140- and 180-kD proteins represent noncovalently associated integrin  $\alpha$  subunits that are coprecipitated with the 120-kD protein. For comparison, NRK fibroblastic cells were also immunoprecipitated with the integrin  $\beta_1$  antiserum (lane 5). Three putative NRK cell integrin  $\alpha$  subunits of  $\sim 150$ , 160, and 180 kD are coprecipitated in addition to a 110-kD  $\beta_1$  subunit. (B) Immunoprecipitation of surface-labeled human placental fibroblast (lane 1), NRK cell (lanes 2 and 3), and PC12 cell (lanes 4 and 5) extracts with the anti-integrin  $\alpha_{\text{FN}}$  serum (lanes 1, 2, and 5) or with preimmune serum (lanes 3 and 4). Integrin  $\alpha_{\text{FN}}$  antibodies recognize an FN-binding heterodimer in the human fibroblastic (lane 1) and NRK (lane 2) cell extracts but not in PC12 cell extracts (lane 5). Arrowheads indicate the position of the  $\alpha_{\text{FN}}$  (160 kD) and  $\beta_1$  (110 kD) subunits. Samples were separated on 6% polyacrylamide gels under nonreducing conditions. Molecular mass markers, in kilodaltons, indicated are myosin (200,000),  $\beta$ -galactosidase (116,000), phosphorylase a (97,000), and BSA (68,000).

1987), the 140- and 180-kD proteins that are coprecipitated by the integrin  $\beta_1$  subunit-specific antibodies and the anti-ECMR serum are likely to be integrin  $\alpha$  subunits that are each associated noncovalently with the 120-kD  $\beta_1$  subunit.

For comparison, integrin  $\beta_1$ -specific antibodies were used to immunoprecipitate extracts of surface-labeled rat fibroblastic cells (NRK). In contrast to PC12 cells, NRK cells attach well to FN-coated substrates, in addition to LN and Col IV (unpublished observations). The integrin  $\beta_1$  antiserum immunoprecipitated a 110-kD  $\beta_1$  subunit in addition to three putative integrin  $\alpha$  subunits of  $\sim 150$ , 160, and 180 kD from NRK cell extracts (Fig. 1 A, lane 5). The two well-resolved  $\alpha$  subunits of 140 and 180 kD appeared to correspond to the two PC12 cell  $\alpha$  subunits, while the diffuse 160-kD NRK  $\alpha$  subunit appeared to be absent from PC12 cell extracts (compare Fig. 1 A, lanes 1 and 5). An identical group of proteins is immunoprecipitated from NRK cell extracts using a polyclonal antiserum to a FN-binding integrin

$\beta_1$  heterodimer purified from chinese hamster ovarian cells (Tomaselli et al., 1987; Brown and Juliano, 1986).

To determine if either of the two putative PC12 cell integrin  $\alpha/\beta$  heterodimers recognized by the integrin  $\beta_1$  and ECMR antibodies is the rat homologue of the previously characterized human and mouse FN receptor heterodimers (Pytela et al., 1985a; Patel and Lodish, 1986; Brown and Juliano, 1986), a rabbit antiserum was raised against a 19-amino acid peptide derived from the COOH-terminal of the cytoplasmic domain of the "human FN receptor"  $\alpha$  subunit (Argaves et al., 1987b; referred to here as the integrin  $\alpha_{FN}$  subunit as described in Hynes, 1987). The specificity and species cross-reactivity of this antiserum were established by immunoprecipitation of detergent extracts of surface-labeled human placental and NRK fibroblastic cells, both cell types that adhere well to FN. The integrin  $\alpha_{FN}$  antibodies specifically immunoprecipitated two cell surface glycoproteins of  $\sim 160$  ( $\alpha_{FN}$ ) and 110 kD ( $\beta_1$ ) from both NRK and human placental fibroblast extracts (Fig. 1 B, lanes 1 and 2). These proteins likely correspond to the FN-binding integrin  $\beta_1/\alpha_{FN}$  heterodimer expressed by many types of mammalian cells (Pytela et al., 1985a; Brown and Juliano, 1986; Patel and Lodish, 1986). Based on the spacing of these proteins in SDS gels, the 110/160-kD heterodimer immunoprecipitated from NRK extracts by anti- $\alpha_{FN}$  appeared to correspond to one of the three putative NRK dimers that are immunoprecipitated by the integrin  $\beta_1$  antibodies (compare Fig. 1 A, lane 5 with Fig. 1 B, lane 2).

In contrast to NRK cell extracts, integrin  $\alpha_{FN}$  antibodies failed to immunoprecipitate any labeled proteins in extracts of PC12 cells (Fig. 1 B, lane 5). From quantitative immunoprecipitations we estimate that PC12 cells express  $<4\%$  of the integrin  $\beta_1/\alpha_{FN}$  dimer as compared to NRK cells, yet express comparable levels ( $\sim 50\%$ ) of total integrin  $\beta_1$  dimers. The lack of detectable integrin  $\beta_1/\alpha_{FN}$  heterodimers on PC12 cells may account for the inability of these cells to attach efficiently to FN-coated substrates (Tomaselli et al., 1987; Turner et al., 1987). It seems likely, therefore, that the PC12 cell integrins recognized by both the integrin  $\beta_1$  and ECMR antibodies function as receptors for ECM proteins other than FN.

