Amyloid β /A4 protein precursor of human glioma cell, Bu-17

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ABSTRACT

For possible *in vitro* experiments of amyloid fibril formation, we have examined the expression of APP in cultured cells. In the present studies, we analyzed APP of Bu-17 glioma cells by immunoelectron microscopic and cell-surface labelling techniques. Immunoelectron microscopic observation revealed that positive reaction products obtained with an antibody to the extracellular domain of APP were located on the cell-surface membranes of Bu-17 cells. APP isolated from Bu-17 cells labelled with sulfo-NHS-biotin reacted on Western blots with an antibody to the cytoplasmic C-terminal domain of APP, and also reacted with avidin-biotinylated peroxidase complex. These results confirm that APP of Bu-17 cells is the transmembrane form.

Amyloid fibril deposition is considered to be the most important chemical and pathological hallmark in the brains of patients with Alzheimer's disease (AD) or Down's syndrome in middle age. The major constituent of amyloid fibrils in the disorder is β /A4 protein (3, 11) which is part of the longer amyloid β /A4 protein precursors (APPs) produced by alternative splicing (7, 8, 14, 16). APP is a family of 90 to 130 K glycosylated, membrane-bound proteins (15, 17) which can be processed to yield soluble derivatives (2, 13), some of which have been identified as protease nexin II (12). Recently, it has been reported that a tissue specific distribution of the secreted form and transmembrane form of APP has been found and this distribution may reflect tissue-specific processing pathways (1). Therefore, aberrant regulation of APP processing or APP degradation is a possible cause of amyloid fibril deposition in the brain in AD. However, the exact post-translational processing mechanisms which generate amyloid fibrils from APP are still unknown.

For possible *in vitro* experiments of amyloid fibril formation, we have examined the expression of APP in cultured cells (6). The Bu-17 (human glioma) cell line, which was one of the analyzed cells, failed to secrete significant quantities of APP

into the culture medium and so appears to synthesize the transmembrane form APP (6). In the present study, we have confirmed the presence of the Kunitz inhibitor-containing transmembrane form of APP on the cell surface of Bu-17 cells using immunoelectron microscopy and cell-surface labelling techniques.

Bu-17 cells were cultured in 10% fetal calf serum/Dulbecco's modified Eagle's medium. For electron microscopic study, these cells were washed with Hanks' balanced solution containing 0.1% NaN₃ and 1% bovine serum albumin (HBSS). Unfixed cells were treated directly with antibodies (1:40) for 30 min at 4°C and were washed with HBSS. After incubation with peroxidase-conjugated goat anti-rabbit IgG (1:30) followed by washing in phosphate-buffered saline, the cells were fixed in 2.5% glutaraldehyde and were treated with 0.05% diaminobenzidine and 0.01% H₂O₂. Finally, the cells were postfixated in a 2% solution of osmium tetroxide, dehydrated, embedded in resin, and processed by the usual method (4).

Bu-17 cell-surface proteins were labelled with sulfo-NHS-biotin (Pierce) as described (10). For the purification of APP, labelled cells or non-labelled cells were homogenized in 10 vol of 10 mM phosphate buffer/0.2 M NaCl, pH 7.2 and centrifuged at

47,400 g for 40 min. The pellets were washed repeatedly and homogenized in 10 mM phosphate/0.2 M NaCl/0.5% Triton X-100, pH 7.2. After centrifugation, the supernatant fractions were applied to an ion-exchange column of DEAE-Sepharose CL-6B (Pharmacia) equilibrated with 10 mM phosphate/0.2 M NaCl, pH 7.2 and eluted with 10 mM phosphate/1.0 M NaCl, pH 7.2. APP immunoreactive fractions were further purified on an anion-exchange column of MA7Q (BioRad) with a linear gradient of increasing NaCl concentration (0.2–0.8 M) in 10 mM phosphate buffer, pH 7.2.

Purified APP was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting and the avidin-biotin complex technique. Antibodies to subsequences of APP (7, 8, 14, 16), N-terminal T97 (residues 18–38 of APP 695), R36 (residues 527–540 of APP 695), β (residues 597–620 of APP 695), C-terminal R37 (residues 681–695 of APP 695) and inhibitor insert R98 (residues 325–337 of APP 770), were produced as described previously (5).

