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An 18-kd heparin-binding protein of developing brain that is distinct from fibroblast growth factors

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An 18-kd heparin-binding protein (p18) was isolated from perinatal rat brain. Although the protein closely resembles the fibroblast growth factors in its strong binding to heparin and in its apparent molecular mass, it has a distinct structure. This was concluded from the amino-terminal sequence analysis that identified a unique structure containing a cluster of lysine residues. Anti-peptide antibodies were raised in rabbits according to the sequence analysis and affinity purified using a synthetic peptide. The antibodies were shown to bind specifically to p18, which was immunochemically distinct from the basic fibroblast growth factor. The antipeptide antibodies detected p18 in brain but not in liver, kidney, heart or skeletal muscle. The content of the protein was shown to undergo a remarkable developmental change corresponding to the time period of rapid sprouting of axons and dendrites in brain. The content of p18 was rapidly increased at the time of birth until the postnatal age of ~1 week, after which it was decreased to values <10% in young adults as compared to the content found in perinatal rats. p18 also enhanced neurite outgrowth in brain neurons *in vitro*. The protein was stained in neurons in cells dispersed from perinatal brain. The properties of p18 suggest that it has a role in the growth and maturation of brain.

Key words: development of brain/growth factors/heparin-binding

Introduction

Although development is a continuous process, various phases can be discerned in the growth and maturation of an organ. In cerebral development of the rat the multiplication of neuronal and glial precursors continues until birth (Jacobson, 1978). After this phase extensive sprouting of axons and dendrites occurs until about the 10th postnatal day, during which time neuronal connections begin to be formed (Jacobson, 1978). The concentrations of neuronal constituents, such as the gangliosides, rapidly increase during this phase (Vanier *et al.*, 1971). The neuronal connections develop further and myelination starts during 1-2 weeks after the perinatal growth phase.

Many proteins that appear to have regulatory functions in development through some form of extracellular signalling, such as adhesive or growth factor effects, bind to heparin. Most notably, the basic and acidic fibroblast growth factors bind strongly to heparin (Klagsbrun and Shing, 1985). These types of growth factors are often isolated from brain (Gospodarowicz *et al.*, 1978), and have been suggested to play a role in the development of brain cells (Schubert *et al.*, 1987; Unsicker *et al.*, 1987).

Because the heparin-binding property is often associated with growth-regulating molecules, we have started the fractionation of heparin-binding proteins that can be solubilized from rat brain at the stage of rapid perinatal development (Rauvala and Pihlaskari, 1987). A 30-kd component (p30) was isolated in these studies as a major neuron-binding protein, and was suggested to have a regulatory role in neuronal growth (Rauvala and Pihlaskari, 1987; Rauvala *et al.*, 1988). p30 is detached from heparin-Sepharose at 0.75-1.0 M NaCl in salt gradients. Another component that is eluted clearly later from heparin-Sepharose in salt gradients was observed in these studies. This protein has properties that are characteristic of the basic fibroblast growth factor (bFGF), namely, the 18-kd molecular weight and strong binding to heparin that is reversed only at 1-1.5 M NaCl (Klagsbrun and Shing, 1985). However, the 18-kd protein (p18) isolated from the octyl glucoside-solubilized perinatal brain was observed to possess a unique amino-terminal sequence against which antibodies could be raised easily in rabbits with the aid of a synthetic peptide. Immunochemical studies using the antisynthetic peptide and anti-bFGF antibodies also indicated that p18 is a distinct molecule. Studies reported in this paper suggest that p18 is also similar to the FGFs in that it is involved in the regulation of cell growth. This inference is based on the observed effects of p18 on brain neurons *in vitro* and on the finding that the occurrence of p18 in brain *in vivo* correlates with the perinatal developmental phase of rat brain characterized by rapid outgrowth of axons and dendrites.

Results

Elution of p18 in a salt gradient on heparin-Sepharose is shown in Figure 1A. It was consistently observed that the protein elutes as the last component at 1.1-1.4 M NaCl (maximal elution at 1.2-1.3 M NaCl), when a linear gradient of 0.05-2.1 M NaCl at pH 8.5 was used. p18 also bound strongly to Affi-Gel blue, and was eluted at 1.5-1.8 M NaCl in a linear gradient of 0.05-2.0 M NaCl at pH 8.5. Analysis on 5-20% SDS-PAGE under reducing or non-reducing conditions indicated that p18 is clearly the major component eluting at 1.2-1.3 M NaCl from heparin-Sepharose, and an apparently pure protein was isolated from early postnatal rat brain by combining the chromatographies on heparin-Sepharose and Affi-Gel blue (Figure 1B).

The amounts of p18 recovered in different isolations varied, obviously depending on the age of the rats used. In an isolation with heparin-Sepharose from 7- to 10-day-old rats, 12 μg of p18 was obtained per 1 g of wet brain tissue.

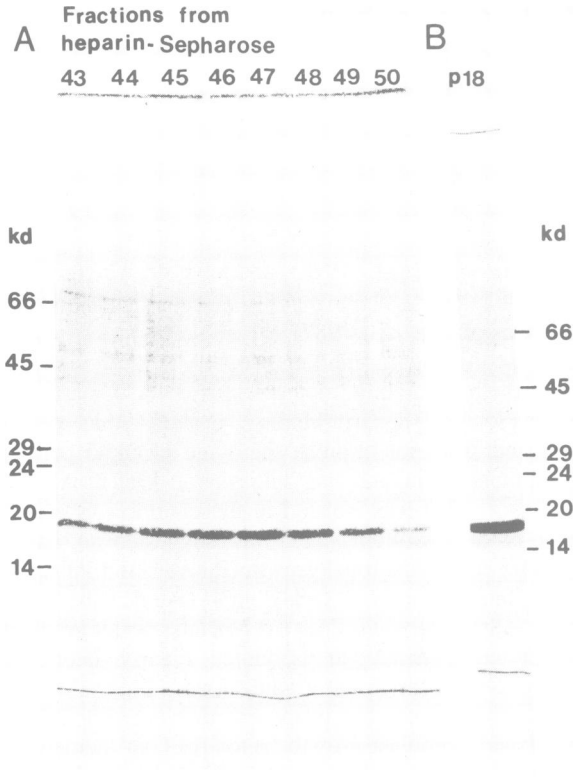

Fig. 1. Isolation of p18. (**A**) Brains from 7- to 10-day-old postnatal rats (45 g of wet tissue corresponding to 55 brains) were used to prepare octyl glucoside-solubilized proteins that were fractionated using a salt gradient on heparin–Sepharose. The total volume of the gradient was 200 ml (from 0.05 to 2.1 M NaCl in 0.5 mM octyl glucoside, 50 mM Tris, pH 8.5), and 2.5-ml fractions were collected. Samples of 45 μl were analysed under reducing conditions on 5–20% gradient SDS–PAGE. The gel was stained with Coomassie brilliant blue. Fraction 43 corresponds to 1.15 M NaCl and fraction 50 to 1.34 M NaCl. (**B**) The fractions from heparin–Sepharose that contain p18 as the major protein were pooled, dialysed and fractionated on Affi-Gel blue. Fractions eluting at 1.5–1.8 M NaCl were pooled, dialysed and p18 was concentrated using heparin–Sepharose (eluted by 1.5 M NaCl in 50 mM Tris, pH 8.5). A sample containing 1.7 μg protein was analysed on SDS–PAGE as in (A).

