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AMYLOID BETA PROTEIN PRECURSOR IS A MITOGEN

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Summary: The form of the secreted amyloid β-protein precursor which contains the protease inhibitor sequence is mitogenic for Swiss 3T3 cells, while the precursor molecule lacking the protease inhibitor domain is not. A ten-fold stimulation of DNA synthesis occurs at 8×10^{-9}M protein.

Amyloid beta protein precursor (ABPP) is both the precursor to the major peptide of filamentous amyloid structures in cores of Alzheimer's plaques (1, 2, 3) and a protein which is synthesized by most cell types (4, 5). A cell associated form of ABPP exists as several molecular species between 110 and 135 kDa (4), while the extracellular forms of the protein in the neuronal PC12 cell line are a pair of tyrosine sulfated proteins of approximately 115 kDa and 140 kDa (6, 7) and a protein of higher molecular weight which may contain heparan sulfate glycosaminoglycan (8). Since one form of ABPP contains an insert with sequence homology to a family of protease inhibitors (9, 10, 11) which can be mitogenic (12), it was asked if the secreted ABPP is mitogenic. The following paragraphs show that the secreted form of ABPP which contains the protease inhibitor domain is mitogenic for Swiss 3T3 cells.

The abbreviation used is: ABPP, amyloid beta protein precursor.

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MATERIALS AND METHODS

Antisera and Immunoblotting. The rabbit antiserum used to identify ABPP with and without the protease inhibitor domain was made against synthetic peptide amino acids 175-186 (GIDKFRGVEFVC) of the predicted ABPP sequence. It was generated after coupling the peptide to keyhole limpet hemocyanin. The specificity of this antiserum, named anti-GID, for ABPP has been documented (6, 13). An antiserum prepared against the protease inhibitor sequence (residues 300-315, 10) is specific for the form of ABPP with the insert (see Fig.1).

To assay for the presence of ABPP, serum free growth conditioned medium was desalted via passage through Sephadex G25 columns in water, lyophilized, dissolved in a $NaDodSO_4$ sample buffer containing 5% 2-mercaptoethanol, and electrophoresed on 7.5% polyacrylamide gels containing $NaDodSO_4$. The proteins were transferred to nitrocellulose and reacted with a 1:1000 dilution of rabbit serum to the ABPP peptides.

Plasmid Construction and Transfection. The permanent cell lines expressing ABPP have been described (4). Briefly, a plasmid was constructed by inserting an Nru I-Spe I fragment of a ABPP cDNA comprising nucleotides -2 to 2358 (3) into the Sma I and Xba I sites of an expression vector (14). Human 293 embryonic kidney cells were each cotransfected with 10 μg of ABPP DNA and 0.5 μg of pSV1-neo DNA by the calcium phosphate precipitate method. The cells were then cultured in selective medium, and after 2-3 weeks, resistant clones were isolated. Serum free growth conditioned medium was prepared by washing the cells twice with serum free medium and incubating for 2 days in serum-free medium. The resulting medium was spun at 1,000 rpm to remove cells and 15,000 rpm to remove membrane debris. Purified ABPP containing the insert domain was prepared by ion exchange and heparin affinity chromatography (6) followed by HPLC chromatography on a C-8 column using a acetonitrile gradient in 0.1% trifluoroacetic acid (8). Purified ABPP without the protease inhibitor was obtained from Drs. D. Schenk and K. Johnson-Wood of Athena Neurosciences.

Mitogenic Assay. Swiss 3T3 fibroblasts were obtained from Tony Hunter (Salk Institute) and were plated at high density in microtiter dishes, starved for two days in 0.5% serum, and then exposed to the mitogen for 24 hrs. H^3-thymidine was then added for 5 hrs and the incorporated isotope determined as described previously (15).

RESULTS AND DISCUSSION

To assay the effect of ABPP on mitogenesis, serum free growth conditioned media from human kidney 293 cells transfected with the form of ABPP with the serine protease inhibitor insert (ABPP 751), without the insert (ABPP 695), or cells transfected with pSV1-neo DNA alone were assayed at a concentration of 12 μg per ml for their effect on tritiated thymidine incorporation in Swiss 3T3 cells. Figure 1 shows by immunoblotting with antisera which recognize both forms of ABPP (anti-GID, lanes 1-3), and

the form containing the protease inhibitor insert (anti-insert, lanes 4-6), that approximately the same amount of amyloid precursor is expressed in the supernatants of the two transfected cell lines. Very little ABPP is secreted by 293 cells alone. When increasing amounts of serum free growth conditioned media are added to the fibroblast cultures, there is a stimulation of DNA synthesis by ABPP 751 but only a slight stimulation by ABPP 695 (Fig.2).

