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β-Amyloid precursor protein binds to the neurite-promoting IKVAV site of laminin

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ABSTRACT **We previously characterized a 110-kDa membrane-associated laminin-binding protein (LBP110) from brain which binds the laminin A chain -Ile-Lys-Val-Ala-Val- (IKVAV) site and increases in injury. Here we demonstrate that antisera directed against different epitopes of β-amyloid precursor protein (APP) recognize LBP110 and that APP is recognized by LBP110 antiserum. APP specifically binds IKVAV and not another biologically active laminin-derived peptide containing the amino acid sequence -Tyr-Ile-Gly-Ser-Arg-. PC-12 cells transfected with antisense APP RNA produce less APP and LBP110, and they form fewer processes when cultured on either laminin or the IKVAV peptide. Thus, LBP110 is a member of the APP family and a function for APP in neurite outgrowth is now defined.**

The basement membrane glycoprotein laminin is active in promoting neurite outgrowth (1–4), terminal differentiation of enteric nervous system precursors (5), and neuron migration during development (6). Using proteolytic fragments, Edgar *et al.* (3) localized a laminin domain which promotes neurite outgrowth to the long arm of the A chain of laminin. Further studies using synthetic peptides from this region demonstrated that -Ile-Lys-Val-Ala-Val- (IKVAV)-containing peptides are potent stimulators of neurite outgrowth for many established and primary neural cells (4, 7). Affinity chromatography was used to isolate a 110-kDa murine newborn brain receptor for the IKVAV laminin site, which was named the 110-kDa laminin-binding protein (LBP110) (8). This receptor was found to be upregulated as migrating neural crest cells contact laminin (5), and it was localized to distinct neuronal subpopulations and fibers in adult brain (9). LBP110-immunoreactive protein also appeared in reactive glia after either a stab wound or brain ischemia (9).

Amyloid proteins derive from a family of larger (110- to 140-kDa) transmembrane β-amyloid precursor proteins (APPs) (refs. 10 and 11; for review see ref. 12). APP is highly enriched in many tissues, including the brain during development, and many different cell types have been shown to synthesize it. The function(s) of APP is not known. In Alzheimer disease (AD), an ≈4.2-kDa fragment from the carboxyl terminus is present in the senile plaques in the brain which are diagnostic for AD (13). The tangles and plaques in the brain of AD patients also contain laminin (14, 15), which has been reported to be bound by APP (16).

APP has been shown to be present in glia (17, 18), particularly after injury to the brain (19), in a pattern very similar to that which was observed with LBP110 (9). Because of similarities in both size and distribution of APP and LBP110, we compared the immunoreactive and functional properties of these two proteins.

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METHODS

Purification of LBP110 and APP and Western Blot Analysis. Purification of LBP110 by IKVAV-affinity chromatography was performed as previously described (8) with an additional column of a mutated IKVAV peptide (CSRARKQAASGKVAVSADR), which has a single amino acid substitution and allows for greater purification before loading on the IKVAV column. Western blot strips of the EDTA-eluted fractions were allowed to react with either rabbit polyclonal antibody AB10 (20) (1:1000 dilution; detected colorimetrically; antibody kindly donated by L. Walker of Johns Hopkins University School of Medicine and E. Koo of Harvard Medical School) or antibody CT (21) (1:1000 dilution; detected by chemiluminescence with the Amersham ECL system) and then goat anti-rabbit IgG conjugated to horseradish peroxidase (1:500 dilution; Kirkegaard & Perry Laboratories). Antibody AB10 is directed against the amino-terminal domain of APP, while antibody CT is directed against the carboxyl terminus of APP.

APP was purified as previously described (22) and 200 ng was electrophoresed on either a reducing 4–20% polyacrylamide/Tris/glycine minigel (Fig. 2) or a nonreducing 7.5% polyacrylamide/Tris/glycine minigel (Fig. 3) and then transferred to nitrocellulose. Rabbit antiserum directed against LBP110 (used at 1:200 dilution) was incubated with the APP-containing nitrocellulose strip and detected with the Amersham ECL system as described above. Ligand blots were performed by blocking with 10% milk in Tris-buffered saline, pH 7.4, containing 0.1% Tween-20 (TBS-T) for 1 hr, then incubating overnight at 4°C with either CYIGSRYIGSRYIGSR ("YIGSR") or CRKQAASIKVAVS ("IKVAV") at 1 mg/ml in TBS-T. Bound peptide was detected with rabbit antiserum (used at 1:500 dilution in TBS-T) raised against these peptides, then goat anti-rabbit IgG conjugated to horseradish peroxidase (1:1000) followed by ECL chemiluminescence detection.