As shown in Fig. 1, the positive immunoperoxidase reaction products obtained with anti-R36 antibody were located on the surface of Bu-17 cell membranes while no positive reaction was obtained with anti-C-terminal R37 antibody. This indicates either that the C-terminal part of APP is located in the cytoplasm or that APP without any C-terminal domain is associated with the cell-surface membranes. To clarify this problem, APP on the cell-surface membranes of Bu-17 cells was labelled with sulfo-NHS-biotin and was purified by anion-exchange chromatography. Purified APP from non-labelled Bu-17 cells reacted with all antibodies to APP subsequences, anti-N-terminal T97, anti-R98, anti-R36, anti- β , and anti-C-terminal R37 antibody (Fig. 2A), while purified APP from Bu-17 cells labelled with sulfo-NHS-biotin reacted with anti-C-terminal R37 antibody and also with avidin-biotinylated peroxidase complex (Fig. 2B). These results indicate that APP obtained from Bu-17 cells is the intact molecule with the inhibitor insert domain and is composed of the extracellular domain labelled with sulfo-NHS-biotin and the cytoplasmic C-terminal domain. Thus, APP on the surface of the Bu-17 cell membrane seems to be the intact transmembrane form.

The secreted form of APP in neuronal R14 cells has been found mostly in the extracellular matrix by electron microscopic observation after immunogold labelling (9). This distribution pattern is differ-

ent from that of APP in Bu-17 cells. The APP-immunoreactivity of Bu-17 cells is localized on the cell-surface membrane, not on the extracellular matrix. Moreover, since the secreted form of APP has been processed within the cytoplasm (1, 2), it does not react with anti-C-terminal R37 antibody. This also suggests that APP on the cell-surface membrane of Bu-17 cells is the transmembrane form.

Recently, it was reported that a tissue-specific distribution of the secreted and transmembrane forms of APP has been found and this distribution pattern may reflect tissue-specific processing pathways (1). Since normal proteolytic processing occurs in the interior of β /A4 protein sequence (2), β /A4 protein seems to be produced by aberrant regulation of the processing pathway of the secreted form of APP or degradation of the transmembrane form of APP in AD and related disorders. Thus, Bu-17 cells with the transmembrane form of APP may be one useful tool to investigate the mechanism of β /A4 protein processing.

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REFERENCES

1. CATTERUCCIA N., WILLINGALE-THEUNE J., BUNKE D., PRIOR R., MASTERS C. L., CRISANTI A. and BEYREUTHER K. (1990) Ultrastructural localization of the putative precursors of the A4 amyloid protein associated with Alzheimer's disease. *Amer. J. Pathol.* **137**, 19–26
2. ESCH F. S., KEIM P. S., BEATTIE E. C., BLACHER R. W., CULWELL A. R., OLTERSDORF T., MCCLURE D. and WARD P. J. (1990) Cleavage of amyloid β peptide during constitutive processing of its precursor. *Science* **248**, 1122–1124
3. GLENNER G. G. and WONG C. G. (1984) Alzheimer's disease: Initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem. Biophys. Res. Commun.* **120**, 885–890
4. ISHII T. and HAGA S. (1984) Immuno-electron-microscopic localization of complements in amyloid fibrils of senile plaques. *Acta Neuropathol.* **63**, 296–300
5. ISHII T., KAMETANI F., HAGA S. and SATO M. (1989) The immunohistochemical demonstration of subsequences of the precursor of the amyloid A4 protein in senile plaques in Alzheimer's disease. *Neuropathol. Appl. Neurobiol.* **15**, 135–147
6. KAMETANI F., HAGA S., TANAKA K. and ISHII T. (1990)

Fig. 1 Electron micrographs of Bu-17 cells with anti-extracellular R36 (A) and anti-C-terminal R37 antibodies (B). (A) Positive immunoperoxidase reaction products are seen on the membrane surface (arrowheads). (B) No positive reaction

Fig. 2 (A) SDS-PAGE of Bu-17 cell membrane proteins and immunoblots with antibodies to subsequences of APP. Lane 1, CBB stain of purified APP; lane 2, immunoblot with anti-N-terminal T97; lane 3, immunoblot with anti-inhibitor domain R98; lane 4, immunoblot with anti-R36; lane 5, immunoblot with anti- β ; lane 6, immunoblot with anti-C-terminal R37. (B) SDS-PAGE of labelled Bu-17 cells and western blotting followed by staining with ABC technique. Lane 1, membrane fraction of labelled Bu-17 cells; lane 2, cytosol fraction of labelled Bu-17 cells; lane 3, purified labelled APP; lane 4, immunoblotting of purified labelled APP with anti-C-terminal R37 antibody. APP is indicated by an arrow. Molecular weights are indicated by bars: 106.0, 80.0, 49.5, 32.5, 27.5 and 18.5 K.