### Affinity Purification of PC12 Cell Integrins

To study the function of the PC12 integrins, these proteins were purified from detergent extracts of PC12 cells using a procedure that combined WGA and ECMR antibody affinity chromatography. The specificity of the ECMR antiserum was established by immunodepletion studies using the integrin  $\beta_1$  subunit-specific antibodies (Fig. 2).  $^{125}\text{I}$ -labeled detergent extracts of PC12 cells were depleted of all detectable 120-, 140-, and 180-kD glycoproteins by sequential immunoprecipitation with integrin  $\beta_1$  antibodies (Fig. 2, lanes 1-3). ECMR serum antibodies failed to immunoprecipitate any residual proteins in the PC12 cell extract that had been depleted of these proteins with integrin  $\beta_1$  antibodies (Fig. 2, lane 5). Since the ECMR antibodies recognize only integrin-related proteins on the surfaces of PC12 cells, they were used to immunopurify the PC12 cell integrins. Octyl-glucoside extracts of surface-iodinated PC12 cells (Fig. 3 A, lane 1) were passed over WGA-Affi-gel 10 and eluted with *N*-acetyl-D-glucosamine. This procedure appeared to enrich for cell surface glycoproteins in the 80-200-kD range

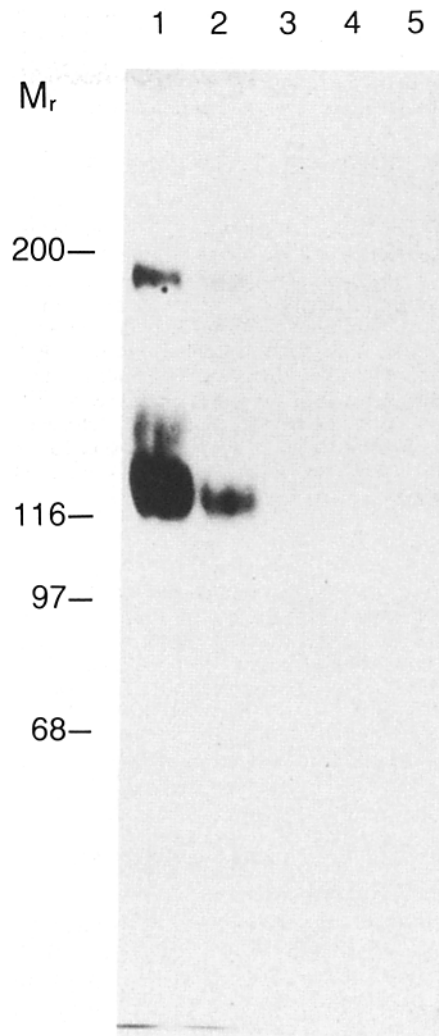
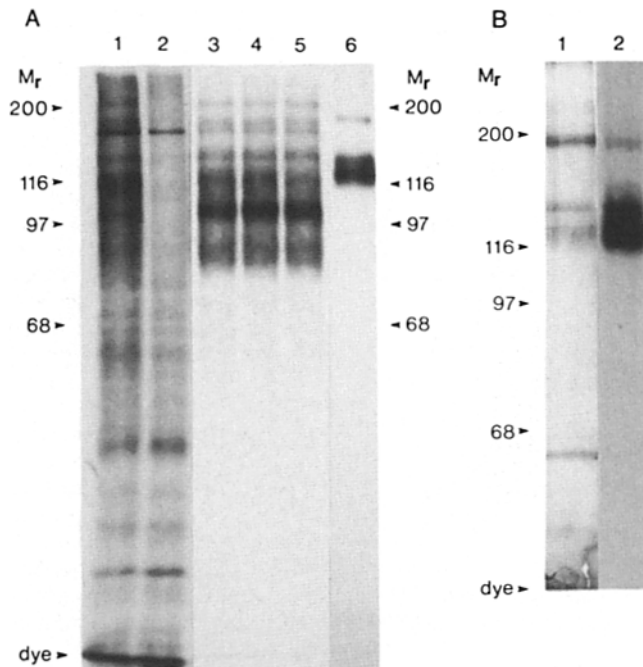


Figure 2. Specificity of the anti-ECMR serum tested by immunodepletion of  $^{125}\text{I}$ -labeled PC12 cell extracts with integrin  $\beta_1$ -specific antibodies. PC12 cell extracts were immunoprecipitated three times with anti-integrin  $\beta_1$  (lanes 1-3), once with protein A-Sepharose alone (lane 4), and then with the anti-ECMR serum (lane 5). Integrin  $\beta_1$  antibodies quantitatively deplete the PC12 cell extract of labeled proteins recognized by ECMR antibodies. Samples were run on 6% polyacrylamide gels under nonreducing conditions. Molecular mass standards, in kilodaltons, indicated are myosin (200,000),  $\beta$ -galactosidase (116,000), phosphorylase a (97,000), and BSA (68,000).

representing  $\sim 5$ - $10\%$  of the total surface-iodinated PC12 cell proteins (Fig. 3 A, lane 3). Subsequent adsorption of the WGA-eluted proteins to anti-ECMR IgG coupled to Sepharose and elution at pH 11.5 yielded three labeled glycoproteins of molecular mass 120, 140, and 180 kD under non-reducing conditions (Fig. 3 A, lane 6). These were the only detectable surface-iodinated proteins purified by this procedure (Fig. 3 A, lane 6, B, lane 2). Silver-stained gels confirmed that the 120-, 140-, and 180-kD glycoproteins were prominent constituents of the cellular proteins purified by this procedure (Fig. 3 B, lane 1). A protein of  $\sim 60$  kD was also seen in addition to other faintly stained proteins (Fig. 3 B, lane 1). These additional proteins were unlabeled by lactoperoxidase-catalyzed cell surface iodination (Fig. 3

