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Plasminogen-binding protein associated with the plasma membrane of cultured embryonic rat neocortical neurons

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To investigate the receptor-like molecule(s) for plasminogen (PGn) on the neuronal surface, the properties of binding of PGn to the plasma membrane of cultured embryonic rat neocortical neurons were investigated. [^{125}I]PGn was found to specifically bind to the plasma membrane depending on the incubation temperature and time. The binding was also affected strongly by ionic strength and slightly by Ca^{2+}. Furthermore, ligand blotting analysis revealed that [^{125}I]PGn binds to a major protein with an apparent molecular weight of 45 kDa among plasma membrane proteins. These results suggest that the 45-kDa protein is a PGn receptor-like molecule on the neuronal surface.

Plasminogen; Plasma membrane; Neuron; Microglia; Receptor

1. INTRODUCTION

Plasminogen (PGn) is an inactive zymogen, which is converted to an active protease, plasmin, by plasminogen activators, and was originally accepted to serve as fibrinolysin [1]. Until recent years, the zymogen was believed to be produced in the liver [2,3]. However, we recently found that PGn is produced in cultured rat microglia [4,5], a type of glial cell in the central nervous system, and secreted extracellularly. Furthermore, we showed that zymogen had potent activity as a neurotrophic factor in vitro [6,7]. PGn promotes neurite outgrowth in neocortical explants [6] and enhances the survival and/or maturation of dopaminergic neurons in mesencephalic neurons [7]. These effects of PGn allow us to speculate that PGn binds to specific receptor-like molecule(s) on the plasma membrane of the neurons and exerts neurotrophic activity. This idea was supported by our previous finding that PGn specifically binds to cultured neurons [6].

In the present studies we have further studied the binding of PGn on the neuronal plasma membrane. The data provide evidence that PGn specifically binds to the neuronal plasma membrane, and that a protein with a molecular mass of 45 kDa, which is associated with the neuronal plasma membrane, is the receptor for binding of PGn.

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2. MATERIALS AND METHODS

2.1. *Materials*

Cytochrome *c* (from horse heart), *P*-nitrophenyl phosphate and 5′-adenosine mono phosphate (5′-AMP) were purchased from Wako Pure Chemical Industries; 6-amino caproic acid, benzamidine and cytochrome *c* oxidase (bovine heart) from Sigma Chemical Co., lysine from Kyowa Hakko Kogyo; glucose-6-phosphate from Nacalai TESQUE; human prothrombin (22.2 U/mg) from Enzyme Research Laboratories; human urokinase (140,000 IU/mg protein) from Mochida Pharmaceutical Co.; Sephadex G-150, Percoll and protein molecular weight markers from Pharmacia LKB; ^{125}I (3.7 GBq/ml) from Amersham Corp. and polyacrylamide gel (10–20% gradient gel) from Daiichi Pure Chemical Co.; X-ray film (XAR5) was supplied by Eastman Kodak Co. Materials and chemicals for neuronal culture were described elsewhere [8,9]. All other reagents were of analytical grade.

2.2. *Neuronal culture*

Neocortical neurons were prepared from 16-day embryonic rat brain as described previously [10]. In brief, the dissociated neurons were cultured in poly-L-lysine-coated plastic culture dishes at a density of $1.5–2.0 \times 10^7/75$ cm^2 for 3 days. The cell purity was over 97%, judged by immunocytochemical study with neurofilament antibody [10].

2.3. *Determination of marker enzyme activity*

The activity of 5′-nucleotidase (EC 3.1.3.5) as a marker for the plasma membrane was determined essentially by the method of Cammer [11]. A reaction mixture containing 100 mM Tris-HCl (pH 7.5), 10 mM $MgSO_4$, 10 mM AMP and a sample (~ 20 μl) solution in a total volume of 100 μl was incubated for 15 min at 37°C. The reaction was stopped by the addition of 100 μl of 20% trichloro acetic acid (TCA), and the unhydrolyzed AMP was precipitated by centrifugation. The amount of phosphate in the supernatant was determined by the method of Chen et al. [12].

As a mitochondrial marker enzyme, cytochrome *c* oxidase (EC 1.9.3.1) activity was measured according to the method of Hodges et al. [13]. The reaction at 25°C was monitored by recording the decrease of absorbance at 550 nm. The enzyme unit was calculated by using

cytochrome *c* oxidase (100 U/7.7 mg of solid) from Sigma, in which one unit was defined as 1 μmol of substrate catalyzed in one minute.

