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'Neuron-specific' protein gene product 9.5 (PGP 9.5) is also expressed in glioma cell lines and its expression depends on cellular growth state

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Protein gene product 9.5 (PGP 9.5), which in the normal nervous system is restricted to certain neurons, has been detected in two glioma cell lines, rat C6 and human GL15, by immunoblotting and immunocytochemistry. Its expression in these cells depends on the cellular growth state, being maximal between the first and second post-plating day. Only a faint PGP 9.5 immunoreactivity can be observed in glioma cells after the eleventh post-plating day, i.e. about one week after confluency has been reached. The present results suggest that PGP 9.5 in cultured glial cells is maximally expressed during the growth phase and that the protein could play a role during brain development in glial cells, in reactive gliosis, or in tumorigenesis of the glial lineage.

Protein gene product 9.5 (PGP 9.5); Glial cell; Immunoblotting; Immunocytochemistry

1. INTRODUCTION

Protein gene product 9.5 (PGP 9.5) is a 26 kDa acidic protein originally purified from human brain [1] and shown to be a marker of certain neurons in the central nervous system [1]. Subsequent studies demonstrated that this protein is also found in axons in the peripheral nervous system [2–6], in a variety of neuroendocrine cells [2,7], in adrenal medulla [8], in human skin cells [5], and in neuroectodermal tumors [9,10]. The analysis of the primary structure of PGP 9.5 and of its cDNA [11] revealed that this protein is highly homologous to ubiquitin carboxyl-hydrolase isozymes [12], and that calf brain PGP 9.5 has ubiquitin carboxyl-terminal hydroxylase activity [12], suggesting that PGP 9.5 might be a member of a multigene family of related isozymes. The mammalian brain is by far the richest source of PGP 9.5 [13]. No evidence for the presence of PGP 9.5 in glial cells in the nervous tissue has been presented thus far by either light [1] or electron [4] immunocytochemistry.

We show here that 2 glioma cell lines, rat C6 and human GL15, express PGP 9.5 and that the expression of PGP 9.5 in these glioma cells depends on the cellular growth state.

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2. MATERIALS AND METHODS

2.1. *Purification of PGP 9.5*

All operations were done at 4°C. Frozen bovine brain tissue (350 g) was homogenized in 5 vols. of buffer A 10 mM Tris-HCl, pH 7.5, 5 mM 2-mercaptoethanol, 1 mM EDTA) and centrifuged at 13 000 × *g* for 1 h. The supernatant was brought stepwise to 100% saturation with solid $(NH_4)_2SO_4$ [14]. Proteins which precipitated between 80 and 100% saturation with $(NH_4)_2SO_4$ were resuspended in 60 ml of buffer A and dialyzed against 5 l of buffer A for 1.5 days with 3 changes of the dialysis buffer. The dialysate was loaded onto a column of DEAE Sephacel (cm 1.5 × 30) equilibrated with buffer A. The column was washed with 250 ml of buffer A and developed with a nine-chamber gradient of NaCl and pH [15]. Fractions of 7.5 ml were collected. The protein peak corresponding to fractions 62–70 was concentrated and loaded onto a column of Sephacryl S-200 (cm 2 × 90) equilibrated with buffer A for final purification of PGP 9.5. See section 3 for further details.

2.2. *Immunochemical analyses*

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10% acrylamide) was done as in [16]. Gels were stained with Coomassie blue. Electrophoretically pure PGP 9.5 was used to raise antisera in rabbits by multisite injections exactly as in [17]. The specificity of antisera was assessed by immunoblotting by the method in [18].

