Trafficking of cell-surface amyloid β-protein precursor

II. Endocytosis, recycling, and lysosomal targeting detected by immunolocalization

Tsuneo Yamazaki1,3, Edward H. Koo2,3 and Dennis J. Selkoe1,3,*

Departments of 1Neurology and 2Pathology, Harvard Medical School, Boston, MA 02115, USA
3Center for Neurologic Diseases, Brigham and Women’s Hospital, Boston, MA 02115, USA
*Author for correspondence at address 3

SUMMARY

Amyloid β-protein (Aβ) is a proteolytic fragment of the amyloid β-protein precursor (βPP). Progressive cerebral deposition of Aβ is an early and invariant feature of Alzheimer’s disease. The cellular trafficking of βPP is of particular interest because understanding the production of Aβ requires a comprehensive elucidation of the metabolic pathways of this protein. In addition, βPP is a type I integral membrane glycoprotein that belongs to a class of molecules with both full length and secreted products. Recent evidence suggests that βPP can be processed in an endosomal/lysosomal pathway. In the latter organelles, a number of βPP carboxy-terminal derivatives are found, but the precise pathway and kinetics of βPP trafficking from the cell surface remain unclear. To address these questions, we visualized directly the βPP internalization pathway by following the localization and distribution of βPP monoclonal antibodies added to intact βPP-transformed Chinese hamster ovary cells. Using immunofluorescence and immunoelectron microscopy, βPP was shown to be rapidly internalized via coated pits and vesicles, after which the molecules were transported to endosomes, prelysosomes, and lysosomes. Using a modified immunodetection protocol, we demonstrated the rapid recycling of endocytosed βPP to the cell surface and its ultimate targeting to lysosomes. Because we recently found that endocytosis of cell surface βPP is one route for the constitutive production of Aβ, the recycling pathway for cell surface βPP demonstrated here is a probable route for production of the critical Aβ fragment.

Key words: Alzheimer’s disease, Amyloid β-protein precursor (βPP), EM, Endocytosis, Recycling

INTRODUCTION

Alzheimer’s disease (AD) is characterized by the progressive formation in the brain of insoluble amyloid plaques and vascular deposits consisting primarily of the ~4 kDa amyloid β-peptide (Aβ). Aβ is derived from a highly conserved and widely expressed integral membrane glycoprotein, the amyloid β-protein precursor (βPP) (Kang et al., 1987). Five major isoforms of βPP have been reported which are derived by alternative splicing of a single gene on human chromosome 21. Besides the 695-amino acid form (βPP695) (Kang et al., 1987), at least four other βPP transcripts have been identified which contain an additional exon encoding a Kunitz-type protease inhibitor domain: βPP563, ‘L-APP’ (βPP751 without residues 618-675), βPP751 and βPP770 (de Sauvage and Octave, 1989; Kitaguchi et al., 1988; Koenig et al., 1992; Ponte et al., 1988; Tanzi et al., 1988). Although the primary structure predicted from the βPP cDNA suggests that βPP might serve as a cell surface receptor (Kang et al., 1987), little direct information about its function as a receptor has been reported.

βPP at the cell surface is known to undergo cleavage within the Aβ region by an as yet unidentified protease (designated α-secretase), resulting in release of the large, soluble ectodomain (βP5) and precluding the formation of an intact Aβ peptide (Esch et al., 1990; Sisodia et al., 1990). In addition to this secretory pathway, cell surface βPP can be internalized and targeted to endosomes and lysosomes, in which an array of Aβ-containing carboxyl-terminal fragments is found (Haass et al., 1992a). These endosomal/lysosomal-derived fragments could potentially serve as intermediates in the formation of Aβ (Cole et al., 1989; Estus et al., 1992; Golde et al., 1992; Haass et al., 1992a). A surprising finding is that the Aβ fragment itself is constitutively produced and released by βPP-expressing cells during normal metabolism (Haass et al., 1992b; Seubert et al., 1992; Shoji et al., 1992). Recent evidence indicates that one source of this secreted Aβ is cell surface βPP that is undergoing endocytosis (Koo and Squazzo, 1994). Specifically, it has been suggested that proteolytic cleavage at the N terminus of Aβ by an enzyme designated β-secretase occurs intracellularly shortly after internalization of βPP, probably in an acidic compartment (Haass et al., 1993; Koo and Squazzo, 1994). Cleavage of the resultant ~12 kDa C-terminal βPP fragment, which has been detected in some cell types (Cai et al., 1993; Golde et al., 1992; Citron et al., 1995), may then be effected at or near the cell surface by an enzyme designated γ-secretase, resulting in rapid release of Aβ into the medium (Higaki et al., 1995). Therefore, it is important to demonstrate the various cellular trafficking routes of βPP in
order to understand both its normal processing and function and the mechanism of generation of its amyloidogenic Aβ fragment.