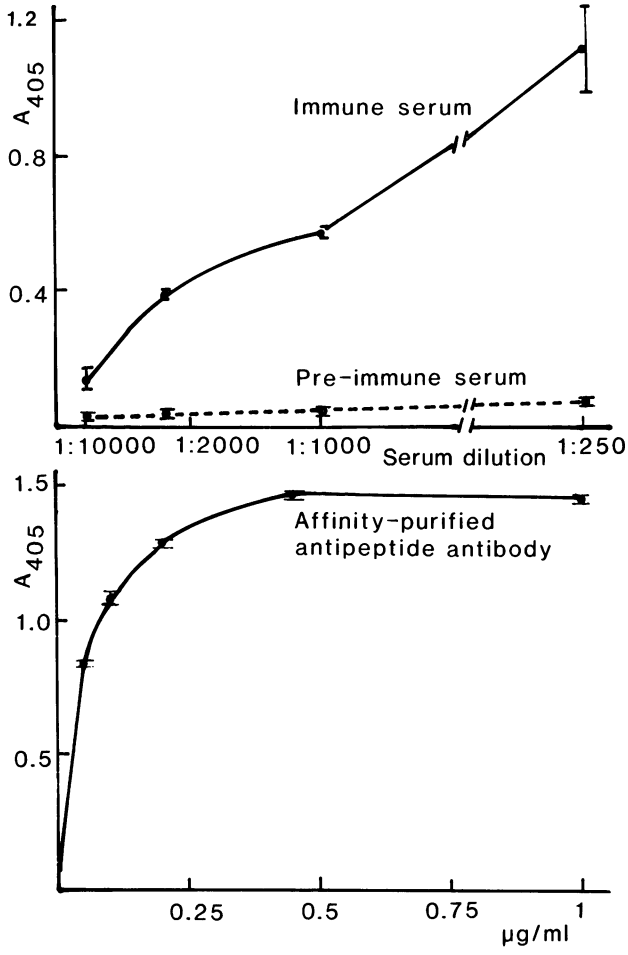

Fig. 2. Binding of antisynthetic peptide antibodies to p18 in ELISA assays. **Upper panel**, the rabbit was immunized with the synthetic peptide coupled to keyhole limpet hemocyanin. The immune serum was taken after the rabbit had received the primary immunization and two booster injections (27 days after the primary immunization). The sera were tested for antibodies that bind to microtitre wells coated with 0.2 μg of purified p18 (see Figure 1B). **Lower panel**, the antipeptide antibodies were purified from the immune serum using the synthetic peptide coupled to thiopropyl–Sepharose. Binding of the affinity-purified antibodies to p18 was assayed as in the upper panel.

This amount agrees with the estimations based on Western blotting of SDS-solubilized rat brains (see below).

The fractions from heparin–Sepharose that contain p18 as the major protein (Figure 1A) were pooled, dialysed and concentrated by rechromatography on heparin–Sepharose (eluted by 1.5 M NaCl). The p18 band was transferred from an SDS gel to a polyvinylidine difluoride membrane (Moos *et al.*, 1988), and analysed by automated Edman degradations. The analysis identified a single amino-terminal sequence that contains seven lysine residues within the first 14 amino acids: Gly-Lys-Lys-Glu-Lys-Pro-Glu-Lys-Lys-Val-Lys-Lys-Ser-Asp.

A synthetic peptide containing the 14-amino-acid sequence (see above) followed by a cysteinamide residue (the SH group was used for coupling purposes) was used to raise antibodies in rabbits. Production of antibodies was observed in two out of two rabbits within 4 weeks, when the sera (diluted 1:250) were tested by Western blotting using the purified p18 as the antigen. The presence of antibodies in the immune serum (at dilutions up to 1:10 000) was also indicated by ELISA assays, in which the purified p18 (Figure 1B) coated to microtitre wells was used as the antigen (Figure 2). The antibodies were purified using the synthetic peptide coupled to thiopropyl–Sepharose. The purified antibodies also bound to p18 both in ELISA assays (Figure 2) and in Western blotting (Figure 3). Samples containing 0.1 μg of p18 could be clearly detected with 0.25 μg/ml of the antibodies in Western blotting. The antibodies did not detect the basic FGF in Western blotting, and polyclonal anti-bFGF antibodies did not detect p18 (shown for 0.5-μg samples of the bFGF and p18 in Figure 3).

Isoelectric focusing of p18 was carried out in polyacrylamide gels. The proteins were then electroblotted to nitrocellulose filters in an SDS-containing buffer and stained for protein with Ponceau S or immunostained with the antipeptide antibodies. Figure 4 shows that the isoelectric point of p18 is close to neutrality as compared to standard proteins analysed in parallel with p18.

Occurrence of p18 in a few tissues of the rat was tested by Western blotting of extracts, from which p18 had been enriched with heparin–Sepharose. Figure 5A shows that p18 was only detected in brain. Figure 5B shows that p18

