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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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 \( \sim 30 \) 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 \( \sim 10 \)-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
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) stained with Coomassic Brilliant Blue.

Amino-Terminal Sequence Analysis

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/mi in the atmosphere of 5% CO2/95% 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 antineuralfilament, antignifiable 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 CaCl2, 0.5 mM MgCl2), 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 cells fixed with methanol at −20°C were used for the staining of permeabilized cells. Antineuralfilament, antignifiable 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 3 min at room temperature, and washed with PBS. The cells were then incubated with 20% normal swine serum (DABOPATTs) in PBS for 20 min at room temperature to block non-specific 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 14C-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 allowed to adhere in microwell plates (Titertek multwell 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 CaCl2, 0.5 mM MgCl2, and 4.5 mg/ml glucose at the cell density of 1.25 × 105/ml (150 μl of cell suspension per well; 67,000 cells/cm²). 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.

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 aminoethyl-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 kDa and a minor band with M_r of ~25 kDa 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](data:image/png;base64,iVBORw0KGgoAAAANSUhEUgAAAAEAAAABCAQAAAC1HAwCAAAAC0lEQVR42mP8/w0AwEAAAAASUVORK5CYII=)
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...
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 Fab 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 heparin-like 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

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**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/cm²) 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 14C-amino acid label from the solubilized cells. The error bars indicate the range of duplicate determinations.
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
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 colomnic 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-
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.

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 neuronal 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.
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 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.
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 noteworthy 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-
poly-L-lysine. We have recently constructed a Lgtll expression experiments (Fig. 10) indicate that p30 and its lysine-rich amino-terminal sequence may represent a physiological analogue of poly-L-lysine. We have recently constructed a lgtl1 expression library using mRNA of perinatal rat brain and isolated using the antigiprotein 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. We thank M. Baumann and P. Mäkinen for help in peptide synthesis in the Department of Biochemistry, University of Helsinki. We are grateful to K. Salmela for excellent technical assistance. This study was supported by grants from the Academy of Finland and the Sigrid Jusélius Foundation. Received for publication 12 January 1988, and in revised form 9 August 1988.

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.

References
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