The Adhesive and Neurite-promoting Molecule p30: Analysis of the Amino-Terminal Sequence and Production of Antipeptide Antibodies that Detect p30 at the Surface of Neuroblastoma Cells and of Brain Neurons

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Abstract. A membrane-bound adhesive protein that promotes neurite outgrowth in brain neurons has been isolated from rat brain (Rauvala, H., and R. Pihlaskari. 1987. *J. Biol. Chem.* 262:16625–16635). The protein is an immunochemically distinct molecule with a subunit size of ∼30 kD (p30). p30 is an abundant protein in perinatal rat brain, but its content decreases rapidly after birth.

In the present study the amino-terminal sequence of p30 was determined by automated Edman degradations. A single amino-terminal sequence was found, which is not present in previously studied adhesive molecules. This unique sequence has a cluster of five positive charges within the first 11 amino acid residues: Gly-Lys-Gly-Asp-Pro-Lys-Lys-Pro-Arg-Gly-Lys. Antisynthetic peptide antibodies that recognize this sequence were produced in a rabbit, purified with a peptide affinity column, and shown to bind specifically to p30.

The antipeptide antibodies were used, together with anti-p30 antibodies, to study the localization of p30 in brain cells and in neuroblastoma cells as follows. (*a*) Immunofluorescence and immunoelectron microscopy indicated that p30 is a component of neurons in mixed cultures of brain cells. The neurons and the neuroblastoma cells expressed p30 at their surface in the cell bodies and the neurites. In the neurites p30 was found especially in the adhesive distal tips of the processes. In addition the protein was detected in ribosomal particles and in intracellular membranes in a proportion of cells. (*b*) The antibodies immobilized on microtiter wells enhanced adhesion and neurite growth indicating that p30 is surface exposed in adhering neural cells. (*c*) Immunoblotting showed that p30 is extracted from suspended cells by heparin suggesting that a heparin-like structure is required for the binding of p30 to the neuronal cell surface. A model summarizing the suggested interactions of p30 in cell adhesion and neurite growth is presented.

Adhesion of neurons to other cells or to extracellular materials is thought to play an important role in the outgrowth and guidance of neurites (Letourneau, 1975). In an attempt to identify adhesive molecules that might play a role in such phenomena, we have fractionated solubilized membranes from young rat brain and monitored the fractionations with brain neurons (Rauvala et al., 1987; Rauvala and Pihlaskari, 1987). These studies have resulted in the isolation of an adhesive molecule that has a subunit size of ∼30 kD (p30). Due to its neuron-binding and neurite outgrowth–promoting properties and the developmental regulation of its content in brain tissue, p30 has been suggested to play a regulatory role in neuronal growth (Rauvala and Pihlaskari, 1987).

To further characterize the structural and functional properties of p30 and its possible relationship to other adhesive and neurite-promoting factors, we have analyzed the amino-terminal amino acid sequence of p30 and produced antipeptide antibodies that specifically detect this unique sequence. The affinity-purified antipeptide and anti-p30 antibodies have been used to stain cultured brain cells. These studies indicate that p30 is mainly associated to neurons in mixed cultures of brain cells. The p30 protein and its lysine-rich amino-terminal sequence are detected as surface-exposed structures in neuroblastoma cells and in brain neurons.

Materials and Methods

Isolation of p30

The p30 protein was isolated from membrane pellets of early postnatal rat brains (from 2 to ∼10-d-old rats) as has been previously described (Rauvala and Pihlaskari, 1987). Protein content of the isolated fractions was determined with a Coomassie Brilliant Blue G-250 dye-binding assay (Bio-Rad Laboratories, Cambridge, MA). The degree of purity of the isolated protein

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was estimated with 5–20% gradient SDS-PAGE (Laemmli, 1970) stained with Coomassie Brilliant Blue.

Amino-Terminal Sequence Analysis

Automated Edman degradations were carried out with either a gas-phase sequencer (model 470A; Applied Biosystems, Inc., Foster City, CA) or with a pulsed liquid-phase sequencer (Model 477A, Applied Biosystems, Inc.). The p30 protein was separated with a reducing 5–20% gradient SDS-PAGE (Laemmli, 1970) in the presence of 0.1 mM sodium thioglycollate (Hunkapiller et al., 1983) and transferred to a glass fiber filter for sequencing (Aebersold et al., 1986). Electrophoretic transfer was carried out in 25 mM Tris, 10 mM glycine, 0.5 mM dithiothreitol (DTT), pH 8.3, at 100 V for 3 h in ice bath. Alternatively, the SDS-PAGE was omitted, and the p30 protein was sequenced directly after the isolation with heparin-Sepharose and Affi-Gel blue (Rauvala and Pihlaskari, 1987).

Synthesis of Peptides

Solid-phase synthesis (Barany and Merrifield, 1979) of peptides I–III (see Table I) was carried out with an automated peptide synthesizer (model 430A; Applied Biosystems, Inc.). The amino acid sequences were confirmed by automated sequencing (see above).

Production and Purification of Antibodies

Production of anti-p30 antibodies and their purification with the p30 polypeptide transferred from SDS-PAGE to nitrocellulose have been previously described (Rauvala and Pihlaskari, 1987). The anti-p30 antibodies were extracted from the protein band at pH 2.5 in the presence of 1 mg/ml of BSA, and neutralized immediately. They were used for Western blotting at the dilutions 1:10–1:100 and for staining of the cells at the dilutions 1:2–1:10.

Antipeptide antibodies were produced in a rabbit immunized with the synthetic peptide I (see table I) after coupling with *m*-bromosuccinimide from the cysteine residue to keyhole limpet hemocyanin (10 μmol of peptide coupled to 10 mg of protein; Green et al., 1982). The antibodies were purified with affinity chromatography using peptide II coupled from carboxyl groups with *N*-ethyl-*N*'-(3-dimethylaminopropyl)-carbodiimide (Sigma Chemical Co., St. Louis, MO; the reagent was used at 20 mg/ml in the coupling) to aminohexyl-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). About 5 μmol of peptide II were coupled to 1 ml of swollen gel at pH 4–5 for ~20 h at room temperature. The gel was alternately washed with 0.1 M sodium acetate, 0.5 M NaCl, pH 4, and with 0.2 M sodium bicarbonate, 0.5 M NaCl, pH 8.3 (four times with each buffer) to block excess amino groups, and was then used for purification of the antibodies. Serum (4 ml/1 ml of swollen gel) was diluted with an equal volume of 1 M NaCl and adsorbed to the column for 3 h at room temperature or for ~20 h at 4°C. The mixture was centrifuged, and the Sepharose pellet was resuspended in PBS (137 mM NaCl, 27 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.4; 10 ml per 1 ml of swollen gel). Washing and centrifuging in PBS was repeated three times, and the Sepharose was further washed three times in the same way in 0.5 M NaCl in PBS. The Sepharose was then packed to a column, which was washed with PBS, and the bound antibodies were eluted with 50 mM glycine-HCl, pH 2.0. The fractions were immediately neutralized with 1 M Na_2HPO_4 (three drops per 1 ml). The antibodies were mostly eluted in one column volume of the eluting buffer, which was routinely used as the solution of antipeptide antibodies. Protein content of the fractions was determined with the Coomassie Brilliant Blue G-250 binding test (Bio-Rad Laboratories). The antibodies were characterized with SDS-PAGE, with ELISA assays (Engvall, 1980) using the p30 protein coated to polystyrene microtiter wells, and with Western blotting experiments (Towbin et al., 1979). They were used for immunoblotting at the concentrations of 0.1–1.0 μg/ml and for cell staining at 1–10 μg/ml.

Cells and Cell Culture

Cells from the N18 neuroblastoma clone were cultured on ordinary tissue culture dishes in DME (Flow Laboratories, Inc., McLean, VA) supplemented with 10% FCS, 100 U penicillin G/ml, and 0.1 mg streptomycin/ml in the atmosphere of 5% CO_2/95% air. Brain cells of the rat were prepared from 17–19-d-old embryos essentially as has been described (Yavin and Yavin, 1974). Briefly, cerebral hemispheres of the embryos were dissected in DME containing 10% FCS, 100 U/ml of penicillin G, 0.1 mg/ml of streptomycin, and 6 mg/ml of glucose. The cells were then dispersed in the serum-containing medium by pressing through a nylon mesh with 106 μm pores. Culture wells for the brain cells were prepared by coating the tissue culture dishes with 5 μg/ml of poly-L-lysine in water (Yavin and Yavin, 1974) followed by washing and further coating for 0.5 h with 10% FCS in DME (see above). The dispersed brain cells adhered nearly quantitatively to such surfaces, as has been previously shown (Yavin and Yavin, 1974). The medium was changed after ~20 h, and the adherent cells were used for the experiments within 2 d, if not otherwise indicated. Immunofluorescence studies (see below) using monoclonal antineurofilament, antiglial fibrillary acidic protein, and antivimentin suggested that ~80–90% of these cells were neurons, and the rest of them were mainly astrocytes. The proportion of astrocytes increased strongly, when the cells were kept in culture for ~1 wk.