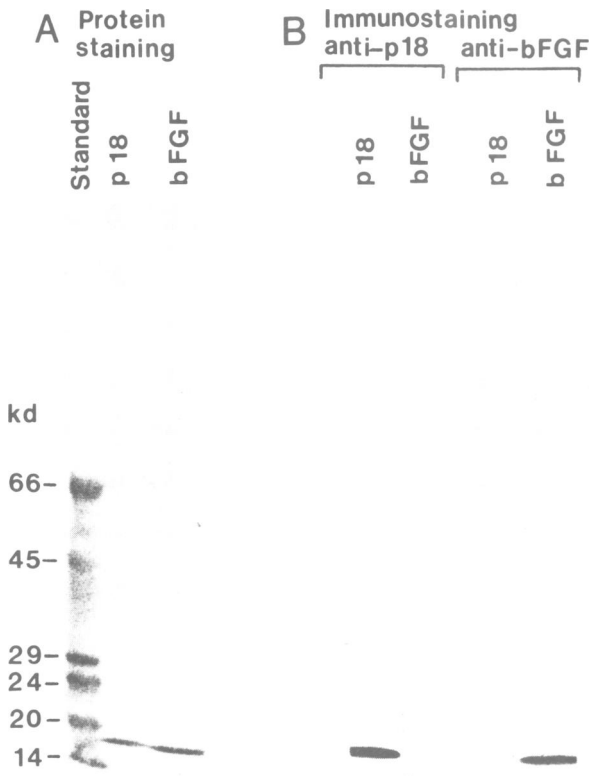

Fig. 3. Demonstration by Western blotting that p18 and bFGF are immunochemically distinct molecules. (**A**) Samples of p18 and bFGF were run on 5–20% gradient SDS–PAGE (0.5 μg each protein; reducing conditions). Proteins were transferred to nitrocellulose and stained with Ponceau S. Both proteins have the apparent molecular mass of ~18 kd. (**B**) Staining of the filter with the affinity-purified antibodies (0.25 μg/ml) to the synthetic amino-terminal sequence of p18 detects p18 but not the bFGF. (**C**) Staining of the filter with affinity-purified anti-bFGF (0.25 μg/ml) detects the bFGF but not p18. Transfer of p18 and bFGF (0.5 μg each) from the gel to the nitrocellulose filter was controlled by protein staining (as in A) before the immunostaining procedures.

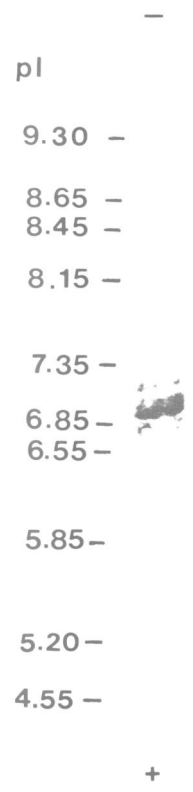

Fig. 4. Isoelectric focusing of p18. A 3.7-μg sample of p18 was run on an ampholine gel, transferred to nitrocellulose in the presence of SDS and stained with the affinity-purified antipeptide antibodies (0.25 μg/ml). The pI values refer to the migration of standard proteins that were analysed in parallel with p18. The centre of the major p18 band corresponds to the pI value of 7.1.

was also specifically detected in early postnatal brain by Western blotting of brain samples solubilized directly in hot SDS. Binding of the antibodies to the 18-kd band in crude extracts was inhibited by the synthetic peptide (Figure 5B).

Figure 6 demonstrates the occurrence of p18 in a few groups of rat brains of different ages. p18 was specifically stained in the samples (Figure 6, upper panel). A developmental regulation of the content of p18 is evident both in peroxidase staining of the immunoblots (Figure 6, upper panel) and in the binding of [^{125}I]protein A to the nitrocellulose strips incubated with the antipeptide antibodies (Figure 6, lower panel). The content of p18 is strongly increased at the time of birth and for a few days after birth. It is then clearly decreased at the age of 14–19 days after birth, and is just detectable in young adults (56 days after birth). Analysis of SDS-solubilized samples from early postnatal rats (the postnatal ages of 0, 1, 2, 3, 4, 6, 8, 9, 10, 11, 14, 17, 18 and 20 days) agreed with the analysis given in Figure 6 (not shown). The content of p18 in 7- to 10-day-old rats was estimated to be 10–15 μg/1 g of wet tissue on the basis of the protein A binding assay (see Materials and methods) that was used for the analysis of brain samples solubilized in SDS (see the text of Figure 5B). Western blotting experiments indicated that the content of p18 is about the same in cerebrum and cerebellum. The major proportion of brain p18 thus comes from cerebrum. For example, in 5- and 7-day-old rats the proportion of cerebral p18 was 80.4 and 84.8% respectively (determined with the antipeptide antibodies and protein A).

p18 clearly enhanced neurite outgrowth of perinatal rat brain neurons in a 20-h assay *in vitro* (Table I). This effect was observed both in tissue culture wells and in ELISA wells. In these assays p18 appears to function as a surface-bound molecule. At least the amino-terminal part of the surface-bound p18 is exposed in the ELISA wells, because it is detected by the antisynthetic peptide antibodies (Figure 2). The neurite-promoting effect was clearly observed at 4–63 ng of p18/well (0.08–1.2 μg/ml used for coating; Table I). Although some process-containing cells were also observed in blanks treated with BSA alone (Table I), the cells did not develop a neuronal morphology on such surfaces (Figure 7). A similar neurite-promoting effect of p18 was also observed in assays with N18 neuroblastoma cells (not shown).

The antipeptide antibodies were used to stain cells dispersed from perinatal brains. p18 was stained in the cell bodies and the neurites of neuron-like cells (Figure 8B) that were also stained with monoclonal anti-neurofilament antibodies (Figure 8A). Astrocytes identified by staining with monoclonal anti-glial fibrillary acid protein (Figure 8C) were

