

Interactions of a Neuronal Cell Line (PC12) with Laminin, Collagen IV, and Fibronectin: Identification of Integrin-related Glycoproteins Involved in Attachment and Process Outgrowth

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Abstract. Neuronal responses to extracellular matrix (ECM) constituents are likely to play an important role in nervous system development and regeneration. We have studied the interactions of a neuron-like rat pheochromocytoma cell line, PC12, with ECM protein-coated substrates. Using a quantitative cell attachment assay, PC12 cells were shown to adhere readily to laminin (LN) or collagen IV (Col IV) but poorly to fibronectin (FN). The specificity of attachment to these ECM proteins was demonstrated using ligand-specific antibodies and synthetic peptides. To identify PC12 cell surface proteins that mediate interactions with LN, Col IV, and FN, two different antisera to putative ECM receptors purified from mammalian cells were tested for their effects on PC12 cell adhesion and neuritic process outgrowth. Antibodies to a 140-kD FN receptor heterodimer purified from Chinese hamster ovarian cells (anti-FNR; Brown, P. J., and R. L. Juliano, 1986, *J. Cell Biol.*, 103:1595-1603) inhibited attachment to LN and FN but not to Col IV.

Antibodies to an ECM receptor preparation purified from baby hamster kidney fibroblastic cells (anti-ECMR; Knudsen, K. A., P. E. Rao, C. H. Damsky, and C. A. Buck, 1981, *Proc. Natl. Acad. Sci. USA.*, 78:6071-6075) inhibited attachment to LN, FN, and Col IV, but did not prevent attachment to other adhesive substrates. In addition to its effects on adhesion, the anti-ECMR serum inhibited both PC12 cell and sympathetic neuronal process outgrowth on LN substrates. Immunoprecipitation of surface-iodinated or [³H]glucosamine-labeled PC12 cells with either the anti-FNR or anti-ECMR serum identified three prominent cell surface glycoproteins of 120, 140, and 180 kD under nonreducing conditions. The 120-kD glycoprotein, which could be labeled with ³²P-orthophosphate and appeared to be noncovalently associated with the 140- and 180-kD proteins, cross reacted with antibodies to the β -subunit (band 3) of the avian integrin complex, itself a receptor or receptors for the ECM constituents LN, FN, and some collagens.

NERVOUS system development is characterized by complex and reproducible patterns of neuronal migration, axon extension, target recognition, and synapse formation, processes that depend on the ability of neurons to extract and respond to information present in the cellular and acellular environments in which they develop. Several recent observations suggest that protein constituents of the extracellular matrix (ECM)¹ influence some aspects of neuronal differentiation. First, purified constituents of the ECM, most notably the basement membrane glycoprotein laminin (LN), are known to promote neuronal process outgrowth and survival in vitro (Manthorpe et al., 1983; Rogers et al., 1983;

Edgar et al., 1984; Lander et al., 1983, 1985a, b; Davis et al., 1985; Calof and Reichardt, 1984, 1985). Fibronectin (FN) and, to a lesser extent, collagen type I and collagen type IV (Col IV) also influence process outgrowth from a variety of avian and mammalian neurons (Akers et al., 1981; Rogers et al., 1983, 1985; Carbonetto et al., 1983; Bozyczko and Horwitz, 1986; Tomaselli et al., 1986a; Hall et al., 1987). Second, neuronal process outgrowth on the surfaces of some nonneuronal cells in vitro depends on the function of neuronal receptors for ECM proteins as well as cell-cell adhesion molecules (Tomaselli et al., 1986a; Bixby et al., 1987). Finally, for neurons in peripheral autonomic ganglia, motoneurons in the spinal cord, and ganglion cells of the retina, axon initiation occurs in regions that are likely to contain LN and/or FN (Rogers et al., 1986; Bronner-Fraser, 1986; Adler et al., 1985; Easter et al., 1984; Halfter and Deiss, 1984, 1986), suggesting a role for these ECM molecules in axon growth in vivo.

1. *Abbreviations used in this paper:* Col IV, collagen IV; CSAT antibody, cell substrate attachment antibody; ECM, extracellular matrix; ECMR, extracellular matrix receptor; FNR, fibronectin receptor; FN, fibronectin; LN, laminin; NGF, nerve growth factor; NRK, normal rat kidney; SCG, superior cervical ganglion.

The influences of ECM constituents on neuronal differentiation are mediated by receptors for these molecules on the neuronal membrane. For example, for avian sensory, parasympathetic, and retinal neurons, attachment and process outgrowth on LN, FN, and Col IV depend on a set of neuronal cell surface glycoproteins recognized by two monoclonal antibodies, cell substrate attachment antibody (CSAT) and JG22 (Bozyczko and Horwitz, 1986; Tomaselli et al., 1986a; Hall et al., 1987). A group of integral membrane glycoproteins of 110–165 kD purified by these antibodies appears to function as a receptor or receptors for FN, LN, and at least some collagens (Horwitz et al., 1985; Akiyama et al., 1986; Buck et al., 1986; Hall et al., 1987). The recent cloning and sequencing of the 110-kD (band 3) component (Tamkun et al., 1986), has established that glycoproteins recognized by the CSAT and JG22 antibodies belong to the integrin family of adhesive protein receptor heterodimers present on a wide variety of avian and mammalian cells (Pytela et al., 1986; Kishimoto et al., 1987; Hemler et al., 1987; reviewed in Hynes, 1987).

The present study was motivated by a search for a neuron-like cell line whose behavior mimics that of neuronal cells to purified ECM molecules. Such a cell line would be useful for biochemical and cell biological studies of neuron/ECM interactions since pure neuronal cultures are difficult to obtain in large quantities. The rat pheochromocytoma cell line, PC12, displays properties of differentiated sympathetic neurons after long-term exposure to nerve growth factor (NGF), including process outgrowth, electrical excitability, and functional synapse formation (Tischler and Greene, 1975; Schubert et al., 1977). In the present study, we used two antisera that recognize mammalian homologs of avian ECM receptors identified by the CSAT antibody to identify PC12 cell surface glycoproteins involved in interactions with the ECM proteins LN, Col IV, and FN: one serum was raised to a heterodimeric FN receptor immunopurified from Chinese hamster ovary (CHO) cells (henceforth referred to as anti-FNR; Brown and Juliano, 1986) and the other was raised to a 120–160-kD ECM receptor preparation purified from baby hamster kidney (BHK) cells (henceforth referred to as anti-ECMR; Knudsen et al., 1981; Damsky et al., 1982). We present evidence for both a structural and functional relationship between the PC12 cell surface glycoproteins recognized by these antisera and ECM receptors belonging to the integrin family of adhesive protein receptors identified by the CSAT antibody on avian cells. Some of these results have been reported in abstract form (Tomaselli et al., 1986b).

Materials and Methods

Animals

Newborn Sprague-Dawley rat pups were purchased from Bantin and Kingman (Fremont, CA).

Chemicals and Reagents

Laminin and collagen IV were purified from Engelbreth-Holm-Swarm (EHS) sarcoma tumors using published methods (Kleinman et al., 1982; Timpl et al., 1982) and were the generous gifts of Drs. A. D. Lander, J. L. Bixby, J. Winter, and D. Hall. Human plasma fibronectin was from Collaborative Research, Inc. (Lexington, MA). Rat tail collagen was prepared as described in Bornstein (1958). Aquasol was purchased from New England Nuclear (Boston, MA). *N*-octyl- β -D-glucopyranoside was purchased

from Calbiochem (La Jolla, CA). Protein A coupled to Sepharose CL-4B and Sepharose CL-4B were purchased from Pharmacia (Piscataway, NJ). Affi-gel 10 was purchased from BioRad Laboratories (Richmond, CA). β -Nerve growth factor (β -NGF) was purified from adult male mouse submaxillary glands as described (Mobley et al., 1976) and was a generous gift from Dr. David Shelton. The Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) and Gly-Arg-Gly-Glu-Ser-Pro (GRGESP) hexapeptides were purchased from Peninsula Laboratories, Inc. (Belmont, CA). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Antibodies

Rabbit anti-LN serum was prepared by Dr. Janet Winter using LN purified from the EHS sarcoma. A rabbit antiserum to the purified E3 fragment of LN (Ott et al., 1982) was the generous gift of Dr. D. Hall. Rabbit anti-N-CAM serum was the generous gift of Dr. J. L. Bixby. Rabbit anti-NILE glycoprotein (or anti-Ng-CAM) was kindly provided by Dr. W. Stallcup. Affinity-purified antibodies to murine collagen IV were purchased from Dr. H. Furthmayr (Yale University, New Haven, CT). An antiserum to a purified preparation of 140-kD adhesion-related, integral membrane glycoproteins purified from BHK cells was generated in goats as previously described by Dr. K. Knudsen (anti-gpl40, here referred to as anti-ECMR; Knudsen et al., 1981; Damsky et al., 1982). F_{ab} fragments of anti-ECMR IgG were prepared as described in Parham (1986). IgG from a goat antiserum to a purified 140-kD FN receptor heterodimer from CHO cells was the very generous gift of Dr. R. Juliano (here referred to as anti-FNR; Brown and Juliano, 1985, 1986). A rabbit serum against the purified β_1 -subunit (band 3) of the avian CSAT/integrin ECM receptor complex was a very generous gift from Dr. C. Buck (Buck et al., 1986).

