Alzheimer Amyloid Protein Precursor Is Localized in Nerve Terminal Preparations to Rab5-containing Vesicular Organelles Distinct from Those Implicated in the Synaptic Vesicle Pathway*

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In order to localize amyloid protein precursor (APP) in nerve terminals, we have immunoisolated vesicular organelles from nerve terminal preparations using antibodies to Rab5 and synaptophysin. These immunoisolates were then analyzed by electron microscopy and by immunoblotting. The synaptophysin immunoisolates represented a nearly homogeneous population of small synaptic vesicles, with less than 10% contamination by other organelles, and very little APP. In contrast, Rab5 immunoisolates contained, in addition to small synaptic vesicles, substantial numbers of large unilamellar vesicles and high levels of APP. Thus, it appears that nerve terminal APP is contained predominantly in large vesicular organelles, distinct from synaptic vesicles and from the synaptic vesicle recycling pathway.

Amyloid protein precursor (APP) is a type I membrane-spanning glycoprotein which is ubiquitously expressed in mammalian cells (1). Proteolytic processing of APP results in the generation of an ~40-residue amyloid (Aβ) fragment which accumulates in the brains of individuals with Alzheimer’s disease (AD). A central role for Aβ in the pathogenesis of AD is indicated by the discovery that various mutations within or flanking the Aβ region of APP cosegregate with affected status in individuals from several families with autosomal dominant AD. For this reason, the localization, trafficking, and processing of APP has generated great interest. Immunocytochemical studies of APP in cultured cells and in brain tissue reveal that a predominant fraction of APP is localized to the endoplasmic reticulum and the Golgi apparatus (2, 3). The localization of APP to biosynthetic organelles can be explained in part by the very high rate of synthesis and turnover of this protein. In axonal and synaptic compartments of brain tissue, APP is localized to large vesicular structures (2, 3). APP is also found on the surface of cultured cells, from where it can be internalized and converted to Aβ (4–7).

APP is transported by fast axonal transport in central and peripheral neurons (8, 9). Soluble APP (sAPP) can be released at synapses, although it is not known whether the soluble fragments are generated at the synapse. Pools of unprocessed APP are also transported retrogradely from axonal or synaptic compartments to neuronal cell bodies and dendrites (10, 11). It is not known which membrane trafficking pathways are involved in anterograde and retrograde transport of APP in neurons or whether APP-containing vesicles are related to the recycling pathway of synaptic vesicles within the nerve terminal.

In an attempt to study the nature of the APP-containing vesicles in nerve terminals, we have isolated APP-containing membranes from synaptosomes of rat forebrain and from PC12 cells. To differentiate vesicles participating in the synaptic vesicle pathway from other endocytic vesicles, organelles were immunoisolated using immobilized monoclonal antibodies directed against either synaptophysin, synaptobrevin, or the endosomal GTPase, Rab5. Synaptophysin and synaptobrevin are found in synaptic vesicle membranes (12–15, 32). Rab5 is a resident of early endosomes that is ubiquitously expressed in all endosomes, irrespective of the nature of the endocytic pathway (16–20). Our data suggest that APP resides on endocytic trafficking organelles which are clearly distinct from organelles involved in synaptic vesicle recycling, suggesting a lighthiero unknown trafficking pathway in nerve terminals of fully differentiated neurons.

MATERIALS AND METHODS

Antibodies—Monoclonal antibodies directed against the synaptic vesicle proteins synaptophysin (c1.7.2) (21), Rab5 (c1.621.1) (19), and synaptobrevin II (c1.69.1) (22) have been described, as has the polyclonal antibody (369) directed against the carboxyl terminus of APP (23). Polyclonal antibody 3129 was raised against a peptide corresponding to Aβ 1–40 and therefore does not cross-react with the APP-like proteins, APLP1 or APLP2.

Preparation of Immunobeads—Immunobeads were prepared as described (19). For coupling to beads, IgG was purified from ascites using Protein G-Sepharose (Pharmacia Biotech Inc.). The purified IgG was dialyzed for 3 days against 150 mM NaCl with 7 changes. Following dialysis, antibodies were centrifuged at 10,000 × g for 15 min, and the supernatant was used for coupling to Eupergit CIZ beads (Rohm Pharma, Darmstadt, Germany) as described (19). Beads were tested for antibody coupling by SDS-PAGE.