- Amyloid β -protein precursor (APP) of cultured cells: secretory and non-secretory forms of APP. *J. Neurol. Sci.* **97**, 43-52
7. KANG J., LEMAIRE H. G., UNTERBECK A., SALBAUM J. M., MASTERS C. L., GRZESCHIK K. H., MULTHAUP M., BEYREUTHER K. and MÜLLER-HILL B. (1987) The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature* **197**, 733-736
 8. KITAGUCHI N., TAKAHASHI Y., TOKUSHIMA Y., SHIOJIRI S. and ITO H. (1988) Novel precursor of Alzheimer's disease amyloid protein shows protease inhibitory activity. *Nature* **331**, 530-532
 9. KLIER F. G., COLE G., STALLCUP W. and SCHUBERT D. (1990) Amyloid β -protein precursor is associated with extracellular matrix. *Brain Res.* **515**, 336-342
 10. LISANTI M. P., SARGIACOMO M., GRAEVE L., SALTIEL A. R. and RODRIGUEZ-BOULAN E. (1988) Polarized apical distribution of glycosyl-phosphatidylinositol-anchored proteins in a renal epithelial cell line. *Proc. Natl. Acad. Sci. USA* **85**, 9557-9561
 11. MASTERS C. L., SIMMS G., WEINMAN N. A., MULTHAUP G., McDONALD B. L. and BEYREUTHER K. (1985) Amyloid plaque core protein in Alzheimer disease and Down syndrome. *Proc. Natl. Acad. Sci. USA* **82**, 4245-4249
 12. OLTERSDORF T., FRITZ L. C., SCHENK D. B., LIEBERBURG I., JOHNSON-WOOD K. L., BEATTIE E. C., WARD P. J., BLACHER R. W., DOVEY H. F. and SINHA S. (1989) The secreted form of the Alzheimer's amyloid precursor protein with the Kunitz domain is protease nexin-II. *Nature* **341**, 144-147
 13. PALMERT M. R., PODLISNY M. B., WITKER D. S., OLTERSDORF T., YOUNKIN L. H., SELKOE D. J. and YOUNKIN S. G. (1989) The β -amyloid protein precursor of Alzheimer dis-

- case has soluble derivatives found in human brain and cerebrospinal fluid. *Proc. Natl. Acad. Sci. USA* **86**, 6338-6342
14. PONTE P., GONZALEZ-DEWHITT P., SCHILLING J., MILLER J., HSU D., GREENBERG B., DAVIS K., WALLACE W., LIEBERBURG I., FULLER F. and CORDELL B. (1988) A new A4 amyloid mRNA contains a domain homologous to serine proteinase inhibitors. *Nature* **331**, 525-527
 15. SELKOE D. J., PODLISNY M. B., JOACHIM C. L., VICKERS E. A., LEE G., FRITZ L. C. and OLDERSDORF T. (1988) β -Amyloid precursor protein of Alzheimer disease occurs as 110- to 135-kilodalton membrane-associated proteins in neural and nonneural tissue. *Proc. Natl. Acad. Sci. USA* **85**, 7341-7345
 16. TANZI R. E., MCCLATCHEY A. I., LAMPERTI E. D., VILLAKOMAROFF L., GUSELLA J. F. and NEVE R. L. (1988) Protease inhibitor domain encoded by an amyloid protein precursor mRNA associated with Alzheimer's disease. *Nature* **331**, 528-530
 17. WEIDEMANN A., KÖNIG G., BUNKE D., FISCHER P., SALBAUM J. M., MASTERS C. L. and BEYREUTHER K. (1989) Identification, biogenesis, and localization of precursors of Alzheimer's disease A4 amyloid protein. *Cell* **57**, 115-126