Cell Culture

Rat pheochromocytoma (PC12) cells (Tischler and Greene, 1975) were grown in monolayer culture in DME with 4.5 g/l glucose (DME H-21; UCSF Cell Culture Facility) supplemented with 10% heat-inactivated horse serum, 5% newborn calf serum, glutamine (2 mM), and penicillin/streptomycin (100 U/ml) and were carried for no more than 10 passages. PC12 cells were "primed" with NGF as described (Greene, 1977; Greene et al., 1982). For studies on process outgrowth, NGF-primed PC12 cells were passaged and mechanically divested of their neurites by trituration through a narrow bore 9-inch pasteur pipet. After washing three times in growth medium, cells were resuspended at a concentration of 10^5 cells/ml in growth medium with or without 50 ng/ml NGF (see Table and Figure legends), and 100 μ l of this cell suspension were added per well (0.28 cm² surface area).

A mouse muscle cell line, C2 (Yaffe and Saxel, 1977), was grown in monolayer culture in DME with 1 g/l glucose (DME H-16; UCSF Cell Culture Facility) supplemented with 20% FCS, 0.5% chick embryo extract (Gibco, Grand Island, NY), glutamine (2 mM), and penicillin-streptomycin (100 U/ml).

Sympathetic neurons were enzymatically dissociated from newborn rat superior cervical ganglia (SCG) as previously described (Lander et al., 1982) and were grown in DME H-21 supplemented with 10% heat-inactivated horse serum, glutamine (2 mM), and penicillin/streptomycin (100 U/ml).

The normal rat kidney (NRK) and BHK fibroblastic cell lines were grown in monolayer culture in DME H-21 supplemented with 10% FCS, glutamine (2 mM), and penicillin/streptomycin (100 U/ml).

Substrate Preparation

LN, FN, and Col IV were diluted in Ca⁺⁺/Mg⁺⁺-free PBS to the concentrations indicated in figure legends. Poly-D-lysine (PDL) was dissolved in H₂O at a concentration of 1 mg/ml. 100 μ l of the appropriate solution were used to coat the 0.28-cm² surface area of a 96-well tissue culture well (cat. No. 76-032-05; Linbro, Flow Laboratories, Hamden, CT) by incubation overnight at 4°C. Plates to be used for PC12 cell process outgrowth assays were washed five times with sterile PBS before the addition of about 10^4 cells/0.28 cm² well in 100 μ l of growth medium. For sympathetic neurite outgrowth assays, neurons were plated in microwells that had first been coated with poly-D-lysine (1 mg/ml), washed with H₂O, and then coated with LN or FN.

Cell Attachment Assay

The assay used to measure attachment of PC12 cells is similar to that used by Hall et al. (1987) to measure retinal cell attachment. Briefly, protein-

coated microwells were rinsed three times with PBS and blocked for 3 h with 10 mg/ml BSA in PBS. 100 μ l of PBS (with 1 mM CaCl₂ and 0.5 mM MgCl₂) or PBS plus 2 \times concentration of the desired antibodies or peptides were added to each well. Antisera used in attachment assays were heat inactivated at 56°C for 1 h before use. PC12 cells to be used in the attachment assays were harvested in 5 mM EDTA in Ca⁺⁺/Mg⁺⁺-free PBS, washed twice in PBS containing 1 mM Ca⁺⁺ and 0.5 mM Mg⁺⁺, and adjusted to a density of 5 \times 10⁵ cells/ml in Ca⁺⁺/Mg⁺⁺-containing PBS. 100 μ l of this cell suspension (5 \times 10⁴ cells) were added to each 96-well plate well. To ensure rapid and even distribution of the added cells onto the bottom of the wells, plates were spun at 100 g for 2 min. Cells were allowed to attach for 90 min at 37°C. Unattached cells were dislodged from the bottom of the well by forceful ejection of 50 μ l of PBS to each of two sides of the microwell and then supernatants and unattached cells were removed. This wash procedure was repeated once to ensure complete removal of unattached cells. Two different methods of quantitation were used in the present study. In one (cf. Fig. 3), PC12 cells that had been labeled overnight with [³⁵S]methionine were used. At the end of the assay, attached cells were extracted in 100 μ l of 1% SDS in PBS, mixed with 5 ml of Aqualol, and counted in a scintillation counter. In the second method (cf. Table II; Figs. 4 and 5), attached cells were fixed and stained with trypan blue as described (Koda et al., 1986). After rinsing with PBS, the A₆₉₀ nm of individual wells was measured using a microtiter plate reader (Flow Laboratories, Inc., McLean, VA). Wells coated with 1 mg/ml poly-D-lysine served as the positive control to which attachment to other substrates was compared. Using either method of quantitation, it was demonstrated that ~60–80% of the input cells attached to poly-D-lysine-coated wells, compared with <2% on BSA-blocked plastic alone. All attachment experiments were repeated at least once with quantitatively similar results.

Radiolabeling of PC12, NRK, and BHK Cells

PC12 cells were metabolically labeled for 16 h with either 50 μ Ci/ml of [³⁵S]methionine (Amersham Corp., Arlington Hts., IL) in methionine-free DME-H21 supplemented with 10% normal growth medium or with 50 μ Ci/ml of [³H-6-D]glucosamine (New England Nuclear, Boston, MA) in normal growth medium. PC12 cells were also labeled metabolically in suspension with 100 μ Ci/ml of ³²P-orthophosphate (Amersham Corp.) in phosphate-free DME H-21 plus 10% normal growth medium and 50 μ M sodium vanadate for 3 h at 37°C with gentle rocking. Lactoperoxidase-catalyzed iodination of PC12, NRK, and BHK cells was carried out as previously described (Pesando et al., 1981).

Immunoprecipitations

For immunoprecipitations with the anti-ECMR serum, radiolabeled PC12 cells were washed three times in PBS and resuspended in PBS with 2 mg/ml hemoglobin at a concentration of ~5 \times 10⁶ cells/ml. 20 μ l of goat anti-ECMR serum or control goat serum were added to a 1-ml aliquot of a suspension of intact cells and the suspension was rocked for 2 h at 4°C (Giancotti et al., 1985). Cells were pelleted by centrifugation at 1,000 g for 5 min and the supernatants were removed. The cell pellets were extracted for 30 min on ice in Ca⁺⁺/Mg⁺⁺-containing PBS plus 100 mM *n*-octyl- β -D-glucopyranoside (octylglucoside) and 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mg/ml pepstatin A, and 1 mg/ml leupeptin. Detergent insoluble material was removed by centrifugation at 12,000 g for 15 min at 4°C. The cleared cell lysates were diluted with an equal volume of PBS to bring the concentration of octylglucoside to 50 mM and were adsorbed twice (2 h each at 4°C) with 100- μ l packed volume of Sepharose CL-4B that had been washed twice in PBS, plus 50 mM octylglucoside and 1 mM PMSF (wash buffer). Immune complexes were recovered from the supernatants by incubation for 30 min at 4°C with 50- μ l packed volume of protein A-Sepharose CL-4B that had been washed twice in wash buffer. Protein A-Sepharose beads were recovered and washed five times with wash buffer by centrifugation at 1,500 g for 1 min. Proteins were eluted by heating at 100°C for 5 min in SDS gel sample buffer (Laemmli, 1970), with or without 5% β -mercaptoethanol, and were frozen at -20°C until SDS gel analysis. Detergent extraction and immunoprecipitation of ³²P-labeled PC12 cell proteins with the anti-ECMR serum was carried out as described above except that the extraction, immunoprecipitation, and washes were done in PBS plus 0.5% Triton X-100, 0.3% sodium deoxycholate, 0.3% SDS, 1 mM PMSF, and 50 μ M sodium vanadate. For immunoprecipitations with rabbit anti-CSAT band 3, rabbit anti-N-CAM, rabbit anti-Ng-CAM, or normal rabbit serum, labeled cells were extracted with 100 mM octylglucoside (anti-CSAT band 3) or 1% Triton X-100 (anti-N-CAM and anti-Ng-CAM) before addition of 20 μ l of the antisera.

Immunoprecipitation of surface-iodinated PC12, NRK, and BHK cells with anti-FNR IgG was performed as described (Brown and Juliano, 1986). Briefly, labeled cells were extracted in 50 mM Tris HCl pH 7.5 with 150 mM NaCl, 1% Triton X-100, and 2 mM PMSF. Cell extracts were spun at 12,000 g for 15 min at 4°C, and the cleared cell lysates were adsorbed twice (2 h each at 4°C) with BSA coupled to Affi-gel 10 (100- μ l packed volume of beads; 10 mg BSA/ml of gel). Anti-FNR IgG or control goat IgG were coupled to Affi-gel 10 at a concentration of 6 mg IgG/ml of gel following manufacturer's instructions. 100 μ l of packed beads were added to precleared cell extracts and incubated for 4 h at 4°C. Antibody-conjugated beads were recovered and washed five times with cell extraction buffer plus 0.5% sodium deoxycholate and 0.1% SDS. Bound proteins were eluted as described above and stored at -20°C until use.

