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Permalink
https://escholarship.org/uc/item/1z4945xx

Journal
Journal of Biological Chemistry, 271(49)

ISSN
0021-9258

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Publication Date
1996-12-17

DOI
10.1074/jbc.271.49.31215

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

(Received for publication, May 8, 1996, and in revised form, September 5, 1996)

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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 neuronal cultures of embryonic day 10 mouse brain cells did not stimulate 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, an inhibitor of glypican 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 [35S]sulfate (specific activity 100 mCi/mmol) was from NEN Research Products (North Ryde, Australia). Sodium [35S]iodide and HyBond-N transfer 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, and aprotinin, pepstatin A, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; CHAPS, 3-(3-cholamidopropyl)di-methyl-ammonio)-1-propane sulfonate; AEBSF, 4-(2-aminoethyl)benzene sulfonfyl fluoride (AEBSF), putrescine, prostaglandin F2α, progesterone, heparan sulfate from bovine kidney, Fast Red TR, naphtol AS-MX glycosyolphosphatidylinositol; β-d-xyloside, 4-methylumbelliferylβ-d-xyloside.

This paper is available online at http://www-jbc.stanford.edu/jbc/
phosphate, 4-methylumbelliferyl-β-D-glucuronide, and bovine conalbumin. Assays were performed using the Nonlinear regression analysis program (GraphPad, San Diego, CA). The samples were scanned on a Beckman series 5 spectrophotometer (Beckman). The specific activity of purified APP was determined as nmol of pNp [3H] released per min per mg of protein.

Expression of Recombinant APP

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 was washed extensively with 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, at a flow rate of 2 ml/min. The column was then eluted with a gradient from 0 to 1.0 M NaCl in Tris-buffered saline containing 0.05% (w/v) Tween 20, and bound protein was eluted 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 cone, and then concentrated further to 50 μg/ml using a PD-10 column. The concentration was determined using a 1600 TR Liquid Scintillation Analyzer (Packard Instrument Co., Downers Grove, IL).

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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 × 106 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 Multitgamma 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 buffer. Nonspecific 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 polysine in

References


2. A. Henry, C. L. Masters, K. Beyreuther, and R. Cappai, manuscript in preparation.
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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 ng/ml purified glypican or 10 ng/ml perlecain in 100 μl of phosphate-buffered saline for 2 h at room temperature. Each was washed twice with 200 μl per well of phosphate-buffered saline. The wells were then incubated with 10 ng/ml of purified human brain APP or 5 μg/ml laminin in 100 μl of phosphate-buffered saline for 2 h and then 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 × 10⁵ 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 10% (v/v) Insulin-transferrin-selenium-X supplement (100 × 100 μm putrescine, 1.67 μg/ml prostaglandin F2α, 6.87 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-phenyl-, 2-glutathione, and 1M NaCl. 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 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. 1A). 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, perlecain, cerebroglycan, and glypican. Western blot and dot blot assays using antibodies to syndecan-1, syndecan-2, syndecan-3, syndecan-4, perlecain, 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. 1A). 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. 1B, 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. 1B, 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 [35S]硫酸 to radiolabel proteoglycans. DEAE-Sepharose Fast Flow chromatography of PC12-conditioned medium was used as a first step to purify glypican (Fig. 2A). 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 milli-units/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. 2B). 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. 2C). Glypican-containing fractions from the Sepharose CL-4B column were pooled, concentrated, deasalted, 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. 3A). 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. 3B). 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).

**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 neurites.
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.

**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>
<thead>
<tr>
<th>Substrate</th>
<th>Percentage of neurons with neurites</th>
<th>Neurite length (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polylysine</td>
<td>3.6 ± 3.0</td>
<td>22 ± 2.7</td>
</tr>
<tr>
<td>Polylysine + glypican</td>
<td>2.3 ± 1.3</td>
<td>19 ± 4.4</td>
</tr>
<tr>
<td>Polylysine – APP</td>
<td>37 ± 6.0</td>
<td>114 ± 10</td>
</tr>
<tr>
<td>Polylysine + glypican + APP</td>
<td>9.0 ± 2.2b</td>
<td>43 ± 10</td>
</tr>
<tr>
<td>Polylysine + laminin</td>
<td>90 ± 15</td>
<td>247 ± 37</td>
</tr>
<tr>
<td>Polylysine + glypican + laminin</td>
<td>92 ± 7.2</td>
<td>223 ± 25</td>
</tr>
<tr>
<td>Polylysine + perlecan + APP</td>
<td>10.7 ± 2.7b</td>
<td>49.5 ± 9.0b</td>
</tr>
<tr>
<td>Polylysine + perlecan + laminin</td>
<td>95 ± 6.3</td>
<td>214 ± 18</td>
</tr>
</tbody>
</table>

* Significantly different from polylysine (p < 0.05).

b Significantly different from polylysine + APP (p < 0.05).
Secreted Glypican Inhibits APP-induced Neurite Outgrowth

Dissimetric neurons were grown on cell culture plates coated with 10 μg/ml APP and labeled with 400 μCi/ml of [35S]sulfate for 48 h in the presence and absence of 1 μM 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 was used. Data represent the mean and standard error of the mean (n = 3).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Incorporation of [35S]sulfate (cpm/10^6 cells)</th>
<th>Control</th>
<th>β-D-xyloside (1 μM)</th>
<th>Control</th>
<th>β-D-xyloside (1 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>Proteoglycans 288,500 ± 31,800</td>
<td>59,400 ± 7,200</td>
<td>21</td>
<td>Glycosaminoglycans 45,800 ± 7,200</td>
<td>1,448,100 ± 120,000</td>
</tr>
<tr>
<td>Cells</td>
<td>Proteoglycans 40,400 ± 6,000</td>
<td>7,000 ± 2,700</td>
<td>17</td>
<td>Glycosaminoglycans 50,500 ± 8,200</td>
<td>68,700 ± 7,500</td>
</tr>
</tbody>
</table>

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 μM 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 was used. Data represent the mean and standard error of the mean.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage of neurons with neurites</th>
<th>Neurite length μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polylysine</td>
<td>7.6 ± 2.1</td>
<td>29 ± 8.4</td>
</tr>
<tr>
<td>Polylysine + β-D-xyloside</td>
<td>5.2 ± 2.0</td>
<td>20 ± 9.0</td>
</tr>
<tr>
<td>Polylysine + APP</td>
<td>45 ± 5.0</td>
<td>119 ± 9.2</td>
</tr>
<tr>
<td>Polylysine + APP + β-D-xyloside</td>
<td>5.3 ± 1.9</td>
<td>25 ± 7.0</td>
</tr>
<tr>
<td>Polylysine + laminin</td>
<td>92 ± 2.4</td>
<td>209 ± 13</td>
</tr>
<tr>
<td>Polylysine + laminin + β-D-xyloside</td>
<td>94 ± 2.2</td>
<td>200 ± 19</td>
</tr>
</tbody>
</table>

Significantly different from polylysine + APP (p < 0.05).

Table II

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 μM 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 was used. Data represent the mean and standard error of the mean.

REFERENCES

Secreted Glypican Inhibits APP-induced Neurite Outgrowth