Fluorescence Microscopy

Adherent cells on glass coverslips were fixed with 4% paraformaldehyde in Ca- and Mg-containing PBS (0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$), pH 7, for 15 min at room temperature, washed with PBS, and used for membrane staining. Alternatively, membrane staining was carried out using adherent live cells. Adherent cultures fixed with methanol at −20°C were used for the staining of permeabilized cells. Antineurofilament, antiglial fibrillary acidic protein, and antivimentin were used according to the manufacturer's guide (Labsystems Inc., Helsinki, Finland) and the antipeptide and anti-p30 antibodies as indicated in each experiment. After washing with 5 or 10 mg/ml of BSA in PBS, the cells were overlaid with FITC-conjugated swine anti-rabbit Ig or with rhodamine-conjugated rabbit anti-mouse Ig (DAKOPATTS, Copenhagen; diluted 1:50 in 5 mg/ml of BSA in PBS), and incubated as indicated in each experiment. After three washes with PBS, the coverslips were mounted on slides, which were studied with a Zeiss microscope (model IM35) equipped for epifluorescence with appropriate filters to specifically detect the FITC or rhodamine staining.

Membrane staining of live cells in suspension was carried out at 0–4°C essentially as has been described (Goding, 1983). Briefly, adherent cells were washed three times with Ca- and Mg-free PBS and incubated in the PBS at 37°C for ~30 min. The cells were dispersed by gentle pipetting, and washed in the PBS. The cell suspensions were allowed to cool in ice bath, and they were then incubated for 90 min with the antibodies, washed and incubated for 60 min with FITC anti-rabbit Ig (1:50; DAKOPATTS). After washing, the cells were centrifuged to slides and studied with phase-contrast and fluorescence microscopy.

Immunoelectron Microscopy

Brain cells dispersed from 17–19-d-old rat embryos were allowed to adhere to poly-L-lysine–coated tissue culture dishes (see above). They were washed three times with PBS, fixed with 1% glutaraldehyde for 5 min at room temperature, and washed with PBS. The cells were then incubated with 20% normal swine serum (DAKOPATTS) in PBS for 20 min at room temperature to block nonspecific binding of the link antiserum. Incubation with the affinity-purified anti-p30 antibodies (1:10 in PBS) was then carried out at 4°C for 48 h. The peroxidase/antiperoxidase method was used as described in detail (Panula et al., 1981). Shortly, unlabeled swine anti-rabbit Ig (DAKOPATTS) and the soluble complex of horseradish peroxidase/rabbit anti-horseradish peroxidase (DAKOPATTS) were diluted 1:100 in PBS and the incubations were carried out for 1 h at room temperature. The cultures were postfixed with 1% osmium tetroxide, dehydrated in a graded ethanol series, and embedded in an Epon-Araldite mixture. Ultrathin sections were viewed and photographed without poststaining in a JEOL 100s electron microscope at 60 kV.

Assays of Cell Adhesion and Neurite Outgrowth

Adhesion assays using neuron-enriched brain cells that had been metabolically labeled with a mixture of ^{14}C-amino acids were carried out as described (Rauvala and Pihlaskari, 1987).

The N18 neuroblastoma cells were dispersed for neurite outgrowth assays by incubating the cultures for ~30 min at 37°C in the Ca- and Mg-free PBS and washed in the same buffer. The cells were assayed for neurite outgrowth in microtiter wells (Titertek® multiwell plates; Flow Laboratories, Inc.) essentially as described (Rauvala, 1984). The assays on the different coated surfaces were carried out in PBS containing 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 4.5 mg/ml glucose at the cell density of 1.25×10^5/ml (150 μl of cell suspension per well; 67,000 cells/cm^2). Assays with rat brain neurons were carried out in the serum-free DME as described (Rauvala and Pihlaskari, 1987).

Results

Amino-Terminal Sequence Analysis

Two approaches were used for sequence analysis. In one method p30 was separated on SDS-PAGE and transferred to activated glass fiber filter for sequencing (Aebersold et al., 1986). In the other method the SDS-PAGE was omitted and the isolated protein was analyzed directly. Both methods gave a single and identical amino acid sequence: Gly-Lys-Gly-Asp-Pro-Lys-Lys-Pro-Arg-Gly-Lys-Met-Ser-X-Tyr-Ala-Phe-Phe-Val-Gln. No clear assignment was obtained for position 14, which is indicated by X. The amount of recovered amino acid derivatives corresponded to the amount of protein loaded on the sequencer.

Production and Characterization of Antipeptide Antibodies

Purification of antibodies obtained by immunizing with p30 (designated anti–p30) has been previously reported (Rauvala and Pihlaskari, 1987). The p30 polypeptide transferred from SDS-PAGE to nitrocellulose was used for the purification of these antibodies.

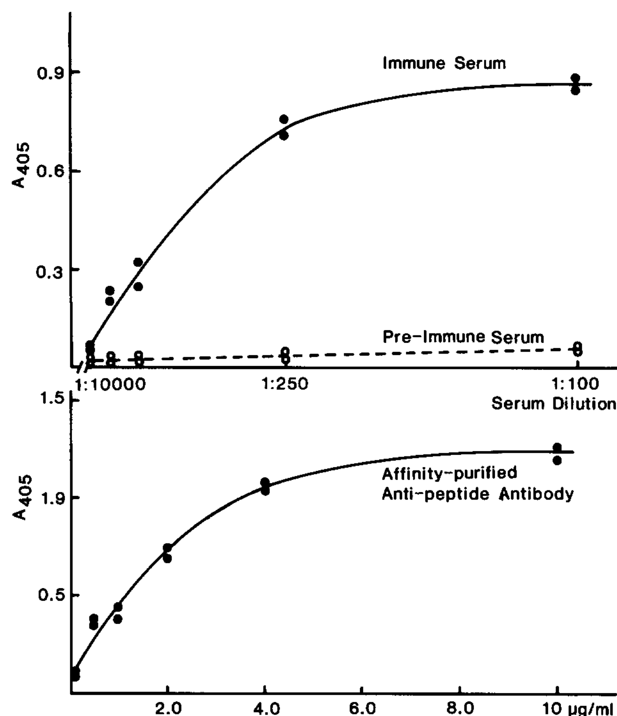

Figure 1. Binding of antipeptide antibodies to p30 in ELISA assays. The rabbit was immunized with 1 mg of peptide I/keyhole limpet hemocyanin conjugate in complete Freund's adjuvant. The immune serum was taken after two 1-mg booster injections given in incomplete Freund's adjuvant (~6 wk after the primary immunization). Affinity purification of the antibodies was carried out with peptide II coupled to aminohexyl-Sepharose (see Materials and Methods). The sera and the affinity-purified antibody (100-μl vol) were tested for binding to 1 μg of p30 (coated in 100 μl of PBS to microtiter wells that were postcoated with 1 mg/ml of BSA). Binding of the antibodies to the wells was assayed with alkaline phosphatase-conjugated anti-rabbit IgG. Alkaline phosphatase was determined with paranitrophenyl phosphate measured in microtiter wells.

An intense immune reaction (Fig. 1) was observed in one rabbit immunized with the synthetic peptide I (Table I) coupled from the cysteine residue to keyhole limpet hemocyanin (Green et al., 1982). This peptide is different from the amino-terminal sequence of p30 in one amino acid residue (No. 9), but the antibody was shown to bind to p30 (Fig. 1) and was therefore further studied.

The antipeptide antibody could be purified with peptide II (lacking the carboxy-terminal cysteine used for coupling to keyhole limpet hemocyanin and not found in p30) coupled with carbodiimide to aminohexyl-Sepharose. About 40 μg of antibody was purified from 1 ml of immune serum. The purified antipeptide antibody gave one major band with M_r of ~50 kD and a minor band with M_r of ~25 kD in SDS-PAGE stained with Coomassie Brilliant Blue. Immunoblotting indicated that the major band is the heavy chain of immunoglobulin (not shown). The peptide affinity column thus isolates essentially pure immunoglobulin in one single step. The purified antibody (designated antipeptide) detected p30 in ELISA (Fig. 1) and in Western blotting using the purified p30, SDS-solubilized rat brains or SDS-solubilized N18 neuroblastoma cells (Fig. 2, *A–C*). Binding of the antibody to the p30 band was completely blocked by the HPLC-purified peptides II and III (Fig. 2 *D*). The inhibition by peptide III (having the same sequence as p30) was observed at the same concentrations (10–100 μM peptide) as by peptide II (used for affinity purification). Thus, the arginine (residue No. 9) is not critical for the recognition by the antipeptide antibody.

In contrast to the antipeptide antibody, binding of the anti–p30 antibody (obtained by immunization with p30 over a period of many months) to the p30 band was not blocked or reduced by the synthetic peptides (not shown). Thus, the amino-terminal sequence of p30 is not a major immunogenic site in the p30 molecule. It is apparent that the binding of the anti–p30 antibodies to p30 does not depend on the conformation of p30, because these antibodies were purified with the denatured polypeptide. The peptide sequences recognized by the anti–p30 antibodies have not been further characterized.