SDS-PAGE and Antigen Blots

Immunoprecipitates were analyzed on 7% polyacrylamide SDS gels (Laemmli, 1970) in the presence (reducing) or absence (non-reducing) of 5% β -mercaptoethanol. Coomassie Blue-stained and dried gels were exposed to Kodak XAR x-ray film at -80°C with intensifying screens (Dupont Lightning Plus, Wilmington, DE). The molecular mass standards used were myosin (200 kD), β -galactosidase (116 kD), phosphorylase a (97 kD), and BSA (68 kD).

For antigen blots using antibodies to purified band 3 of the avian CSAT/ECM receptor complex (anti-CSAT band 3), ~5 \times 10⁷ surface-labeled PC12 cells were incubated with 70 μ l of the anti-ECMR serum and then extracted for immunoprecipitation as described above. Immune complexes recovered with protein A-Sepharose were run in a single lane of a 7% SDS gel under nonreducing conditions and electrophoretically transferred to nitrocellulose paper by the method of Towbin (1979). The area of the nitrocellulose corresponding to the immunoprecipitated and transferred PC12 cell proteins was cut into two strips and incubated 3 h at 25°C with either the anti-CSAT band 3 serum or a control rabbit antiserum diluted 1:100 in 50 mM Tris HCl pH 7.5, 150 mM NaCl, and 5% nonfat dry milk (Carnation Co., Los Angeles, CA). Unbound antibody was removed by washing five times (5 min each) in 50 mM Tris HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS. Bound antibody was detected by incubating the nitrocellulose for 1 h at 25°C in goat anti-rabbit IgG coupled to horseradish peroxidase diluted 1:1,000 in 50 mM Tris HCl pH 7.5, 150 mM NaCl, and 5% nonfat dry milk (Carnation Co.). Unbound second antibody was removed by washing as described above, and the antigen blot was developed using H₂O₂ and 4-chloronaphthol as described by Hawkes et al. (1982). The dried antigen blot was then subjected to autoradiography to visualize the PC12 cell proteins originally immunoprecipitated by the anti-ECMR serum.

Microscopy

Cultured cells were fixed in 2.5% glutaraldehyde in PBS plus 5% sucrose for 1 h at 25°C, washed twice with PBS, and then viewed and photographed using a 40 \times water immersion lens (na = 0.70) on a Zeiss IM microscope using phase contrast optics.

Results

Characterization of PC12 Cell Interactions with Purified ECM Proteins

The ECM proteins LN and FN stimulate adhesion and process outgrowth by many types of neurons (Manthorpe et al., 1983; Rogers et al., 1983, 1985; Hall et al., 1987). The ability of the neuronlike PC12 cell line to extend neurites on substrates coated with LN and FN was compared with the response of neonatal rat sympathetic neurons on these same substrates. To study neurite extension, PC12 cells were first differentiated by growth in 50 ng/ml NGF for 10 d. These "NGF-primed" PC12 cells (Greene, 1977) were then passaged onto substrates coated with either poly-D-lysine, LN, or FN and cultured for 24 h in the absence of added NGF. Under these conditions, <10% of individual cells or small clumps of cells regenerated neurites on poly-D-lysine or FN,

Table I. Percentage of Cells with Neurites

Cells	Poly-D-lysine	LN	FN
NGF-primed PC12 cells	7 ± 1	44 ± 2	9 ± 3
Rat sympathetic neurons	1 ± 1	55 ± 4	6 ± 1

Process outgrowth from NGF-primed PC12 cells and neonatal rat sympathetic neurons on substrates coated with poly-D-lysine, LN, or FN in the absence of NGF. NGF-primed PC12 cells or dissociated rat sympathetic neurons were cultured 24 and 16 h, respectively, on substrates coated with poly-D-lysine (1 mg/ml), LN (10 µg/ml), or FN (20 µg/ml). Fixed cells were scored for the percentage of individual cells (or small clumps of <10 cells in the case of PC12 cells) with a process >2-cell-diameter long. Values represent the average and range of determinations made on duplicate cultures run in parallel. At least 200 cells or clumps of cells were counted for each value.

compared with 44% on LN (Table I; Fig. 1, A-C). Dissociated sympathetic neurons showed a similar response to these substrates. In the absence of added NGF, <10% of the neurons extended processes in 16 h on either poly-D-lysine or FN, while LN substrates supported process outgrowth from 55% of the neurons (Table I; Figure 1, D-F). In contrast to previous studies using PC12 cells or sympathetic neurons (see Akesson and Warren, 1986; Rogers et al., 1983, 1985), process outgrowth was studied here in the absence of added NGF, since NGF itself has neurite outgrowth-promoting activity. Based on the apparent similarity between PC12 cells and sympathetic neurons with respect to process outgrowth on these adhesive substrates, experiments were done to characterize PC12 cell interactions with LN and other ECM constituents in more detail.

A quantitative assay was used to study attachment of undifferentiated PC12 cells to substrates coated with adhesive proteins. Using this assay, PC12 cells were shown to attach readily to poly-D-lysine, LN, or Col IV (Table II). By comparison, PC12 cells attached poorly to substrates coated with FN and failed to attach appreciably to substrates coated with BSA (Table II). Attachment of PC12 cells to LN or Col

Table II. PC12 Cell Attachment

Substrate	Attachment: percentage of positive control
Poly-D-lysine	100
BSA	3 ± 0.5
Laminin	100 ± 4
Col IV	106 ± 21
FN	19 ± 2

PC12 cell attachment to substrates coated with poly-D-lysine (1 mg/ml), BSA (1 mg/ml), laminin (5 µg/ml), Col IV (2 µg/ml), or FN (20 µg/ml). Each value represents the mean and standard deviation of determinations made on triplicate cultures run in parallel and is expressed as the percentage of attachment relative to the positive control, poly-D-lysine.

IV was accompanied by spreading and flattening of a large percentage of the cells within 2 h (Fig. 2, A and B). On FN or poly-D-lysine, PC12 cells did not spread or flatten, but remained round and phase bright (Fig. 2, C and D).

PC12 cell attachment to LN and Col IV was concentration dependent (Fig. 3). Half-maximal attachment to LN or Col IV was attained at substrate-coating concentrations of ~200 and 60 ng/ml for LN and Col IV, respectively (Fig. 3). Since Col IV is adsorbed to the plastic plates used in this assay about threefold more effectively than LN (Hall et al., 1987), Col IV and LN appear to be equally effective on a weight basis in promoting PC12 cell attachment. Although only a small percentage of PC12 cells attached to FN, attachment was concentration dependent, with half-maximal attachment occurring at ~1 µg/ml of FN (Fig. 3).

The specificity of PC12 cell attachment to LN and Col IV was demonstrated using specific antibodies. Attachment to LN was largely inhibited by antisera either to intact LN or to the E3 fragment of LN (Fig. 4 a), but was not affected by preimmune serum or by affinity-purified antibodies to murine Col IV (Fig. 4 a). Previous studies have demonstrated that the interactions of both sympathetic neurons and adrenal

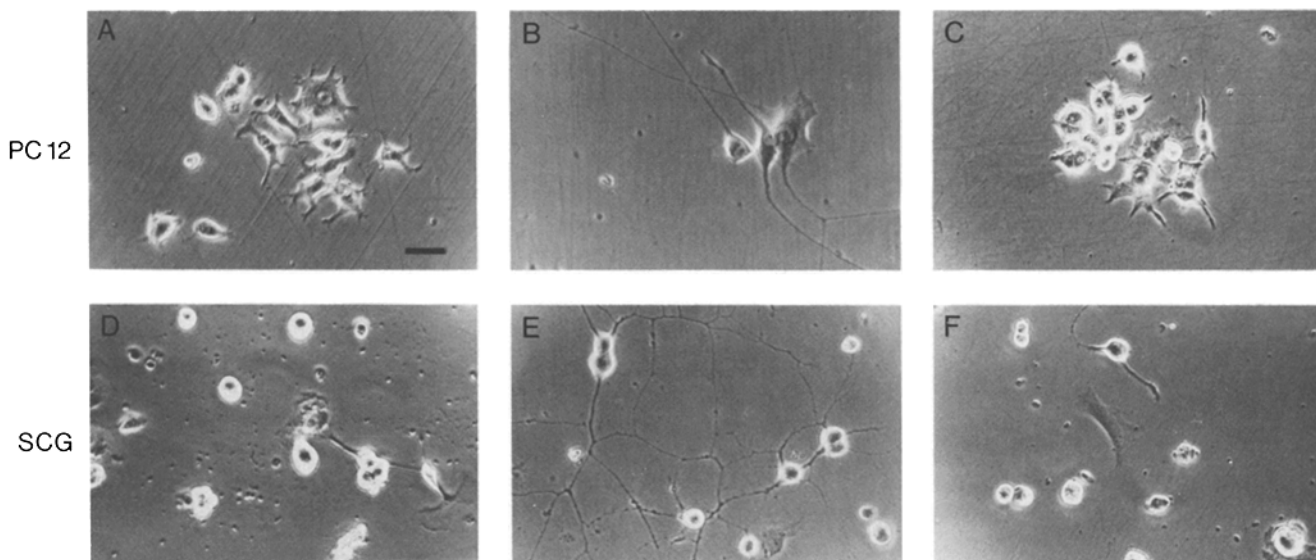


Figure 1. Process outgrowth by NGF-primed PC12 cells or dissociated neonatal rat sympathetic neurons (SCG) in the absence of NGF. NGF-primed PC12 cells were passaged and grown 24 h in the absence of added NGF on substrates coated with 1 mg/ml poly-D-lysine (A), 10 µg/ml LN (B), or 20 µg/ml FN (C). Rat sympathetic neurons were cultured 16 h in the absence of added NGF on substrates coated with 1 mg/ml poly-D-lysine (D), 10 µg/ml LN (E), or 20 µg/ml FN (F). Bar, 10 µm.

