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Secreted Glypican Binds to the Amyloid Precursor Protein of Alzheimer's Disease (APP) and Inhibits APP-induced Neurite Outgrowth*

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The amyloid precursor protein (APP) of Alzheimer's disease has been shown to stimulate neurite outgrowth *in vitro*. The effect of APP on neurite outgrowth can be enhanced if APP is presented to neurons in substrate-bound form, in the presence of heparan sulfate proteoglycans. To identify specific heparan sulfate proteoglycans that bind to APP, conditioned medium from neonatal mouse brain cells was subjected to affinity chromatography with recombinant APP_{695} as a ligand. Glypican bound strongly to the APP affinity column. Purified glypican bound to APP with an equilibrium dissociation constant of 2.8 nM and inhibited APP-induced neurite outgrowth from chick sympathetic neurons. The effect of glypican was specific for APP, as glypican did not inhibit laminin-induced neurite outgrowth. Furthermore, treatment of cultures with 4-methylumbelliferyl-β-D-xyloside, a competitive inhibitor of proteoglycan glycanation, inhibited APP-induced neurite outgrowth but did not inhibit laminin-induced neurite outgrowth. This result suggests that endogenous proteoglycans are required for substrate-bound APP to stimulate neurite outgrowth. Secreted glypican may act to inhibit APP-induced neurite outgrowth *in vivo* by competing with endogenous proteoglycans for binding to APP.

Alzheimer's disease is a progressive dementia that is characterized by neuronal degeneration, synaptic loss, and the deposition of amyloid fibrils in the brain. The major constituent of the amyloid is the Aβ protein (1, 2), which is derived from the amyloid precursor protein (APP).[1] APP has the structure of a transmembrane glycoprotein (3).

The function of APP is not clearly understood. APP expression is coordinately regulated with neuronal differentiation, neurite outgrowth, and synaptogenesis in the developing brain (4–7). A number of studies have implicated APP in the regulation of neurite outgrowth *in vitro* (8–16).

The binding of APP to heparan sulfate proteoglycans (HSPGs), purified from postnatal day 3 mouse brain cells, has been shown to stimulate process outgrowth from central and peripheral neurons (17–19). Not all HSPG fractions were found to stimulate this action of APP. A preparation of HSPG from embryonic day 10 mouse brain cells did not stimulate APP-induced neurite outgrowth (17). This suggests that a specific, developmentally regulated HSPG may be responsible for activating the trophic function of APP.

A large number of developmentally expressed proteoglycans have been implicated in the regulation of neurite outgrowth. Proteoglycans are capable of providing signals that either stimulate or inhibit axonal growth in the developing nervous system (20). HSPG expression is generally associated with neuronal differentiation and synaptogenesis. The exact mechanism by which HSPGs facilitate neurite outgrowth is not clearly understood. Perlecan has been shown to have neurite outgrowth promoting activity *in vitro* (21). However, other HSPGs may stimulate neurite outgrowth by activating growth-enhancing proteins (22–25) or by presenting growth factors to their cellular receptors (26, 27).

The aim of the present study was to identify and purify HSPGs that bind specifically to APP and to examine the effect of these HSPGs on the neurite outgrowth-promoting activity of APP. We show that the HSPG glypican (28) can bind strongly to APP and may regulate the neurite outgrowth-promoting properties of APP.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium and Ham's F12 medium were purchased from ICN Biomedicals Australasia Pty. Ltd. (Seven Hills, Australia). IODO-GEN iodination reagent and BCA Protein Assay Reagent were purchased from Pierce. Sodium [^{35}S]sulfate (specific activity 100 mCi/mmol) was from NEN Research Products (North Ryde, Australia). Sodium [^{125}I]iodide and HyBond-N+ membranes were from Amersham Australia Pty. Ltd. (Castle Hill, Australia). Immobilon-P transfer membrane was from Millipore Corp. (Bedford, MA). Polylysine, leupeptin, aprotinin, pepstatin A, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), putrescine, prostaglandin F2α, progesterone, heparan sulfate from bovine kidney, Fast Red TR, naphthol AS-MX

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[1] The abbreviations used are: APP, amyloid precursor protein; HSPG, heparan sulfate proteoglycan(s); E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; CHAPS, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propane sulfonate; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; GPI, glycosylphosphatidylinositol; β-D-xyloside, 4-methylumbelliferyl β-D-xyloside.