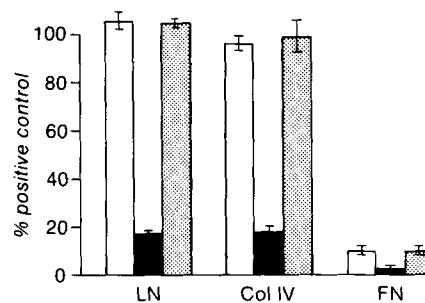


**Figure 3.** SDS-PAGE analysis of PC12 cell integrin purification. (A) Autoradiogram of an 8% nonreducing SDS gel showing fractions from different stages of the purification. Lane 1, surface-iodinated and detergent-extracted PC12 cell starting material (100,000 cpm); lane 2, material that flowed through WGA-Sepharose (100,000 cpm); lane 3, material that bound to WGA-Sepharose and was eluted with *N*-acetyl-D-glucosamine (30,000 cpm); lane 4, material that flowed through normal goat IgG-Sepharose (30,000 cpm); lane 5, material that flowed through the anti-ECMR IgG-Sepharose (30,000 cpm); lane 6, proteins eluted from anti-ECMR IgG-Sepharose at pH 11.5 (2,000 cpm). Longer exposures of autoradiograms of this and other purifications failed to identify any labeled proteins other than the 120-, 140-, and 180-kD proteins shown in lane 6. (B) Protein silver stain and autoradiographic analysis of purified material used for ECMR antiserum neutralization and liposome reconstitution and binding experiments. Proteins were separated on a 6.8% SDS gel under nonreducing conditions. Lane 1, silver stain of material eluted from the anti-ECMR IgG column. Lane 2, autoradiogram of material eluted from the anti-ECMR IgG column. Note the silver-stained 60-kD contaminating protein was not labeled by cell surface iodination. Molecular mass markers, in kilodaltons, indicated are myosin (200,000),  $\beta$ -galactosidase (116,000), phosphorlylase a (97,000), and BSA (68,000).

B, lane 2) and, therefore, are likely to be contaminating intracellular or serum proteins. Immunopurified PC12 cell integrins were used for further studies, as described below.

#### Neutralization of the Anti-ECMR Serum with Purified PC12 Cell Integrins

The purified PC12 cell integrins were tested for their ability to neutralize the adhesion-blocking activity of the anti-ECMR serum. We showed previously that a 0.6% concentration of the anti-ECMR serum inhibits the high levels of PC12 cell attachment to LN and Col IV, as well as the low levels of attachment to FN (Tomaselli et al., 1987). When the anti-ECMR serum was premixed with the purified 120-, 140-, and 180-kD PC12 cell integrins that had been dialyzed free of detergent, it was rendered incapable of inhibiting

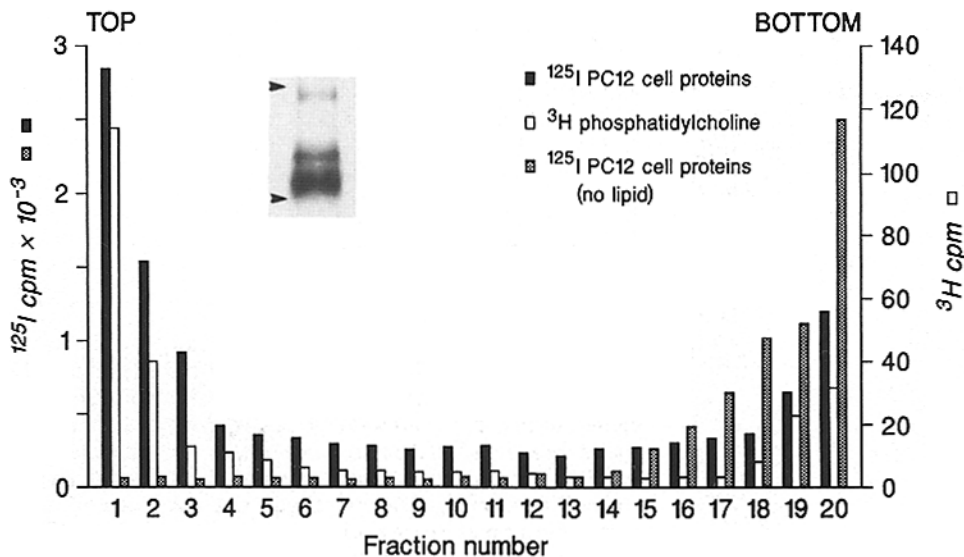


**Figure 4.** Neutralization of the adhesion-blocking activity of the anti-ECMR serum by immunopurified PC12 cell integrins. PC12 cell attachment to substrates coated with LN (5  $\mu$ g/ml), Col IV (2  $\mu$ g/ml), or FN (20  $\mu$ g/ml) was tested in the absence of added antibodies (*open bars*), in the presence of a 0.6% concentration of the anti-ECMR serum that had been mixed with antibody column elution buffer containing 20% normal goat serum as a control (*solid bars*), or in the presence of a 0.6% concentration of the anti-ECMR serum that had been mixed with the immunopurified and dialyzed 120-, 140-, and 180-kD PC12 cell proteins in addition to 20% normal goat serum (*stippled bars*). Values represent the average and range of determinations made on duplicate cultures run in parallel and are expressed as percent of cell attachment to the highly adhesive substrate, poly-D-lysine. Note: PC12 cells attach to FN at ~10% of the levels of attachment to LN and Col IV.

PC12 cell attachment to any of these ECM proteins (Fig. 4, *stippled bars*). In contrast, when the anti-ECMR serum was mixed with control proteins, it remained fully active in inhibiting PC12 cell attachment to LN, Col IV, and FN (Fig. 4, *solid bars*). Thus, all of the adhesion-blocking antibodies in the anti-ECMR serum recognize epitopes on either the 120-, 140-, or 180-kD PC12 cell surface glycoproteins.

#### Incorporation of PC12 Cell Integrins into Liposomes

To determine if the surface-labeled, purified PC12 cell integrins could be incorporated into artificial phosphatidylcholine vesicles, the purified proteins were mixed with [ $^3$ H]phosphatidylcholine and liposomes were formed by detergent dialysis (Mimms et al., 1981). Liposomes were separated on 10–45% sucrose gradients and fractions were analyzed for [ $^3$ H]phosphatidylcholine and [ $^{125}$ I]-labeled PC12 cell proteins. Under these conditions, liposomes were recovered at the top of the sucrose gradient as reflected by the presence of a peak of  $^3$ H in fractions taken from the top of the gradient (Fig. 5, *open bars*). When [ $^{125}$ I]-labeled, immunopurified PC12 cell proteins were mixed with [ $^3$ H]phosphatidylcholine before liposome formation, ~70% of the labeled proteins comigrated with the liposomes to the top of the gradient (Fig. 5, *solid bars*). When phosphatidylcholine was omitted, virtually all of the [ $^{125}$ I]-labeled PC12 cell proteins could be recovered in the bottom fractions of the sucrose gradient (Fig. 5, *stippled bars*). In additional control experiments, <10% of added BSA comigrated with [ $^3$ H]-phosphatidylcholine-containing liposomes to the top of the gradient, indicating that little protein-trapping during liposome formation occurs (not shown). SDS gel analysis of liposome-containing fractions from the top of the gradient demonstrated that the 120-, 140-, and 180-kD PC12 cell glycoproteins were incorporated into the lipid vesicles (Fig. 5, *inset*).