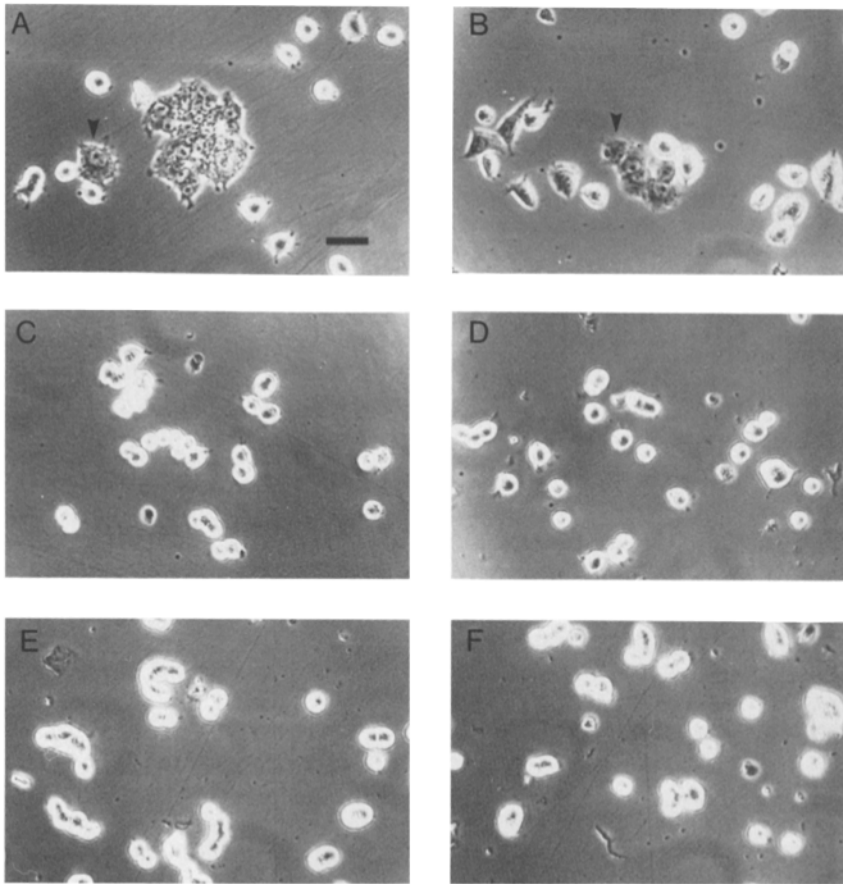


Figure 2. PC12 cells spread on LN or Col IV but not on FN or poly-D-lysine. PC12 cells were passaged with PBS/5 mM EDTA and grown in serum-free medium with 1 mg/ml BSA for 2 h on (A) LN (10 μ g/ml), (B) Col IV (5 μ g/ml), (C) FN (100 μ g/ml), or (D) poly-D-lysine (1 mg/ml). Arrowheads in A and B mark phase-dark cells that have spread on LN and Col IV, respectively. Cells on FN or poly-D-lysine (C and D) do not spread appreciably as indicated by their round, phase-bright appearance. (E and F) The anti-ECMR serum causes detachment of PC12 cells that have spread on LN or Col IV. After PC12 cells were allowed to attach and spread for 5 h in serum-containing medium on LN (E) or Col IV (F), the anti-ECMR serum was added to a final concentration of 1%. Cells rounded up within 1 h after addition of the anti-ECMR serum. Normal goat serum had no effect on cell morphology (not shown). Bar, 10 μ m.

chromaffin cells with intact LN are inhibited by antibodies to the E3 fragment of LN (Edgar et al., 1984; Acheson et al., 1986). Our results extend these observations to PC12 cells and suggest a common specificity with which PC12 cells and sympathetic neurons interact with LN. PC12 cell attachment to Col IV was inhibited by affinity-purified anti-Col IV antibodies but was largely unaffected by anti-LN or anti-LN fragment E3 sera (Fig. 4 a).

PC12 cell attachment to LN, Col IV, and FN was studied in the presence of a hexapeptide (GRGDSP), derived from a cell binding domain of FN, and a control hexapeptide (GRGESP) which lacks blocking activity (Pierschbacher and Ruoslahti, 1984a, b). In separate experiments, the GRGDSP peptide, but not GRGESP, inhibited the attachment of BHK fibroblastic cells and primary rat muscle-derived fibroblasts to FN substrates (unpublished observations). The RGD-containing hexapeptide inhibited the small percentage of PC12 cells that attached to FN-coated substrates, while the RGE-containing hexapeptide had no detectable blocking activity (Fig. 4 b). Neither peptide had noticeable effects on PC12 cell attachment to LN or Col IV (Fig. 4 b).

Effects of Antisera to Purified Mammalian ECM Receptor Preparations on PC12 Cell Attachment to ECM Proteins

Two different antisera to mammalian cell surface proteins involved in adhesion to ECM proteins were tested for their effects on PC12 cell attachment to LN, Col IV, and FN. The anti-ECMR serum was prepared against a purified prepara-

tion of adhesion-related 140-kD BHK cell surface glycoproteins and induces the detachment of a wide variety of adherent murine and rat cell types when added to their culture medium at a concentration of 1% (Knudsen et al., 1981;

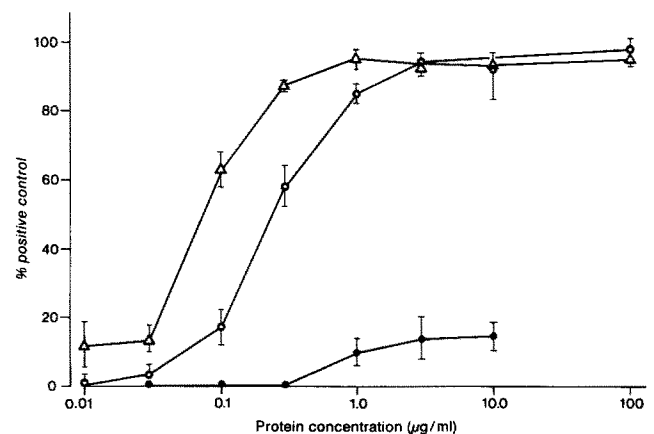


Figure 3. Concentration-dependent attachment of PC12 cells to LN, Col IV, and FN. [35 S]Methionine-labeled PC12 cells were allowed to attach for 90 min at 37°C to substrates coated with varying concentrations of LN (open circles), Col IV (open triangles), or FN (solid circles). After removal of nonadherent cells, attached cells were solubilized in 1% SDS and counted. Each value represents the mean and standard deviation of determinations made on triplicate cultures run in parallel and is expressed as percent of attachment to the positive control, poly-D-lysine.

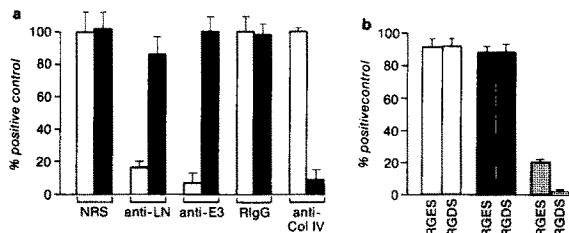


Figure 4. Specificity of PC12 cell attachment to LN, FN, and Col IV. (A) PC12 cell attachment to substrates coated with LN (1 $\mu\text{g}/\text{ml}$; open bars) or Col IV (0.5 $\mu\text{g}/\text{ml}$; solid bars) was studied in the presence of preimmune rabbit serum (NRS; 1:20 dilution), a rabbit anti-LN serum (1:20 dilution), a rabbit antiserum to the E3 fragment of LN (anti-E3; 1:20 dilution), normal rabbit IgG (RlgG; 50 $\mu\text{g}/\text{ml}$), or affinity-purified rabbit anti-Col IV IgG (50 $\mu\text{g}/\text{ml}$). Attachment is expressed as percent of the positive control, poly-D-lysine. The mean and standard deviation of triplicate points is shown for each condition. (B) Effects of RGD-containing hexapeptides on PC12 cell attachment. Cells were allowed to attach to LN (10 $\mu\text{g}/\text{ml}$; open bars), Col IV (2 $\mu\text{g}/\text{ml}$; solid bars), or FN (20 $\mu\text{g}/\text{ml}$; stippled bars) in the presence of 2 mg/ml of the control hexapeptide, GRGESP (RGES), or in the presence of 2 mg/ml of the hexapeptide GRGDSP (RGDS). Values represent the mean and standard deviation of determinations made on triplicate cultures run in parallel and are expressed as percent of the positive control, poly-D-lysine. Attachment to LN, Col IV, or FN in the presence of GRGESP was not significantly different from attachment in the absence of added peptides.