phosphate, 4-methylumbelliferyl-β-D-xyloside (β-D-xyloside), and bovine serum albumin were purchased from Sigma-Aldrich Pty. Ltd. (Castle Hill, Australia). Econo-Pac Q cartridge (5 ml), Silver Stain Kit, and Affi-Gel 15 were purchased from Bio-Rad Laboratories Pty. Ltd. (North Ryde, Australia). Insulin-transferrin-selenium-X supplement (100 ×) was from Life Technologies, Inc. Heparitinase (heparin-sulfate lyase, EC 4.2.2.8) was purchased from Sapphire Bioscience (Alexandria, Australia). Laminin and perlecan were from Collaborative Biomedical Products (Bedford, MA). Cell culture plates were purchased from Nunc A/S (Kamstrup, Denmark). Nerve growth factor, chondroitinase ABC (EC 4.2.2.4), and CHAPS were from Boehringer Mannheim Australia Pty. Ltd. (Box Hill, Australia). Fetal calf serum and horse serum were obtained from Commonwealth Serum Laboratories Ltd. (Parkville, Australia). Dimethylmethylene blue was from Serva Feinbiochemica (Heidelberg, Germany). PD-10 columns, DEAE-Sepharose Fast Flow, and Sepharose CL-4B were purchased from AMRAD-Pharmacia Biotech (North Ryde, Australia). Rat monoclonal antibody 1948 which recognizes an epitope on the core protein of perlecan was purchased from Chemicon International Inc. (Temecula, CA). Rat monoclonal antibody 281–2 which recognizes an epitope on the core protein of mouse syndecan-1 (29) was a generous gift from Dr. Markku Jalkanen (University of Turku, Finland). Rabbit antisera MSE-2, MSE-3, and MSE-4 raised to the ectodomains of syndecan-2, syndecan-3, and syndecan-4 (30), respectively, were a generous gift from Dr. Merton Bernfield (Harvard Medical School, MA). Affinity-purified rabbit antisera 343-1 and 521-2[2] were raised to synthetic peptides homologous to amino acids 343–360 of rat glypican (31) and amino acids 521–535 of rat cerebroglycan (32), respectively. Goat anti-rabbit and anti-rat IgG conjugated to alkaline phosphatase were from Promega Corp. (Rozelle, Australia).

Expression of Recombinant APP_{695}—Human APP_{695} was expressed in *Pichia pastoris.*[3] Briefly, cDNA encoding APP_{695} from leucine 18 to glutamine 611 was cloned into the expression vector pHIL-S1 and transfected into *P. pastoris* cells. Cells were grown in shaker flasks and APP_{695} purified from the culture medium using Q-Sepharose, Mono-Q anion-exchange chromatography, and phenyl-Superose hydrophobic interaction chromatography (33).

APP Affinity Chromatography—Recombinant human APP was covalently coupled to Affi-Gel 15 by combining 4 mg of APP and 2 ml of gel in the presence of 100 mM Hepes buffer, pH 7.4. The gel slurry was gently mixed at 4 °C for 4 h. Active esters remaining on the gel were blocked by adding 200 μl of 1 M ethanolamine-HCl, pH 8.0, and gently mixing the gel for 1 h at 4 °C. The gel was poured into a 1 × 5-cm column and washed extensively with 150 mM NaCl, 20 mM Tris-HCl, pH 7.4 (Tris-buffered saline). Conditioned medium from cultures of dissociated postnatal day 3 mouse brain cells (19) was collected and centrifuged (1000 × *g* for 15 min at room temperature). A mixture of proteinase inhibitors was added to the medium (10 ml) to give a final concentration of 1 mM EDTA, 0.4 mM AEBSF, 2 μg/ml pepstatin A, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 5 μM E-64. The medium was applied to the APP affinity column which was washed with 20 ml of Tris-buffered saline. A linear salt gradient from 0 to 1.0 M NaCl in 20 mM Tris-HCl, pH 7.4, containing 0.02% (v/v) Tween 20 was applied to the APP affinity column over 30 ml at a flow rate of 1.0 ml/min and 1.0-ml fractions were collected. An aliquot (100 μl) from each fraction eluting from the APP affinity column was blotted onto HyBond-N+ membrane using a Bio-Dot Microfiltration Apparatus (Bio-Rad). Aliquots from each fraction were dialyzed against Tris-buffered saline containing 0.05% (v/v) Nonidet P-40 using a CF-35 Centriflo ultrafiltration membrane cone (Amicon, Danvers, MA) and then incubated with 50 milliunits/ml heparitinase in the presence of 1 mM calcium acetate, 0.4 mM AEBSF, 2 μg/ml pepstatin A, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 5 μM E-64 for 2 h at 37 °C. The samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (34) containing 8.5% (w/v) acrylamide and then electrophoretically transfered to Immobilon-P transfer membrane. Both Immobilon-P and HyBond N+ membranes were incubated in 0.5% (w/v) hydrolyzed casein in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) for 1 h. Membranes were then incubated with primary antibodies in Tris-buffered saline containing 0.05% (w/v) Tween 20 and 5% (v/v) goat serum. Antisera MSE-2, MSE-3, and MSE-4 were used at a dilution of 1:500; monoclonal antibodies 281-2 and 1948 and the affinity-purified 343-1 and 52--2 antisera were used at a concentration of 10 μg/ml. Anti-rabbit and anti-rat antibodies conjugated to alkaline phosphatase were used at 1 μg/ml in Tris-buffered saline containing 0.05% (w/v) Tween 20 and 5% (v/v) goat serum. The secondary antibody was visualized using Fast Red TR and naphthol AS-MX phosphate. The concentration of NaCl in the eluted fractions was determined by measuring conductivity in each fraction using an Activon model 301 conductivity meter (Activon, Carlton, Australia). Protein concentrations were determined with the BCA Protein Assay Reagent using bovine serum albumin as a standard.