Acid phosphatase (EC 3.1.3.2) activity as a lysosomal marker enzyme was determined by measuring at 405 nm the amount of *P*-nitrophenol released from *P*-nitrophenyl phosphate as substrate [14].

As an endoplasmic reticulum marker enzyme, glucose-6-phosphatase (EC 3.1.3.9) activity was determined according to the method of Heymann et al. [15]. The reaction was stopped by the addition of 0.1 ml of 50% TCA. The liberated phosphate was determined by the method of Chen et al. [12].

The amounts of protein were measured by the method of Lowry et al. [16] by using bovine serum albumin as a standard protein.

2.4. *Iodination of rat PGn*

Rat PGn was isolated from adult rat plasma as described previously [4,5]. The purified rat PGn was iodinated by a slight modification of the chloramine T method [6,17]. The iodinated plasminogen was separated from free ^{125}I by Sephadex G-150 gel filtration with 20 mM Tris-HCl (pH 7.5) buffer containing 100 mM NaCl. The specific activity was 6.7×10^5 dpm/μg of plasminogen. The [^{125}I]PGn showed a single peak at around 90–100 kDa in glycerol gradient centrifugation (2–10% glycerol, 257,600 g_{av}, 6 h, Hitachi PRS 65T) (Fig. 1A), and could be activated by urokinase as shown by casein-urokinase-zymography [4] (Fig. 1B), indicating that [^{125}I]PGn retains the native properties as a zymogen without aggregation or degradation.

2.5. *Cell fractionation*

Three day-cultured neurons were harvested by scraping with a rubber policeman and precipitated by low-speed centrifugation. The collected neurons were homogenized in HO buffer [20 mM Tris-HCl (pH 7.5), 0.2 M KCl, 5 mM EDTA, 0.32 M sucrose] by using a Teflon homogenizer (clearance – 0.2 mm) with 10 up-and-down strokes by hand. The homogenate was centrifuged for 10 min at $1000 \times g$, and the supernatant (S_1) was then centrifuged for 30 min at $35,000 \times g$. The supernatant (S_2) was removed and the resulting pellet was suspended with the HO buffer and used as the crude plasma membrane fraction (P_2). Plasma membrane and mitochondria in the crude plasma membrane fraction were separated by Percoll gradient centrifugation according to the method of Belsham [18]. The P_2 fraction (about 2 mg of protein/200 μl) was layered over 3 ml of a 20% Percoll solution containing 20 mM Tris-HCl (pH 7.5), 0.2 M KCl, and 5 mM EDTA, and centrifuged at $30,000 \times g$, for 17 min with slow-accele starting (Beckman TL100, TLA 3 rotor). The resultant Percoll gradient was divided into 17 fractions by aspirating the solution from the bottom of the tube, and assayed for enzyme activity. For further experiments, resultant layers (plasma membrane in the upper position and mitochondria in the lower position) were collected with a Pasteur pipette, and precipitated by centrifugation of $250,000 \times g$ for 60 min. The precipitates of plasma membrane and mitochondria were resuspended with HO buffer, and washed once with the same buffer. The plasma membrane preparation was stored at −80°C until used.

2.6. *PGn binding assay by centrifugation method*

The standard reaction mixture, containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 200 nM [^{125}I]PGn and 10 μg of protein of the plasma membrane in a total volume of 50 μl, was incubated at 22°C for ~ 24 h with or without an excess of unlabeled rat PGn (40 μM), and then centrifuged for 5 min at $18,500 \times g$. The resulting pellet was resuspended in 50 μl of the washing solution (20 mM Tris-HCl (pH 7.5), 100 mM NaCl) with a vortex mixer for 10 s, and immediately centrifuged as above. After removal of the supernatant, the radioactivity in the pellet was measured in a gamma counter (Packard cobra II). Specific binding of PGn was calculated by subtracting non-specific binding measured in the presence of excess cold rat PGn (200 times the [^{125}I]PGn) from the total binding. In some experiments, various buffers with different concentrations of NaCl (50–600 mM), $CaCl_2$ (0–4 mM) and $MgCl_2$ (0–4 mM) were used for the reaction mixture and corresponding washing solution.