Fig. 3 shows that the purified antipeptide antibody is able to inhibit the adhesive effect of p30 under the conditions that favor binding of the antibody to p30 instead of the plastic culture substrates (coating of the plastic substrates with the antibodies enhances adhesion, see below). Inhibition of the adhesive effect of p30 with the affinity-purified anti–p30 antibody has been shown in the previous study (Rauvala and Pihlaskari, 1987). Thus, both the antipeptide and the anti–p30 antibodies detect an adhesive molecule.

Staining of Cells with the Antibodies

The N18 neuroblastoma cells cultured on glass coverslips and fixed shortly with paraformaldehyde were clearly stained with the antipeptide antibodies at the protein concentration

Table I. Synthetic Peptides Used in the Study

Peptide	Sequence
I	Gly-Lys-Gly-Asp-Pro-Lys-Lys-Pro-Ser-Gly-Lys-Met-Ser-Cys
II	Gly-Lys-Gly-Asp-Pro-Lys-Lys-Pro-Ser-Gly-Lys
III	Gly-Lys-Gly-Asp-Pro-Lys-Lys-Pro-Arg-Gly-Lys

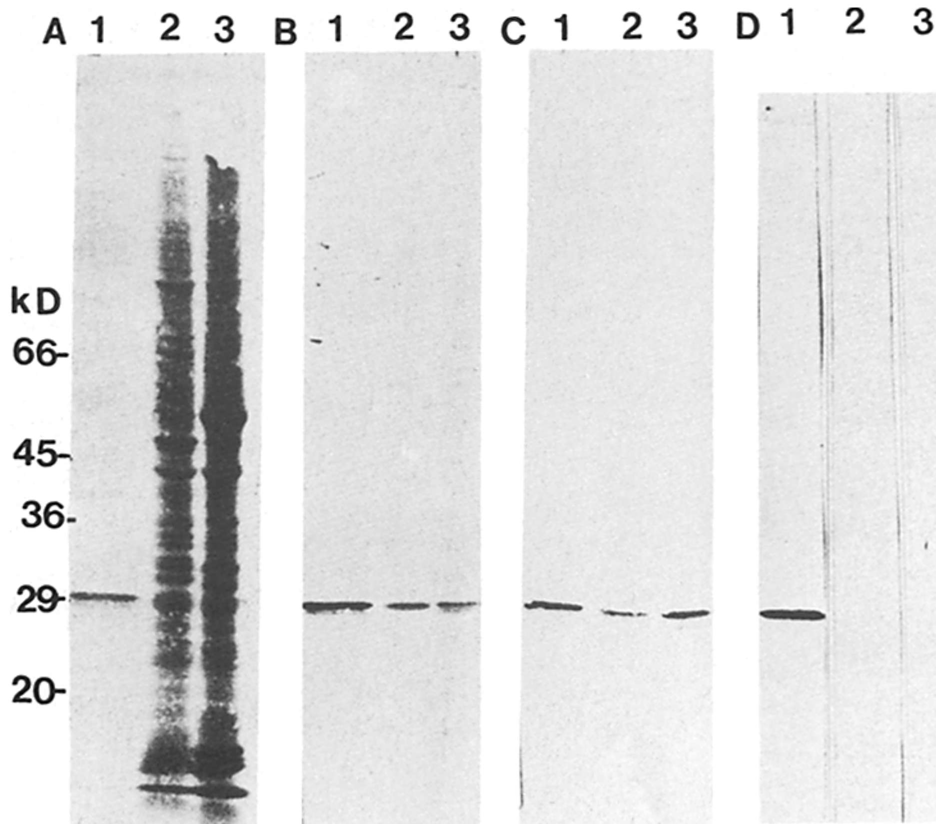

Figure 2. Immunoblots suggesting specific binding of anti-p30 and antipeptide antibodies to p30. (*A*) Amido black staining of the samples transferred from 5–20% reducing SDS-PAGE to nitrocellulose. Lane *1*, 1.5 μg of purified p30; lane *2*, 25 μl of SDS-solubilized N18 neuroblastoma cells (~100 mg of cells were solubilized for 0.5 h at 100°C in 2.5 ml of reducing SDS gel buffer); lane *3*, 25 μl of SDS-solubilized rat brain (100 mg of brain from 17–19-d-old rat embryos were solubilized for 0.5 h at 100°C in 2.5 ml of reducing SDS gel buffer). *B* and *C* show replicas containing samples as in *A*, lanes *1-3*. *B* was stained with anti-p30 antibodies (1:100). *C* was stained with the affinity-purified antipeptide antibody (0.2 μg/ml). (*D*) Each lane contained 1.5 μg of p30. Lane *1*, staining with 1 μg/ml of affinity-purified antipeptide antibody; lane *2*, staining as in lane *1* in the presence of 10 μM peptide II (see Table I); lane *3*, staining as in lane *1* in the presence of 10 μM peptide III. Transfer of the proteins to all lanes was controlled by staining with Ponceau S. before other staining procedures. Marker proteins: BSA (66 kD), ovalbumin (45 kD), glyceraldehyde-3-phosphate dehydrogenase (36 kD), carbonic anhydrase (29 kD), and soybean trypsin inhibitor (20 kD).

1-10 μg/ml (Fig. 4). Membrane staining was observed in most cells in both the cell bodies and the neuritic processes. The proximal parts of the neurites often lacked the staining, whereas the distal adhesive tips were clearly stained (Fig. 4). Staining of the cells could not be inhibited by 20% nonimmune swine serum used in some experiments instead of BSA (Fig. 4) to block nonspecific binding of the antibody to the cells. The staining pattern was similar when the antibody was incubated with the cells for ~20 h at 4°C or for 1 h at room temperature. The surface staining of both the paraformaldehyde-fixed N18 cells and the brain cells could be inhibited by 100 μM synthetic peptide, shown for the brain cells in Fig. 5, *a–d*. Specificity of the membrane staining was also suggested by the finding that nonimmune rabbit IgG (10 μg/ml) did not stain the cell surface.

Adherent live cells could be also stained with the antipeptide antibody (Fig. 4, *e–h*), but it was difficult to retain the extended morphology of the neurites during the staining procedure. Thus, the neurites apparently retract during staining on ice bath giving rise to multiple varicosity-type formations, which were not seen before the staining procedure or in the cells stained after short fixation with paraformaldehyde (Fig. 4, *a–d*). However, the surface staining of adherent live cells is also seen in both the cell bodies and the neurites (Fig. 4, *e–f*). The patchy staining outside the cells (Fig. 4 *f*) probably corresponds to the substrate-attached material left behind by the moving cells. The adherent live cells were stained with the anti-p30 antibody in a similar way (not shown).

Fig. 5 shows that also the adherent brain cells can be specifically stained with the antipeptide antibody. As in the neuroblastoma cells, the staining was found in both the cell bodies and the neurite-type processes, especially the varicosities of the neurites (Fig. 5, *a–b*). The cell bodies and the processes of these cells were also stained by monoclonal antineurofilament antibodies after permeabilization with methanol (not shown).

In addition to adherent cells, p30 is found at the surface after the cells have been suspended using Ca- and Mg-free PBS. Fig. 6 shows an example of staining of live cells in suspension using the anti-p30 antibody. In controls, in which the first antibody was omitted, only some broken cells were fluorescent.

Immunoelectron microscopy detected p30 on the plasma

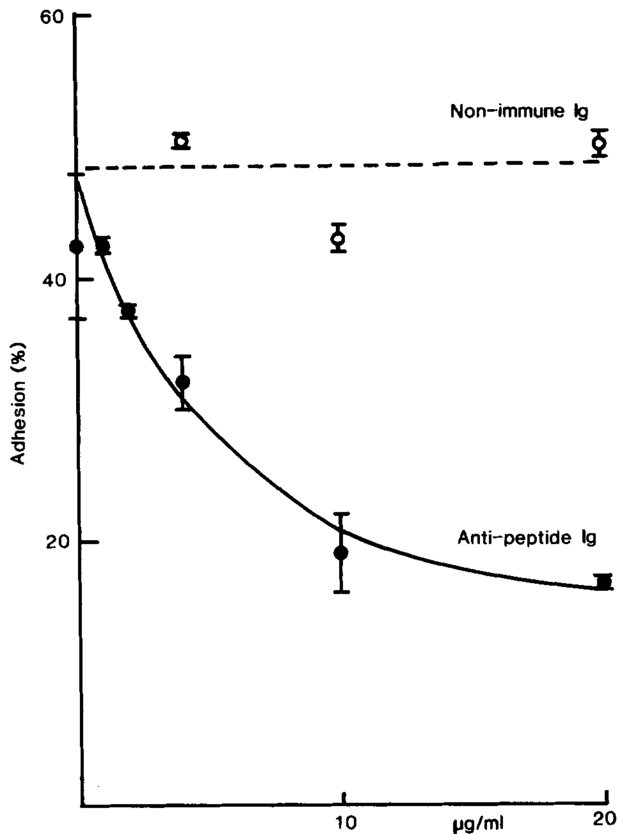

Figure 3. Inhibition of brain cell adhesion to p30-coated surfaces by the affinity-purified antipeptide antibody. Polystyrene wells (Greiner, 3.5 cm diam) were coated with 4 μg/ml of p30 (750 μl vol) at 4°C for ∼20 h. The wells were washed with PBS and incubated with 1 mg/ml of BSA (crystalline BSA, essentially globulin-free, Sigma Chemical Co.) in PBS or with the indicated concentrations of immunoglobulins in the BSA-containing buffer (750 μl vol; BSA was used to inhibit coating of the plastic with the antibodies). The nonimmune immunoglobulin was purified from rabbit serum with protein A–Sepharose and was of similar purity as the antipeptide immunoglobulin (that also binds to protein A–Sepharose), as assessed with SDS-PAGE stained with Coomassie Blue. After 1 h incubation at room temperature with the BSA-PBS buffer or the antibodies, the wells were washed two times with PBS, and the brain cells were incubated on the wells (52,000 cells/cm^2) for 30 min at 37°C. The wells were washed, rotated on an orbital shaker at 140 rpm for 3 min, washed, and estimated for cell binding with phase-contrast microscopy. Quantification of cell binding was based on the counting of ^{14}C-amino acid label from the solubilized cells. The error bars indicate the range of duplicate determinations.