Purification of Soluble Glypican from PC12 Cells—HSPG was purified from the conditioned medium of rat pheochromocytoma (PC12) cells following a method for the purification of HSPG from the conditioned medium of human lung fibroblasts (35, 36). Rat pheochromocytoma (PC12) cells (37) were cultured for 4 days in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum and 5% (v/v) horse serum in 600-cm^2 cell culture dishes. Conditioned medium (1400 ml) was combined with medium (10 ml) from cells that had been labeled for 48 h with 200 μCi/ml of sodium [^{35}S]sulfate. Urea and Tris were added to the medium to a concentration of 6 M and 50 mM, respectively, and the final pH was adjusted to pH 8.0. The medium was applied to a column (2.5 × 20 cm) containing of DEAE-Sepharose Fast Flow (80 ml) at a flow rate of 2 ml/min. The column was washed extensively with a buffer containing 6 M urea, 1 mM EDTA, 0.1% (w/v) Triton X-100 in 20 mM Tris-HCl, pH 8.0, and bound protein was eluted with a gradient from 0 to 1.0 M NaCl over 800 ml in the same buffer. Fractions (10 ml) were collected. The amount of radioactivity in each fraction was determined using a 1600 TR Liquid Scintillation Analyzer (Packard International, Mt. Waverley, Australia). Fractions containing glypican were identified by dot blot and Western blot analysis using affinity-purified 343-1 antiserum. Glypican-containing fractions were pooled (120 ml), concentrated, and washed into Tris-buffered saline containing 0.05% (w/v) Nonidet P-40 using a CF-35 Centriflo ultrafiltration membrane cone (Amicon, Danvers MA). The sample was treated with chondroitinase ABC (100 milliunits/ml) for 3 h at 37 °C in the presence of 1 mM EDTA, 2 μg/ml pepstatin A, 0.4 mM AEBSF, 2 μg/ml leupeptin, and 5 μM E-64. The sample was then applied to a 5-ml Econo-Pac Q cartridge, and bound protein was eluted with a gradient from 0 to 1.0 M NaCl in 20 mM Tris-HCl, pH 7.4, containing 0.02% (w/v) Tween 20 over 30 ml. One-ml fractions were collected. Glypican-containing fractions were pooled (10 ml), concentrated, and washed into 4 M guanidine HCl, 1 mM EDTA, 1 mM benzamidine, 0.5% (w/v) CHAPS in 50 mM acetate buffer, pH 5.8, using a CF-35 Centriflo ultrafiltration membrane cone. The sample (600 μl) was applied to a column (1 × 100 cm) containing Sepharose CL-4B (78 ml) and eluted with the same buffer at a flow rate of 10 ml/h with 1-ml fractions collected. Glypican-containing fractions were pooled (16 ml) concentrated using a CF-35 Centriflo ultrafiltration membrane cone, and then the sample (600 μl) was desalted into 100 mM ammonium hydrogen carbonate, pH 8.0, using a PD-10 column. The sample was then lyophilized. Proteins were separated by SDS-PAGE containing 8.5% (w/v) acrylamide and protein bands visualized using a Coomassie Blue-silver stain method (38). The concentration of glycosaminoglycan was determined using dimethylmethylene blue (39) with heparan sulfate as a standard.

Binding Assay—Purified glypican (1 μg) was iodinated using IODOGEN iodination reagent (Pierce). Radiolabeled glypican was separated from free iodide using a PD-10 column. The specific radioactivity of the radiolabeled glypican was 2.9×10^5 cpm/ng. Purified human brain APP (33) was added to a 96-well enzyme-linked immunosorbent assay plate (Greiner GmbH, Frickenhausen, Germany) at a concentration of 50 μg/ml in Tris-buffered saline; 100 μl was added to each well and incubated overnight at 4 °C. Nonspecific-binding sites were then blocked with 200 μl of 1% (w/v) bovine serum albumin in Tris-buffered saline for 2 h. Iodinated glypican was added to each well in 100 μl of 1% (w/v) bovine serum albumin in Tris-buffered saline for 1 h at room temperature. The wells were then washed five times with 150 μl of Tris-buffered saline containing 0.05% (w/v) Tween 20. Bound protein was then released from the well by adding 150 μl of 10% (w/v) SDS to each well and incubating at 60 °C for 30 min. The amount of radioactivity was determined using a 1261 Multigamma gamma counter (Wallac Oy, Turku, Finland). Specific binding was defined as the amount of glypican bound to a well incubated with APP minus the amount of glypican bound to a well incubated with blocking solution alone. Molar concentrations of glypican were determined by estimation of the protein concentration with the BCA Protein Assay Reagent with bovine serum albumin as a standard, assuming a molecular mass for the core protein of rat glypican of 62 kDa (40).

Neurite Outgrowth Assay—Cell culture plates (96-well) were prepared by incubating each well with 100 μl of 0.1 mg/ml polylysine in

[2] E. D. Litwack, J. K. Ivins, A. Kumbasar, C. S. Stipp, and A. D. Lander, manuscript in preparation.

[3] A. Henry, C. L. Masters, K. Beyreuther, and R. Cappai, manuscript in preparation.