Although the 293 cells were transfected with only a single ABPP gene, it is possible that the expression of ABPP 751 induces the expression of another protein which is mitogenic. To rule out this possibility, 10 ml of the serum-free growth conditioned medium expressing ABPP 751 was run on a reverse phase column, the resultant fractions assayed for mitogenic activity,

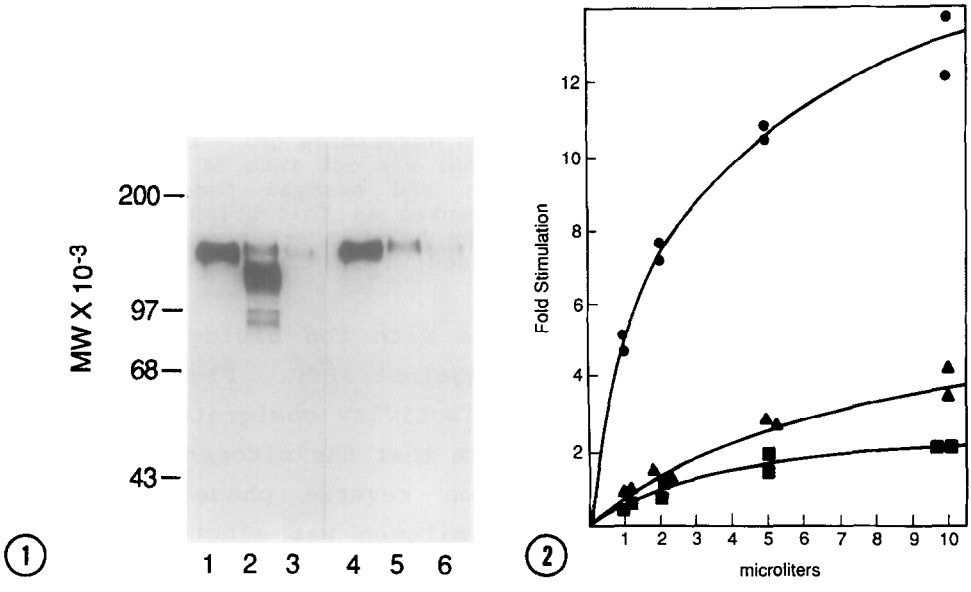

Figure 1. Expression of ABPP. The indicated cell lines were incubated in serum-free medium for 48 hours, and the medium immunoblotted with antibody against ABPP residues 175 to 186 (anti-GID) or anti-insert antibody. Supernatants from: Lanes, 1. 293 cells transfected with ABPP plus insert (anti-GID). 2. 293 cells transfected with ABPP minus insert (anti-GID). 3. 293 cells (anti-GID). 4. 293 cells transfected with ABPP plus insert, (anti-insert). 5. 293 cells transfected with ABPP minus insert, (anti-insert). 6. 293 cells, (anti-insert).

Figure 2. Mitogenic Stimulation of Swiss 3T3 Cells. The ability of serum-free growth conditioned media from the transfected cell lines to stimulate [^{3}H]thymidine incorporation into DNA was assayed as described in Materials and Methods. Increasing amounts of media are plotted against the stimulation of thymidine incorporation as compared with unstimulated cells (550 dpm). (■ - ■) 293 (● - ●) 293 transfected with ABPP 751; (▲ - ▲) 293 transfected with ABPP 695.