**Figure 5.** Incorporation of surface-labeled, immunopurified PC12 cell integrins into phosphatidylcholine liposomes. Purified proteins in 50 mM octylglucoside were either mixed with [ $^3\text{H}$ ]phosphatidylcholine and dialyzed against PBS (*open and solid bars*) or just dialyzed against PBS without added phosphatidylcholine (*stipled bars*). The dialysates were fractionated by sucrose gradient centrifugation and aliquots of fractions were analyzed for [ $^3\text{H}$ ]phosphatidylcholine (*open bars*) or [ $^{125}\text{I}$ ]labeled protein (*solid and stipled bars*). Fraction number 1 represents the top of the sucrose gradient. About 70% of the [ $^{125}\text{I}$ ]labeled proteins are associated with the [ $^3\text{H}$ ]labeled liposomes at the top of the gradient. (*Inset*) Au-

toradiogram of [ $^{125}\text{I}$ ]labeled proteins recovered in fraction 1 and run on a 7% SDS-polyacrylamide gel under nonreducing conditions. Arrowheads indicate the position of the molecular mass markers, in kilodaltons, myosin (200,000; *upper arrowhead*) and  $\beta$ -galactosidase (116,000; *lower arrowhead*).

### Binding of Reconstituted Liposomes to ECM Proteins

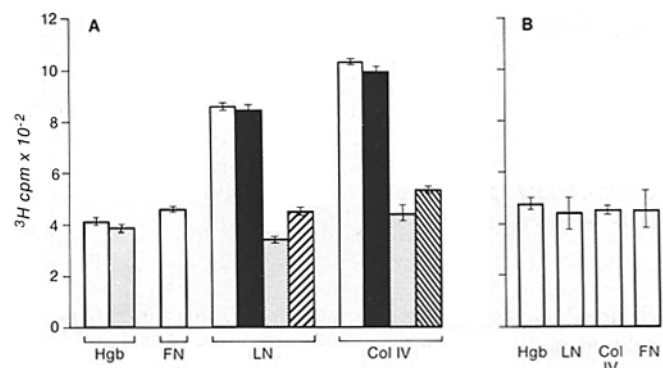
$^3\text{H}$ -labeled liposomes prepared containing the purified PC12 cell integrins were tested for their ability to bind to ECM protein-coated substrates. Integrin-containing liposomes bound to LN-coated substrates at a level of twice the background binding to hemoglobin-coated wells (Fig. 6 A). Binding of liposomes to LN was inhibited either by Fab fragments of anti-ECMR IgG or by anti-LN serum, but was not inhibited by preimmune IgG (Fig. 6 A). Integrin-containing liposomes also bound to Col IV-coated substrates at a level of  $\sim 2.3$  times background levels of binding to hemoglobin (Fig. 6 A). Liposome binding to Col IV was inhibited either by Fab fragments of anti-ECMR IgG or by antibodies to Col IV, but not by preimmune IgG (Fig. 6 A). In contrast, integrin-containing liposomes did not bind to FN-coated substrates significantly above background binding to hemoglobin (Fig. 6 A).  $^3\text{H}$ -labeled liposomes formed in the absence of purified PC12 cell integrins did not bind significantly to either LN, Col IV, or FN (Fig. 6 B). The background levels of binding of empty liposomes to these ECM proteins was not reduced by anti-ECMR Fab or by either LN or Col IV antibodies (not shown). SDS-PAGE analysis confirmed the presence of the purified PC12 integrins in the liposomes that bound to LN and Col IV (not shown). Thus, liposomes containing the 120-, 140-, and 180-kD PC12 cell integrins bound specifically but at low levels to LN and Col IV but not to FN. The low levels of binding of the integrin-containing liposomes to LN and Col IV may be accounted for, in part, by irreversible denaturation of the integrin heterodimers into monomers during the pH 11.5 elution from the ECMR IgG column (see below).

### Comparison of the Hydrodynamic Properties of the Purified PC12 Cell Integrins with Those of Other Integrin Heterodimers

Since the 140- and 180-kD PC12 cell glycoproteins are both noncovalently associated with the 120-kD  $\beta_1$  subunit (Fig. 1