In a companion report (see accompanying paper, Koo et al., 1996), we described an analysis of the rate of βPP release and internalization from the cell surface using a radioiodinated monoclonal antibody against the extracellular domain of βPP. In this report, we adapted this approach to morphologically characterize the βPP internalization pathway from the cell surface. The antibodies were added to transfected Chinese hamster ovary (CHO) cells and the trafficking route of surface βPP after internalization was immunolocalized. By light and electron microscopy, cell surface βPP is internalized in the receptor mediated endocytic pathway where the molecules can be detected in coated pits and endosomes, after which βPP undergoes rapid recycling to the cell surface or is transported to the lysosomal system for degradation.

MATERIALS AND METHODS

Antibodies
The affinity-purified polyclonal antibody B5 (Oltersdorf et al., 1990) is directed against a recombinantly expressed protein of βPP19-667 (numbering of βPP770). The monoclonal antibodies 5A3 and 1G7, and goat polyclonal antibody 207 (a gift from Dr B. Greenberg) were raised to human βPP, purified from βPP-transfected CHO cells (Koo and Squazzo, 1994) or baculovirus infected cells, respectively (Shoji et al., 1992). On the basis of competition studies, the two βPP monoclonal antibodies are directed to non-overlapping epitopes within the midregion of the βPP ectodomain between residues 380 and 665, excluding the Aβ region. 1G7 was fully characterized in the companion study and was shown not to alter βPP trafficking (Koo et al., 1996). Because the staining patterns observed with either 5A3 or 1G7 antibody are very similar, the two monoclonal antibodies were generally used together (1:1) to obtain a higher signal on immunostaining. Thus, for the immunocytochemical experiments described herein, we routinely used purified 5A3 and 1G7 antibodies together.

At the immuncytochemical level, these anti-βPP antibodies did not recognize amyloid precursor like protein 2 (AβPP2) expressed in CHO cells (not shown). Monovalent Fab fragments of 5A3 and 1G7 were obtained by papain digestion and purified by Protein A Sepharose chromatography to remove Fc fragments and undigested IgGs. Some experiments were carried out with peroxidase-conjugated monoclonal antibodies. Maleimide-activated horseradish peroxidase (HRP) was crosslinked to reduced antibody according to the manufacturer’s instructions (Pierce, Rockford, IL). In brief, the IgG molecule was exposed to mild reduction by 2-mercaptoethylamine prior to HRP coupling in order to generate free sulphydryl groups at the hinge region. Therefore, the resultant labeled antibodies were monovalent rather than bivalent species. Two additional monoclonal antibodies were used in the studies: human lysosome associated membrane protein (LAMP-2) (Developmental Studies Hybridoma Bank, Baltimore, MD) and rat transferrin receptor, clone Ox-26 (BioSource International, Camarillo, CA).