membranes of both cell bodies (Fig. 7 *a*) and of neuritic processes (Fig. 7, *c–e*). Intensely stained structures were observed in areas of membrane–membrane contact (Fig. 7, *c–e*). Intracellular cytoplasmic staining was seen in many cells. The intensity of the intracellular staining varied from moderate to intense, and its location suggested association to polyribosomes (Fig. 7 *b*). Intracellular staining was also seen in cross sections (Fig. 7 *c*) and in longitudinally cut sections (Fig. 7 *d*) of cellular processes, which were in addition stained on the plasma membranes. In some processes the staining was confined to the plasma membrane (Fig. 7 *e*). The results were similar when either anti–p30 or antipeptide antibodies were used, but no staining was observed when the primary antibodies were omitted. However, binding of the antipeptide antibody to p30 was strongly reduced when the protein was treated with glutaraldehyde used as the fixing reagent in the staining method (not shown). The anti–p30 antibodies were therefore mainly used in the immunoelectron microscopy.

Neurite-promoting Effect of the Immobilized Antibodies

To study whether the p30 protein is surface exposed in living adhering cells, the effect of the immobilized antibodies on cell behavior was tested. Fig. 8 shows that the immobilized antipeptide antibody has a dramatic effect on the morphology of neuroblastoma cells. Extensive neurite initiation with flattened growth cones is observed in a 3-h assay. This effect is clearly more pronounced than that of p30 itself for the neuroblastoma cells. In an assay of 20 h, the effect of p30 on the neuroblastoma cells is already clearly observed, but the outgrowth of neurites is still more pronounced on surfaces coated with the antipeptide antibody. In contrast to the neuroblastoma cells, rat brain neurons favor p30 over the antipeptide antibody, although this also has some effect (not shown).

The effect of the antipeptide antibody was reduced by the synthetic peptide (Fig. 8), which did not inhibit neurite growth on laminin when tested at the same concentrations. However, the peptides also have some effects of their own on neuronal adhesion (not shown), which jeopardizes interpretation of the results. The effects of the peptides synthesized according to the p30 sequences thus require further studies before they can be evaluated in a more detailed way. Specificity of the effect of the antipeptide antibody was clearly indicated by experiments, in which its effect was compared to that of nonimmune immunoglobulin of similar purity. The antipeptide antibody was similar to the purified nonimmune immunoglobulin in that it was quantitatively bound to protein A–Sepharose (not shown). A difference of several orders of magnitude was observed (Fig. 9) when the dose-response relationships of the two immunoglobulins for neurite initiation were studied. In agreement with this finding, the F_{ab} fragments of the antipeptide antibody had the same effect as the native antibody. The anti–p30 antibody had a similar effect on the cells as the antipeptide antibody (not shown), but it has been only purified as dilute solutions in the presence of BSA, which has prevented a more detailed characterization of the effect.

Extraction of p30 from Suspended Cells by Heparin

The isolated molecule p30 binds to heparin-Sepharose rather strongly, and is eluted from the column at 0.75–1.0 M NaCl in salt gradients (Rauvala and Pihlaskari, 1987). The possibility was therefore studied that p30 also binds to a heparinlike structure in living cells. We observed that p30 can be displaced from living brain cells by heparin. In the immunoblotting experiment shown in Fig. 10 *A*, the amount of p30 released from the cells to the supernatant was found to be comparable to that released by SDS, whereas the amount released to PBS (with or without Ca and Mg) was low or undetectable. Some detachment of p30 probably occurred in the presence of 1 mM EDTA (Fig. 10 *A*). Fig. 10 *B* shows

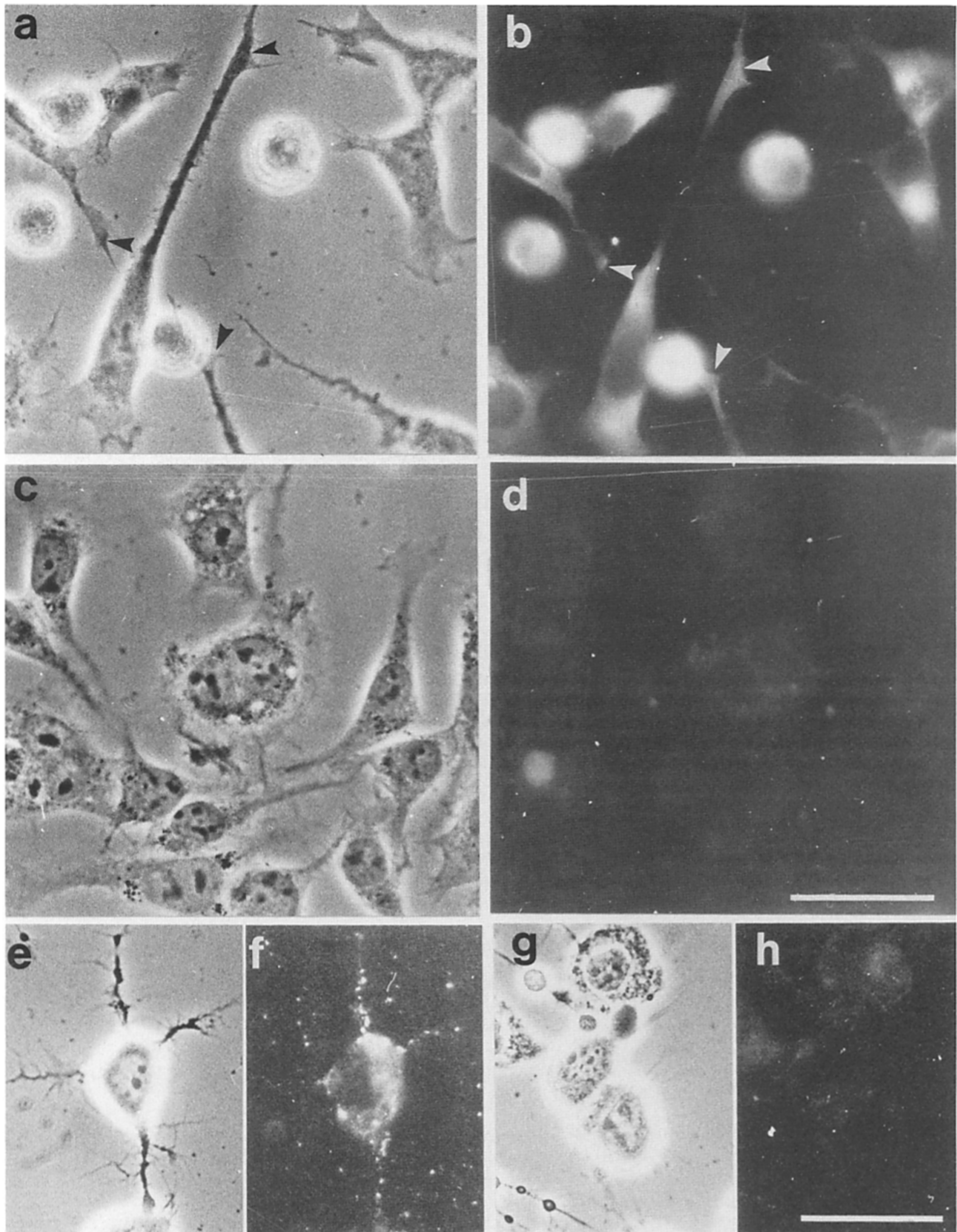

Figure 4. Indirect immunofluorescence staining of N18 neuroblastoma cells by the affinity-purified antipeptide antibody. Phase-contrast microscopy (*a*, *c*, *e*, and *g*) and fluorescence microscopy (*b*, *d*, *f*, and *h*) of the respective fields. (*a–d*) The cells grown on glass coverslips were fixed with 4% paraformaldehyde in PBS, pH 7, for 15 min at room temperature, washed with PBS, and incubated for 0.5 h with 5 mg/ml of BSA in PBS. The cells were then incubated for ~20 h at 4°C with 10 μg/ml of the antipeptide antibody in the BSA-PBS buffer (*a* and *b*) or without the first antibody in the BSA-PBS (*c* and *d*). An intense staining is seen in cell bodies and in the distal tips of neuritic processes (*arrowheads*). (*e–h*) Staining of live cells on ice bath. The cells were washed with PBS, incubated for 60 min with 10 mg/ml