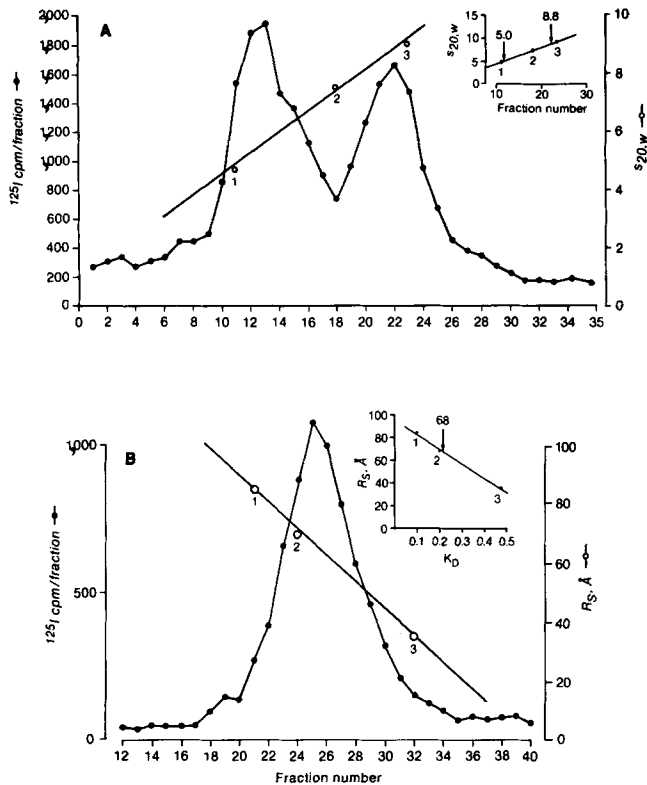
A and Tomaselli et al., 1987), these proteins exist in detergent solution as either a heterotrimer or as two heterodimers with a shared 120-kD  $\beta_1$  subunit. To determine if the PC12 cell integrins have the physical properties expected of heterodimers, the hydrodynamic properties of the purified PC12 cell integrins were studied and compared to the known hydrodynamic properties of other well characterized integrin  $\alpha/\beta$  heterodimers.

When [ $^{125}\text{I}$ ]labeled, immunopurified PC12 cell integrins were subjected to centrifugation in a 5–20% sucrose density gradient, two peaks centered at 5.0S and 8.8S were observed



**Figure 6.** Binding of [ $^3\text{H}$ ]labeled liposomes formed in the presence of (A) or absence (B) of the immunopurified PC12 cell integrins. Binding of [ $^3\text{H}$ ]labeled liposomes to hemoglobin (*Hgb*; 10  $\mu\text{g}/\text{ml}$ ), FN (20  $\mu\text{g}/\text{ml}$ ), LN (20  $\mu\text{g}/\text{ml}$ ), or Col IV (10  $\mu\text{g}/\text{ml}$ ) was measured in the absence of added antibodies (*open bars*), or in the presence of either 1 mg/ml of preimmune goat IgG (*solid bars*), 1 mg/ml anti-ECMR Fab (*stipled bars*), a 1:20 dilution of rabbit anti-LN serum (*hatched bar on LN*), or 100  $\mu\text{g}/\text{ml}$  of anti-Col IV IgG (*hatched bar on Col IV*). Values from one experiment are shown here and represent the average and range of [ $^3\text{H}$ ]cpm bound to duplicate wells run in parallel. Liposome binding experiments were repeated twice using different preparations of immunopurified PC12 cell glycoproteins with quantitatively similar results.

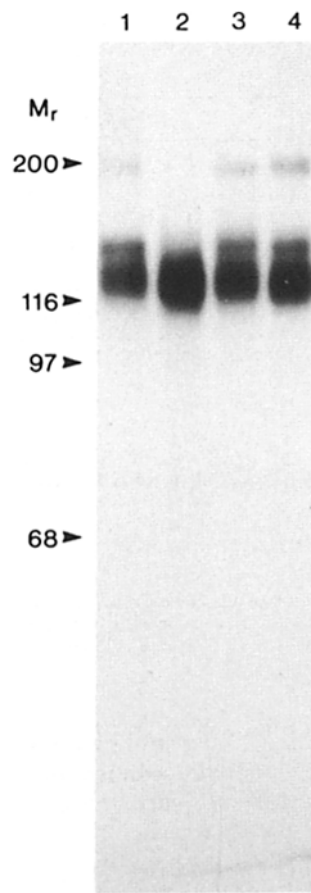




**Figure 7.** Hydrodynamic properties of PC12 cell integrins. (A) 5–20% sucrose density gradient analysis of immunopurified  $^{125}\text{I}$ -labeled PC12 cell integrins. Fractions were analyzed for  $^{125}\text{I}$ -labeled proteins (solid circles) and for sedimentation standards (open circles); 1: BSA, 4.7S; 2: aldolase, 7.35S; 3: fumarase, 9.09S. (Inset) plot of the sedimentation coefficients ( $s_{20,w}$ ) of the standards versus fraction number. Arrows indicate the positions of the two PC12 cell protein peaks. (B) Molecular sieve chromatography of purified  $^{125}\text{I}$ -labeled PC12 cell integrins on Sephacryl S-300. Proteins were run on a column that had been precalibrated with protein standards of known Stokes radii (open circles; 1: thyroglobulin, 85 Å; 2:  $\beta$ -galactosidase, 69 Å; 3: BSA, 36 Å). Fractions were analyzed for  $^{125}\text{I}$ -labeled PC12 cell proteins (solid circles). (Inset) plot of the distribution coefficients ( $K_D$ ) versus Stokes radius ( $R_s$ ) of the calibration standards. The arrow indicates the position of the PC12 cell protein peak.

(Fig. 7 A). SDS gel analysis of fractions from this gradient demonstrated that the purified 120-, 140-, and 180-kD glycoproteins were present in both peaks (Fig. 8, lanes 3 and 4). The presence of all three proteins in both peaks suggests that the heavier 8.8S peak contains proteins that are either aggregated or noncovalently associated with each other, perhaps as dimers or larger oligomers, while the 5.0S peak contains monomeric proteins. Consistent with this interpretation, the pooled trailing fractions of the 5.0S peak were found to contain predominantly the smaller 120-kD protein (Fig. 8, lane 2), while the pooled peak and leading fractions from the 5.0S peak contained all three proteins (Fig. 8, lane 3). This is what one might expect if the 5.0S peak represented three partially superimposed peaks corresponding to the positions of integrin subunit monomers of 120, 140, and 180 kD. In contrast, all three proteins appeared to be present in both the leading and trailing fractions from the 8.8S peak.