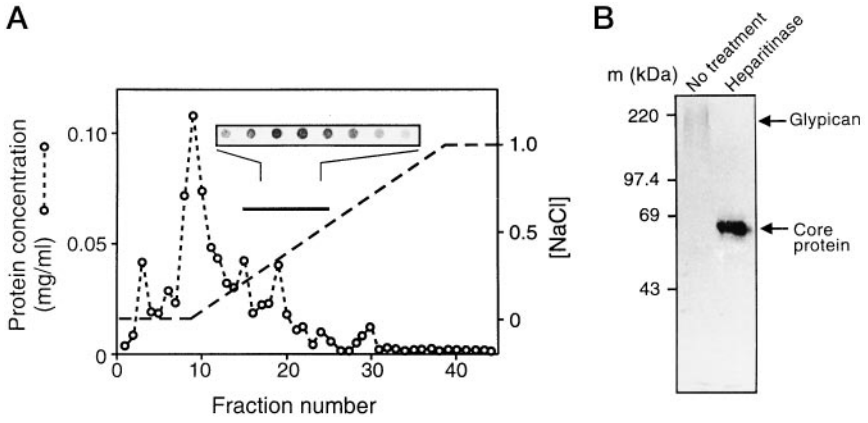

FIG. 1. **Affinity chromatography of the conditioned medium, from cultures of postnatal day 3 mouse brain cells, on the APP affinity column (*A*).** Eluted fractions were analyzed for protein concentration (*open circles*) and 343-1 immunoreactivity by dot blots (*A*, *inset*, fractions 17–24); the *solid bar* represents fractions that contained 343-1 immunoreactivity. The fraction containing peak 343-1 immunoreactivity (fraction 20) was analyzed by Western blot (*B*, *lane 1*) and pretreated with heparitinase (*B, lane 2*), with affinity-purified 343-1 antiserum.

sterile distilled water for 15 min. The wells were then washed three times with 200 μl of sterile distilled water. The proteins of interest were then coated onto cell culture plates in order to be presented to the neurons in a substrate-bound form (substrate-bound). Wells were incubated with 10 μg/ml purified glypican or 10 μg/ml perlecan in 100 μl of phosphate-buffered saline for 2 h at room temperature. Each well was washed twice with 200 μl per well of phosphate-buffered saline. The wells were then incubated with 10 μg/ml of purified human brain APP or 5 μg/ml laminin in 100 μl of phosphate-buffered saline for 2 h and then wells were washed twice with 200 μl of phosphate-buffered saline. The order in which the proteins were added to the wells did not affect the results. Approximately 2×10^3 cells, prepared from embryonic day 12 chick paravertebral sympathetic ganglia (41), were added to each well in 100 μl of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 media containing 1.0% (v/v) Insulin-transferrin-selenium-X supplement (100 ×) 100 μM putrescine, 1.67 μg/ml prostaglandin F2α, 6.67 ng/ml progesterone, 10 ng/ml NGF, and 0.5 mg/ml bovine serum albumin. In some experiments, cultures were allowed to grow for 1 h after plating, and then the medium was removed and replaced with fresh medium containing 1 mM β-D-xyloside. Cultures were grown for 48 h, fixed with 2% (w/v) glutaraldehyde in phosphate-buffered saline, and then examined by phase-contrast microscopy. Selected fields were captured for computer-assisted image analysis (MD30 Plus Image analysis system, Adelaide, Australia). The percentage of neurons with neurites was determined in nine separate fields around the center of each well. On average 60 neurons were counted per well. For the analysis of neurite length, the longest neurite on 20 neurons bearing neurites in each well was measured. A minimum of four wells for each treatment group was analyzed. Differences between the means of controls and each treatment group were analyzed by a two-tailed Student's *t* test. Means were assumed to be significantly different when the *p* value for the null hypothesis was less than 0.05.

RESULTS

APP Affinity Chromatography—An affinity column containing recombinant APP_{695} as a ligand was used to purify HSPGs from neonatal mouse brain which bind strongly to APP. Conditioned medium from postnatal day 3 mouse brain cultures was applied to the APP affinity column, and bound protein was eluted with a linear salt gradient (Fig. 1*A*). Aliquots of each fraction eluted from the column were assayed by Western blotting and dot blotting using antibodies to a number of well characterized HSPGs. The antibodies tested were specific to syndecan-1, syndecan-2, syndecan-3, and syndecan-4, perlecan, cerebroglycan, and glypican. Western blot and dot blot assays using antibodies to syndecan-1, syndecan-2, syndecan-3, syndecan-4, perlecan, or cerebroglycan did not detect any immunoreactivity in fractions eluted from the column. However, antibodies to glypican (affinity-purified 343-1 antiserum) detected immunoreactivity eluting between fractions 15 and 25 (Fig. 1*A*). The peak of 343-1 immunoreactivity eluted at 0.39 M NaCl (fraction 20).

Western blot analysis of the eluate revealed a diffuse immunoreactive band migrating with an apparent molecular mass of 200 kDa (Fig. 1*B, lane 1*). Pretreatment of the eluate with heparitinase resulted in the loss of the 200-kDa band and the appearance of an immunoreactive band with an apparent molecular mass between 60 and 67 kDa (Fig. 1*B, lane 2*), consistent with the apparent molecular mass of the core protein of rat glypican (31).

Purification of Glypican from PC12 Cells—To prepare a sufficient amount of glypican for binding and cell culture studies, glypican was purified from the conditioned medium of rat pheochromocytoma (PC12) cells. The conditioned medium of the PC12 cells contains a soluble form of glypican which has probably been cleaved from the cell-membrane adjacent to its glycosylphosphatidylinositol (GPI) anchor (42). PC12 cells were cultured in the presence of [^{35}S]sulfate to radiolabel proteoglycans. DEAE-Sepharose Fast Flow chromatography of PC12-conditioned medium was used as a first step to purify glypican (Fig. 2*A*). The amount of radioactivity in each fraction was determined. The presence of glypican in the fractions was determined by dot blot and Western blot analysis using the affinity-purified 343-1 antiserum. Glypican eluted between 0.25 and 0.35 M NaCl.