2.3. *Immunocytochemical analyses*

C6 or GL15 [19] glioma cells plated on glass coverslips in tissues culture cluster plates at a cell density of 5×10^4 cells/well were cultured in DMEM supplemented with 20% heat-inactivated calf serum and 100 iu penicillin and 0.1 mg streptomycin/ml. The culture medium was changed every other day. At intervals, cells were washed free of the culture medium with 20 mM Tris-HCl, pH 7.5, 0.9% (w/v) NaCl (TBS) and fixed with 4% (w/v) paraformaldehyde in TBS for 20 min at room temperature. After several washings with TBS, cells were permeabilized with 0.1% (v/v) Triton X-100 in TBS for 30 min at room temperature, and washed with TBS. Before immunocytochemistry, fixed cells

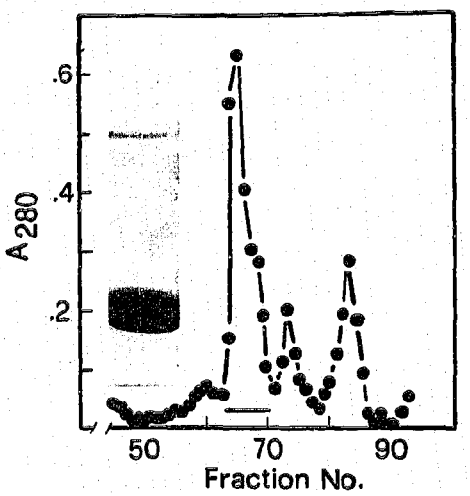

Fig. 1. Purification of bovine brain PGP 9.5. DEAE Sephacryl chromatography of proteins that precipitated between 80 and 100% saturation with $(NH_4)_2SO_4$. The column was developed with a nine-chamber gradient of NaCl and pH [15]. Fractions 62–70 (underlined) contained a 26 kDa polypeptide in large amounts plus a ~70 kDa contaminant, as investigated by SDS-PAGE (inset).

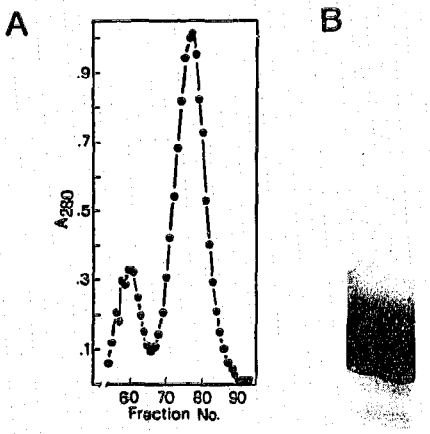

Fig. 2. Purification of bovine brain PGP 9.5. (A) Sephacryl S-200 chromatography of fractions 62–70 from the DEAE Sephacel chromatography illustrated in Fig. 1. The major protein peak contained the 26 kDa polypeptide in pure form. (B) SDS-PAGE of fraction No. 76 of the chromatogram in (A). In those cases where still-contaminated 26 kDa protein was recovered after chromatography on Sephacryl S-200, final purification was accomplished by chromatography of the major protein peak in (A) on DEAE Sephacel (cm 1 × 5) in buffer A with elution by means of a 0–0.25 M gradient of NaCl in buffer A (not shown).

were treated with 3% (v/v) H_2O_2 in methanol for 15 min at room temperature to block any endogenous peroxidase activity, and washed extensively with TBS. Cells were then incubated with 3% (w/v) bovine serum albumin (BSA) in TBS for 3 h at room temperature, followed by the rabbit anti-PGP 9.5 antiserum (1:50 in 1% BSA in TBS) at 4°C overnight. This step was followed by sequential incubations of cells with a sheep-anti-rabbit IgG antiserum (1:40 in 1% BSA in TBS) and with a rabbit peroxidase–anti-peroxidase complex (1:100 in 1% BSA in TBS), each for 1 h at room temperature. Washing of cells after each step was done with 0.1% Triton X-100 in TBS (twice for 5 min) followed by TBS (twice for 5 min). Cells were finally incubated with 0.3 mg of diaminebenzidine/ml of 50 mM Tris-HCl, pH 7.5, containing 0.02% H_2O_2, in the dark for 20 min. Cells were washed with TBS and mounted in permanent aqueous mounting medium on glass slides for light microscopy. In control experiments, the anti-PGP 9.5 anti-serum was omitted, replaced by the pre-immune antiserum, or substituted for by anti-PGP 9.5 antiserum previously absorbed with PGP 9.5. Identical results were obtained irrespective of the procedure used, i.e. no immune reaction product could be seen (not shown).