The sedimentation coefficient of the putative PC12 cell



**Figure 8.** Nonreducing 7% SDS gel analysis of  $^{125}\text{I}$ -labeled PC12 cell proteins in fractions from the sucrose gradient shown in Fig. 7 A. Lane 1, immunopurified starting material which contains three proteins of 120, 140, and 180 kD; lane 2, fractions 10–12 from the 5.0S peak; lane 3, fractions 13–16 from the 5.0S peak; lane 4, fractions 20–24 from the 8.8S peak. Molecular mass markers, in kilodaltons, indicated are myosin (200,000),  $\beta$ -galactosidase (116,000), phosphorylase (97,000), and BSA (68,000).

dimers (8.8S) is similar to that for the human platelet integrin  $\beta_3$  heterodimer, IIb/IIIa (Table I), and the avian integrin  $\beta_1$  heterodimers recognized by the CSAT monoclonal antibody (Table I). The sedimentation coefficient of the more slowly sedimenting form of the PC12 cell proteins (5.0S; putative monomers) is similar to that determined for the monomeric  $\alpha$  (IIb) and  $\beta$  (IIIa) subunits of the platelet integrin  $\beta_3$  dimer and that of the avian integrin  $\beta_1$  subunit monomer (Table I).

$^{125}\text{I}$ -labeled, immunopurified PC12 cell proteins were also subjected to molecular sieve chromatography on Sephacryl S-300. Under these conditions, a single broad peak with a Stokes radius centered at 68 Å was observed (Fig. 7 B). The Stokes radius of the PC12 cell integrins is similar to that of the platelet IIb/IIIa heterodimer (Table I) and the avian integrin  $\beta_1$  dimers recognized by the CSAT monoclonal antibody (Table I). The presence of a single peak by molecular sieve chromatography is in contrast to the presence of two peaks in sucrose density gradients (compare Fig. 7, A and B). A possible explanation for this is that the Stokes radii of the two populations of PC12 cell proteins seen in sucrose density gradients (putative monomers and dimers) are too similar to be resolved on the Sephacryl column. A consistent observation is that the Stokes radii of the isolated platelet integrin monomers IIb and IIIa are quite similar to the Stokes radius observed for the IIb/IIIa heterodimer complex (Table I). This close similarity in the effective Stokes radii of monomers and dimers is what one would expect if the dimers were composed of monomers with the shapes of rods or prolate ellipsoids (Tanford, 1961). One would expect, however,

**Table I. Comparison of the Hydrodynamic Properties of the Purified PC12 Cell Integrins with Those of Other Integrin Heterodimers**

Integrins	Dimers			Monomers	
	$R_s(\text{\AA})$	$s_{20,w}$	$M_r$	$R_s(\text{\AA})$	$s_{20,w}$
PC12 cell integrins	68	8.8	269,000 <sup>†</sup>	—	5.0
Platelet IIb/IIIa*	71	8.6	265,000	61 <sup>§</sup> , 67 <sup>  </sup>	4.7 <sup>§</sup> , 3.2 <sup>  </sup>
Avian integrin $\beta_1$ ‡	60	8.6	235,000	—	4.0

The sedimentation coefficients ( $s_{20,w}$ ), Stokes radii ( $R_s$ ), and estimated relative molecular mass ( $M_r$ ) of the purified PC12 cell integrins were compared with the published values for the human integrin  $\beta_3$  heterodimer, platelet proteins IIb/IIIa, and the avian integrin  $\beta_1$  heterodimers recognized by the CSAT monoclonal antibody.

\* Data from Jennings and Phillips, 1982.

‡ Data from Horwitz et al., 1985.

§ Values for platelet IIb monomers.

|| Values for platelet IIIa monomers.

† An estimate of the relative molecular mass ( $M_r$ ) of the PC12 cell integrins was calculated from the formula:

$$M_r = \frac{6\pi NR_s s_{20,w}}{1 - \rho\bar{v}}$$

where  $R_s = 68 \text{ \AA}$ ,  $s_{20,w} = 8.8S$ , and the value for the partial molar volume ( $\bar{v}$ ) of the antigen was taken to be equivalent to that of the avian integrin  $\beta_1$  heterodimers (0.75 ml/g) determined experimentally in Horwitz et al. (1985).

monomers and dimers with similar effective Stokes radii to be resolved in sucrose density gradients, since sedimentation in such gradients depends, in part, on molecular mass.

## Discussion

In the present study, we have used antibodies directed against synthetic peptides derived from integrin  $\alpha$  and  $\beta$  subunit protein sequences to characterize integrin  $\beta_1$ -class heterodimers expressed by the rat neuronal cell line, PC12. In addition, we have characterized the ligand-binding specificity and hydrodynamic properties of these PC12 cell integrins after immunopurification using an adhesion-perturbing antiserum (anti-ECMR). We provide evidence that these PC12 cell integrins are structurally and functionally distinct from the previously characterized FN-binding integrin heterodimers and, thus, can account for the ability of these cells to attach to LN and Col IV but not FN.

PC12 cells express two distinct integrin  $\beta_1$ -class cell surface heterodimers. Immunoprecipitation of detergent extracts of surface-labeled PC12 cells with antibodies to the cytoplasmic domains of the human and avian integrin  $\beta_1$  subunit monomers yields three surface-labeled proteins of apparent molecular mass 120, 140, and 180 kD under non-reducing conditions (cf. Fig. 1 A). These proteins are identical to three cell surface glycoproteins recognized by the adhesion-perturbing antiserum, anti-ECMR, in PC12 cell extracts (cf. Fig. 1 A and Fig. 2; see also Tomaselli et al., 1987). We have previously shown that polyclonal antibodies to the purified avian integrin  $\beta_1$  subunit specifically recognize only the 120-kD PC12 cell protein in antigen blots yet also coprecipitate both the 140- and 180-kD proteins (Tomaselli et al., 1987). Taken together with the hydrodynamic data (see below), these observations implicate the 140- and 180-kD proteins as integrin  $\alpha$  subunits that are each noncovalently associated with a common 120 kD  $\beta_1$  subunit to form two  $\alpha/\beta$  heterodimers of 120/140 and 120/180 kD on the sur-

faces of PC12 cells. At present, it is difficult to say if the PC12 cell integrin  $\alpha$  subunits correspond to any of the five human  $\alpha$  subunits that are known to form dimers with the human integrin  $\beta_1$  subunit (VLA 1-5  $\alpha$ 's; Hemler et al., 1987; Takada et al., 1987b). However, based on the mobilities of the 180- and 140-kD PC12 cell proteins in SDS gels under reducing and nonreducing conditions, it seems plausible that the 180-kD protein corresponds to the 200-kD human VLA 1  $\alpha$  subunit and the 140-kD protein corresponds to the 150-kD human VLA 3  $\alpha$  subunit.