Immunoisolation of Vesicular Organelles from Nerve Terminal Preparations—Nerve terminal preparations (synaptosomes) were purified as described by Nicholls (24). Purified synaptosomes were pelleted, resuspended in a minimal volume of 140 mM NaCl, 10 mM glucose, 5 mM KCl, 1 mM MgCl2, 1.2 mM Na2HPO4, 20 mM HEPES, pH 7.4, and hypotonically lysed by diluting 10-fold in ice-cold H2O and homogenizing at 2,000 rpm, using 8 strokes in a glass/Teflon homogenizer. HEPES and NaCl were added to final concentrations of 10 mM and 100 mM, respectively, and the lysed synaptosomes were spun for 15 min at

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† The abbreviations used are: APP, amyloid protein precursor; AD, Alzheimer’s disease; PAGE, polyacrylamide gel electrophoresis.
18,700 × g to separate the synaptic vesicular organelles from synaptosomal membrane debris. The supernatant from this fraction was used for the immunooisolation of vesicular organelles. Immunooisolation was conducted as described previously (19). Briefly, 2 ml of the supernatant from the nerve terminal preparation were incubated with 10-μl bed volume of immunobeads for 45 min at 4 °C under slow rotation. Beads with bound material were pelleted for 10 min at 360 × g and washed 3 times with 0.32 m sucrose, 10 mM HEPES, pH 7.4. Bead-bound organelles were then analyzed by immunoblotting (see below) and/or electron microscopy (25).

Immunooisolation of Vesicular Organelles from PC12 Cells—Immunooisolation of vesicles from PC12 cells was carried out as described (22). Briefly, cells were homogenized by passing 20 times through a ball bearing cell cracker with a 0.0006-inch clearance. Homogenates were centrifuged at 10,000 × g for 10 min at 4 °C. The beads were sedimented and washed as described above, and bound material was analyzed by SDS-PAGE followed by immunoblotting.

Semi-quantitative and Quantitative Immunoblotting—For immunoblot analysis, washed beads were resuspended in Laemmli loading buffer, boiled for 5 min, and spun down. The supernatant was subjected to SDS-PAGE followed by immunoblotting. Membranes were probed for synaptophysin with antibody C17.2 and for APP with antibodies 369 or 3129. Detection was carried out using peroxidase-labeled secondary antibodies and enhanced chemiluminescence (ECL, Amersham) or with radiiodinated Protein A followed by PhosphorImager (Molecular Dynamics, Mountain View, CA) autoradiography.

RESULTS

Previous research has shown that APP is transported to the nerve terminal, and that full-length APP can be transported retrogradely from the nerve terminal (8, 10, 11, 26). The relationship of these pathways for the transport of APP to the trafficking pathways for small synaptic vesicles was investigated in the present study. Immunoblotting of a conventionally purified (27) small synaptic vesicle fraction showed that there was a small amount, but no enrichment, of APP immunoreactivity (Fig. 1A, Ves). In order to localize APP and synaptophysin in this vesicle preparation, immunolabeling of frozen ultrathin sections was carried out using either anti-synaptophysin (c17.2) (Fig. 1B) or anti-APP (369) (Fig. 1C) antibodies. Immunolabeling with anti-synaptophysin antibodies demonstrated that the vast majority of the small synaptic vesicles contained synaptophysin, as expected. In contrast, only very few structures, which were typically larger than small synaptic vesicles, contained APP (Fig. 1C, arrowheads). The paucity of profiles which were immunoreactive for APP is consistent with the results of immunoblotting and presumably represents trace amounts of contaminating vesicles of unknown origin. Thus, these results indicate that APP is virtually absent from small synaptic vesicles.

To further characterize APP-containing organelles in the synapse, we chose a two-step procedure to isolate synaptic organelles. First, synaptosomes were prepared using a combination of differential centrifugation and Ficoll density gradient centrifugation. This procedure yields a fraction that is highly enriched in nerve terminals, with only low levels of contamination by soma-derived organelles, myelin, or mitochondria (28, 29). Second, these purified nerve terminals were lysed by osmotic shock to release internal organelles, followed by immunooisolation of organelles using methacrylate beads coated with antibodies directed against synaptophysin, synaptobrevin II, or Rab5.