Generation of Stable APP Antisense PC-12 Clonal Cell Lines and Analysis of APP/LBP110 Protein Expression Levels. To construct the APP antisense recombinant, an *Eco*RI fragment representing bp 1795–2857 of the Kang *et al.* (23) sequence was inserted in the antisense orientation downstream of the SCMV IE94 promoter of the vector pJ7Ω (24). Clonal PC-12 rat adrenal pheochromocytoma cells stably transfected with the APP antisense recombinant and with the vector alone were selected and amplified. Equal numbers of cells were harvested for analysis of soluble proteins as described above. Equal amounts of protein blotted to nitrocellulose were incubated with rabbit polyclonal antibodies to LBP110 (1:1000 in TBS-T) or APP (1:4000 in TBS-T), then goat

Abbreviations: LBP110, 110-kDa laminin-binding protein; APP, β-amyloid precursor protein; AD, Alzheimer disease; NGF, nerve growth factor.

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anti-rabbit IgG conjugated to horseradish peroxidase (1:1000 in TBS-T), and detected by chemiluminescence with the ECL system.

Neurite Outgrowth Assay. PC-12 cells were cultured with or without nerve growth factor (NGF) for 1 day as previously described (25, 26), then 14,000 cells were seeded onto 16-mm wells uncoated or coated with either 10 μg of laminin, 200 μg of IKVAV 13-mer (CRKQAASIKVAVS), or 200 μg of scrambled IKVAV 12-mer (KKSAVQARIVAS) for 1 day. Cells with neurites at least 3 cell body diameters in length were counted (Fig. 5). No neurites were observed in cells cultured on plastic. The mean percentages of cells with neurites (±SEM) are indicated from a total of 150 cells counted per data point. Each experimental condition was repeated at least three times.

RESULTS

APP and LBP110 Are Immunologically Related and Bind the Laminin-Derived IKVAV Peptide. We analyzed pure LBP110 and APP for their immunologic properties and IKVAV binding. The laminin-binding protein was purified by IKVAV-affinity chromatography and assayed by Western blot with antisera directed against either the amino or the carboxyl terminus of APP (Fig. 1). The antisera reacted strongly with this purified protein, confirming that LBP110 shares epitopes with APP. Another APP antibody (see ref. 27; from Athena Neurosciences, San Francisco), directed against human APP-(590–695), also recognized purified LBP110 (data not shown). Converse Western blots were performed with two different preparations of APP. Purified APP (PN-2) was immunopositive when incubated with LBP110 antiserum (Fig. 2). APP obtained from a different source (ref. 28; Athena Neurosciences; APP751 from a baculovirus expression system) was also immunopositive with the LBP110 antiserum (data not shown).

Ligand blots were next performed to determine if purified APP binds IKVAV. APP (PN-2) specifically bound an IKVAV-containing peptide but not a -Tyr-Ile-Gly-Ser-Arg-(YIGSR) containing peptide, another active site on laminin (Fig. 3). IKVAV-ligand blots with another APP source (Athena Neurosciences' APP751) were also positive (data not shown). Although we suspect, due to a specific nervous system localization pattern in mouse, that our protein is most like APP695, it is clear that APP forms containing the Kunitz-type protease inhibitor (KPI) domain can also bind this site in laminin. It has been reported that APP695, APP751, and APP770 can all bind whole laminin with high affinity ($K_d \approx 0.1$ nM; ref. 16). The above data demonstrate that APP specifically binds to the IKVAV-containing site of laminin.

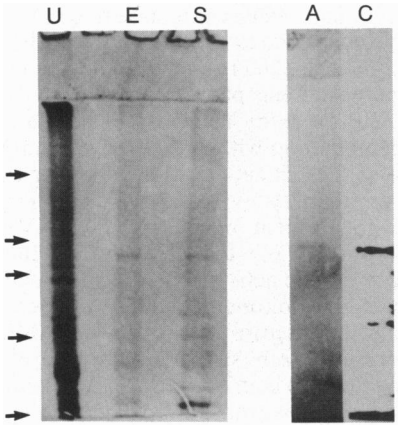

FIG. 1. Purification of LBP110 from neonatal mouse brain and Western blots with APP antisera. The left three lanes demonstrate Coomassie blue-stained fractions after IKVAV-affinity chromatography. High molecular mass markers (200, 116.25, 97.4, 66.2, and 45 kDa) are indicated by arrows. Lane U, the unbound fraction; lane E, the EDTA-eluted fraction; and lane S, the 1.0 M NaCl-eluted fraction. LBP110 is highly purified and located predominantly in the EDTA fraction. This fraction was used in the Western blot strips on the right and reacted positively with antiserum directed against amino-terminal APP epitopes (lane A; AB10) or against carboxyl-terminal APP epitopes (lane C; CT).