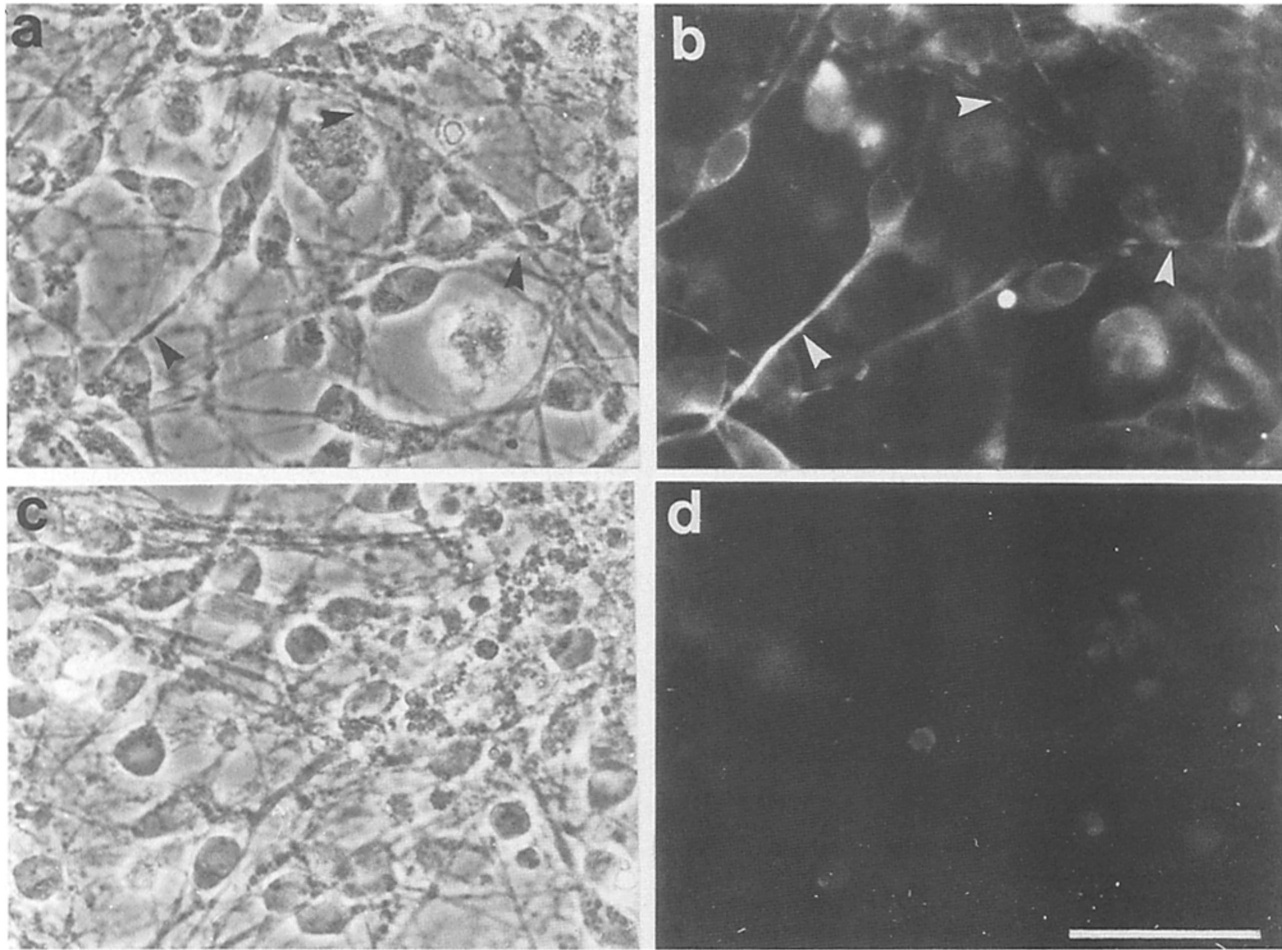

Figure 5. Indirect immunofluorescence staining of brain cell cultures with the antipeptide antibody. Phase-contrast microscopy (*a* and *c*) and fluorescence microscopy (*b* and *d*) of the respective fields. The cells dispersed from the hemispheres of 17–19-d-old rat embryos were cultured for 4 d on poly-L-lysine–coated glass coverslips and fixed with 4% paraformaldehyde in PBS, pH 7, for 15 min at room temperature. The fixed cells were washed with PBS and incubated for 1 h at room temperature with 20% nonimmune swine serum in PBS. The cells were then incubated for 1 h at room temperature with 10 μg/ml of the antipeptide antibody in the swine serum–PBS in the absence (*a* and *b*) or presence (*c* and *d*) of 100 μM synthetic peptide III (Table I). The peptide was preincubated for 1 h with the antibody before the mixture was applied to the cells. The arrowheads point to neurites. The cell bodies and the varicosities of the neurites are clearly stained, and this staining is inhibited by the lysine-rich amino-terminal peptide. Bar, 50 μm.

that heparin concentrations of 1.0 and 0.25 mg/ml released p30, whereas 0.1 mg/ml of heparin was rather ineffective. The low molecular weight heparin was more effective than heparin on weight basis. Thus, 0.1 mg/ml of low molecular weight heparin still effected an apparently maximal release from the cells (Fig. 10 *B*). Fig. 10 *C* shows that dextran sulfates (especially those of higher molecular weight) were also able to release p30, whereas the chondroitin sulfates used (4 mg/ml of chondroitin sulfate from whale or shark cartilage) or colominic acid (polysialic acid; 4 mg/ml) did not release p30 from the brain cells. In addition to some polyanionic substances shown in Fig. 10 *C*, the following compounds (tested at 4 mg/ml) did not extract p30 from the brain cells: polyadenylic acid, poly-L-glutamic acid (degree of polymerization 20,000), and poly-L-glutamic acid (degree of polymerization 21,000). Thus, sulfate-containing polyanionic substances, especially heparin-like structures, appear to release p30 from the cells. A high polymeric size is not necessarily needed, but depolymerized heparin (mol wt of 4,000–6,000) also displaces p30 from the cells.

Discussion

In a previous study (Rauvala and Pihlaskari, 1987) the hepa-

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of BSA in PBS on ice bath and then for 90 min with 10 μg/ml of the antipeptide antibody in the BSA-PBS in the absence (*e* and *f*) or presence (*g* and *h*) of 100 μM synthetic peptide III (Table I). The peptide was preincubated for 1 h with the antibody before the mixture was applied to the cells. A respective inhibition of the antibody with the synthetic peptides is shown in a Western blotting experiment in Fig. 2 *D*. Bars, 50 μm.

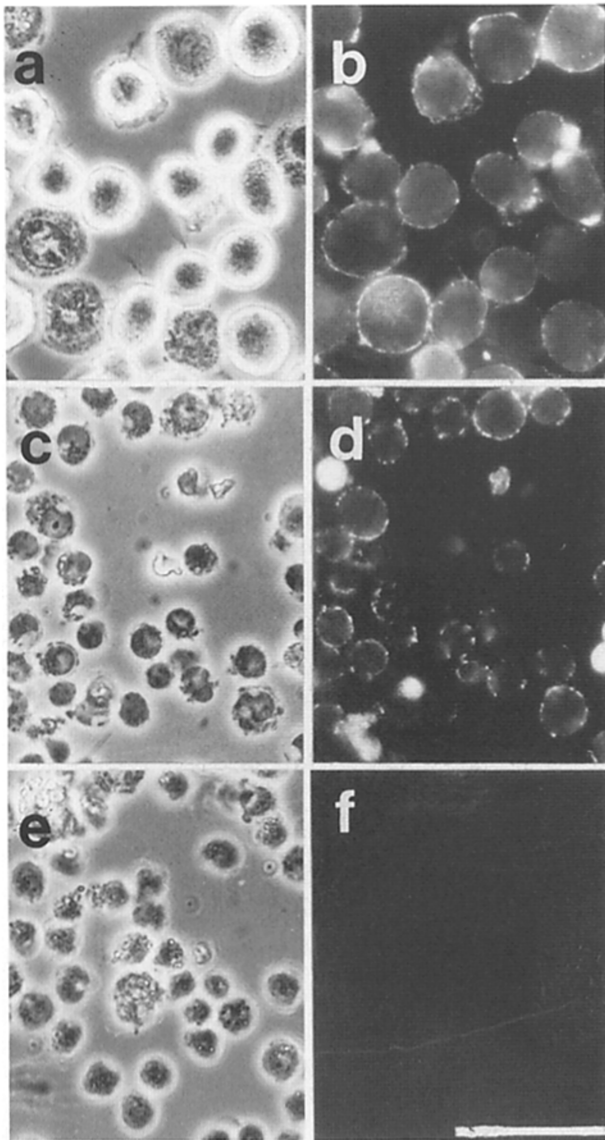

Figure 6. Indirect immunofluorescence staining of live N18 neuroblastoma cells (*a* and *b*) or live rat brain cells (*c–f*) in suspension. Phase-contrast microscopy (*a*, *c*, and *e*) and fluorescence microscopy (*b*, *d*, and *f*) of the respective fields. The anti–p30 antibodies extracted from the p30 band (1:2) were incubated for 90 min with the cells in ice bath. A patchy cell surface staining is observed in both the neuroblastoma cells (*a* and *b*) and the brain cells (*c* and *d*). The staining was not observed in cells incubated without the first antibody (shown for the brain cells in *e* and *f*). Bar, 50 μm.

rin-binding protein p30 was isolated from cell membranes of rat brain. Because p30 binds efficiently brain neurons and in addition enhances neurite growth in these cells, it has been called an adhesive molecule. The inference that p30 has a role in a developmental process in brain as an adhesive and/or growth-promoting molecule has been further supported by the finding that it is abundantly present in the perinatal brain of rat, but the content is rapidly decreased after birth (Rauvala and Pihlaskari, 1987).