Comparison of vesicular organelles immunooisolated with Rab5 and synaptophysin antibodies revealed significant differences. The synaptophysin immunooisolates contained predominantly (>90%) small synaptic vesicles (typically <60 nm in diameter, Fig. 2A). Rab5 immunooisolates contained, in addition to small synaptic vesicles (Fig. 2B), a substantial number of other distinct vesicles, including large unilamellar vesicles (Fig. 2, C and D), large bilamellar vesicles (Fig. 2, E and F), and multivesicular bodies (Fig. 2, G and H). It should be noted that the distinction between unilamellar and bilamellar vesicles was not always clear-cut (e.g. Fig. 2, C and D); in these cases, the profiles were counted as unilamellar. There was a 4-fold increase in the proportion of large unilamellar vesicles and a 2-fold increase in the proportion of large bilamellar vesicles, in Rab5 immunooisolates relative to synaptophysin immunooisolates (Fig. 2, lower panel). Whereas the small synaptic vesicles in both synaptophysin and Rab5 immunooisolates were heavily immunoreactive with anti-synaptophysin as shown by immunogold labeling of immunooisolates (Fig. 2, A and B), the large unilamellar vesicles, bilamellar vesicles, and multivesicular bodies showed negligible immunoreactivity (data not shown). We were unable to obtain satisfactory immunolabeling of the Rab5 immunooisolates with 369 due to nonspecific adsorption of antibody to the beads.

The levels of APP were determined in vesicular organelles immunooisolated from nerve terminal preparations. Since the immunooisolates contained significant amounts of immunoglobulins derived from the isolation procedure, total protein could not be used as a basis for comparing the various preparations. Instead, we used the levels of synaptophysin for comparison. Synaptophysin is found in small synaptic vesicles in synaptophysin, synaptobrevin, and Rab5 immunooisolates (e.g. Fig. 2, A and B). Since the frequency of small synaptic vesicles in the various types of immunooisolates was so high (78–91%; Fig. 2, lower panel), it enabled us to use synaptophysin levels as the basis for comparison. Samples of each immunooisolate, containing equivalent amounts of synaptophysin, were subjected to immunoblotting with an antibody (369) raised against a peptide corresponding to the cytoplasmic domain of APP or an...
### DISCUSSION

Previous studies in brain tissue using immunoelectron microscopy have shown that APP resides in large vacuolar structures in axonal and synaptic compartments (2, 3). However, the nature of these APP-containing vesicles was unknown. In the present study, we have characterized APP-containing vesicular organelles obtained by immunosolubilization from purified synaptosomes of rat brain. The use of synaptosomal preparations made it possible to study the distribution of APP in organelles derived from nerve terminals without significant contamination by trafficking organelles from other sources. Immunolocalizations were carried out using immobilized monoclonal antibodies directed against either the synaptic vesicle proteins synaptophysin or synaptobrevin or against the endosomal GTPase, Rab5. Synaptophysin and synaptobrevin are membrane proteins specific for synaptic vesicles and are thus expected to be present in all organelles participating in the pathways of synaptic vesicle recycling and regulated exocytosis. Rab5 is a resident of early endosomes of all endocytic pathways studied to date. We found that APP was highly enriched in Rab5-

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**Fig. 2.** Rab5 immunoisolates contain large multilamellar vesicles. Vesicular organelles were immunosolubilized from lyed nerve terminal preparations using bead-coupled antibodies to Rab5 or synaptophysin. Upper panel, electron microscopic analyses were carried out on Epon-embedded immunoisolates. A, synaptophysin immunoisolate showing small synaptic vesicles; B–H, Rab5 immunoisolates showing small synaptic vesicles (B), large unilamellar vesicles (C and D), large bilamellar vesicles (E and F), and multivesicular bodies (G and H). In A and B, the immunoisolates were reacted with affinity-purified antisa-ynaptophysin antiserum (G95) and gold-labeled Protein A, before em-

**Fig. 3.** APP is present in Rab5 immunoisolates from rat brain nerve terminals and from PC12 cells. Vesicular organelles were immunosolubilized from lyed nerve terminal preparations (A–C) or PC12 cells (D), using bead-coupled antibodies to either synaptophysin, or Rab5. A, visualization of APP, by immunoblotting with antibodies 369 and 3129, and of synaptophysin, in immunoisolates, after normalization for synaptophysin. B, quantification of APP levels in synaptophysin and Rab5 immunoisolates, after normalization for synaptophysin. Data represent means ± S.E. for five separate experi-