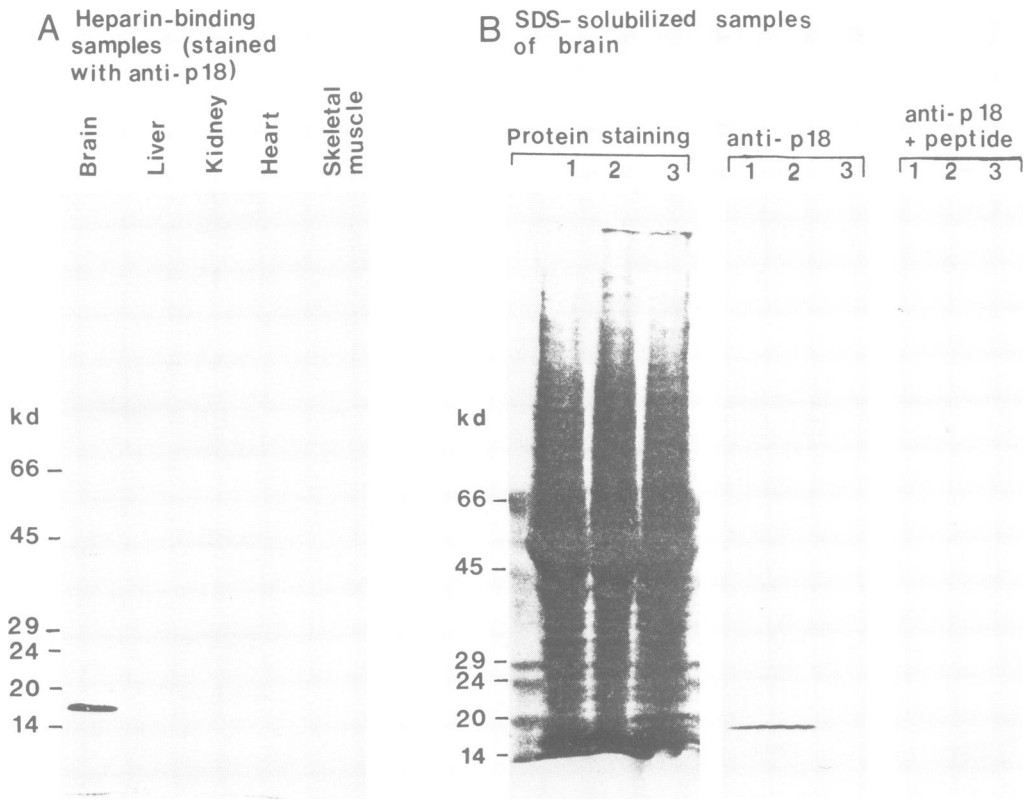

Fig. 5. Occurrence of p18 in different tissues. (**A**) p18 was extracted from 0.9 g tissue samples of 14-day-old rats, enriched with heparin–Sepharose (eluted with 1.5 M NaCl) and analysed by Western blotting (as in Figure 3). All lanes correspond to 18 mg of wet tissue. The antipeptide antibodies detect p18 only in brain. (**B**) Pieces of minced brain were solubilized in reducing SDS sample buffer at 100°C for 10 min and analysed by Western blotting (as in Figure 3). A 4-mg sample of SDS-solubilized brain was loaded per lane. **Lanes 1**, newborn rats (0-day postnatal age); **2**, 9-day-old rats; **3**, 20-day-old rats. The nitrocellulose filters were stained for protein with Ponceau S that was eluted off, and the filters were then immunostained. Immunostaining with the anti-p18 (the same filter that is shown for protein staining) clearly detects p18 in newborn and 9-day-old rats (**lanes 1** and **2**) but only faintly in 20-day-old rats (**lane 3**). This immunostaining is clearly inhibited by 1 μM synthetic peptide.

not stained by the anti-peptide antibodies (Figure 8D). Double-immunofluorescence staining was also carried out by using monoclonal anti-vimentin and anti-cytokeratin antibodies, but these did not show a correlation with the p18 staining (not shown). The staining of the neurons was also observed with the antipeptide immune serum (diluted 1:1000) but not by the pre-immune serum. The neuron staining was strongly reduced if the antibodies were adsorbed with the synthetic peptide–Sepharose (Figure 8E and F), or if the primary antibodies were omitted from the staining protocol. Specificity of the antibodies was also suggested by the finding that p18 was specifically detected by Western blotting of complex protein mixtures from perinatal brain (Figure 5B). Treatment of the fixed cells with acetone, methanol or Triton X-100 clearly enhanced the staining with the antipeptide antibodies (not shown). However, it did not reveal location of p18 in other parts of the cell as compared to the staining pattern shown (Figure 8B, E).

Discussion

An 18-kd heparin-binding protein was isolated from perinatal rat brain. The isolated p18 may not be a subunit of a more complex protein because SDS–PAGE detects it as an apparently pure polypeptide after isolation under non-denaturing conditions (Figure 1). This inference includes the assumption that the 18-kd polypeptide is not dissociated from a more complex protein under the conditions, including the high salt concentrations, used in the isolations. p18 does not either appear to be an artefactual proteolytic product from a larger polypeptide because it is detected as the 18-kd species in immunoblotting of heavily loaded samples of brain solubilized directly in a hot, SDS-containing buffer (Figure 5B).

p18 closely resembles the fibroblast growth factors in its strong binding to heparin and in its molecular mass (Figures 1 and 3). The salt concentrations required to elute p18 from heparin–Sepharose (1.1–1.4 M NaCl at pH 8.5) are very close to those (1.0 and 1.4–1.6 M NaCl) reported for the brain FGFs (Klagsbrun and Shing, 1985). The reason why p18 has not been previously reported in studies on the brain FGFs may be differences in the solubilization and isolation procedures (Gospodarowicz *et al.*, 1978) as compared to the present study, or to a rather short time period when p18 can be found in brain (Figure 6).

Despite the similarities of p18 as compared to the FGFs, it is clearly a distinct molecule. The isoelectric point of p18 appears to be close to neutrality (Figure 4), whereas acidic (pI ~5) and basic (pI ~8) values have been reported for the brain-derived FGFs (Klagsbrun and Shing, 1985). Although this difference could be due to post-translational modifications, the polypeptide structure of p18 is different from that of the acidic and basic FGFs. The amino-terminal sequence of p18 is not similar to sequences found in the FGFs or related sequences described recently (Esch *et al.*, 1985; Klagsbrun *et al.*, 1987; Yoshida *et al.*, 1987;

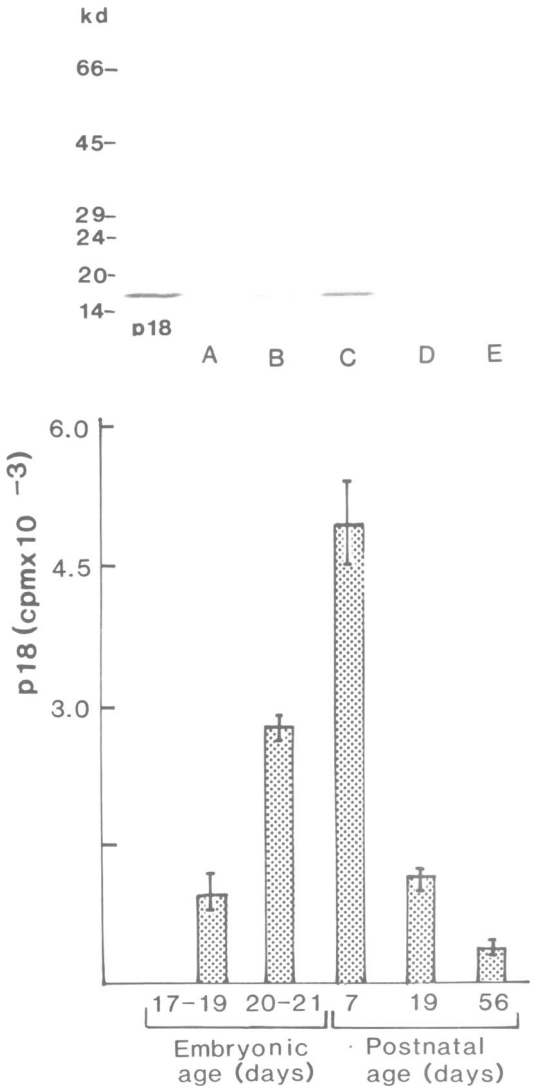

Fig. 6. Occurrence of p18 in the brains of rats of different age groups. **Upper panel**, Western blotting (see Figure 3) of brain samples. The minced brain tissue (0.9 g) was extracted with octyl glucoside, and p18 was enriched from the extracts with heparin–Sepharose (eluted with 1.5 M NaCl). Samples that correspond to 27 mg of wet brain tissue from the ages A–E (the ages are given in the lower panel) were loaded per lane. A 1.2-μg sample of p18 was analysed as a standard. p18 is clearly detected in embryos close to birth (B) but only faintly in the group of embryos 2–4 days before birth (A). A strong detection is seen in 7-day-old postnatal brains (C) but only a faint detection in 19- and 56-day-old postnatal brains (D–E; see also Figure 5B). **Lower panel**, quantification of p18 with the aid of the antipeptide antibodies and [^{125}I]protein A (see Materials and methods and Figure 9) gives a similar developmental profile as compared to the immunostaining (upper panel). The bars indicate the range from triplicate determinations.