2.7. *Ligand blotting for PGn*

The plasma membrane fraction was solubilized with an equal volume of SDS sample buffer (125 mM Tris-HCl (pH 6.8), 4.6% SDS, 20% glycerol). After being heated at 90°C for 3 min, samples were subjected to SDS-PAGE with 10–20% gradient gels according to the method of Laemmli [19], and their proteins were transferred to Immobilon P with a semi-dry transblot apparatus in the presence of 0.01% SDS and 20% methanol. As a standard condition, the Immobilon membrane strips were blocked with 3% BSA in TNMC buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$) for 1 h and incubated with 5 μg of [^{125}I]PGn per 10 ml of TNMC buffer containing 3% BSA. After being washed with TNMC buffer for 2 h, the membrane was dried, and autoradiographed with X-ray film. On

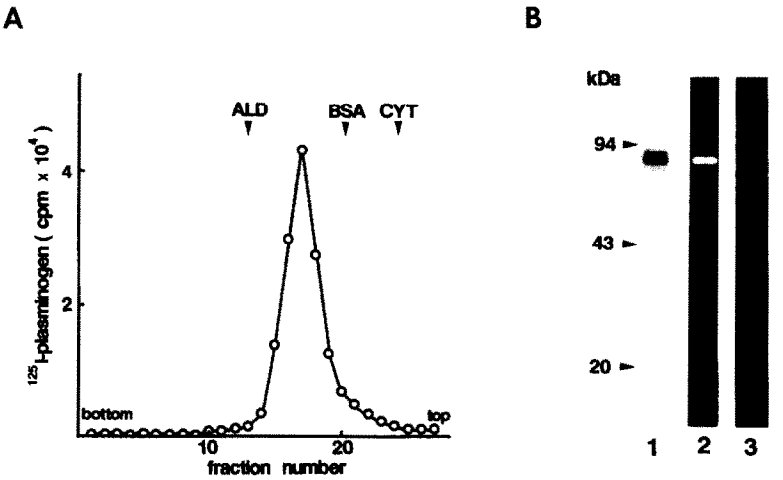

Fig. 1. Properties of [^{125}I]PGn. (A) Glycerol gradient centrifugation. [^{125}I]PGn (6×10^6 cpm/10 μl) was overlayed on 5 ml of a glycerol gradient (2–10%) containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 1 mM $MgCl_2$, and centrifuged at $240,000 \times g_{av}$, for 6 h. The gradient was fractionated by releasing 5 drops (about 130 μl) from the bottom, and a sample was removed and counted. ALD, BSA and CYT are the positions where aldolase (MW 158 kDa), bovine serum albumin (MW 68 kDa) and cytochrome *c* (MW 12.5 kDa) were eluted, respectively. (B) Casein-zymography for PGn. [^{125}I]PGn (1.0 μg) was subjected to SDS-PAGE with three lanes. One was stained with Coomassie blue (1). The others were washed for 1 h with 50 mM Tris-HCl (pH 7.5), 2.5% Triton X-100, to eliminate SDS and subjected to casein-zymography in the presence (2) or absence (3) of urokinase as described previously [4].

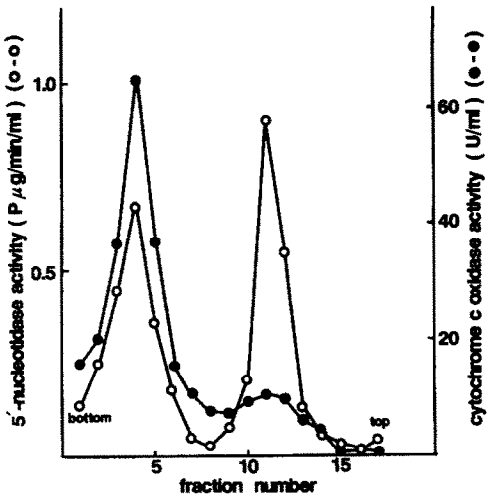

Fig. 2. Preparation of plasma membrane by Percoll gradient centrifugation. The crude plasma membrane fraction (P_2) was subjected to Percoll gradient centrifugation as described in section 2. After centrifugation, the gradient was fractionated into 17 tubes and each fraction was assayed for 5'-nucleotidase (○-○) and cytochrome *c* oxidase activity (●-●).

the other hand, for protein staining of the transblotted proteins, the membrane was soaked in 0.1% Coomassie brilliant blue R250 for 5 min, and destained with an adequate concentration of methanol.