Cell culture
CHO cells were grown in Dulbecco’s modified Eagle’s medium containing 10% Fetal Clone II serum (HyClone Laboratories, Logan, UT). CHO cell lines stably expressing wild-type βPP 751 were generated with expression vector pcMV751 using the Lipofectin (Life Technologies, Bethesda, MD) transfection method and selected by G418 resistance. Two other mutant βPP constructs were generated by oligonucleotide-directed mutagenesis of the parental βPP751 plasmid and stably transfected into CHO cells: a construct with a Val717Phe amino acid substitution (V717F) found in kindred with familial (autosomal dominant) AD (Murrell et al., 1991) and a C-terminal truncated βPP construct (AC) deleting almost the entire cytoplasmic tail (residues 729-770; βPP770 numbering). The putative coated pit-mediated internalization signal (NPTY) at residues 757-762 is deleted in the latter construct.

Immunofluorescence microscopy
βPP-transfected CHO cells grown on coverslips were incubated with anti-βPP antibodies for 1 hour on ice, washed 5 times with cold DPBS and then warmed to 37°C for various times (0 to 60 minutes). Cells were then fixed with cold 4% formaldehyde (freshly prepared from paraformaldehyde) in PBS, permeabilized for 5 minutes with 0.3% Triton X-100 in PBS, and incubated with goat anti-rabbit or antimouse IgG conjugated to fluorescein isothiocyanate (FITC) (Boehringer Mannheim, Indianapolis, IN) for 1 hour at room temperature. For control staining, the transfected cells were incubated with mouse anti-rabbit IgG or cold Dulbecco’s phosphate buffer saline (DPBS) instead of the primary antibody; untransfected CHO cells were used as well. In all the experiments, results obtained by whole IgGs were similar to those by Fab fragments, however, the intensity of Fab immunoreaction was significantly lower. Therefore, the results shown here were obtained in experiments using IgG from a mixture of both antibodies.

In control experiments with saponin pretreatment, βPP751 transfected CHO cells were incubated with 5A3/1G7 for 15-60 minutes after internalization or normal mouse IgG (2.5 mg/ml) for 0.5-2 hours at 37°C to allow for antibody internalization. The concentration of the non-immune mouse IgG is such that the antibody should be internalized by fluid phase uptake. Cultures were then treated with 0.01% saponin in DPBS for one minute at room temperature. After washing, fixation, and permeabilization, cells were incubated with secondary antibodies as described above.

In colocalization of internalized βPP and transferrin receptor, cells on coverslips were cultured in the presence of human holotransferrin-Texas red (20 μg/ml) (Molecular Probes, Eugene, OR) for 2 hours at 37°C and, then incubated with anti-βPP monoclonal antibodies for 1 hour at 4°C. After brief washing with cold DPBS, coverslips were transferred to prewarmed medium at 37°C containing holotransferrin-Texas red for five minutes. Following fixation and permeabilization, the cells were incubated with anti-mouse IgG conjugated to FITC to visualize the βPP antibodies. In a second approach, CHO cells were fixed and permeabilized after the internalization of 5A3/1G7 monoclonal antibodies for five minutes, and then incubated with an anti-rat transferrin receptor antibody. FITC- and Texas red-conjugated secondary antibodies were used to visualize βPP and transferrin receptor antibodies, respectively. In this latter experiment, digital images obtained from confocal microscopy scanned at a single 0.5 μm thick section were used for colocalization. Controls from individually labeled samples were carried out to exclude signal crossover.

For the recycling study, cultures were labeled with βPP antibodies for 1 hour at 4°C. After washing with cold DPBS, labeled βPP was allowed to internalize for 15 minutes in prewarmed medium at 37°C. Cells were chilled with cold DPBS and treated with DPBS at pH 2.8 for 2 minutes. After washing with DPBS, cultures were incubated with warmed medium in the presence of secondary antibodies at ~1 μg/ml for 30-60 minutes. At this concentration, the resultant signal was not derived by fluid phase uptake of the secondary antibodies. No signal was detected when the primary antibodies were omitted.