P30 was isolated as an immunochemically distinct molecule suggesting that the subunit 30-kD polypeptide is not a fragment of a previously characterized adhesive or growth-promoting polypeptide of higher molecular weight. The amino-terminal sequence analyzed in the present study confirms this inference. Thus, this sequence is not found in a computer homology search (DNASIS of LKB containing the sequences of Protein Identification Resource, data base R 12.0, dated March 1987). The most clearcut similarity is found in a viral nucleoprotein (Herr, 1984) that has the same six–amino acid sequence (Pro-Lys-Lys-Pro-Arg-Gly), but is otherwise a clearly different protein as compared to p30.

The extracellular glycoprotein laminin (Timpl et al., 1979) is a neurite-promoting molecule (Baron van Evercooren et al., 1982; Manthorpe et al., 1983; Rogers et al., 1983) and might give a cell-active fragment in the isolation procedure. In fact, the heparin-binding fragment of laminin is known to enhance neurite growth (Edgar et al., 1984). However, the amino-terminal sequence of p30 is not similar to published laminin sequences in computer homology searches, and it is not found in the recently published sequences of laminin (Pikkarainen et al., 1987; Sasaki et al., 1987). The data base used in computer homology searches contains the fibronectin sequences, but the amino-terminal sequence of p30 is not similar to any of these sequences. The sequences of the neural cell adhesion molecule (Barthels et al., 1987; Cunningham et al., 1987) do not either contain the p30 sequence analyzed in this study. The p30 protein also appears different from basic fibroblast growth factor that has various growth-promoting effects when tested with neural cells (Schubert et al., 1987; Unsicker et al., 1987). Western blotting of SDS-solubilized N18 cells and embryonic rat brain (Fig. 2, *A–C*) further supports the idea that p30 is a distinct novel molecule.

The sequence data were used to synthesize peptides (Table I) for the production of antibodies. An antisynthetic peptide antibody was affinity purified and shown to bind to p30 in ELISA assays (Fig. 1), Western blotting experiments (Fig. 2), and in cell adhesion assays (Fig. 3). These experiments confirm that the amino-terminal sequence described in this study derives from the adhesive molecule p30.

The N18 neuroblastoma cells were previously shown to express p30 (Rauvala and Pihlaskari, 1987) and were therefore used to study the cellular localization of this molecule. The neuroblastoma cells can be regarded as "autonomous" with respect to the substrate required in neurite growth, although the rate of neurite growth in these cells can be enhanced by certain proteins, especially laminin (Jousimaa et al., 1984; Rauvala et al., 1987). Thus, the neuroblastoma cells are able to initiate and extend neurites even on an inert or artificial surface (Seeds et al., 1970; Schubert et al., 1971). Interestingly, in the neuroblastoma cells grown on glass the antipeptide antibody stains the surface in the cell bodies and in the distal growth cone–like parts of the neurites (Fig. 4, *a–b*). This localization is consistent with the adhesive function of p30, because the growing neurites are known to adhere to external surfaces with their distal tips (Letourneau, 1975). Thus, the adhesive molecule p30 might function in an "autocrine manner" in neurite growth: the protein produced by the cell is secreted in the growth cone to facilitate adhesion and neurite growth on an external surface. Immunoelectron microscopy (see below) is consistent with the proposal that p30 is produced by the neurons and transported to the growth cone within the neurite. Conversely, when the antipeptide

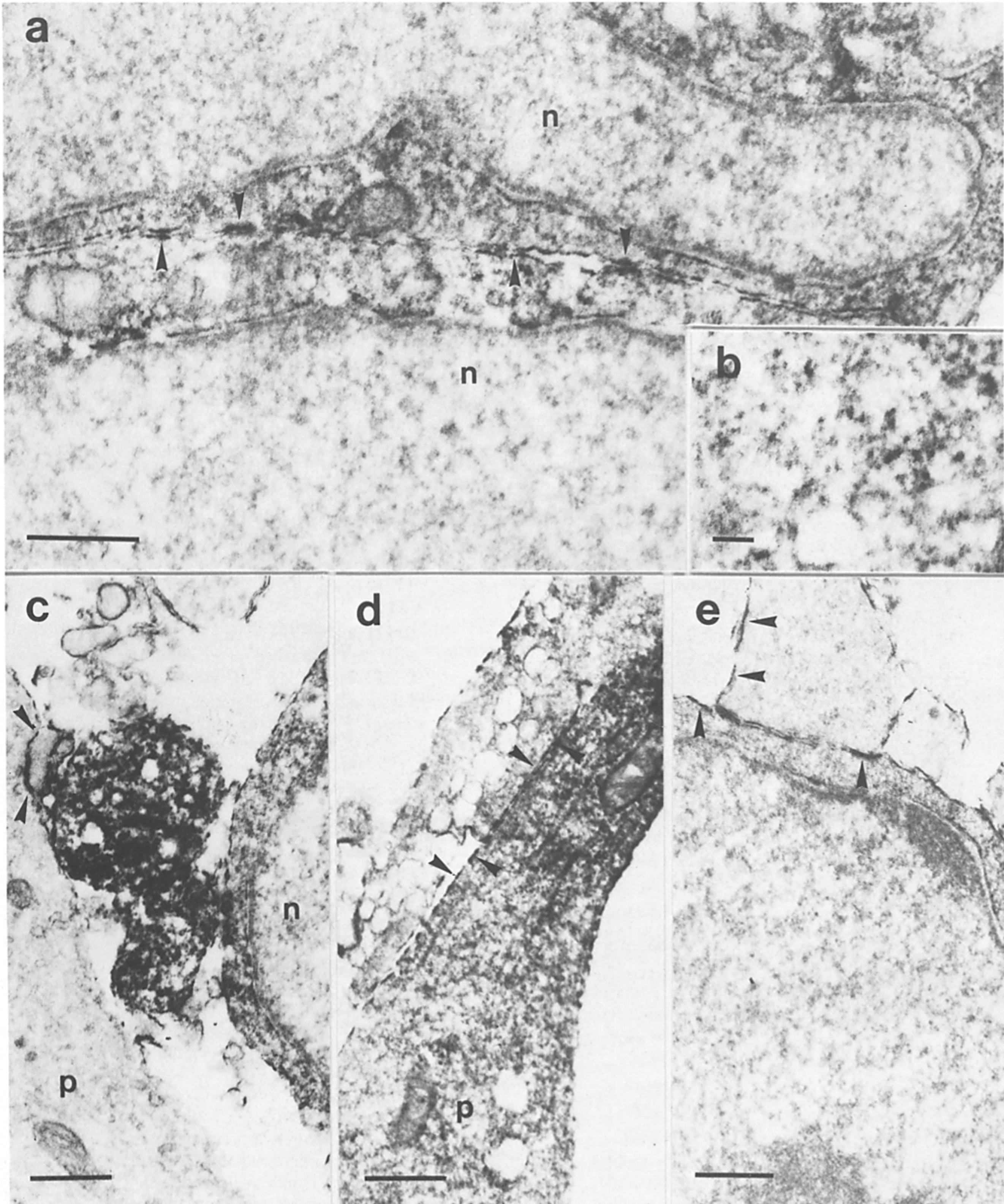

Figure 7. Immunoelectron microscopy of cell bodies and processes of rat brain cells. Cells were dispersed from 17–19-d-old rat embryos, allowed to adhere to poly-L-lysine–coated glass coverslips, and prepared for electron microscopy as described in Materials and Methods. Affinity-purified anti–p30 antibodies (1:10) were used for staining. *n*, nucleus. (*a*) Two cells stained at their plasma membranes (*arrowheads*) and moderately in cytoplasmic structures. (*b*) A higher magnification of the areas of cytoplasmic immunoreaction shows aggregation of the reaction product in polyribosomes. (*c*) Cross sections of two heavily stained processes making contact with a cellular process and an immunoreactive cell. The process (*p*) has only plasma membrane immunoreaction near the contact area (*arrowheads*). (*d*) A longitudinal section of a process (*p*) that displays immunoreaction both in cytoplasmic structures and at the plasma membrane (*arrowheads*). (*e*) A cellular process in contact with a cell. Both are only stained at their plasma membranes (*arrowheads*). Note the intense staining in several areas of membrane–membrane contact in *c–e*. Bars: (*a* and *c–e*) 500 nm; (*b*) 100 nm.