Damsky et al., 1982). A 1% concentration of the anti-ECMR serum largely prevented PC12 cell attachment to LN, Col IV, and FN, while the same concentration of the control serum did not (Fig. 5 A). F_{ab} fragments of anti-ECMR IgG also inhibited attachment to LN, Col IV, and FN by >90% (Fig. 5 A), and this inhibition was concentration dependent (not shown). The effects of the anti-ECMR serum were specific for ECM-derived substrates: attachment to poly-D-lysine or to the surfaces of a mouse muscle cell line (C2) was not affected by concentrations of serum fourfold higher than those needed to effectively inhibit attachment to LN, Col IV, or FN (Fig. 5 A). In addition to preventing the initial adhesion of PC12 cells to LN or Col IV, the anti-ECMR serum caused a rapid detachment of cells that had already spread on LN or Col IV (Fig. 2 E and F).

Antibodies to a purified FN receptor heterodimer maximally inhibit CHO cell attachment to FN at a concentration of 10 mg/ml (Brown and Juliano, 1986). This high concentration of anti-FNR IgG specifically inhibited PC12 cell attachment to FN and LN without noticeably affecting attachment to either Col IV or poly-D-lysine (Fig. 5 B). Attachment to FN was also inhibited ~90% by concentrations of the anti-FNR IgG (1 mg/ml) that had little effect on attachment to LN (Fig. 5 B). Anti-FNR IgG inhibition of PC12 cell attachment to LN and FN was also concentration dependent (not shown).

Effects of the Anti-ECMR Serum on Process Outgrowth by PC12 Cells and Sympathetic Neurons

To study the effects of the anti-ECMR serum on PC12 cell process outgrowth, NGF-primed PC12 cells were passaged onto LN-coated substrates in the presence of the anti-ECMR serum or a control serum. Cells passaged onto LN substrates

in the continued presence of 50 ng/ml NGF extended neurites rapidly and profusely (Fig. 6 A). Process outgrowth on LN in the presence of NGF was more pronounced than that seen on LN in the absence of NGF illustrated in Fig. 1 B. This response was completely inhibited by a 1% concentration of the anti-ECMR serum (Fig. 6 B). When dose dependence was examined, half-maximal inhibition of process outgrowth occurred at a 0.2% concentration of the anti-ECMR serum (not shown), and appeared to be primarily a result of inhibiting cell attachment, spreading, and filopodial extension on the LN substrate (arrowhead in Fig. 6 B). Process outgrowth by NGF-primed PC12 cells on poly-D-lysine substrates in the presence of 50 ng/ml NGF was modest, though clearly less than on LN substrates (compare Fig. 6, A and C). A 2% concentration of the anti-EMCR serum completely inhibited NGF-stimulated process outgrowth on poly-D-lysine substrates (Fig. 6 D), while the same concentration of the control serum had no effect (Fig. 6 C). PC12 cell attachment to poly-D-lysine was not affected by the anti-ECMR serum, however, as is apparent from the presence of microspikes in contact with the substrate (arrowheads in Fig. 6 D). The effects of the anti-ECMR serum on process outgrowth on either LN or poly-D-lysine substrates appeared to be reversible after removal of the antibody (Fig. 6 E and F).

Sympathetic neuronal process outgrowth on LN substrates was also sensitive to the anti-ECMR serum. Neurons cul-

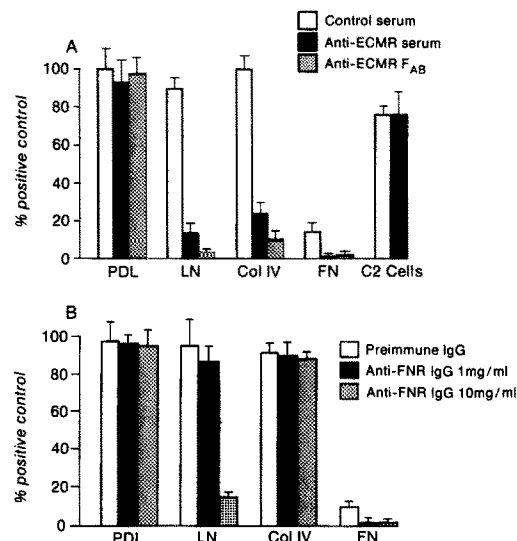


Figure 5. Effects of anti-ECMR and anti-FNR on PC12 cell attachment to adhesive substrates. Substrates were coated with either poly-D-lysine (PDL; 1 mg/ml), LN (3 $\mu\text{g}/\text{ml}$), Col IV (2 $\mu\text{g}/\text{ml}$), FN (20 $\mu\text{g}/\mu\text{l}$), or C2 cells (1,000 cells/mm²). (A) PC12 cells were allowed to attach to various substrates in the presence of normal goat serum (1% concentration; 4% concentration on poly-D-lysine and on C2 cells; open bars), the anti-ECMR serum (1% concentration; 4% concentration on poly-D-lysine and on C2 cells; solid bars), or anti-ECMR F_{ab} (1 mg/ml; stippled bars). (B) PC12 cells were allowed to attach to various substrates in the presence of preimmune goat IgG (open bars; 10 mg/ml on poly-D-lysine, LN, and Col IV; 1 mg/ml on FN), or anti-FNR IgG (solid bars: 1 mg/ml; stippled bars: 10 mg/ml). Values represent the mean and standard deviation of determinations made on triplicate cultures run in parallel and are expressed as percent of the positive control, poly-D-lysine, in the absence of added antibodies.