Table I. Effect of p18 on neurite outgrowth of rat brain neurons

p18 (ng/well)	% of cells with processes
63	53 ± 7.9
16	28 ± 4.9
4	13 ± 6.9
0	3.7 ± 2.6

P18 was coated in a 50 μl volume to microtitre ELISA wells for 2 h at 37°C, after which the wells were washed and incubated with 1 mg/ml of BSA in the DMEM assay medium for 1 h at 37°C. The wells were then assayed with rat brain neurons at the density of 2.5×10^5 cells/ml in the final volume of 200 μl in the serum-free DMEM containing 1 mg/ml BSA. The cells were fixed after 20 h with 2% glutaraldehyde and stained with toluidine blue. The percentages of cells having one or more processes >10 μm were then estimated. The values are given as the averages ± SD from seven random microscopic fields (on average 39 cells per microscopic field).

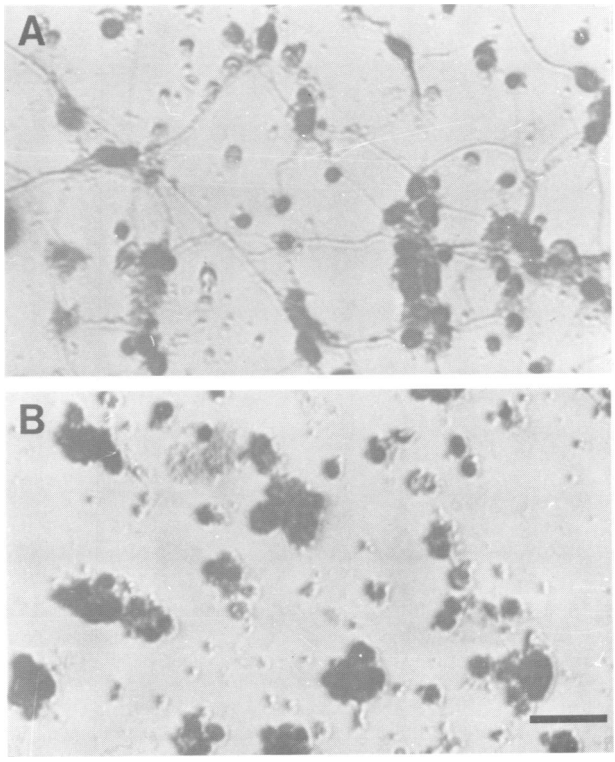

Fig. 7. Neurite outgrowth in rat brain neurons in the presence (**A**) or absence (**B**) of p18. p18 (32 ng in 50 μl) was coated to microtitre wells, which were then washed and treated with 1 mg/ml of BSA. The blank wells were incubated without p18 and treated with 1 mg/ml of BSA. The assay with rat brain neurons (2.5×10^5 cells/ml in the final volume of 200 μl) was carried out as explained in Table I. Scale bar, 50 μm.

Moscatelli *et al.*, 1987; Sommer *et al.*, 1987; Bovi *et al.*, 1987; Zhan *et al.*, 1988). Neither was it found in computer searches of protein sequences (the Swiss-prot protein sequence databank, the NBRF protein libraries). Furthermore, antibodies raised against the synthetic amino-terminal sequence bind specifically to p18 (Figures 2, 3 and 5) but not to the bFGF (Figure 3). Polyclonal anti-bFGF antibodies bind to the FGF but not to p18 (Figure 3), also indicating a marked difference in the structures. The experiments reported in this paper do not, however, rule out the possibility that the p18 sequence would have some similarity as compared to the structures of the FGF family. Further studies are in progress to resolve this question and whether the p18 structure is similar to any neuronal growth-associated molecule characterized before (Berg, 1984; Thoenen and Edgar, 1985) or to some other previously studied protein sequence.

The properties of p18 are similar to the p30 isolated previously from perinatal rat brain (Rauvala and Pihlaskari, 1987). This protein has been more recently called as 'amphoterin' due to its dual structure (a cluster of cationic