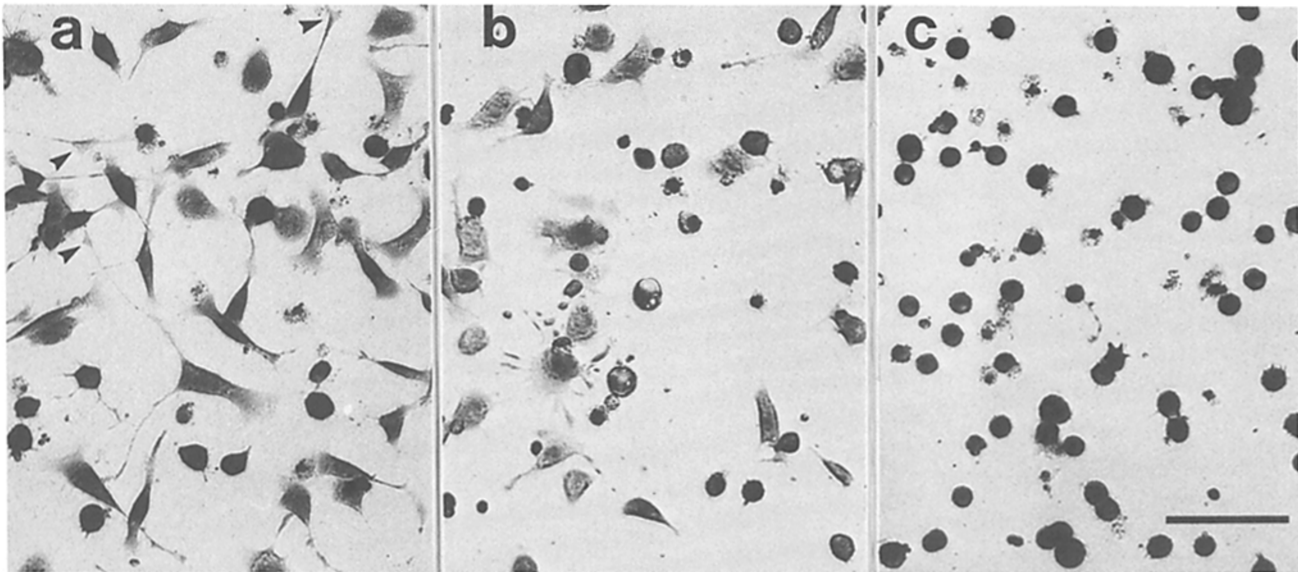

Figure 8. Neurite outgrowth in N18 neuroblastoma cells on a surface coated with 10 μg/ml of the affinity-purified antipeptide antibody. The cells were incubated for 3 h at 37°C on the antibody-coated surfaces (67,000 cells/cm²) in the absence (*a*) or presence (*b*) of 400 μM synthetic peptide, fixed with 2% glutaraldehyde and stained with 0.1% toluidine blue. The HPLC-purified peptide II (Table I) was dissolved in PBS and passed through a Sephadex G-25 column in PBS before it was diluted in the assay medium. It was allowed to bind to the antibody-coated surface for 1 h before the cells were added. The antibody coating was omitted in *c*; other conditions were as in *a*. Flattened growth cones are seen in several cells in *a* (*arrowheads*). Bar, 100 μm.

antibody is immobilized on the substrate, the neuroblastoma cells respond in a dramatic way extending rapidly neurites that end in flattened growth cones (Figs. 8 and 9). Taken together, these data suggest that the antipeptide antibody binds to an adhesive molecule of the cell surface that is involved in neurite growth.

Culturing of embryonic brain cells for a few days results in a characteristic structure, in which the neurons and the aggregates of neurons are connected to each other with neurites

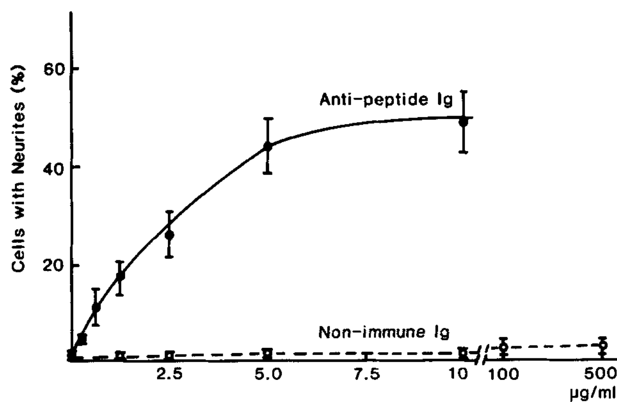

Figure 9. Dose-response curves showing neurite outgrowth in N18 neuroblastoma cells on surfaces coated with the affinity-purified antipeptide antibody or the nonimmune immunoglobulin of similar purity. The cells were incubated for 3 h at 37°C on the different surfaces (67,000 cells/cm²) and fixed with glutaraldehyde. The proportion of cells having one or more neurites >10 μm long was calculated for each concentration of the immunoglobulins. The results are expressed as the average value ±SD from six microscopic fields. Each microscopic field contained on the average 158 cells.

and bundles of neurites, as has been shown before (Yavin and Yavin, 1974). In this kind of mixed cultures neurons are frequently located on top of a cell monolayer consisting mainly of astrocytes (on the basis of staining with antiglial fibrillary acidic protein). Staining of the mixed brain cell cultures with the antipeptide antibody detects p30 mainly in neurons (Fig. 5). As in the neuroblastoma cells, p30 is found both in the cell bodies and the neurites. The varicosities are clearly stained in the neurites (Fig. 5, *a–b*). Immunoelectron microscopy suggests that p30 is clearly stained in the contact-forming areas of the processes (Fig. 7; see below). Thus, the localization of p30 in neurons would appear to be analogous to that seen in the neuroblastoma cells.

Two groups of stained cells are discerned by the anti-p30 antibody used in immunoelectron microscopy. In one cell type the staining is restricted to the plasma membrane, especially in the areas of membrane–membrane contact, whereas in the other cell type the staining is seen in addition in intracellular structures (Fig. 7). Immunofluorescence staining of live cells with the same antibody (the anti-p30 staining shown for suspended cells in Fig. 6) supports the surface localization seen in electron microscopy. The intracellular stained structures would appear to be ribosomes and intracellular membranes in cells containing neurotubule-like structures. In the cells expressing neurites both the surfaces of neurites and the intracellular membrane structures of neurites are clearly stained (Fig. 7, *c–e*). It would thus appear that p30 is synthesized in neurons and transported in the growing neurite together with other newly synthesized membrane structures, which are known to be transported within the neurite to the growth cone where the transported vesicles fuse with the plasma membrane. However, the site of p30 synthesis in different cell types in brain remains to be elucidated with in situ hybridization experiments.