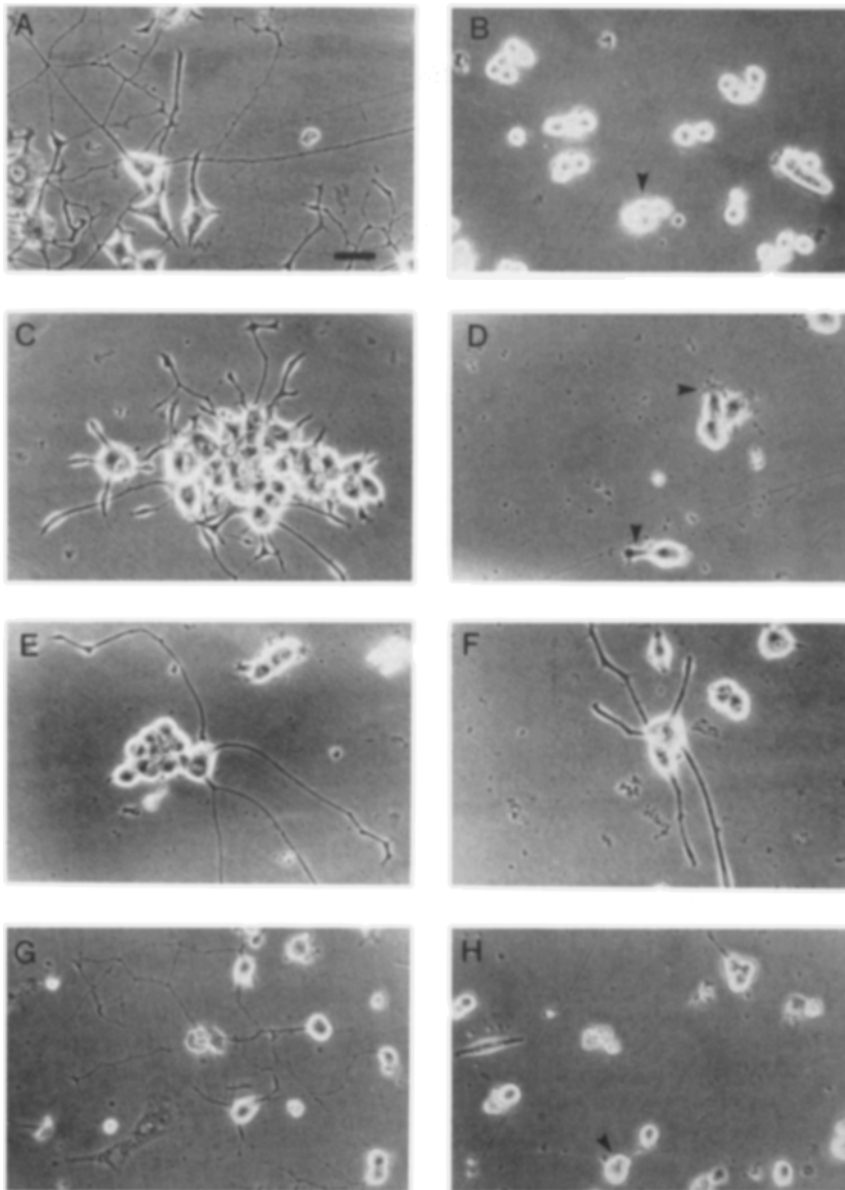


Figure 6. Effects of the anti-ECMR serum on process outgrowth by NGF-primed PC12 cells or neonatal rat sympathetic neurons. PC12 cells that were primed for 10 d in 50 ng/ml NGF were passaged in the continued presence of 50 ng/ml NGF onto substrates coated with LN (10 μ g/ml; A, B, and E) or poly-D-lysine (1 mg/ml; C, D, and F) and grown 24 h in the presence of normal goat serum (2% concentration; A and C) or goat anti-ECMR serum (1% in B; 2% in D). Arrowhead in B denotes a clump of cells that are not well attached or spread and have failed to extend neurites on the LN substrate in the presence of the anti-ECMR serum. Arrowheads in D denote cells that are apparently well attached to the poly-D-lysine substrate but lack neurites in the presence of the anti-ECMR serum. Note NGF-stimulated process outgrowth is more pronounced on LN than on poly-D-lysine (compare A and C). PC12 cells recovered the ability to grow neurites when cultured in the continued presence of 50 ng/ml NGF an additional 24 h on LN (E) or poly-D-lysine (F) after removal of the anti-ECMR serum. G and H: Effects of anti-ECMR serum on sympathetic neuronal process outgrowth. Neonatal rat sympathetic neurons were grown 12 h on mixed LN/poly-D-lysine substrates in the presence of control goat serum (0.6% concentration; G) or the anti-ECMR serum (0.3% concentration, H). Arrowhead in H marks one of several neuronal cell bodies that has been prevented from growing neurites on LN in the presence of the anti-ECMR serum. Bar, 10 μ m.

tured on a mixed substrate of LN and poly-D-lysine in the presence of control serum exhibited profuse process outgrowth (Fig. 6 G). This response was completely prevented by the anti-ECMR serum (Fig. 6 H) and when dose dependence was examined, half-maximal inhibition was achieved at a 0.04% concentration of the anti-ECMR serum (not shown). The anti-ECMR serum appeared not to affect neuronal adhesion to the mixed LN/poly-D-lysine substrates (Fig. 6 H). However, when LN was used to coat a substrate to which neurons did not readily adhere, namely glass coverslips, neuronal attachment to LN was inhibited \sim 80% by the anti-ECMR serum (not shown).

Characterization of PC12 Cell Proteins Recognized by the Anti-ECMR and Anti-FNR sera

PC12 cell proteins recognized by anti-ECMR and anti-FNR antibodies were identified by immunoprecipitation of radio-labeled cells followed by SDS-PAGE analysis (Fig. 7). When

octylglucoside extracts of surface-labeled PC12 cells were immunoprecipitated with the anti-ECMR serum, three prominently labeled protein bands were identified. Under non-reducing conditions these proteins migrated as diffuse bands with apparent molecular masses centered at 120, 140, and 180 kD (Fig. 7, lane 2). Long autoradiographic exposures of some of these immunoprecipitates revealed the presence of a very faintly labeled protein band at \sim 60 kD (Fig. 7 A, lane 3). A faint protein band at \sim 170 kD under nonreducing conditions was nonspecifically immunoprecipitated in equal amounts by the anti-ECMR serum and a control goat serum (Fig. 7 A, lanes 1 and 2). Under reducing conditions, the mobility of this nonspecific protein band appeared to shift from 170 to \sim 40 kD (Fig. 7 A, compare lanes 1 and 4). When the proteins bound by the anti-ECMR serum were separated under reducing conditions, the 120- and 140-kD bands appeared to merge into a diffuse band centered at \sim 130 kD and the faint 60-kD protein shifted to \sim 75 kD (Fig. 7 A, lane 5).

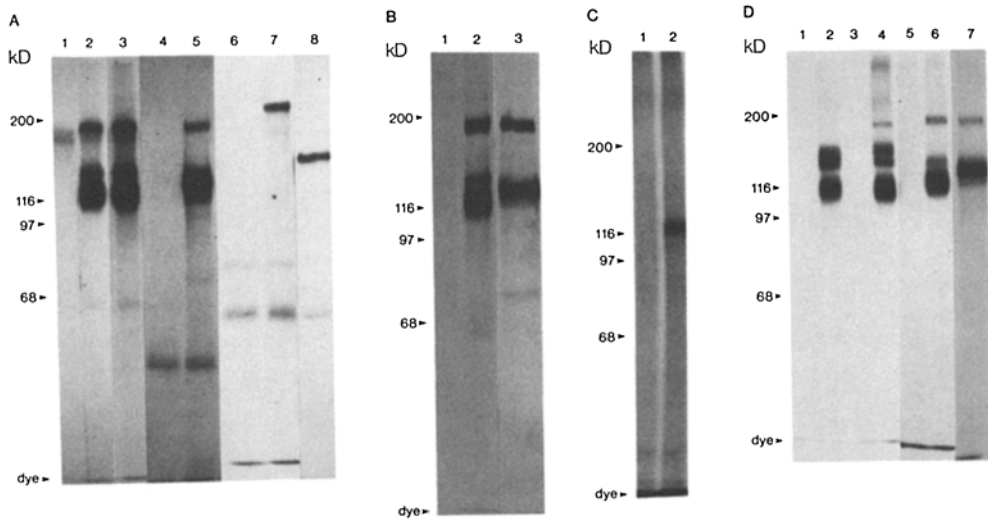


Figure 7. Immunoprecipitation and 7% SDS-PAGE analysis of surface-iodinated (A and D), [^3H]glucosamine-labeled (B), or [^{32}P]labeled (C) proteins with the anti-ECMR serum (A-C) or the anti-FNR IgG (D). (A) Surface-labeled PC12 cells were immunoprecipitated with control goat serum (lane 1, nonreduced; lane 4, reduced), anti-ECMR serum (lanes 2 and 3, nonreduced; lane 5, reduced; lane 3 is an overexposure of lane 2 to facilitate visualization of a faintly labeled 60-kD protein), preimmune rabbit serum (lane 6, reduced), anti-Ng-CAM (lane 7, reduced) or anti-N-

CAM (lane 8, reduced). (B) [^3H]glucosamine-labeled PC12 cells were immunoprecipitated with control goat serum (lane 1, nonreduced) or anti-ECMR serum (lane 2, nonreduced; lane 3, reduced). (C) [^{32}P]labeled PC12 cell proteins were immunoprecipitated with control goat serum (lane 1, nonreduced) or anti-ECMR serum (lane 2, nonreduced). (D) Immunoprecipitation of surface-labeled BHK (lanes 1 and 2), NRK (lanes 3 and 4), or PC12 (lanes 5-7) cells with preimmune IgG (lanes 1, 3, and 5, nonreduced) or anti-FNR IgG (lanes 2, 4, and 6, nonreduced; lane 7, reduced). Molecular mass markers indicated are myosin (200 kD), β -galactosidase (116 kD), phosphorylase a (97 kD), and BSA (68 kD).

Merging of the 120- and 140-kD bands upon reduction appeared to be the result of a slight increase in the mobility of the 140-kD protein and a concomitant decrease in the mobility of the 120-kD protein. Identical proteins were immunoprecipitated by the anti-ECMR serum from extracts of labeled PC12 cells prepared with 1% Triton X-100 (not shown). The proteins recognized by the anti-ECMR serum were distinct from cell-cell adhesion molecules recognized by anti-N-CAM or anti-NILE (anti-Ng-CAM) antibodies (Fig. 7 A, lanes 6-8).

To determine if proteins recognized by the anti-ECMR serum are glycoproteins synthesized by PC12 cells, [^3H]glucosamine-labeled cells were immunoprecipitated as above (Fig. 7 B). The anti-ECMR specifically recognized three prominently labeled glycoprotein bands of 120, 140, and 180 kD and a weakly labeled glycoprotein of 60 kD under nonreducing conditions (Fig. 7 B, lane 2). Under reducing conditions, the 120- and 140-kD bands appeared to migrate as a single diffuse band at \sim 130 kD and the 60 kD protein shifted to \sim 75 kD (Fig. 7 B, lane 3). The iodinated 170-kD protein that was nonspecifically immunoprecipitated by the control goat serum (Fig. 7 A, lane 1) was unlabeled by [^3H]glucosamine (Fig. 7 B, lane 1) and, therefore, may be a serum protein adsorbed to PC12 cell surfaces.

To determine if any of the PC12 cell glycoproteins recognized by the anti-ECMR serum are phosphoproteins, [^{32}P]labeled PC12 cells were immunoprecipitated with the anti-ECMR serum or with control goat serum. A single labeled protein migrating at \sim 120 kD under nonreducing conditions was specifically immunoprecipitated by the anti-ECMR serum (Fig. 7 C, lane 2). Based on its ability to label with [^{32}S], [^{32}P], and [^3H]glucosamine, the 120-kD glycoprotein is likely to be an integral membrane glycoprotein with exposed cytoplasmic and extracellular domains.