3. RESULTS

3.1. *Plasma membrane from cultured neurons*

A neuronal plasma membrane was prepared by Percoll gradient centrifugation as described in section 2. The isolation profiles are shown in Fig. 2. The plasma membrane fraction (numbers 10–12) showed hardly any contamination with cytochrome *c* oxidase, while the mitochondria fraction (numbers 4–6) were contained with a considerable amount of 5'-nucleotidase.

The activities of marker enzymes in the recovered plasma membrane and other subcellular fractions are summarized in Table I. Hardly any cytochrome *c* oxidase activity was present in the membrane fraction, as is consistent with the results shown in Fig. 2. In addition, relatively low glucose-6-phosphatase and acid phosphatase activity in the plasma membrane fraction showed that the fraction was not contaminated with microsomes or lysosomes. These results indicate that a highly enriched fraction of the neuronal plasma membrane can be obtained by Percoll gradient centrifugation. From 0.6–0.7 g (wet weight) of cultured neurons, about 1–2 mg of protein of the plasma membrane could be obtained.

3.2. *Characterization of specific binding of PGn to the plasma membrane*

To characterize the PGn binding on the neuronal plasma membrane in detail, a small-scale centrifugation method was employed. As shown in Fig. 3, about 7 pmol of [^{125}I]PGn bound to the plasma membrane (10 μg of protein). The binding of [^{125}I]PGn to the plasma membrane was inhibited in the presence of unlabeled rat PGn (100- to 200-fold), but was not affected by prothrombin or urokinase. Lysine and its analogue, 6-amino caproic acid, and benzamidine also inhibited PGn binding, suggesting that PGn specifically binds to the neuronal membrane through the lysine-binding domain as found in the case of the binding to fibrin [20].

The specific binding of PGn to the plasma membrane was also examined at different temperatures (Fig. 4) and was found to be higher at 37°C. At 22°C, PGn binding was only about 50% of that observed at 37°C. However, the binding tended to decrease from 6 h to 12 h at 37°C.

Fig. 5A shows the effect of ionic strength on the specific PGn binding to the plasma membrane. Changes in NaCl concentration caused marked alteration of PGn binding. The PGn binding was found to be higher at lower NaCl concentrations. Furthermore, to examine the requirement of divalent cations in the PGn binding, Ca^{2+} or Mg^{2+} was added to the reaction mixture (Fig. 5B). The specific binding of PGn increased slightly depending on Ca^{2+} concentration up to 4 mM. Although not shown, no further increase was observed at over 5 mM Ca^{2+}. In addition, no significant stimulation was seen when Mg^{2+} was present.

Table I

Marker enzyme activities showing the purity of subcellular fractions

Fraction	5'-Nucleotidase (nmol P/min/mg protein)	Cytochrome *c* oxidase (Unit/mg protein)	Acid phosphatase (nmol P/min/mg protein)	Glucose-6-phosphatase (nmol P/min/mg/protein)
Homogenate	6.5 ± 0.8	8.04 ± 1.46	57.6 ± 3.6	3.87 ± 0.43
S_1	5.6 ± 1.0	14.58 ± 0.00	45.0 ± 2.9	n.d.
P_2	13.9 ± 1.0	37.92 ± 2.92	91.2 ± 3.2	3.62 ± 0.18
S_2	0.1 ± 0.0	1.46 ± 1.20	21.2 ± 3.1	n.d.
Plasma membrane	12.3 ± 0.9	4.38 ± 2.62	39.7 ± 1.9	1.09 ± 0.07
Mitochondria	14.0 ± 3.2	87.50 ± 16.04	66.5 ± 1.7	7.61 ± 0.24

The marker enzyme activities in each fraction were determined as described in section 2. The values are expressed as the mean of three independent experiments. n.d.: not determined.