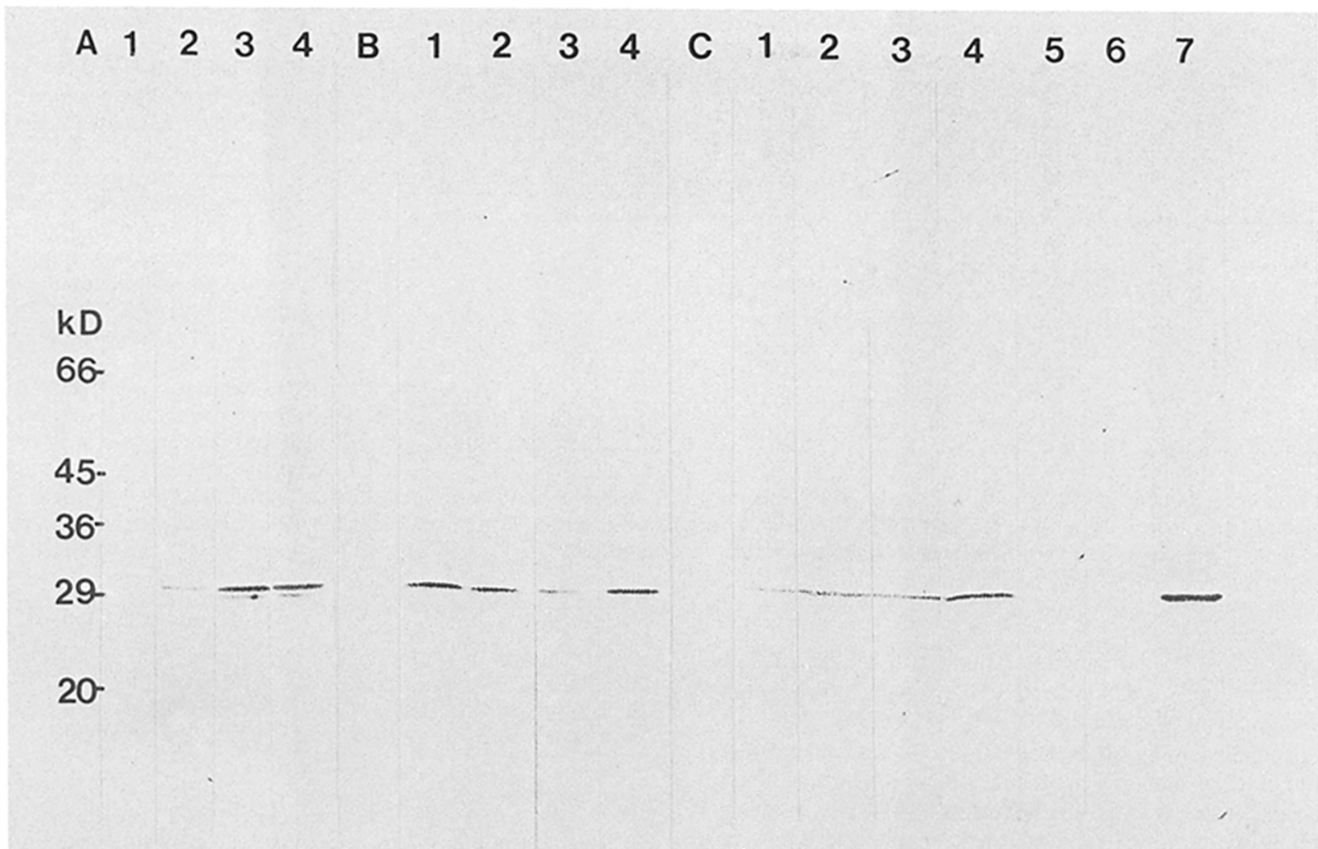

Figure 10. Immunoblots showing the extraction of p30 from suspended brain cells by heparin and by low molecular weight heparin (mol wt of 4,000–6,000). The brain cells were extracted for 15 min at room temperature under different conditions, centrifuged, and the supernatants were separated on 5–20% gradient SDS-PAGE, transferred to nitrocellulose, and analyzed for binding of anti–p30 or antipeptide antibodies. Each lane corresponds to an extract from 1×10^6 cells. (*A*) Adherent brain cells cultured overnight on poly-L-lysine–coated tissue culture dishes were dispersed in Ca- and Mg-free PBS (see Materials and Methods) and washed in PBS. The suspended cells were extracted in PBS (lane *1*), 1 mM EDTA in PBS (lane *2*), 1 mg/ml heparin (Sigma Chemical Co.) in PBS (lane *3*), or in the reducing SDS gel sample buffer (lane *4*); 1 mM PMSF was added to all extraction buffers from a 100-mM solution in ethanol. Detection, anti–p30 (1:100). (*B*) The extractions were carried out essentially as in *A*, but the cells dispersed from 17–19-d-old rat brain hemispheres were only allowed to recover for 1 h in suspension before they were extracted. The extractions were carried out in PBS containing 0.1 mg/ml of BSA without PMSF. Heparin was tested at 1 mg/ml (lane *1*), 0.25 mg/ml (lane *2*), and 0.1 mg/ml (lane *3*); the concentration of low molecular weight heparin (mol wt of 4,000–6,000; Sigma Chemical Co.) was 0.1 mg/ml (lane *4*). Detection, antipeptide (0.25 μg/ml). (*C*) Extraction of brain cells dispersed from adherent cultures (the cells were cultured for 2 d after they had been dispersed from brain) with different polyanions (Sigma Chemical Co.) at 4 mg/ml. Dextran sulfates with mol wt of 5,000 (lane *1*), 8,000 (lane *2*), and 500,000 (lane *3*) are compared to heparin (lane *4*), chondroitin sulfate from whale cartilage (lane *5*), and colominic acid (lane *6*). Immunoblotting of the purified p30 (1.45 μg) is shown as a standard (lane *7*). Detection, anti–p30 (1:100).

The antipeptide antibody immobilized on microtiter wells strongly enhances neurite growth in the neuroblastoma cells (Figs. 8 and 9) and to a lower extent in the brain cells. This finding is consistent with the immunofluorescence localization of p30 in the neuroblastoma cells (Fig. 4, *a* and *b*), and further indicates that the lysine-rich amino-terminal sequence of p30 is surface exposed and available for adhesive interactions in living adhering cells. The data do not exclude the possibility that an antibody directed to another structure occurring at the surface of neurons and their growth cones would also enhance neurite growth. It is, however, noteworthy that tens of different cell-binding surfaces have been previously studied using this assay (Rauvala, 1984; Rauvala et al., 1987). Of these surfaces only laminin has a comparable neurite-promoting effect on the neuroblastoma cells.

Fig. 11 envisions the effects of p30 (*top*) and of the antipeptide antibody that binds to the amino-terminal sequence of p30 (*bottom*). It is noteowrthy that p30 stays at the cell surface after the cells have been dispersed using Ca- and Mg-free PBS (Fig. 6). It was previously suggested that p30 is either an integral membrane component or associated to an integral membrane component (Rauvala and Pihlaskari, 1987). The latter possibility is depicted in Fig. 11 because p30 can be displaced from suspended cells by heparin-like structures (Fig. 10). In cell contact with the substrate-bound p30 (Stage I, top of Fig. 11) the putative receptors of p30 are immobilized (Stage II), which initiates neurite growth (Stage III). In the bottom of Fig. 11 the substrate-bound antibody acts through the same receptors by binding to the receptor-associated p30. The extent to which different cells would re-

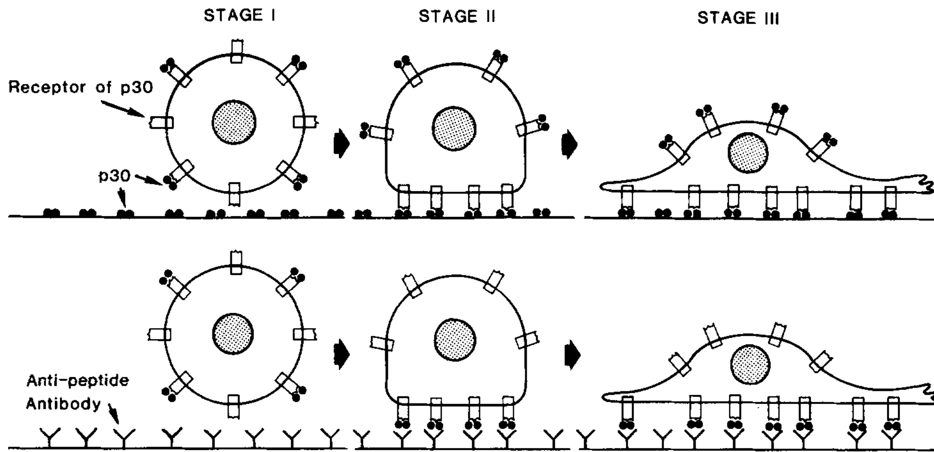

Figure 11. A schematic model for the interaction of neuronal cells with immobilized p30 (*top*) or with the immobilized antibody that binds to the lysine-rich amino-terminal sequence of p30 (*bottom*). Cells dispersed for the assay contain surface-associated p30 (shown in Fig. 6) that binds to an integral membrane component (*receptor of p30*). P30 coated on the culture surface immobilizes the surface receptors (Stage II), which triggers neuritic growth. In this step the substrate-bound p30 binds to free receptors, but it may also bind to p30 associated to the cell surface receptors. This possibility (a homophilic binding mechanism) is suggested by the finding that p30 forms polymeric structures (Rauvala and Pihlaskari, 1987). P30 is depicted as a dimer in the model, although higher polymeric forms may also occur. The immobilized antibody stimulates the cells through the same sequence of components by binding to the cell surface–associated p30.

spond either to the substrate-bound p30 or the substrate-bound antibody might depend, at least in part, on the degree of occupancy of the receptors by p30 (see Fig. 11).

The cell staining experiments (Figs. 4–7), the neurite outgrowth experiments (Figs. 8 and 9), and the heparin extraction experiments (Fig. 10) indicate that p30 and its lysine-rich amino-terminal sequence are associated to the surface of neural cells and are available for adhesive interactions. It is worth noting that synthetic poly-L-lysine has been known for a long time to be essential for the survival of central nervous system neurons in tissue culture (Yavin and Yavin, 1974). The adhesive molecule p30 and its lysine-rich amino-terminal sequence may represent a physiological analogue of poly-L-lysine. We have recently constructed a λgt11 expression library using mRNA of perinatal rat brain and isolated using the antipeptide antibody, a cDNA clone that encodes the amino-terminal part of p30 (Merenmies, J., and H. Rauvala, unpublished results). The deduced amino acid sequence of this clone contains a sequence that exactly matches with the amino-terminal peptide reported in this study. Furthermore, the lysine-rich sequence of p30 contains at least ~60 amino acids in the amino-terminal part of the molecule (lysine plus arginine comprise 25% of the amino acids in this sequence). Elucidation of the whole sequence of p30 is under way, and should give the possibility to study the various peptide sequences of p30 for their effects on neurons.

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Note Added in Proof: After this study was completed, the amino-terminal sequence that we report for p30 was found in a computer search (Swiss-prot protein sequence data bank, May 1988). This sequence and the sequences of the isolated cDNA clones (Merenmies, J., and H. Rauvala, unpublished results) closely correspond to the primary structure of the high mobility group protein 1 (HMG 1) that has been previously studied as a DNA-binding protein. The adhesive and heparin-binding properties of this protein (Figs. 3 and 10) as well as its localization in the extracellular and cytoplasmic compartments (Figs. 4–11) strongly suggest that extranuclear mechanisms should be considered as the basis of the role of p30 in cell growth.

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