Proteins recognized by the anti-FNR IgG were identified in 1% Triton X-100 extracts of surface-labeled BHK, NRK,

and PC12 cells using anti-FNR IgG coupled to Affi-gel 10 (Fig. 7 D). Immunoprecipitation of a control hamster cell line, BHK, with anti-FNR IgG yielded two diffuse protein bands of M_r 110 and 130 kD under nonreducing conditions (Fig. 7 D, lane 2). These two proteins are probably identical to proteins recognized by this antiserum on CHO cells (Brown and Juliano, 1986) and constitute an FN receptor heterodimer (Brown and Juliano, 1985, 1986; see also Pytela et al., 1985). Immunoprecipitation of surface-labeled PC12 cell extracts with the anti-FNR IgG identified three labeled PC12 cell protein bands migrating at 120, 140, and 180 kD under nonreducing conditions (Fig. 7 D, lane 6). Upon reduction of disulfide bonds, the 120- and 140-kD bands appeared to migrate as a single diffuse band centered at 130 kD (Fig. 7 D, lane 7). The PC12 cell proteins recognized by the anti-FNR IgG comigrated with those recognized by the anti-ECMR serum under both reducing and nonreducing conditions. Immunoprecipitation of another rat cell line, NRK, with the anti-FNR IgG identified predominantly four labeled proteins (Fig. 7 D, lane 4). Three of these appeared to correspond to the 120-, 140-, and 180-kD proteins in PC12 cells. The fourth, a diffuse band centered at \sim 160 kD under nonreducing conditions, was absent from PC12 cell extracts (Fig. 7 D, compare lanes 4 and 6).

The 120-kD PC12 Cell Glycoprotein Is Related to the β -Subunit (Band 3) of the CSAT/Integrin ECM Receptor Complex

To determine if any of the PC12 cell proteins recognized by the anti-FNR or anti-ECMR sera are related to characterized components of the avian CSAT/integrin ECM receptor complex, surface-iodinated PC12 cells were extracted in octylglucoside and immunoprecipitated with the anti-ECMR serum. Bound proteins were separated on an SDS gel under nonreducing conditions, transferred electrophoretically to nitrocellulose paper, and overlaid with an antiserum to the

purified β_1 subunit (band 3) of the avian ECM receptor complex recognized by the CSAT mAb (Buck et al., 1986). The CSAT mAb itself could not be used in this protocol since it does not react with SDS-denatured proteins and does not cross react with rat proteins. Autoradiography of the nitrocellulose revealed proteins of 120, 140, and 180 kD that were immunoprecipitated by the anti-ECMR serum (Fig. 8 A, lanes 1 and 2). Specific binding of the anti-CSAT band 3 serum to proteins immunoprecipitated by the anti-ECMR serum, monitored with a horseradish peroxidase-conjugated secondary antibody, was confined to a single protein band centered at 120 kD (Fig. 8 A, lanes 3 and 4). Thus, the 120-kD glycoprotein recognized by the anti-ECMR serum cross reacts with antibodies to band 3 of the avian CSAT/integrin ECM receptor complex.

When the anti-CSAT band 3 serum was used to immunoprecipitate proteins from octylglucoside extracts of surface-labeled PC12 cells, the 140- and 180-kD glycoproteins were brought down in addition to the 120-kD glycoprotein (Fig. 8 B, lanes 2 and 4). The mobilities of the 120- and 140-kD proteins under nonreducing conditions were distorted due to the presence of nonreduced rabbit IgG running at \sim 120–150 kD. An additional protein at 95 kD under nonreducing conditions was also recognized by this serum (Fig. 8 B, lane 2). Under reducing conditions, the 120- and 140-kD protein bands appeared to merge into a single diffuse band centered at \sim 130 kD and the 180-kD protein was clearly visible (Fig. 8 B, lane 4). These observations, coupled with the fact that the anti-CSAT band 3 serum recognized only the 120-kD glycoprotein in antigen blots (Fig. 8 A, lane 3), suggest that the 140- and 180-kD proteins are noncovalently associated with the 120-kD protein in detergent extracts of PC12 cells.

Discussion

Studies aimed at the molecular characterization of neuronal interactions with ECM proteins are made difficult by the need for pure neuronal preparations in quantities suitable for cell biological and biochemical analysis. Some of these difficulties can be circumvented by studying cell lines with neuronal characteristics. Observations presented here show that the interactions of the PC12 cell line with LN and other ECM proteins approximate those of neurons. Firstly, rapid, NGF-independent process outgrowth by NGF-primed PC12 cells in response to LN and FN substrates is similar to the response of sympathetic neurons on these substrates (cf. Table I and Fig. 1). Secondly, PC12 cells and sympathetic neurons both interact selectively with distinct structural domains of LN and FN, suggesting that they share receptors with common specificities. In the case of LN, both cell types interact with a domain at or near the end of the long arm of the cruciform LN molecule (cf. Fig. 4 A; see also Edgar et al., 1983; Acheson et al., 1986; Engvall et al., 1986). Cell surface receptors for LN on PC12 cells and sympathetic neurons may be structurally homologous, since the anti-ECMR serum inhibits attachment and process outgrowth of both cell types on LN substrates (cf. Fig. 6). In the case of FN, PC12 cells and sympathetic neurons appear to interact with a domain that contains the sequence RGDS (Fig. 4 B; see also Rogers et al., 1985). Finally, PC12 cell attachment to the surfaces of a mouse muscle cell line, C2, is not inhibited by the anti-ECMR serum (cf. Fig. 5). Although C2 cells make LN

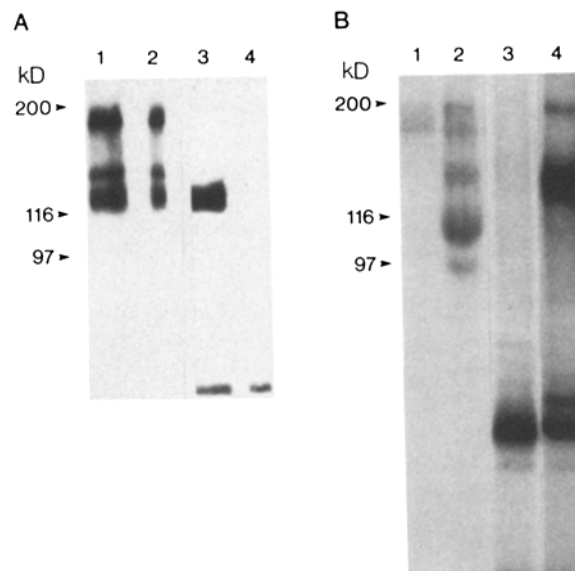


Figure 8. (A) Relationship of the 120-kD PC12 cell surface glycoprotein recognized by the anti-ECMR serum to the integrin β_1 -subunit (band 3) of the CSAT/ECM receptor complex. Surface-iodinated PC12 cell proteins were immunoprecipitated with the anti-ECMR serum, separated on a 7% polyacrylamide gel under nonreducing conditions, and transferred electrophoretically to nitrocellulose paper. The nitrocellulose was cut into two unequal strips and overlaid with either the anti-CSAT band 3-specific serum or a control rabbit serum. Lanes 1 and 2: Autoradiograms of nitrocellulose strips showing iodinated PC12 cell proteins immunoprecipitated by the anti-ECMR serum. Lane 3: Horseradish peroxidase-reaction product identifying anti-CSAT band 3 antibodies bound to nitrocellulose strip shown in lane 1. Lane 4: Horseradish peroxidase-reaction product identifying control rabbit serum antibodies bound to the nitrocellulose strip shown in lane 2. Note that the anti-CSAT band 3 antibodies specifically recognize only the labeled 120-kD protein bound by the anti-ECMR serum. A second unlabeled protein band is recognized by both the anti-CSAT band 3 and normal rabbit serum (lanes 3 and 4). (B) Immunoprecipitation of detergent extracts of surface-iodinated PC12 cells with the anti-CSAT band 3 serum. Lanes 1 and 3: Proteins immunoprecipitated by control rabbit serum analyzed under nonreducing (lane 1) or reducing (lane 3) conditions. Lanes 2 and 4: PC12 cell proteins immunoprecipitated with anti-CSAT band 3 serum analyzed under nonreducing (lane 2) or reducing (lane 4) conditions. In lane 2, the juxtaposition of the 120- and 140-kD proteins is distorted due to the presence of nonreduced anti-CSAT band 3 IgG migrating at \sim 120–150 kD. Molecular mass markers indicated are myosin (200 kD), β -galactosidase (116 kD), phosphorylase a (97 kD), and BSA (68 kD).

and can express it on their surfaces (Olwin and Hall, 1985), other proteins, most notably cell-cell adhesion molecules, are likely to contribute to PC12 cell attachment to C2 cell surfaces. In these types of interactions, PC12 cells may also behave like neurons, whose interactions with a variety of nonneuronal cells (e.g., Schwann cells, myotubes, and astrocytes) are mediated by a combination of both ECM receptors and cell-cell adhesion molecules (Tomaselli et al., 1986a; Bixby et al., 1987).