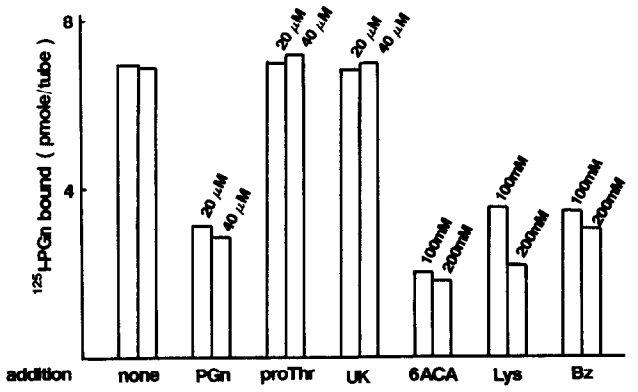

Fig. 3. Specificity of PGn binding to the neuronal plasma membrane. The binding of [^{125}I]PGn to the plasma membrane was examined in the presence of the indicated concentration of rat PGn, prothrombin (pro Th), urokinase (UK), 6-amino caproic acid (6ACA), lysine (Lys) or benzamidine (B_Z) under the standard reaction condition. The incubation was carried out at 22°C for 12 h.

3.3. *Ligand blotting analysis*

As PGn was suggested to specifically bind to the plasma membrane, the molecules for PGn binding were surveyed by ligand blotting under the standard condition (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$) (Fig. 6). [^{125}I]PGn bound mainly to a protein with a molecular weight of about 45 kDa (lane 2). This binding was clearly inhibited when an excess amount of cold PGn or 6ACA was present in the reaction mixture (lanes 3 and 4), suggesting that PGn specifically binds to the 45-kDa protein through the lysine-binding domain(s).

4. DISCUSSION

As previously reported, microglia-secretory plasminogen has potential activity as a neurotrophic factor, such as stimulation of neurite outgrowth [6] and dopamine uptake [7], in cultured neurons. Together with these biological activities, PGn was found to specifically bind to cultured neurons. These findings have suggested that PGn, like established growth factors, binds to certain specific receptor protein(s) on the neuronal surface, through which PGn-specific signal transductions are induced in neurons.

In the present study, we first characterized the specific binding of PGn to neuronal plasma membranes. High purity plasma membranes can be isolated by Percoll gradient centrifugation. The method is simple and reproducible, and has the advantage of eliminating mitochondria from the crude plasma membrane fraction (P_2). Like other cultured cells [21], the plasma membrane has higher 5′-nucleotidase activity than the other fractions. Such plasma membranes would be suitable starting materials for purification of a PGn-binding protein.

We investigated the characteristics of PGn binding to the neuronal membrane by using a simple centrifugation binding assay. Specific binding of PGn to the plasma membrane was demonstrated to be dependent on incubation temperature and incubation time. Furthermore, the binding was highly affected by ionic strength and slightly affected by Ca^{2+}. In addition, although the data are not shown, we observed that the binding was also affected by the pH (6.5–8.5) of the reaction mixture. The amount of specific binding was higher in the acidic condition. It seems that PGn binding is delicately modulated by a variety of inoic conditions including ionic strength and divalent cations, and these effectors in combination with pH may act as physiological modulators of PGn binding in the extracellular microenvironment. However, we think that the most

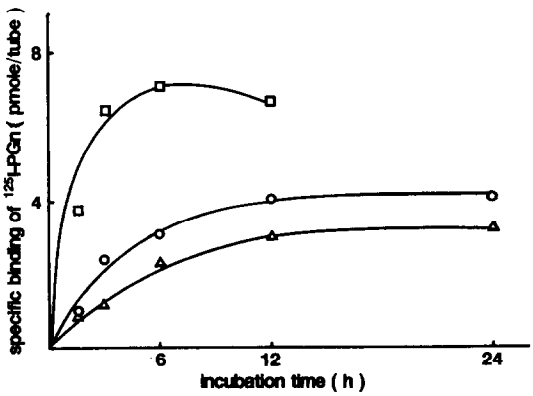

Fig. 4. Time and temperature dependency of the PGn binding. The specific binding of [^{125}I]PGn to the plasma membrane was examined at 37°C (□-□), 22°C (○-○) and 4°C (△-△) under the standard reaction conditions.