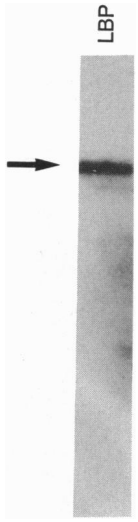

FIG. 2. Western blot of purified APP with antiserum directed against LBP110.

APP and LBP110 Are Involved in PC-12 Neurite Outgrowth on Laminin and on IKVAV. Production and release of APP isoforms by PC-12 rat pheochromocytoma cells have been shown to be modulated by addition of NGF to the culture media (26). To examine the role of APP/LBP110 in neurite outgrowth, we compared neurite outgrowth in PC-12 cells expressing antisense to APP RNA with that seen in control cells. PC-12 cells, two PC-12 lines transfected with a cytomegalovirus (CMV) vector expressing antisense to APP RNA (APPAS #7 and #9) or control vector-transfected PC-12 cells were treated with NGF for 1 day, then the cells were plated on plastic, laminin, an IKVAV-containing 13-mer, or a scrambled IKVAV peptide (Fig. 4). Both APP and LBP110 levels are reduced in the antisense cell lines relative to the parental PC-12 and vector-transfected cells, although APP expression is more completely suppressed in the APPAS #7 cell line (Fig. 4). An identical pattern of suppression of the

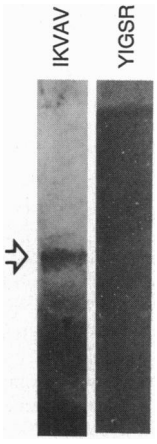

FIG. 3. Ligand blot with purified APP using either laminin-derived IKVAV-containing or YIGSR-containing synthetic peptide.

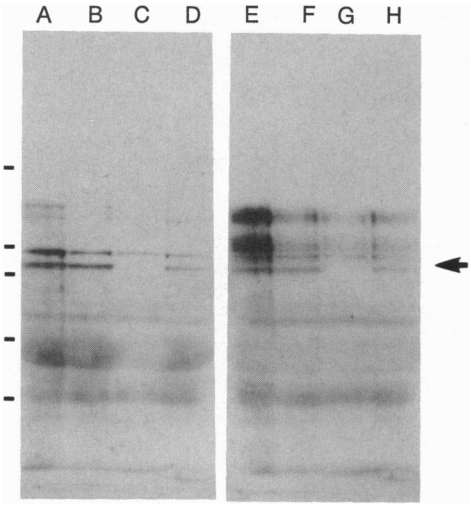

FIG. 4. Expression of APP and LBP110 in PC-12 cells transfected with antisense to APP. Soluble proteins from cell lysates were analyzed for APP (lanes A–D) and LBP110 (lanes E–H) by Western blot analysis. Lanes A and E are PC-12 lysates, lanes B and F are control vector transfectant cells, lanes C and G are APPAS #7 (APP antisense) lysates, and lanes D and H are APPAS #9 (APP antisense) lysates. An identical pattern of immunoreactive protein was found in the ≈110-kDa range (arrow).

110-kDa protein reactive with the two antisera in the transfected antisense lines indicates that LBP110 is related to APP.

Culture of the NGF-primed cells on laminin promoted significant neuritic process formation in both the PC-12 parental cells and the vector control cells. The APP antisense lines produced very few neurites on laminin, and the ones produced were short. Quantitation of neurite processes on laminin, plastic, IKVAV 13-mer, or scrambled IKVAV 12-mer (KKSAVQARIVAS) demonstrated that expression of LBP110/APP695 (Fig. 4) correlated with the ability of the cells to grow neurites on laminin or IKVAV peptide, since the APPAS #7 cell line showed the least protein expression and least ability to form processes (Fig. 5). These experiments suggest that, in addition to sharing structural epitopes and binding activities, LBP110 and APP both function in neurite outgrowth, lending further strength to the supposition that they are related (identical).