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containing vesicles, but virtually absent from synaptophysin- or synaptobrevin-containing vesicles. Based on these results, we hypothesize that Rab5 immunosolates contain at least two classes of organelles. One consists of small synaptic vesicles, which contain synaptophysin but not APP. The second consists of a previously unidentified class of endocytic trafficking organelles, which contain APP but little or no synaptophysin. This would account for the high levels of APP in Rab5 immunosolates as compared with that in synaptophysin immunosolates (Fig. 3B), as well as for the intense synaptophysin staining (Fig. 1B) and virtual absence of APP staining (Fig. 1C) of conventionally purified small synaptic vesicles.

Several studies suggest that synaptic vesicles arise after endocytosis from the presynaptic membrane (19, 30, 31). The apparent exclusion of APP from synaptic vesicles implies that APP resides on an organelle which is part of a separate endocytic pathway. The fact that most of the APP in the synapse is associated with a Rab5-positive compartment indicates that APP spends most of its time in the endocytic rather than the exocytic limb of its trafficking pathway. This means that APP, which reaches the nerve terminal by fast anterograde transport (8, 9), is transiently exposed to the synaptic plasma membrane surface (7), reinternalized, and targeted to organelles distinct from membranes involved in synaptic vesicle recycling.

A similar example of specialized sorting in nerve terminals is provided by the polymeric immunoglobulin receptor (pIgR), which mediates transport of polymeric IgA and IgM across epithelial surfaces (33, 34). When expressed in differentiated PC12 cells (31) and primary neuronal cultures (35), pIgR is primarily sorted to neuritic or axonal processes, where it is found in specialized endosomes, but is excluded from purified synaptic vesicle-like structures (31). In transfected neurons, pIgR undergoes transcytosis from the somatodendritic compartment to the axonal compartment (35). Full-length APP, which, unlike the pIgR, is an endogenous neuronal protein, also undergoes transcytosis, albeit from the axonal to the somatodendritic compartment (7, 11). Thus, APP might have a similar role as a transcytotic carrier of an as yet unidentified ligand which would bind to APP on the presynaptic terminal and/or axonal membrane. The APP-containing organelles which we have isolated in the present study would represent the earliest stages in this transport pathway, since they still contain Rab5, which is only transiently associated with membranes (16–20) and is, therefore, a stage-specific marker.

A more complete biochemical characterization of these Rab5/APP vesicles would be greatly facilitated by the development of high affinity monoclonal antibodies directed against the cytoplasmic domain of APP, which would allow the direct immunosolation of APP-containing vesicular organelles. This would also enable the study of the fate of the APP carrier vesicles during their retrograde or transcytotic transit.

In summary, we have biochemically and ultrastructurally characterized APP-containing vesicles in rat brain synaptosomal preparations. These vesicles contain the endosomal GTPase Rab5 and only very low levels of synaptophysin. We therefore conclude that these APP carriers are derived from an endocytic pathway distinct from that involved in synaptic vesicle recycling. The presence of APP in synaptic terminals, in proximity to synaptic vesicle-rich areas, may indicate a relation between synaptic activity and APP function. Since most of the APP in cultured cells is produced via an endocytic pathway (5), the Rab5/APP-containing endocytic organelles described here may play a crucial role in APP formation and deposition in the brain.