PC12 cells attach to and extend neurites efficiently on substrates coated with LN and Col IV, but attach poorly to FN (Tomaselli et al., 1987; Turner et al., 1987). Thus, it seems unlikely that either of the two PC12 cell integrin heterodimers described here corresponds to the FN-binding integrin  $\beta_1/\alpha_{FN}$  heterodimer previously characterized in human and rodent cells (Pytela et al., 1985a; Brown and Juliano, 1986; Patel and Lodish, 1986; see Hynes, 1987, for integrin nomenclature). Immunoprecipitation studies with the integrin  $\beta_1$  peptide antibodies and with antibodies to the cytoplasmic domain of the human integrin  $\alpha_{FN}$  subunit are in agreement with this interpretation. Integrin  $\alpha_{FN}$  antibodies immunoprecipitate two cell surface proteins of  $\sim 160$  kD ( $\alpha_{FN}$ ) and 110 kD ( $\beta_1$ ) from both NRK and human fibroblastic cells (Fig. 1 B), both cell types that adhere well to FN. In contrast to NRK cells, PC12 cells appear to express little or no integrin  $\beta_1/\alpha_{FN}$  FN receptor since integrin  $\alpha_{FN}$  antibodies failed to immunoprecipitate detectable amounts of the rat 160-kD  $\alpha_{FN}$  protein from PC12 cell extracts (Fig. 1 B, lane 5). Quantitative estimates based on the specific activities of the labeled PC12 and NRK cell proteins indicates that PC12 cells express <4% of the levels of  $\beta_1/\alpha_{FN}$  dimers expressed by NRK cells, despite having comparable total levels of integrin  $\beta_1$  dimers. Although we cannot detect  $\beta_1/\alpha_{FN}$  dimers on the surfaces of PC12 cells, even after long autoradiographic exposures, the ability of PC12 cells to attach weakly to FN-coated substrates in an integrin-dependent (cf. Fig. 4) and RGD peptide-sensitive manner (Tomaselli et al., 1987; Akeson and Warren, 1986) suggests that they may express low levels of this FN receptor. Alternatively, one of the two prominent integrin heterodimers on PC12 cells may directly interact with FN and thus mediate low levels of PC12 cell attachment to FN.

To study the structure and function of the PC12 cell integrins recognized by the integrin  $\beta_1$  peptide antibodies and the ECMR serum antibodies, the 120-, 140-, and 180-kD proteins were purified using combined lectin and ECMR antibody affinity chromatography (cf. Fig. 3). The specificity of the ECMR antiserum used in the purification was established by immunodepletion studies using the integrin  $\beta_1$  subunit-specific antibodies (Fig. 2). The immunopurified PC12 cell integrins completely neutralize the ability of the anti-ECMR serum to inhibit PC12 cell attachment to LN and Col IV (cf. Fig. 4), demonstrating that these proteins function in PC12 cell adhesion to LN and Col IV. To directly assay ECM receptor function, the purified integrins were tested for their ability to bind to ECM protein-coated substrates after incorporation into lipid vesicles. Integrin-containing liposomes bound specifically, but at low levels, to substrates coated with LN or Col IV but failed to bind significantly to FN or hemoglobin (cf. Fig. 6). In contrast, "empty" liposomes did not bind significantly to any of these

ECM protein-coated substrates. This pattern of integrin-reconstituted liposome binding to purified ECM proteins reflects the adhesive specificity of intact PC12 cells, namely: LN = Col IV >> FN (Tomaselli et al., 1987; Turner et al., 1987).

The observed binding of PC12 cell integrin-containing liposomes to LN and Col IV provides evidence that these proteins function directly as receptors for LN and Col IV. These findings are also consistent with previous observations that (a) integrin  $\beta_1$  antibodies (CSAT) inhibit neuronal attachment to both LN and Col IV (Bozyczko and Horwitz, 1986; Hall et al., 1987) and (b) avian integrin complexes immunopurified using the integrin  $\beta_1$  subunit-specific monoclonal antibody, CSAT, are capable of interacting with LN in equilibrium gel filtration assays (Horwitz et al., 1985). There are several possible explanations for the low levels of binding (two- to threefold above background) exhibited by the integrin-containing vesicles in the present study. First, elution of the glycoproteins from the ECMR antibody column was effected at pH 11.5, a condition that has been reported to denature one of the human integrin  $\beta_1$  heterodimers, VLA 4, into monomers (Hemler et al., 1987a, b). Thus, a proportion of the purified PC12 cell integrins may have been denatured by the conditions used to elute them from the ECMR antibody affinity column. A consistent observation is the finding that ~50% of the eluted proteins have a sedimentation coefficient (5.0S) expected of integrin subunit monomers (cf. Fig. 7 A and Table I). Since interactions of purified avian integrins with LN and FN have been shown to require the integrity of the oligomeric complexes (Buck et al., 1986), dissociated PC12 cell integrin subunits are probably not capable of ligand binding. Another possibility is that the binding affinities of the PC12 cell integrins for LN and Col IV are quite low. Detergent-solubilized avian integrin  $\beta_1$  receptors bind LN and FN with an estimated  $K_d$  of  $\sim 10^{-7}$  M (Horwitz et al., 1985). Due to the "weak" nature of these interactions, one would expect the binding of liposomes containing these receptors to depend critically on the incorporation of large numbers of intact, functional receptors. We estimate from silver-stained gels of purified material that each liposome contained  $\sim 10$ – $20$  molecules of each of the 120-, 140-, and 180-kD proteins. Since only some of these are expected to be inserted in the right orientation (extracellular domains facing outward) and since some of the receptor complexes are probably denatured (see above), the actual number of functional receptors per liposome may be quite low. In previous studies, liposomes containing comparable amounts of integrin  $\beta_1$  or  $\beta_3$  receptor heterodimers to those used here were shown to bind to FN and vitronectin at levels ranging from  $\sim 5$ – $10$ -fold above background (Pytela et al., 1985a, b; Cardarelli and Piersbacher, 1987). However, it is likely that smaller proportions of these receptor heterodimers were denatured, since they were purified on ECM protein affinity columns using milder RGDS-containing peptide elution conditions (Pytela et al., 1985a, b). In addition, the liposomes in these earlier studies probably contained only one type of receptor heterodimer. Since RGDS peptides have no noticeable inhibitory effect on PC12 cell attachment to either LN or Col IV (Tomaselli et al., 1987), it is not clear that RGDS peptide elution will be useful for receptor purification from LN or Col IV affinity columns.