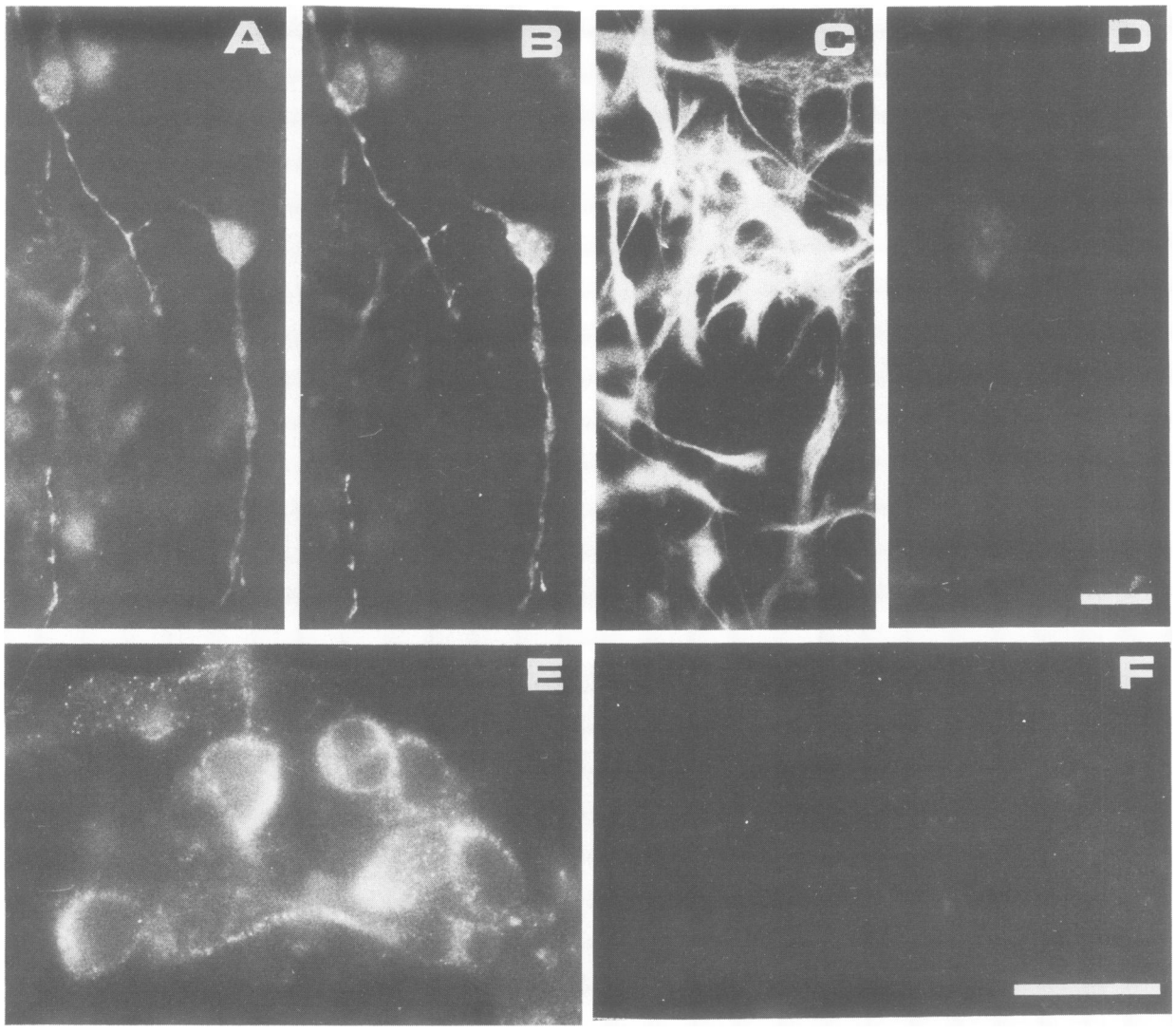

Fig. 8. Immunofluorescence staining of p18 in cells dispersed from rat brain. (**A**–**D**) Double-immunofluorescence staining of rat brain cells cultured for 7 days and fixed with paraformaldehyde. (**A**) Monoclonal anti-neurofilament (1:50) detected with TRITC-conjugated anti-mouse Ig. (**B**) The same field stained with affinity-purified antipeptide antibodies (10 μg/ml). The antipeptide antibodies were detected with FITC-conjugated anti-rabbit Ig (1:50). p18 is stained both in the cell bodies and the processes of neurons. (**C**) Monoclonal anti-glial fibrillary acid protein (1:50). (**D**) p18 (stained as in B) is not stained in the astrocytes (the same field as in C). (**E**–**F**) Binding of the neuron-staining antibodies by the synthetic peptide. The antipeptide antibodies (2 ml, 10 μg/ml in PBS containing 10 mg/ml BSA) were passed through a 250-μl thiopropyl–Sepharose column that had been pre-treated with pre-immune serum (**E**) or through the synthetic peptide/thiopropyl–Sepharose column pre-treated wtih pre-immune serum (**F**). Staining of brain cells cultured for 2 days and fixed with paraformaldehyde–glutaraldehyde (followed by reduction) shows that the neuron-staining antibodies are bound by the synthetic peptide. At the resolution used in this experiment (E–F) the staining pattern is mainly granular. Bars, 25 μm.

residues at the amino-terminal sequence and a cluster of anionic residues at the carboxy-terminal sequence; Rauvala *et al.*, 1988; J.Merenmies *et al.*, in preparation). p18 also has a cluster of lysine residues at its amino-terminal sequence, although this sequence is distinct from that of the amphoterin. The carboxy-terminal sequence of p18 has not yet been analysed. Both amphoterin and p18 can be solubilized with a neutral detergent but also with salt extraction. We have previously suggested that amphoterin can associate to membrane structures, but is not an integral membrane component (Rauvala *et al.*, 1988). The properties of p18 are consistent with a similar type of association to the cell. In the isolation procedures we have preferred detergent extraction over salt extractions, because some other charged molecules expected to jeopardize the isolations, like the histones, are not solubilized in the method used. The affinity properties of amphoterin and p18 are also similar: both proteins bind strongly to heparin–Sepharose and to Affi-Gel blue. p18 binds somewhat more strongly to both matrices as compared to amphoterin.

Both amphoterin (Rauvala *et al.*, 1988) and p18 (Figure 8) are mainly stained in neurons in cells dispersed from perinatal rat brain. We have previously detected amphoterin in the cytoplasm and at the plasma membranes of neuronal cells (Rauvala *et al.*, 1988). p18 is also stained in the cytoplasm of the neurons (Figure 8). A granular type staining shown for p18 can be also demonstrated for amphoterin (unpublished data).

The content of both amphoterin and p18 is developmentally regulated in brain. The expression of amphoterin precedes that of p18, which is consistent with early phases of process outgrowth or other neuronal growth phenomena

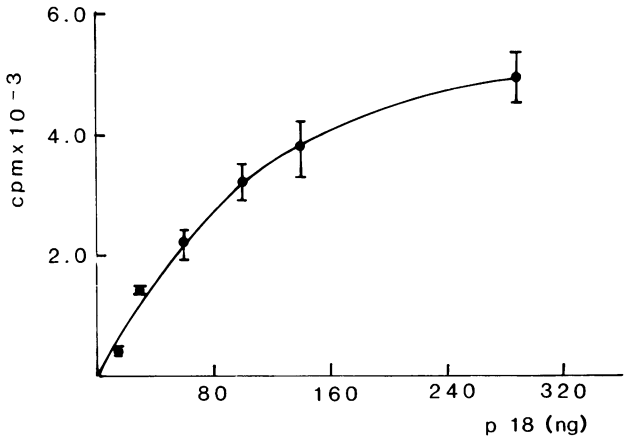

Fig. 9. Dose–response curve showing the binding protein A to p18-antipeptide antibody complexes. The amounts of p18 refer to samples loaded per lane in 5–20% reducing SDS–PAGE. Binding of [^{125}I]protein A to the antibodies was measured as described in Materials and methods. The experiment shows that the method can be used to estimate the amounts of p18. The bars indicate the range of duplicate determinations.

suggested earlier for amphoterin (Rauvala and Pihlaskari, 1987). Amphoterin was previously shown to enhance process outgrowth of central neurons *in vitro* (Rauvala and Pihlaskari, 1987). A similar effect was also observed for p18 (Figure 7, Table I). This effect could be due to lysine cluster sequences of the proteins. Synthetic poly-L-lysines are generally used to enhance differentiation and survival of brain neurons in tissue culture (Yavin and Yavin, 1974). The effects of amphoterin and p18 *in vitro* would thus appear to be adhesive in nature, although the possibility that the proteins would be taken into the cells during the assays has not been rigorously excluded. It has been also shown that the bFGF is able to enhance neurite outgrowth after it has been adsorbed to the culture surface (Schubert *et al.*, 1987; Unsicker *et al.*, 1987).