To remove chondroitin sulfate proteoglycans, glypican-containing fractions (fractions 32–46 from the DEAE-Sepharose Fast Flow column) were pooled and treated with 100 milliunits/ml chondroitinase ABC and then applied to an Econo-Pac Q cartridge (Bio-Rad). Glypican eluted between 0.6 and 0.8 M NaCl (Fig. 2*B*). Glypican-containing fractions (fractions 28–39 from the Econo-Pac Q cartridge) were pooled and concentrated and then applied to a Sepharose CL-4B gel filtration column (1×100 cm). Glypican eluted between fractions 50 and 65 (Fig. 2*C*). Glypican-containing fractions from the Sepharose CL-4B column were pooled, concentrated, desalted, and lyophilized.

Purified glypican was analyzed by SDS-PAGE and Western blot. Glypican did not stain strongly using a Coomassie Blue-silver stain which may be due to its high level of glycosylation and the diffuse nature of the band. Heparitinase treatment of glypican generated two bands that were not observed in the heparitinase preparation alone, with one band migrating at 64 kDa and the other migrating at 50 kDa (Fig. 3*A*). Western blot analysis of the purified glypican with the 343-1 antibodies revealed a high molecular weight immunoreactive band migrating between 140 and 220 kDa. Pretreatment of the purified glypican with heparitinase, or a combination of heparitinase and chondroitinase ABC, resulted in the loss of the 140- to 220-kDa band and the appearance of an immunoreactive band between 58 and 70 kDa and an immunoreactive band at 49 kDa (Fig. 3*B*). The 49-kDa band detected by Western blot analysis and the 50-kDa band detected by the Coomassie Silver stain may represent a proteolytic breakdown product of the core protein of glypican. The recovery of glypican from the conditioned medium was 0.18 μg of bovine serum albumin eq/ml and

0.07 μg of heparan sulfate eq/ml.

Binding of APP to Glypican—The binding of APP to purified glypican was examined using a solid-phase binding assay. A range of glypican concentrations (0 to 4.0 nM) was added to the APP-coated enzyme-linked immunosorbent assay plates. Binding of glypican to APP was saturable, and Scatchard analysis of the binding indicated one class of binding site with an equilibrium dissociation constant (K_D) of 2.8 nM (Fig. 4).

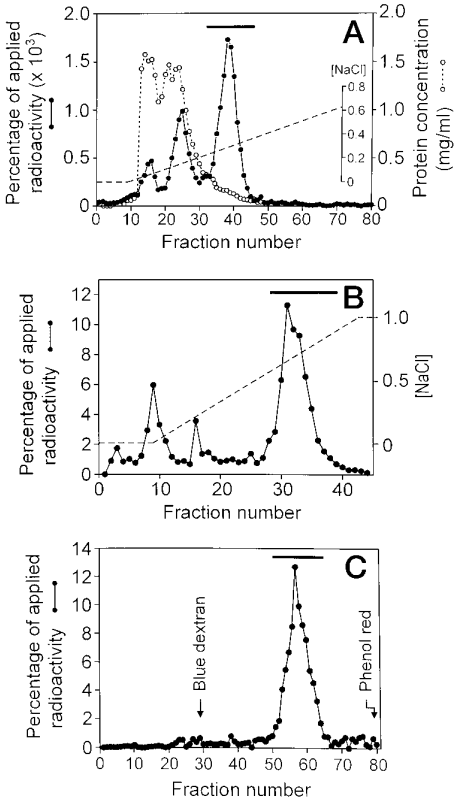

FIG. 2. **Chromatography of PC12-conditioned medium.** Conditioned medium was applied to DEAE-Sepharose Fast Flow; bound protein was eluted with a linear gradient of 0–1.0 M NaCl in 6 M urea, 0.1% (w/w) Triton X-100, 20 mM Tris-HCl, pH 8.0, and 10-ml fractions were collected (*A*). The protein concentration (*open circles*) and the amount of radioactivity (*closed circles*) were determined in each fraction. Glypican-containing fractions were incubated with chondroitinase ABC before being applied to an Econo-pac Q cartridge; bound protein was eluted with a linear gradient from 0 to 1.0 M NaCl in 0.02% (v/v) Tween 20, 20 mM Tris-HCl, pH 7.4, and 1-ml fractions were collected (*B*). Glypican-containing fractions were pooled, concentrated, and applied to Sepharose CL-4B, and 1-ml fractions were collected (*C*). Radioactivity in each fraction is expressed as a percentage of the total radioactivity applied to the column. The *solid bars* represent fractions containing glypican immunoreactivity.