Our immunoprecipitation studies using antibodies (anti-integrin  $\beta_1$ ) that recognize only the 120-kD PC12 cell glycoprotein in antigen blots demonstrate that the 120-kD integrin  $\beta_1$ -like subunit is noncovalently associated with both the 140- and 180-kD glycoproteins (cf. Fig. 1 A and Tomaselli et al., 1987). Thus the 120-, 140-, and 180-kD PC12 cell proteins are likely to comprise either a single heterotrimer or two noncovalently associated  $\alpha/\beta$  heterodimers with a shared 120-kD subunit. Data presented here support the latter interpretation: the sedimentation coefficients, Stokes radii, and estimated molecular masses of the putative PC12 cell integrin dimers are very similar to those of both the human integrin  $\beta_3$  heterodimer, platelet glycoproteins IIb/IIIa, and the avian integrin  $\beta_1$  heterodimers recognized by the CSAT monoclonal antibody (cf. Table I). These measurements are most consistent with a dimeric structure for the purified integrins. In preliminary studies we have found that when the PC12 cell integrins are cross-linked using dithiobissuccinimidylpropionate (as in Hemler et al., 1985; 1987a), the cross-linked products migrate in SDS gels with apparent molecular weights expected of cross-linked dimers (260 kD and 320 kD) and not a cross-linked trimer ( $\sim 450$  kD; Tomaselli, K., unpublished observations). Thus it appears likely that the 120-, 140-, and 180-kD proteins are organized as two dimers of 120/140 and 120/180 kD. Immunoprecipitations with  $\alpha$  subunit-specific antibodies would provide the most direct evidence that the 140- and 180-kD glycoproteins are each separately associated with the 120-kD integrin  $\beta_1$  subunit.

Our results implicate two integrin heterodimers as receptors for LN and Col IV on PC12 cells. It is not yet certain whether distinct heterodimers bind separately to LN and Col IV, or whether a single heterodimer binds to both ECM proteins. In favor of the first possibility, PC12 cell attachment to LN is much more sensitive to an antibody to a hamster fibronectin receptor than is attachment of the same cells to Col IV (Tomaselli et al., 1987). Furthermore, in retinal neurons, the functions of integrin class receptors for LN and Col IV appear to be regulated independently (Hall et al., 1986). Consistent with the second possibility, integrin heterodimers in the  $\beta_3$  and  $\beta_1$  families that bind to more than one ECM protein have been described previously (Pytela et al., 1986; Plow et al., 1985; Wayner and Carter, 1987). Most relevant to this study, a monoclonal antibody that recognizes a single VLA 3-like  $\alpha$  subunit associated with the integrin  $\beta_1$  subunit has been shown to inhibit attachment of HT 1080 human fibrosarcoma cells to LN, Col IV, and FN (Wayner and Carter, 1987). Studies using individual PC12 integrin heterodimers should make it possible to distinguish between these possibilities.

It is important to note that additional PC12 cell surface proteins may interact with LN or Col IV, both of which are large, multidomain adhesive proteins. LN, for example, has more than one cell-binding domain (Goodman et al., 1987; Graf et al., 1987; Engvall et al., 1986), as does FN (Humphries et al., 1986, 1987; Bernardi et al., 1987; Rogers et al., 1985, 1987). Recently, several LN-binding cell surface proteins have been described, some of which are expressed in cell lines of mixed neuronal and glial origin (cf. Graf et al., 1987; Smalheiser and Schwartz, 1987; Kleinman et al., 1988). Preliminary characterization of these putative LN receptors indicates that they are unrelated to integrin-class

ECM protein receptors. It will be important in the future to further examine the relationship of these binding proteins to integrins and to establish their functions in the response of neurons to LN-containing substrates. Similarly, several collagen receptors have recently been described (Santoro, 1986; Wayner and Carter, 1987; Dedhar et al., 1987), two of which appear to be integrin heterodimers. The platelet Ia/IIa heterodimer binds type I collagen in a  $Mg^{++}$ -dependent fashion (Santoro, 1986; Santoro, S. A., unpublished observations) and a similar, if not identical, VLA 2-like heterodimer on human fibrosarcoma cells appears to interact with several collagens, including Col IV (Wayner and Carter, 1987).

In summary, we have immunopurified three PC12 cell surface glycoproteins of 120, 140, and 180 kD that belong to the integrin family of adhesive protein receptor heterodimers. These glycoproteins likely comprise two  $\alpha/\beta$  heterodimers with a shared 120-kD integrin  $\beta_1$  subunit. Liposomes containing these integrin heterodimers bind specifically to LN and Col IV, and thus mediate, in part, PC12 cell attachment and process outgrowth on LN and Col IV. As the ECMR antiserum inhibits rat sympathetic neuronal attachment and process outgrowth on LN substrates (Tomaselli et al., 1987), mammalian neurons are likely to use similar receptors.

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