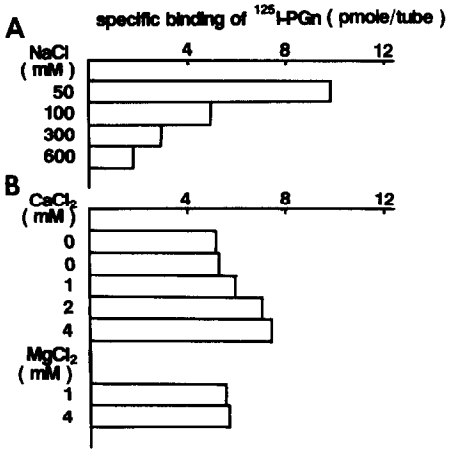

Fig. 5. Effect of ionic strength and Ca^{2+}, Mg^{2+} on the specific binding of [^{125}I]PGn. (A) Effect of ionic strength. The specific binding of [^{125}I]PGn to the plasma membrane was examined at the indicated NaCl concentrations in the standard reaction mixture. Buffer solution containing the same concentration of NaCl was also used for washing. (B) Effect of Ca^{2+} or Mg^{2+}. The specific binding of PGn was examined in the presence of $CaCl_2$ (~ 4 mM) or $MgCl_2$ (~ 4 mM) in the standard reaction mixture. The same concentration of $CaCl_2$ or $MgCl_2$ was used for washing.

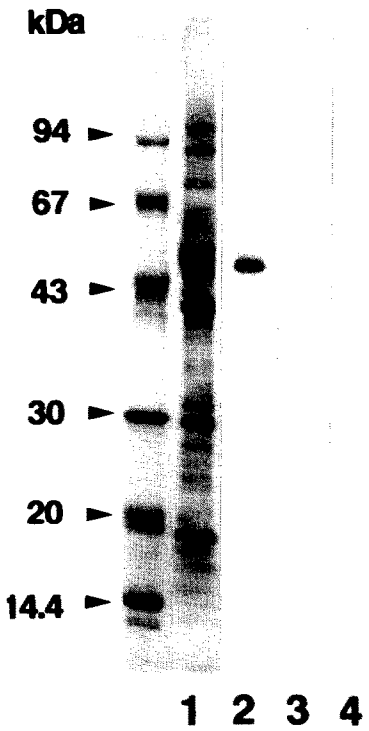

Fig. 6. Ligand blotting for PGn binding proteins. The plasma membrane (20 μg of protein/lane) was subjected to SDS-PAGE under the non-reduced condition, and the proteins were transferred to Immobilon. One strip was stained with Coomassie blue (lane 1). The others were incubated with [^{125}I]PGn in the absence (lane 2) or presence of 200-fold amounts of PGn (lane 3) or 0.2 M 6ACA (lane 4) as described in section 2.

significant and important binding condition is the physiological circumstance such as phosphate buffered saline (Dulbecco's, Earl's or Hanks' solution).

The presence of specific binding protein(s) for PGn in the neuronal plasma membrane was examined by ligand blotting, and mainly a 45-kDa protein was demonstrated to bind PGn in the physiological condition. Although a few minor proteins other than 45-kDa were detected in cell homogenates of neurons, these proteins were not associated with the plasma membrane. From these results, a 45-kDa protein is focused on as a primary candidate for a PGn receptor-like molecule in the neuronal plasma membrane. The binding of [^{125}I]PGn to a 45-kDa protein of the plasma membrane was strongly affected by ionic strength. The intensity of ^{125}I tended to be high at lower concentrations of NaCl (< 50 mM), consistent with the result obtained in the centrifugation binding assay. In addition, the presence of Ca^{2+} or a change in pH also changed the degree of PGn-binding to the 45-kDa protein. Recently, it was shown that PGn binds to a variety of cells such as platelets [17], monocytes, granulocytes and lymphocytes [22], endothelial cells [23–25], hepatocytes [26], a tumor cell line [27], a fibroblast cell line, a monocytic cell line [28] and glioma cells [29]. Among these cells, endothelial cells [30,31] and U937 monocytoid [32] were reported to have 45- to 46-kDa and 54-kDa proteins for interacting with PGn, respectively. Whether there is a correlation among those proteins and neuronal 45-kDa protein in the present study is interesting.

The results involving the method of preparation of neuronal plasma membrane and binding characteristics of PGn may be valuable for obtaining further information about the regulation of PGn binding to the neurons and for the purification of the PGn-binding protein.

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