The occurrence of p18 in different tissues and cells is clearly more limited as compared to amphoterin. Thus, in 14-day-old rats, p18 was found in brain but not in liver, kidney, heart or femoral muscle (Figure 5A). In contrast, amphoterin was found in all these tissues when the same extracts were immunoblotted using anti-amphoterin antibodies (J.Merenmies *et al.*, in preparation). The content of amphoterin appeared to be higher in brain and kidney as compared to the other tissues studied. Amphoterin thus appears to be a rather generally occurring molecule, whereas p18 might represent a specifically neural variant on the same theme.

The time period of the rapid p18 increase in rat brain (Figures 5B and 6), from birth until postnatal days 7–10, correlates to the rapid growth spurt of brain, when axons and dendrites grow out and glial growth is initiated (Jacobson, 1978). The content in 7- to 10-day old rats is ~10 μg/1 g of wet tissue, which corresponds to a ratio of ~1:10 000 as compared to total protein. The respective ratio is 1:100 000 or less in 56-day-old rats. This suggests that p18 is a regulatory rather than a structural component. The developmental profile shown for p18 (Figure 6) mainly reflects cerebral development, because ~80% of the brain p18 is found in the cerebrum. The content of p18 is similar in cerebellum as compared to that in cerebrum. This finding and the immunofluorescence staining of dissociated brain cells suggest that p18 is a generally occurring constituent in neurons of the central nervous system at the developmental stage described. However, further studies are clearly warranted to define more accurately the occurrence of p18 in different parts of the developing brain. It has not yet been studied whether p18 also occurs in peripheral neurons.

p18 appears to be a neuronal growth-associated molecule, but its mechanism of action is not yet known. The granular type of staining in the cytoplasm of developing neurons (Figure 7) suggests that the protein is a secretory component of neural cells. This would be consistent with some form of extracellular signalling in developing brain, such as an autocrine or paracrine growth-promoting effect on developing axonal processes.

Materials and methods

Isolation of p18

Brains from Wistar rats were minced, washed and homogenized as described (Rauvala and Pihlaskari, 1987). The samples were centrifuged at 100 000 *g* for 1 h in 1 mg/ml of BSA in 10 mM Tris, pH 7.4, and the pellets were solubilized in 50 mM octyl glucoside, 50 mM NaCl, 50 mM Tris, 1 mM phenylmethylsulphonyl fluoride, pH 8.5. p18 could be also solubilized with 0.5–1 M NaCl, but detergent extraction was routinely used in order to reduce solubilization of proteins expected to interfere with the subsequent isolation steps. The solubilized samples were centrifuged at 100 000 *g* for 1 h, and the supernatants were adsorbed to heparin–Sepharose (Pharmacia, Sweden) that was washed and then eluted with 0.05–2.1 M NaCl at pH 8.5 as described (Rauvala and Pihlaskari, 1987). Fractions containing p18 as the major protein were pooled, dialysed and fractionated on Affi-Gel blue (Bio-Rad, 100–200 mesh) using a linear gradient of 0.05–2 M NaCl at pH 8.5. p18 was concentrated by adsorption to heparin–Sepharose that was washed with 100 mM NaCl and 250 mM NaCl, and then eluted with 1.5 M NaCl in 50 mM Tris, pH 8.5. Protein of the isolated fractions was measured with Coomassie brilliant blue G-250 (Bradford, 1976) using a commercially available assay (Bio-Rad).

Sequencing of immobilized proteins

The fractions containing p18 in the salt gradient from heparin–Sepharose (Figure 1) were pooled, dialysed and concentrated on heparin–Sepharose (eluted with 1.5 M NaCl). The concentration of NaCl was diluted to 0.75 M with water, and the samples were separated with a neutral SDS–PAGE system essentially as described (Moos *et al.*, 1988). The samples were incubated with 1 vol of the sample digestion buffer (2% SDS, 2% 2-mercaptoethanol, 20% sucrose, 0.5% bromophenol blue) at 100°C for 3 min and run on neutral gels at 8–10°C in the presence of 0.1 mM sodium thioglycolate. Electroblotting to polyvinylidine difluoride membranes (Immobilon^{TM}, Millipore) was carried out in 10 mM CAPS, 10% methanol, pH 9.0 (Moos *et al.*, 1988). The protein bands on the filter were stained with Coomassie brilliant blue R-250, destained with 50% methanol and excised for sequencing. About 300–400 pmol of p18 on the filter was used for sequencing. A pulsed liquid-phase sequencer (model 477A, Applied Biosystems), modified to be used both as a liquid-phase and a gas-phase sequencer (M.Baumann, in preparation), was used. The samples on the polyvinylidene difluoride membrane were analysed using the gas-phase modification of the automated sequencer on line with a PTH-amino acid analyser (model 120A, Applied Biosystems).

Production and purification of antipeptide antibodies

The peptide that corresponds to the 14-amino-acid amino-terminal sequence of p18 followed by a cysteinamide residue (Gly-Lys-Lys-Glu-Lys-Pro-Glu-Lys-Lys-Val-Lys-Lys-Ser-Asp-Cysamide) was purchased from Multiple Peptide Systems (San Diego, CA). The sequence of the peptide was confirmed by the pulsed liquid-phase sequencer (see above). The peptide (12.5 mg) was coupled through the cysteinamide residue to *m*-bromosuccinimide-treated keyhole limpet haemocyanin (10 mg; Sigma) as described (Green *et al.*, 1982). Primary immunization was carried out by multiple s.c. injections with ~1 mg of the peptide–haemocyanin complex in Freund's complete adjuvant. Booster injections in incomplete Freund's adjuvant were given 2 and 3 weeks after the primary immunization and at 2- to 3-week intervals after that. A 10-mg sample of the peptide was

coupled through the thiol groups in the cysteinamide residue to 1.5 ml of swollen activated Thiol–Sepharose 4B (Pharmacia, Sweden) according to the manufacturer's instructions. Non-specific binding sites of the affinity column were blocked by pre-immune serum of the rabbit. The immune serum (10 ml/1.5 ml of the gel) was diluted by 1 vol of 1 M NaCl to inhibit non-specific binding of proteins to the lysine-rich peptide, and the antibodies were affinity purified as described previously (Rauvala *et al.*, 1988).