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REFERENCES

1. Sisodia, S. S., and Price, D. L. (1995) FASEB J. 9, 366–370
2. Caporaso, G. L., Takei, K., Gandy, S. E., Matteoli, M., Mundyigl, O., Greengard, P., and De Camilli, P. (1994) J. Neurosci. 14, 3122–3128
3. Tomimoto, H., Akiyoshi, I., Wakiha, H., Nakamura, S., and Kimura, J. (1995) Brain Res. 672, 167–195
4. Haass, C., Koo, R. H., Mollen, A., Hung, A. Y., and Selkoe, D. J. (1992) Nature 357, 500–503
5. Koo, E. H., and Squazzo, S. L. (1994) J. Biol. Chem. 269, 17386–17389
6. Kelly, E. H., Squazzo, S. L., Selkoe, D. J., and Koo, E. H. (1996) J. Cell Biol. 109, 991–998
7. Yamazaki, T., Koo, E. H., and Selkoe, D. J. (1996) J. Cell Sci. 109, 1089–1098
8. Koo, E. H., Sisodia, S. S., Archer, D. R., Martin, L. J., Weidemann, A., Reyterreuth, K., Fischer, P., Masters, C. L., and Price, D. L. (1996) Proc. Natl. Acad. Sci. U.S.A. 87, 1561–1565
9. Morin, P. J., Abraham, C. R., Amarutunga, A., Johnson, R. J., Huber, G., Sandell, J. H., and Fine, R. E. (1995) J. Neurochem. 61, 464–473
10. Yamazaki, T., Selkoe, D. J., and Koo, E. H. (1995) J. Cell Biol. 129, 431–442
11. Simons, M., Ikonen, E., Tiernan, P. J., Cryl-Arregui, A., Monnig, U., Beyreuther, K., and Dotti, C. G. (1995) J. Neurosci. Res. 41, 121–128
12. Sudhof, T. C., Petrenko, A. G., Whitaker, V. P., and Jahn, R. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 235–240
13. John, R., and Sudhof, T. C. (1994) Annu. Rev. Neurosci. 17, 219–246
14. Mundigl, O., and De Camilli, P. (1994) Curr. Opin. Cell Biol. 6, 561–567
15. Chilcote, T. J., Galli, T., Mundigl, O., Edelmann, L., McPherson, P. S., Takei, K., and De Camilli, P. (1995) J. Cell Biol. 129, 219–231
16. Bucci, C., Parton, R. G., Mather, I. H., Stunnenberg, H., Simons, K., Hofack, B., and Zerial, M. (1992) Cell 70, 715–728
17. Zerial, M. (1993) Cytotocology 11, 847–849
18. Bucci, C., Wunderinger-Ness, A., Lutcke, A., Chiarelli, M., Bruni, C. B., and Zerial, M. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 5061–5065
19. Fischer von Mollard, G., Stahl, B., Walch-Solimena, C., Takei, K., Daniels, L., Kholkatchev, A., De Camilli, P., Sudhof, T. C., and Jahn, R. (1994) Eur. J. Cell Biol. 65, 319–326
20. de Hoop, M. J., Huber, L. A., Stenmark, H., Williamson, E., Zerial, M., Parton, R. G., and Dotti, C. G. (1994) Neuron 13, 11–22
21. John, R., Schiebler, W., Quinet, M., and Greengard, P. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 4317–4414
22. Chilcote, T. J., Galli, T., Mundigl, O., Edelmann, L., McPherson, P. S., Takei, K., and De Camilli, P. (1995) J. Cell Biol. 129, 219–231
23. Buxbaum, J. D., Gandy, S. E., Ciecheti, P., Ehrlich, M. E., Czernik, A. J., Fracasso, R. P., and Ramabhadran, T. V., Unterbeck, A. J., and Greengard, P. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 6003–6006
24. Nichols, D. G. (1978) Biochem. J. 176, 511–522
25. Cameron, P. L., Sudhof, T. C., John, R., and De Camilli, P. (1991) J. Cell Biol. 115, 151–164
26. Sisodia, S. S., Koo, E. H., Hoffmann, P. N., Perry, G., and Price, D. L. (1993) J. Neurosci. 13, 3130–3142
27. Huttner, W. B., Schiebler, W., Greengard, P., and De Camilli, P. (1983) J. Cell Biol. 96, 1374–1388
28. Booth, R. F., and Clark, J. B. (1978) Biochem. J. 176, 365–370
29. Maycox, P. R., Link, E., Reetz, A., Morris, S. A., and Jahn, R. (1992) J. Cell Biol. 118, 1379–1388
30. Kelly, R. B., and Grote, E. (1993) Annu. Rev. Neurosci. 16, 85–127
31. Konig, L., and De Camilli, P. (1995) J. Cell Biol. 127, 1603–1616
32. Fratelli, T. L., Cameron, P., De Camilli, P., and Banker, G. (1991) J. Neurosci. 11, 1617–1620
33. Mostov, K., Apodaca, E., Arneti, B., and Okamoto, C. (1992) J. Cell Biol. 116, 577–583
34. Mostov, K. E. (1994) Annu. Rev. Immunol. 12, 63–84
35. de Hoop, M., van Poser, C., Lange, C., Ikonen, E., Hunsiker, W., and Dotti, C. G. (1995) J. Cell Biol. 130, 1447–1459