The ability of F_{ab} fragments of anti-ECMR IgG to inhibit attachment of PC12 cells to LN, FN, and Col IV suggests that PC12 cell surface proteins recognized by this antiserum are

involved either directly or indirectly in attachment to LN, Col IV, and FN. Two lines of evidence suggest that the anti-ECMR serum acts directly to inhibit receptors for ECM proteins: (a) We have used the anti-ECMR serum to purify the 120-, 140-, and 180-kD glycoproteins from PC12 cells. When preincubated with the anti-ECMR serum, these purified proteins completely neutralize the adhesion-blocking activity of this serum, demonstrating that all of the adhesion-blocking activity can be accounted for by proteins that are immunoprecipitated by the anti-ECMR serum. (Tomaselli, K., C. H. Damsky, and L. F. Reichardt, manuscript in preparation). (b) The proteins immunoprecipitated from PC12 cells by the anti-ECMR serum bear a strong resemblance to receptors for ECM proteins purified from other sources, in particular to glycoproteins of ~110, 140, and 160 kD immunoprecipitated from avian myoblasts, fibroblasts, and retinal neurons by the CSAT and JG22 antibodies (Chapman, 1984; Knudsen et al., 1985; Horwitz et al., 1985, 1986; Hasegawa et al., 1985; Akiyama et al., 1986; Bozyczko and Horwitz, 1986; Hall et al., 1987). The ECM receptor proteins recognized by the CSAT and JG22 antibodies on avian cells belong to the integrin family of adhesive protein receptors. Members of this receptor family are characterized by a heterodimeric structure in which different α -subunits are noncovalently complexed to a common β -subunit (Pytela et al., 1986; Kishimoto et al., 1987; Hemler et al., 1987; reviewed in Hynes, 1987). Sequence homology of cDNA clones encoding the CSAT band 3 glycoprotein with cDNAs encoding a human FN receptor β -subunit and the β -subunit of the leukocyte adhesion receptors has established that the 110-kD CSAT band 3 glycoprotein is a β_1 -subunit of the integrin family (Tamkun et al., 1986; Kishimoto et al., 1987; Hynes, 1987). A functional association between band 3 and one or more putative α -subunits (either the 140- [band 2] or 160-kD [band 1] proteins) is required for LN and FN binding (Buck et al., 1986). The CSAT mAb, which binds to band 3 in equilibrium gel filtration experiments (Buck et al., 1986), inhibits cell attachment to LN, FN, and Col IV (Horwitz et al., 1985; Hall et al., 1987).

By analogy to the ECM receptor proteins recognized by the CSAT antibody on avian cells, the PC12 cell glycoproteins described here and identified by the anti-ECMR and anti-FNR sera appear to belong to the integrin family of adhesive protein receptors. The 120-kD glycoprotein appears to be a β_1 -like subunit since it is recognized by antibodies to avian integrin β_1 (anti-CSAT band 3; cf. Fig. 8 A). Also, like CSAT-band 3, the 120-kD PC12 cell protein is a phosphoprotein (cf. Fig. 7 C; Hirst et al., 1986). The 140- and 180-kD glycoproteins may correspond to α -subunits that are noncovalently associated with the 120-kD β_1 -subunit, since antibodies that recognize only the 120-kD glycoprotein band (anti-CSAT band 3) coprecipitate the 140- and 180-kD proteins (cf. Fig. 8 B). In a similar fashion, antibodies directed against the common 130-kD β_1 -subunit of the human T cell VLA protein heterodimers coprecipitate five different α -subunits in the 150–200-kD range (Hemler et al., 1987). Some of the very late activation (VLA) protein heterodimers have recently been shown to cross react with polyclonal antibodies to the avian CSAT/integrin complex and to be involved in cell attachment to LN and FN (Takada et al., 1987). Although initially discovered and characterized on human T cells (Hemler et al., 1985, 1987), the VLA protein

heterodimers are also present on human neuronal cell lines (Pischel et al., 1986) where they may subserve neuronal adhesion to ECM proteins. Although the observations presented above suggest that the PC12 cell proteins described here function as receptor heterodimers for ECM proteins, definitive statements concerning structure and ligand binding await experiments with the purified receptor proteins.

PC12 cells attach and spread poorly on FN in comparison with LN and Col IV. One possible explanation for this observation is that PC12 cells express a low level of FN receptors on their surface relative to other cell types that adhere well to FN (e.g., fibroblasts). This is suggested by the fact that anti-FNR IgG inhibits PC12 cell attachment to FN at concentrations (1 mg/ml) that have little effect on CHO cell attachment to FN (see Brown and Juliano, 1986). Since mammalian FN receptors are α/β heterodimers (Pytela et al., 1985; Patel and Lodish, 1986; Brown and Juliano, 1986), absence or low level expression of either the α - or β -subunit could account for the low level of PC12 cell attachment to FN. The rat fibroblastic cell line, NRK, attaches well to FN, in addition to LN and Col IV (Tomaselli, K., unpublished observations). Immunoprecipitation of surface-labeled NRK cells with the anti-FNR IgG identifies a set of proteins similar to those recognized in PC12 cell extracts except for an additional prominent band centered at 160 kD that is absent from PC12 extracts (cf. Fig. 7 D). The correlation between the absence or low levels of this surface protein on PC12 cells and the relative inability of these cells to attach to FN merits further investigation. Alternatively, PC12 cells could express a normal level of FN receptor heterodimers that are unable to bind FN, as appears to be the case for the II_b/III_a FN-binding heterodimer on unactivated platelets (Gardner and Hynes, 1985). Although a previous study (Akeson and Warren, 1986) demonstrated apparently high levels of attachment of PC12 cells to FN, differences in the cell line or the conditions used to measure attachment could account for the quantitative differences in levels of attachment to FN seen in the present study.

A distinguishing feature of several recently purified receptors for adhesive proteins is that they recognize the tripeptide sequence, Arg-Gly-Asp (RGD), present in the cell-binding domains of their cognate ligand(s) (reviewed in Ruoslahti and Pierschbacher, 1986). Consistent with these observations, the low level of PC12 cell attachment to FN is inhibited by an RGD-containing hexapeptide (cf. Fig. 4 B; see also Akeson and Warren, 1986). This peptide has no effect, however, on PC12 cell attachment to LN or Col IV, suggesting that receptors for these matrix proteins are functionally distinct from FN receptors. Avian neural retinal cells also appear to have distinct receptors for these matrix proteins (Hall et al., 1987; see also Pytela et al., 1985). Although Col IV apparently contains several RGD sequences, the same RGD-containing peptides that block cell attachment to FN do not inhibit attachment to Col IV (Pierschbacher et al., 1984a, b; see also Dedhar et al., 1987). It is presently unknown whether LN contains an RGD sequence, however, a nonapeptide (CDPGYIGSR) derived from a LN BI chain sequence has recently been shown to inhibit the attachment of epithelial cells to intact LN (Graf et al., 1987). This peptide appears to compete for the binding of a high affinity 68-kD LN-binding protein to LN (Graf et al., 1987). The nonapeptide sequence, and presumably the 68-kD LN receptor-binding

site, is located near the intersection of the two arms of the LN molecule. This site is distinct from the PC12 cell and neuronal-binding site located near the end of the long arm of LN (Edgar et al., 1984; Engvall et al., 1986). PC12 cell interactions with LN appear not to involve a 68-kD LN-binding protein similar to that described on other cell types (Malinoff and Wicha, 1983; Lesot et al., 1983; Terranova et al., 1983), since antibodies to an endothelial cell 68-kD LN-binding protein failed to detect a 68-kD protein in PC12 cell extracts by immunoprecipitation or antigen blotting (unpublished observations).

PC12 cell and neuronal surface proteins recognized by the anti-ECMR serum appear to be involved in two related but separable phenomena: attachment and process outgrowth on LN-coated substrates. We have previously shown that homologous receptors defined by the CSAT antibody on avian neurons are required for LN-dependent process outgrowth on mixed LN/poly-D-lysine substrates where neuronal adhesion is unaffected by the CSAT antibody (Tomaselli et al., 1986a). Process outgrowth thus appears to require more selective interactions than does adhesion. Somewhat surprisingly, results here show that the anti-ECMR serum inhibits NGF-stimulated process outgrowth from NGF-primed PC12 cells even on poly-D-lysine when attachment is unaffected by the anti-ECMR serum (cf. Fig. 6). There are two possible explanations for this result. First, since PC12 cells do secrete some laminin (Lander et al., 1985a), process outgrowth on poly-D-lysine could conceivably require ECM receptor interactions with PC12 cell-derived LN. In light of this possibility, it is interesting that antibodies to an LN-containing protein complex inhibits NGF-dependent sympathetic neuronal process outgrowth on poly-D-lysine substrates (Coughlin and Kessler, 1982). Alternatively, a direct interaction of ECM receptors with poly-D-lysine may be required for PC12 process outgrowth on this substrate. ECM proteins are clearly not the only ligands that can activate the functions of ECM receptors, since substrate-bound CSAT antibody can itself promote attachment and process outgrowth by avian neurons (Hall et al., 1987).

In summary, we have shown that PC12 cells are a useful model for studying neuronal interactions with ECM constituents. Antibodies that perturb PC12 cell interactions with LN, Col IV, and FN recognize predominantly three glycoprotein bands of 120, 140, and 180 kD. Based on structural and functional similarities to avian ECM receptors recognized by the CSAT antibody, we propose that glycoproteins recognized by the anti-ECMR and anti-FNR sera are members of the integrin family of adhesive protein receptors and may be responsible, in part, for PC12 cell and neuronal interactions with LN, Col IV, and FN.

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