The Effect of Glypican on APP-induced Neurite Outgrowth—Previous studies (19) have shown that substrate-bound APP can stimulate neurite outgrowth in the absence of exogenously added HSPG, provided that cells are cultured for 48 h or longer in serum-free medium. The effect of glypican on neurite outgrowth from sympathetic neurons cultured on substrate-bound APP and laminin was examined (Fig. 5). Cultures were grown for 48 h, and the percentage of neurons with neurites and neurite length were determined (Table I). Laminin and polylysine induced a 25-fold increase in the number of neurons with neurites and an 11-fold increase in neurite length compared with neurons cultured on polylysine alone. Neurons cultured on APP and polylysine exhibited a 10-fold increase in the number of neurons with neurites and a 5-fold increase in neurite length compared with neurons cultured on polylysine alone. Glypican inhibited APP-stimulated neurite outgrowth 76%, as measured by the number of neurons with neurites, and 62%, as measured by neurite length. Glypican did not significantly affect the number of neurons with neurites or neurite length of neurons cultured on laminin. Neurons cultured on polylysine and glypican did not exhibit any increase in both the number of neurons with neurites or neurite length above that observed on the polylysine alone.

The effect of glypican on APP- and laminin-induced neurite outgrowth was compared with the effect of the basement membrane HSPG, perlecan. Perlecan inhibited APP-stimulated neurite outgrowth 71%, as measured by the number of neurons with neurites, and 56%, as measured neurite length. Perlecan did not significantly affect the number of neurons with neurites or neurite length of neurons cultured on laminin.

Effect of β-D-Xyloside on APP-induced Neurite Outgrowth—The effect of inhibiting endogenous proteoglycan on APP- and laminin-induced neurite outgrowth was investigated. Cultures were grown in the presence or absence of 1 mM β-D-xyloside (Fig. 5), a competitive inhibitor of proteoglycan glycanation (43). The presence of 1 mM β-D-xyloside resulted in an 80% inhibition of proteoglycan glycanation as determined from the incorporation of [^{35}S]sulfate in secreted and cell-associated proteoglycans (Table II). The percentage of neurons with neurites and neurite length were determined (Table III). Neurons cultured on substrate-bound APP in the presence of β-D-xyloside exhibited an 88% reduction in the number of neurons with

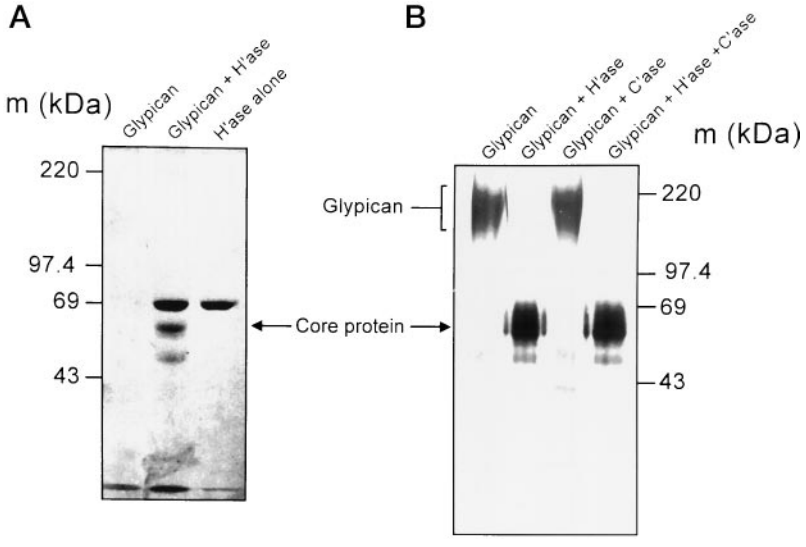

FIG. 3. **Coomassie Silver stain (*A*) and Western blot analysis using affinity-purified 343-1 antiserum (*B*) of purified glypican.** Glypican was incubated in the presence or absence of heparitinase (*H'ase*) and/or chondroitinase ABC (*C'ase*) before SDS-PAGE. Molecular mass standards used were myosin (220 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (69 kDa), and ovalbumin (43 kDa).

neurites and a 79% reduction in neurite length compared with neurons cultured in the absence of β-D-xyloside. The presence of β-D-xyloside did not affect the percentage of neurons with neurites and did not affect neurite length of neurons cultured on substrate-bound laminin compared with neurons cultured in the absence of β-D-xyloside.

DISCUSSION

There is increasing evidence that the physiological effects of secreted APP are mediated through an interaction with components of the extracellular matrix such as HSPGs. HSPGs constitute a majority of binding sites for APP in the extracellular matrix (5) and up to 50% of the binding sites for APP on the cell surface (13). Previous studies have shown that specific, developmentally regulated HSPGs can modulate the neurite outgrowth-promoting activity of APP (17, 18). In this study, affinity chromatography using recombinant APP as a ligand showed that the HSPG glypican can bind to APP. The equilibrium dissociation constant for this interaction was low (2.8 nM) suggesting that this is a strong interaction of biological significance. The equilibrium dissociation constant for the binding of APP to glypican was similar to that reported for the binding of APP to the basement membrane form of HSPG (perlecan) (44). Perlecan was not detected in the eluate from the APP affinity column. However, this is likely to have been due to the low

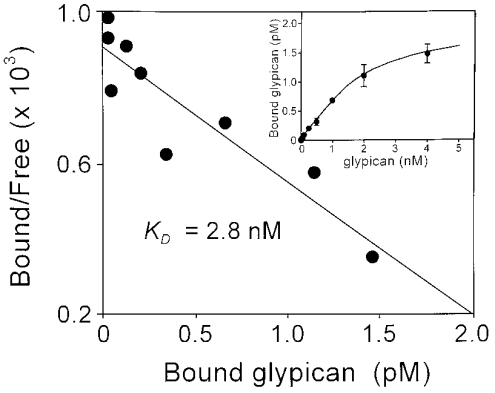

FIG. 4. **Scatchard analysis of glypican binding to purified APP.** The *inset* shows the concentration dependence of glypican binding to APP. Specific binding represents nonspecific binding subtracted from total binding. Data represent the mean ± the standard error of the mean in quadruplicate wells.