3. RESULTS AND DISCUSSION

By DEAE chromatography of brain proteins that precipitated between 80 and 100% saturation with $(NH_4)_2SO_4$, several protein peaks could be resolved (Fig. 1). The protein peak corresponding to fractions 62–70 contained a 26 kDa polypeptide in large amounts plus a contaminant of ~70 kDa (Fig. 1, inset), as investigated by SDS-PAGE. This protein peak was concentrated and chromatographed on Sephacryl S-200. Two major protein peaks were obtained (Fig. 2A), the second of which contained the 26 kDa polypeptide in pure form (Fig. 2B). The chromatographic behavior of the 26 kDa polypeptide indicated that the purified polypeptide was a monomeric protein. An antiserum raised against the 26 kDa protein proved specific to this protein by immunoblotting (Fig. 3). This antiserum decorated cell bodies and the dendritic tree of Purkinje cells in rat cerebellum and no other elements in the cerebellar cortex [20], axons in the cerebellar white matter (not shown), and rat cerebellar Purkinje cells and

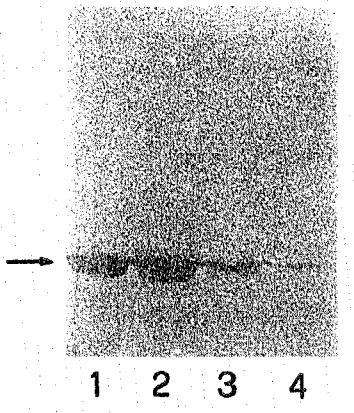

Fig. 3. Immunochemical characterization of PGP 9.5. Rat brain PGP 9.5 (lane 1), bovine brain PGP 9.5 (lane 2), SDS-extracts from GL15 (lane 3) and C6 (lane 4) glioma cells were subjected to SDS-PAGE and electroblotted onto nitrocellulose paper for immunostaining with the anti-26 kDa (PGP 9.5) antiserum (1:1000). The arrow points to the position of PGP 9.5. Note that the antiserum is specific to the protein. GL15 or C6 glioma cells grown on 35-mm plastic petri dishes (2×10^5 cells/dish) were cultivated for 2 days, washed free of the culture medium with TBS, and extracted with 0.2 ml of 3% SDS in 50 mM Tris-HCl, pH 7.5, for 15 min at room temperature. 2-Mercaptoethanol was then added to a final concentration of 5% before boiling for 5 min and electrophoresis.

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26 kDa protein   MET GLN LEU LYS PRO MET GLU ISO ASP PRO GLU
                                              10
PGP 9.5

26 kDa protein   MET LEU ASN LYS VAL LEU THR ARG LEU GLY VAL
                                              20
PGP 9.5          MET LEU ASN LYS VAL LEU SER ARG VAL GLY VAL

26 kDa protein   ALA GLY GLN
PGP 9.5          ALA GLY GLN
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Fig. 4. Partial amino acid sequence analysis of the purified 26 kDa protein. Comparison with the amino acid sequence of PGP 9.5 [11]. Aside from a leader sequence of eleven residues, the 26 kDa protein is 86% homologous, in the sequenced portion, to PGP 9.5. The two substitutions are conservative. Identical residues are underlined.

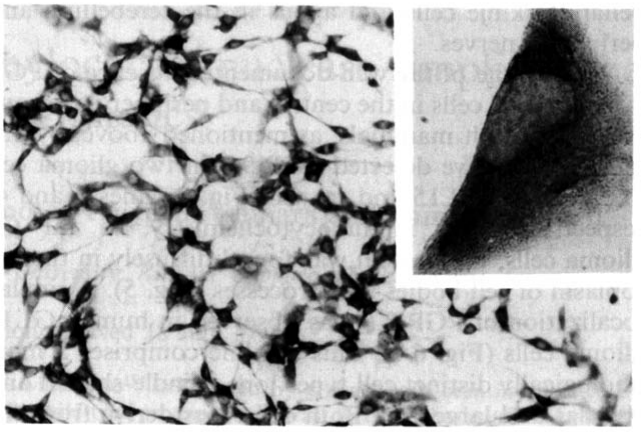

Fig. 5. Immunocytochemical localization of PGP 9.5 in rat C6 glioma cells. At low magnification (×90), C6 cells appeared as spindle-shaped cells displaying immunoreactivity in the cytoplasm of cell bodies and processes. At a higher magnification (×900) (inset) the PGP 9.5 immunoreactivity is clearly seen in the cytoplasm.

axons in rat sciatic nerves by immunocytochemistry at the electron microscope level [4].