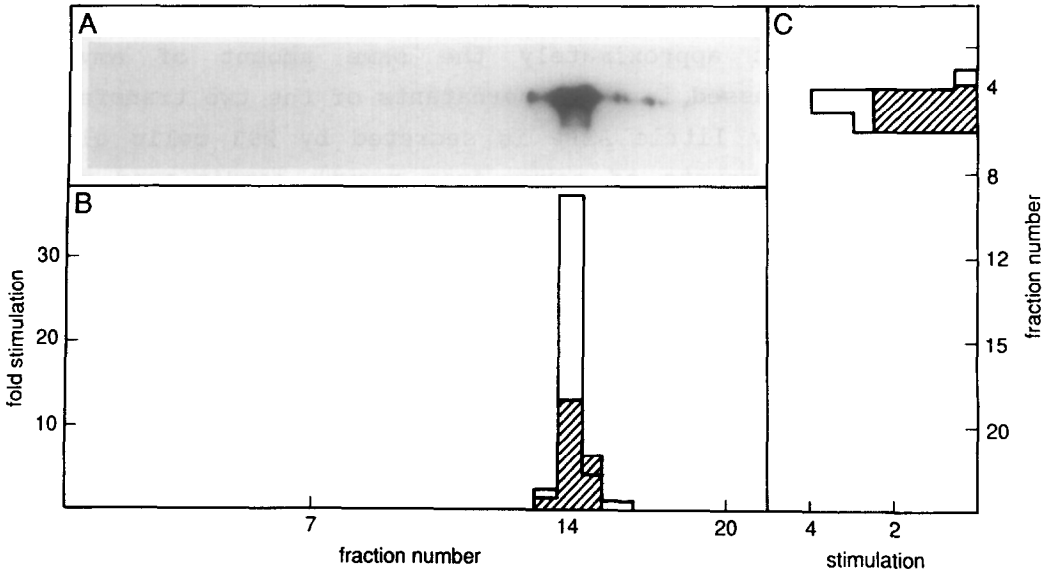

Figure 3. Comigration of ABPP with Mitogenic Activity. Ten ml of serum-free growth conditioned medium were loaded onto a C8 reverse phase column in 0.1% TFA and the protein eluted with a gradient of zero to 80% acetonitrile in 2 hrs (8). The column fractions were assayed for mitogenic activity (B) and immunoblotted for ABPP after electrophoresis on acrylamide gels (A). The remainder of fraction 14 was dried, taken up in sample buffer and run on 2 lanes of an SDS acrylamide gel. One lane was immunoblotted for ABPP and the other was cut into 24 pieces, the proteins eluted into 0.02% SDS, and assayed for biological activity (C). The data are presented as fold-stimulation over unstimulated cells (420 cpm). The hatched areas are duplicate assays using one half the amount used in the clear bars.

and then the fractions near those with the biological activity immunoblotted with an antiserum against ABPP. Figures 3A and B show that ABPP and the mitogenic activity comigrate on the high resolution HPLC column. To confirm that the mitogen was ABPP and not a protein which coeluted on reverse phase, the column fraction 14 which contained the mitogen was electrophoresed on $NaDodSO_4$ acrylamide gels under nonreducing conditions, one lane of the gel cut into 24 pieces and the proteins eluted and assayed for mitogenic activity. The other lane was immunoblotted for ABPP 751. Figure 3C shows that the mitogen comigrated on $NaDodSO_4$ gels with the ABPP antigen. To determine how much ABPP 751 is required to stimulate DNA synthesis, increasing amounts of the purified protein were added to Swiss 3T3 cultures and the stimulation of [^{3}H]thymidine incorporation relative to control cultures determined. Figure 4 shows that a ten-fold stimulation of DNA synthesis occurs at 8×10^{-9}M ABPP 751. Although ABPP 751 is clearly a mitogen, it is approximately 100 fold less potent on Swiss 3T3 cells than basic fibroblast growth factor

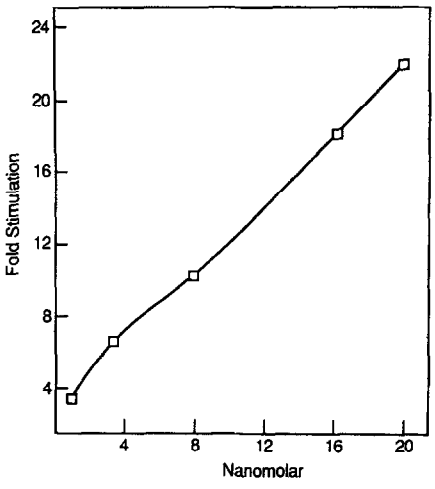

Figure 4. Dose-response Curve of ABPP Stimulated Thymidine Incorporation. Increasing amounts of purified ABPP 751 were added to Swiss 3T3 cells and the incorporation of [^{3}H]thymidine determined after 20 hrs. The stimulation of incorporation relative to unstimulated cells (320 cpm) is plotted as a function of the concentration of protein added.

(15). However, 3T3 cells are not likely to be the correct target, and it is probable that the activity will be higher on the appropriate cell type. Alternatively, the specific activity of the protein may have been reduced during the HPLC purification step. The form of the precursor molecule without the protease inhibitor (ABPP 695) did not stimulate DNA synthesis when tested at concentrations up to 15 x 10^{-9}M.

The above data show that ABPP is mitogenic for Swiss 3T3 cells. The form of ABPP which is mitogenic contains an insert specifying the synthesis of a Kunitz-type serine protease inhibitor (9, 10, 11). A protein with sequence homology to this class of protease inhibitors has been isolated from the growth conditioned media of hepatoma cells on the basis of its mitogenic activity (12). It is therefore not unlikely that the form of ABPP containing this sequence can act as a mitogen. In addition, ABPP may modulate the adhesive interactions of cells (13), and perturbations in adhesion are known to stimulate mitosis in most anchorage dependent cells (16). It has not been ruled out, however, that ABPP has a specific cell surface receptor which can generate a mitotic signal. The relationship between the biological activity of ABPP and Alzheimer's pathology remains to be defined. However, the observations that ABPP is synthesized by most cell types and that it can act as a mitogen suggest that it has a critical role in the biology of the cell.

ACKNOWLEDGMENTS

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