TABLE I

Effect of glypican on neurite outgrowth from sympathetic neurons cultured on substrate-bound APP and laminin

Neurons were cultured for 48 h on cell culture plates coated with 10 μg/mL APP and 5 μg/ml Laminin with and without coating with 10 μg/ml glypican or 10 μg/ml perlecan. Computer-assisted image analysis (MD30 Plus Image) was performed on the cultures. The percentage of neurons with neurites was determined in nine separate fields around the center of each well. The length of the longest neurite on 20 neurons was measured. A minimum of four wells for each treatment group was analyzed. Data represent the mean and standard error of the mean.

Substrate	Percentage of neurons with neurites	Neurite length
		μm
Polylysine	3.6 ± 3.0	22 ± 2.7
Polylysine + glypican	2.3 ± 1.3	19 ± 4.4
Polylysine − APP	37 ± 6.0[a]	114 ± 10[a]
Polylysine + glypican + APP	9.0 ± 2.2[b]	43 ± 10[b]
Polylysine + laminin	90 ± 15	247 ± 37
Polylysine + glypican + laminin	92 ± 7.2	223 ± 25
Polylysine + perlecan + APP	10.7 ± 2.7[b]	49.5 ± 9.0[b]
Polylysine + perlecan + laminin	95 ± 6.3	214 ± 18

[a] Significantly different from polylysine ($p < 0.05$).
[b] Significantly different from polylysine + APP ($p < 0.05$).

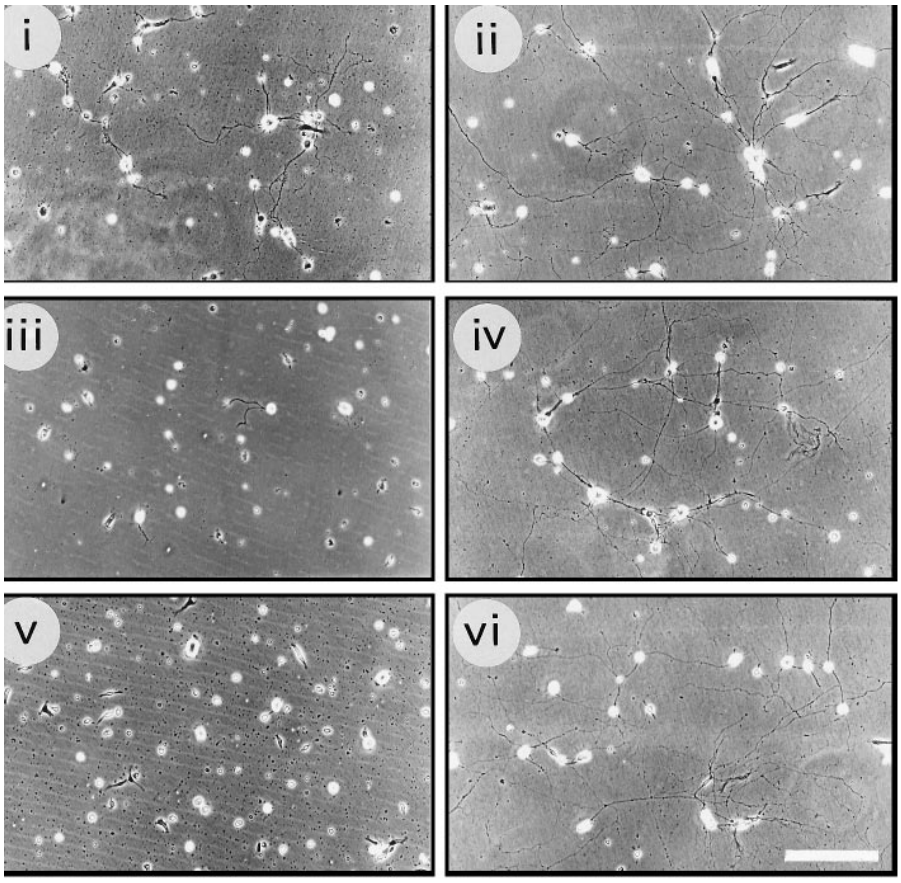

FIG. 5. **Phase-contrast micrographs of primary cultures of embryonic day 12 chick sympathetic neurons cultured for 48 h on substratum-bound APP and laminin in the presence or absence of glypican or in the presence and absence of 1 mM β-D-xyloside.** Culture dishes were coated with APP (*i*), laminin (*ii*), glypican + APP (*iii*), and glypican + laminin (*iv*). Neurons were cultured on substrate-bound APP in the presence 1 mM β-D-xyloside (*v*) and on substrate-bound laminin in the presence of 1 mM β-D-xyloside (*vi*), *bar*, 100 μm.