Partial amino acid sequence analysis of electrophoretically pure 26 kDa protein (done in the laboratory of Professor Robert H. Kretsinger, Charlottesville, VA) revealed that, aside from a leader sequence of 11 residues, the next 14 residues of the protein were 86% identical to the corresponding sequence of human brain PGP 9.5 [11], with 2 conservative substitutions (Fig. 4).

Three lines of evidence suggested that our 26 kDa protein was the bovine brain form of human PGP 9.5: (i) both proteins were acidic, precipitated between 80 and 100% saturation with $(NH_4)_2SO_4$, and had an M_r of ~26 kDa; (ii) a high sequence homology was observed between the portion of our 26 kDa protein so far sequenced and PGP 9.5; and (iii) an antiserum raised against the bovine brain 26 kDa protein decorated cere-

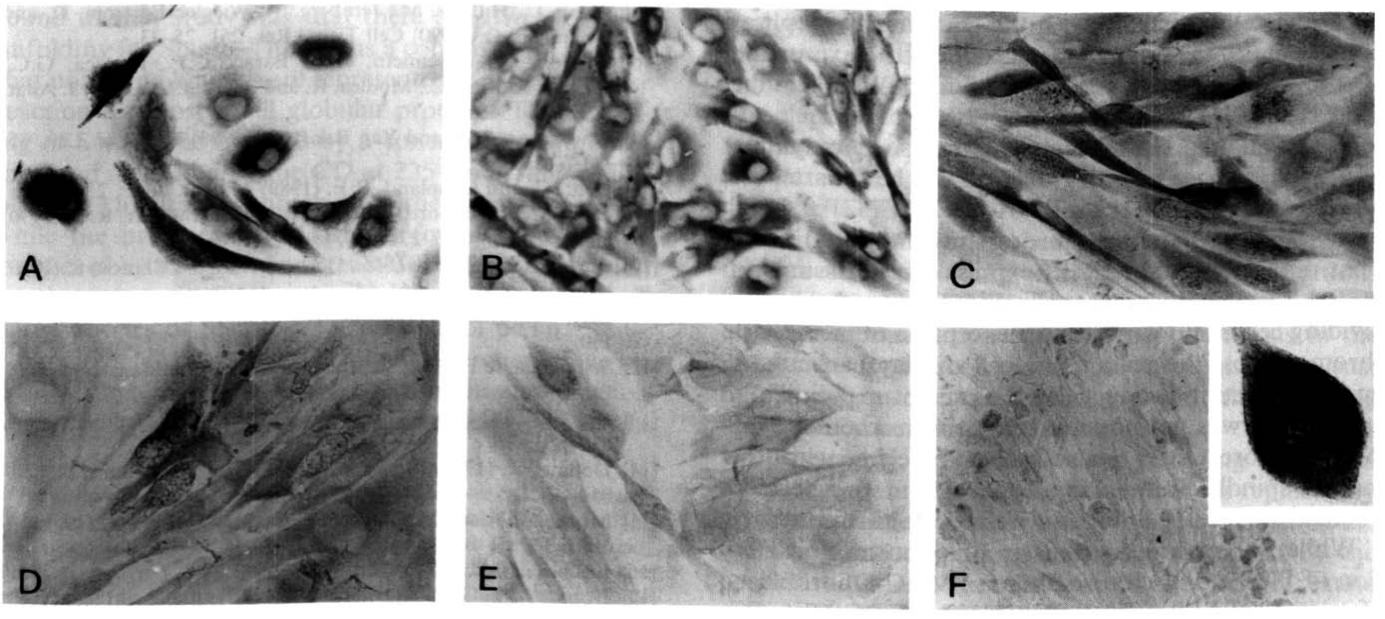

Fig. 6. Immunocytochemical localization of PGP 9.5 in human GL15 glioma cells as a function of cellular growth state. At day 1 after plating (A) both cell types constituting this cell line were immunostained. Generally, the spindle-shaped cells displayed a more intense immunoreactivity than the large and flat ones. Under the present experimental conditions, cells attained confluency between the second (B) and fourth (C) post-plating day. By this time most of cells were spindle-shaped and the immunostaining per cell appeared to decrease. At days 7 (D) and 11 (E) after plating, only a faint immunoreactivity was seen, and an even fainter immunostaining was observed at the eighteenth post-plating day (F). The inset in (F) shows a dividing cell at metaphase displaying an intense immune reaction product in the cytoplasm, excepting the region of metaphase chromosomes. Less intense immunoreactivity seems to be present in the regions of spindle poles. Original magnifications: A–E = ×160; F = ×90; F (inset) = ×400.

bellar Purkinje cells and axons in the cerebellum and peripheral nerves.

Yet, in spite of the well-documented absence of PGP 9.5 from glial cells in the central and peripheral nervous system of adult mammals, as mentioned above, by immunoblotting we detected PGP 9.5 in two glioma cell lines, human GL15 and rat C6 (Fig. 3, lanes 3 and 4, respectively). By immunocytochemistry, in rat C6 glioma cells, the protein was found diffusely in the cytoplasm of cell bodies and processes (Fig. 5). A similar localization of PGP 9.5 was observed in human GL15 glioma cells (Fig. 6A). This cell line comprises 2 morphologically distinct cell types, one spindle-shaped and one flat and large [19]. Both cell types derive from the same parental cell and display the same chromosomal abnormalities [19]. Generally, the spindle-shaped cells appeared more intensely immunostained than the flat and large cells. Following this observation, we investigated the expression of PGP 9.5 in the two cell lines as a function of cellular growth state. Identical results were obtained