Electrophoretic methods

SDS–PAGE was carried out as described (Laemmli, 1970). The kd values refer to the following standard proteins (Sigma): bovine serum albumin (66 kd), ovalbumin (45 kd), carbonic anhydrase (20 kd), trypsinogen (24 kd), soybean trypsin inhibitor (20 kd) and α-lactalbumin (14 kd). For Western blotting the samples were separated on 5–20% gradient SDS–PAGE and transferred to nitrocellulose (Towbin *et al.*, 1979) in a buffer containing 0.2% SDS, 20% methanol, 5 mM Tris, 192 mM glycine, pH 8.3. The transfers were carried out in a Bio-Rad Trans-Blot cell at 100 V for 2 h on an ice bath. The nitrocellulose sheets were stained with Ponceau S to control the transfer of proteins and to locate the markers. The nitrocellulose strips were washed with 0.5 M NaCl, 20 mM Tris, pH 7.5, and then incubated overnight in 10 mg/ml of BSA in the NaCl–Tris solution to remove the protein stain and to block non-specific binding sites. The filters were washed with 0.05% Tween-20 in 0.5 M NaCl, 20 mM Tris, pH 7.5, and incubated with the antibodies in the Tween-20–NaCl–Tris buffer for 3 h at room temperature. The rabbit antibodies to bFGF and a recombinant human bFGF that were compared to the antipeptide antibodies and p18 in the immunoblotting were kindly provided by Dr Olli Saksela (Department of Virology, University of Helsinki) and Dr Andreas Sommer (Synergen Inc., Denver, CO) respectively. The bound antibodies were detected with horseradish peroxidase-conjugated anti-rabbit IgG which was stained with 4-chloro-1-naphthol (Bio-Rad).

For quantitative determination of p18, immunoblotting was carried out as described above. The areas corresponding to p18 (~15–20 kd) were cut off after the Ponceau staining and treated with BSA as in the Western blotting. The nitrocellulose pieces were washed and incubated with the antibodies and protein A at room temperature using constant shaking. Washing was carried out with 0.05% Tween-20 in 0.5 M NaCl, 20 mM Tris, pH 7.5, three times for 5 min. Incubation with 2 μg/ml of the affinity-purified antipeptide antibodies was for 3 h, and the washing was as above. The nitrocellulose pieces were then incubated with 250 μl of [^{125}I]protein A (Amersham; 100 μCi/ml, diluted 1:400) for 1 h and washed with the Tween-20-containing NaCl–Tris and NaCl-Tris (each three times for 5 min). An analysis of nitrocellulose pieces in a gamma-counter is shown in Figure 9.

Isoelectric focusing was carried out using commercially available ampholine gels (Ampholine® Pagplate, pH 3.5–9.5, Product no. 1804-101; LKB, Sweden) according to the manufacturer's instructions. The gel was separated from the plastic support after isoelectric focusing, and the proteins were transferred at 150 V for 0.5 h in the SDS-containing buffer to nitrocellulose as in the Western blotting. Ponceau S staining and immunostaining were as in the immunoblotting procedure (see above). The pI values refer to the following standard proteins (Pharmacia, Sweden): trypsinogen (9.30), lentil lectin (8.65, 8.45 and 8.15), horse myoglobin (7.35 and 6.85), human carbonic anhydrase B (6.55), bovine carbonic anhydrase B (5.85), β-lactoglobulin (5.20) and soybean trypsin inhibitor (4.55). Location of the markers was based on the staining of the gels with Coomassie blue R-250 and on Ponceau S staining of the proteins transferred to nitrocellulose filters.

ELISA assays

For ELISA assays (Engvall, 1980) p18 was coated to microtitre wells (Titertek® multiwell plates; Flow Laboratories, Inc.) overnight at +4°C. The plates were post-coated with 10 mg/ml of BSA and incubated with the sera or purified antibodies for 1 h at 37°C. The bound antibodies were assayed with the aid of alkaline phosphatase-conjugated anti-rabbit IgG (Sigma). Alkaline phosphatase was determined with paranitrophenyl phosphate that was measured in the microtitre wells.

Neurite outgrowth assays

The neurite outgrowth assays were carried out in ELISA microtitre wells (see above) essentially as described (Rauvala and Pihlaskari, 1987). The cells were dispersed from cerebral hemispheres of 17- to 19-day-old rat embryos in 10% fetal calf serum in a DMEM medium. Cells containing mainly neurons (75–80%) were prepared for the assays as described (Rauvala and Pihlaskari, 1987). All neurite outgrowth assays were carried out in a serum-free DMEM medium containing 1 mg/ml of BSA at 37°C in an atmosphere of 5% CO_2–95% air.

Fluorescence microscopy

Brain cells from 17- to 19-day-old rat embryos were dispersed and allowed to adhere to poly-L-lysine-treated glass coverslips for immunostaining (Rauvala and Pihlaskari, 1987). Monoclonal anti-neurofilament was from Sera-Lab (Sussex, UK), and monoclonal anti-glial fibrillary acid protein, anti-cytokeratin and anti-vimentin were from Labsystems (Helsinki, Finland). The antipeptide antibodies that bind to p18 were detected with FITC-conjugated swine anti-rabbit Ig, and the monoclonal antibodies were detected with TRITC-conjugated rabbit anti-mouse Ig (Dako, Copenhagen, Denmark). In double-immunofluorescence staining the cells were fixed with 3% paraformaldehyde in PBS, pH 7, for 10 min at room temperature. The cells were treated with 10 mg/ml of BSA in PBS for 30 min at room temperature and then incubated for 30 min with the antipeptide antibodies followed by the FITC-conjugated anti-rabbit Ig. To enhance the cytoskeletal staining the cells were treated wtih methanol (−20°C, 5 min), incubated with 10 mg/ml of BSA and then with the monoclonal antibodies followed by the TRITC-conjugated anti-mouse Ig. Controls, in which antibodies to p18 or the cytoskeletal proteins were omitted from the staining protocol, indicated that the staining patterns shown are not due to spillover of the FITC or TRITC fluorescence between the different channels. In addition to fixing with paraformaldehyde, the following fixing systems were tested in the staining of p18: 0.4% parabenzoquinone, +4°C, 0.5 h (Pearse and Polak, 1975), 4% paraformaldehyde–0.025% glutaraldehyde at room temperature for 30 min followed by reduction (two times with 1 mg/ml of $NaBH_4$ for 5 min) and the paraformaldehyde–glutaraldehyde reduction followed by acetone (−20°C, 5 min), methanol (−20°C, 5 min) or 0.1% Triton X-100 (room temperature, 5 min). The samples were studied with a Zeiss microscope (model IM 35) equipped for epifluorescence with appropriate filters to detect specifically the FITC or TRITC staining.

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