TABLE II

The effect of β-D-xyloside on the incorporation of [^{35}S]sulfate into proteoglycans and glycosaminoglycans produced by sympathetic neurons in culture

Sympathetic neurons were grown on cell culture plates coated with 10 μg/ml APP and labeled with 400 μCi/ml of [^{35}S]sulfate for 48 h in the presence and absence of 1 mM 4 methylumbelliferyl-β-D-xyloside. The incorporation of [^{35}S]sulfate into low molecular weight glycosaminoglycan and high molecular weight proteoglycans was determined by size exclusion high performance liquid chromatography (5). Data represent the mean and standard error of the mean ($n = 3$).

Fraction	Incorporation of [^{35}S]sulfate (cpm/10^3 cells)		
	Control	β-D-Xyloside (1 mM)	Control
			(%)
Medium			
Proteoglycans	288,500 ± 31,800	59,400 ± 7,200	21
Glycosaminoglycans	45,800 ± 7,200	1,448,100 ± 120,000	3,162
Cells			
Proteoglycans	40,400 ± 6,000	7,000 ± 2,700	17
Glycosaminoglycans	50,500 ± 8,200	68,700 ± 7,800	136

TABLE III

Effect of xyloside on neurite outgrowth from sympathetic neurons cultured on substratum-bound APP and laminin

Neurons were cultured for 48 h in the presence or absence of 1 mM 4-methylumbelliferyl-β-D-xyloside (β-D-xyloside). Computer-assisted image analysis (MD30 Plus Image) was performed on the cultures. The percentage of neurons with neurites was determined in nine separate fields around the center of each well. The length of the longest neurite on 20 neurons was measured. A minimum of four wells for each treatment group was analyzed. Data represent the mean and standard error of the mean.

Treatment	Percentage of neurons with neurites	Neurite length
		μm
Polylysine	7.6 ± 2.1	29 ± 8.4
Polylysine + β-D-xyloside	5.8 ± 2.0	26 ± 9.0
Polylysine + APP	45 ± 5.0	119 ± 9.2
Polylysine + APP + β-D-xyloside	5.3 ± 1.9[a]	25 ± 7.0[a]
Polylysine + laminin	92 ± 2.4	209 ± 13
Polylysine + laminin + β-D-xyloside	94 ± 2.2	200 ± 19

[a] Significantly different from polylysine + APP ($p < 0.05$).

expression of perlecan in the cultured mouse brain cells, rather than to any difference in the relative affinities of glypican and perlecan for APP during affinity chromatography.

Cell culture studies demonstrated that exogenous glypican can specifically inhibit the neurite outgrowth-promoting activity of APP. Glypican did not inhibit neurite outgrowth on laminin, indicating that this effect may be specific for APP. In addition, neurite outgrowth on substrate-bound APP was significantly reduced by inhibiting proteoglycan glycanation with β-D-xyloside. However, β-D-xyloside did not significantly reduce neurite outgrowth on laminin. Taken together, these results suggest that endogenous proteoglycans either on the cell surface, or secreted by the neuron, are required for the promotion of neurite outgrowth on substrate-bound APP. Therefore, exogenous glypican may inhibit APP-induced neurite outgrowth by competing with endogenous proteoglycans for binding to APP. This view is supported by the inhibitory effect of perlecan on APP-induced neurite outgrowth. APP has been shown to bind to perlecan with high affinity (44) which suggests that perlecan, like exogenous glypican, inhibits APP-stimulated neurite outgrowth by competing with endogenous proteoglycans for binding to APP. The endogenous proteoglycan remains to be identified; it could be GPI-anchored glypican. However, other cell-surface or secreted proteoglycans could also be involved.

The results presented in this study provide further evidence of the importance of proteoglycans in APP-stimulated neurite outgrowth. The present study suggests that glypican may be involved in this mechanism. Glypican is found either as a GPI-anchored cell-surface HSPG or, when cleaved from its GPI anchor, a soluble HSPG (28). The glypican used in this study would be the secreted form as it was purified from conditioned medium. Glypican is found in high levels in the brain where it is primarily expressed by neurons (31, 45). The view that glypican may be involved in neurite outgrowth is consistent with a recent study that has shown that glypican is expressed on the surface of most axons during periods of major axon growth.[2]

The precise mechanism by which APP promotes neurite outgrowth remains to be elucidated. The neurite outgrowth-promoting domain of APP has been associated with the RERMS sequence contained within the ectodomain of the protein (14). However, other studies place the neurite outgrowth-promoting domain closer to the carboxyl terminus of the ectodomain of APP (16), a region of APP implicated in modulating intracellular calcium levels (46). A peptide homologous to the RERMS-containing domain of APP has been shown to stimulate neurite outgrowth in a manner that is independent of heparan sulfate binding (13). However, the present study suggests that full-length APP requires the presence of endogenous proteoglycans in order to promote neurite outgrowth.

A number of proteoglycans, including perlecan, have been localized to the amyloid plaques of Alzheimer's disease (47–50). Little is known about the effects these proteoglycans have on the function and metabolism of APP which may be secreted in the region of the amyloid plaques. As proteoglycans have been shown to modulate the neurite outgrowth-promoting activity of APP, they may perturb the normal function of APP in the region of the amyloid plaque. What significance, if any, such a perturbation may have for the pathogenesis of Alzheimer's disease remains to be determined.

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