with either cell line. Specifically, in both C6 cells (not shown) and GL15 cells (Fig. 6 A–F) the maximum of immunostaining/cell was observed between the first and second post-plating day (Fig. 6A,B) under our culture conditions. At the eleventh post-plating day, i.e. about one week after confluency (Fig. 6E), most of cells appeared spindle-shaped and only a faint immunostaining was detected. An even fainter immunostaining was seen at the eighteenth post-plating day (Fig. 6F). Thus, it appears that the maximum of expression of PGP 9.5 in glioma cells coincides with the maximum of replication, whereas as confluency is attained, the expression of the protein progressively declines. Whether the decreased PGP 9.5 immune reaction product in post-confluent glioma cells depends on decrease in its synthesis and/or increase in its degradation will be a matter for future research. Also the mRNA levels of PGP 9.5 will be investigated under the same experimental conditions. That replicating glioma cells express large amounts of PGP 9.5 is also documented in Fig. 6F (inset) where a dividing cell is shown. With the exception of metaphase chromosomes, the rest of the cell displays an intense immune reaction product. By the present experimental approach it was not possible to ascertain whether PGP 9.5 immunoreactivity is also present on elements of the mitotic spindle. Immunocytochemistry at the electron microscope level will hopefully elucidate this point.

While this manuscript was in preparation, localization of PGP 9.5 in cultured human MRC-5 fibroblasts of normal origin was reported [21]. Also, PGP 9.5 was shown to be strongly down-regulated in the SV40-transformed counterparts of these cells [21]. These data were interpreted as suggesting that culturing of fibroblasts might have induced the expression of PGP 9.5 in these cells, since fibroblasts in their normal environment in vivo do not express the protein. Similarly, it is possible that culturing might have induced the expression of PGP 9.5 in a cell type, the glial cell, which does not express the protein in the nervous tissue. Significantly, the maximum of expression of the protein in glioma cells was observed during the proliferation phase, suggesting that PGP 9.5 might be involved in the regulation of as yet unknown activities in these cells during the growth phase. Immunochemical and immunocytochemical analyses of PGP 9.5 during development of the nervous system would shed light on the pattern of expression of this protein in nervous cells, as well as on the possible biological role(s) of this protein. As a member of a family of enzymes with ubiquitin carboxyl-terminal hydroxylase activity [12], PGP 9.5 could play an important role during proliferation of glial cells in the nervous system, during reactive gliosis, and/or in connection with tumorigenesis of the glial lineage.

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