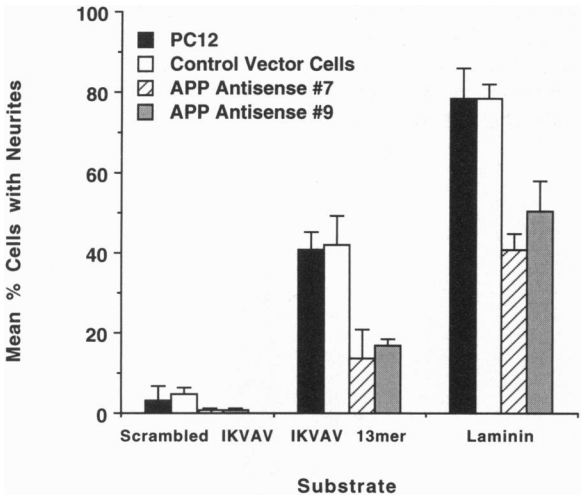

FIG. 5. Neurite production by APP antisense cell lines. Quantitation of neurites (at least 3 cell body diameters in length) by the four cell lines on 10 μg of laminin per well, 200 μg of IKVAV 13-mer per well, or 200 μg of control scrambled IKVAV 12-mer per well (indicated on the *x*-axis) indicated that the ability of cells to produce neurites correlated with their production of LBP110/APP (Fig. 4).

DISCUSSION

It is clear from many studies (refs. 29–31; for review see ref. 12) that while APP may be important in AD pathology, it is also expressed in normal cells. Therefore, elucidation of APP's normal function is critical to understanding its role in dementia. We present data indicating that APP binds the IKVAV site on the laminin A chain which has been shown to promote neurite outgrowth and cell attachment. Antisense cell lines which no longer express significant amounts of APP/LBP110 are unable to form long neurites when cultured on either laminin or IKVAV peptide. Although the APP antisense transfectant data indicate that most of the laminin neurite-promoting activity resides in the IKVAV site, other sites in laminin also have neurite-promoting activity and may be active with PC-12 cells (32).

It will be important to examine LBP110/APP expression during development and neuronal migrations. Pomeranz *et al.* (5, 33) have already demonstrated that LBP110 immunoreactivity is elevated on the surface of neural crest cells which have reached their final destination in the gut to form the enteric ganglia. Neural crest cells which have been selected on the basis of possession of surface LBP110 form predominantly neurons and glia, particularly after culture on laminin (33). Developmental studies utilizing antibodies to the many APP family members may find that different APP forms are expressed at different times during development. For example, APP695 may have a more neural-specific function due to its restricted distribution, while APP PN-2 may have a generalized protease inhibition function throughout the body. It is clear, however, from studies done by Kisilevsky's group (16) that these larger APP forms containing the KPI insert can still bind laminin. In the APP literature, there are some disparities in characterization and localization of APP in studies utilizing antibodies (10, 11, 34–36). These results may be explained by noting that this protein is subject to complex processing and various antibodies recognize only specific epitopes or conformations due either to transient exposure during development or to handling of the tissue. It is also possible that additional members of the APP family exist. We have compared expression of the LBP110 to that of APP (using antibody 22C11) in brain and found essentially identical immunostaining patterns (37). Elucidation of expression of APP forms by using antibodies to unique APP epitopes in combination with *in situ* hybridization may help answer some of the critical questions described above.

We have preliminary evidence which indicates that in normal resting basement membrane the IKVAV site on laminin is cryptic. A key feature of AD is thought to be unbalanced proteolytic activity (38). In such a disease state, this normally hidden epitope of laminin may be exposed and stimulate abnormal neurite outgrowth. IKVAV-containing peptides have also been shown to possess a number of activities in addition to neurite outgrowth, including promoting metastasis and collagenase IV production (39), stimulation of plasminogen activation (40), increased growth and vascularization of tumors, and *in vivo* angiogenesis (41). Deposition of APP or amyloid in plaques may be nucleated by fragments of laminin containing the IKVAV site. Laminin has already been shown to be a component of senile plaques (15). Recently it has been shown that the amyloid β protein can interact with laminin and this substrate can enhance neurite outgrowth over that observed with laminin alone (42). Investigations of the interactions between laminin and IKVAV fragments and LBP110/APP in normal and AD brains may provide clues to the pathogenesis of this disease.

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