In some experiments, double labeling immunocytochemistry was carried out with two mouse monoclonal antibodies (Negoescu et al., 1994; Yamazaki et al., 1995). In this case, the cells were first labeled with one monoclonal antibody, washed extensively, and incubated with an excess of Fab fragments of goat anti-mouse IgG (1:10) following manufacturer’s instructions (Jackson Immunoresearch) so that all the primary antibodies (usually anti-βPP monoclonal anti-
bodies) were stericly covered by goat Fab fragments. Subsequently, the cells were incubated with the second monoclonal antibody, followed by anti-goat and anti-mouse secondary antibodies to visualize the two different primary antibodies. Two control experiments confirmed the effectiveness of this approach. First, no reaction can be detected with a control FITC-conjugated anti-mouse antibody in the presence of excess Fab fragments of goat anti-mouse antibody, the latter used to sterically mask the primary mouse monoclonal antibody. Second, the pattern of the double labeled cultures is identical to immunostaining with each of the respective monoclonal antibodies alone. In all experiments save one mentioned above, the immunostained cells were viewed by conventional fluorescence microscopy.

**Immunoelectron microscopy**

Two methods were used to visualize directly the localization of βPP during trafficking. In the first method, βPP-transfected CHO cells grown in 60 mm dishes were incubated with monovalent βPP monoclonal antibodies conjugated to goat F(ab')2 and rabbit peroxidase (HRP) conjugated with Epon embedding. Ultrathin sections were stained with aqueous uranyl acetate and lead citrate and observed at 80 kV. As a control, cells were incubated in cold PBS or with nonimmune mouse monoclonal antibody to visualize the location of the primary antibodies. The staining with little to no intracellular staining was observed at all time points after internalization of βPP transfected cells (Fig. 1e). In the absence of the primary antibodies, cells showed a fine punctate staining pattern over the cell surface (Fig. 1f). After 30 minutes, almost no cell surface staining was observed in wild-type βPP transfected cells (Fig. 1g). In the ΔC transfected cells, more robust cell surface staining with little to no intracellular staining was observed at all time points after internalization of βPP as compared to wild-type transfecants. At 30 minutes, there was generally no staining remaining on the cell surface (Fig. 1g), although a few rare cells in the ΔC cell line showed diminished but still detectable surface immunoreactivity (Fig. 1h). In untransfected cells, the staining was virtually negative, with rare scattered cells showing immunoreactivity most likely representing endogenous βPP (data not shown).

To determine whether βPP shares the same trafficking compartments with other molecules known to undergo receptor mediated endocytosis, cells were incubated for five minutes at 37°C to initiate internalization of βPP antibodies and then stained with transferrin receptor antibody. Virtually all the structures immunolabeled by βPP antibodies (Fig. 2a) were also positive for transferrin receptor (Fig. 2b). Similarly, the internalized βPP antibodies colocalized with Texas red-conjugated human holotransferrin (Cameron et al., 1991) (Fig. 2c.d). These results suggested that at this early time point, internalized βPP is located predominantly in early endosome.

To investigate the perinuclear compartments in which βPP antibodies were localized after 15 and 30 minutes of incubation, cells were double-labeled with βPP monoclonal antibodies and the lysosomal membrane marker, LAMP2 (Chen et al., 1985) After the βPP antibodies were allowed to internalize at 37°C as above, the cells were fixed, permeabilized and incubated with the LAMP2 (H4B4) antibody. After 15 minutes, βPP positive granules were distributed diffusely within the cell (Fig. 2e), whereas LAMP2 staining was predominantly in a perinuclear distribution (Fig. 2f). In the perinuclear region, some of the βPP immunoreactive vesicles were also positive for LAMP2 staining (Fig. 2e,f), indicating the localization of same βPP molecules to lysosomes within 15 minutes. After 30 minutes, most structures immunolabeled by βPP antibodies were also positive for the LAMP2 antibody (Fig. 2g,h), and the peripheral staining was lost.

The polyclonal βPP antibodies, B5 and 207, produced the same pattern of βPP immunolocalization as the monoclonal antibodies (not shown). Cell-surface βPP labeled with Fab fragments of the monoclonal antibodies was also internalized and produced a similar staining pattern, however, the signal was distinctly weaker than with IgG. This observation provides additional confirmation that the bivalent antibody did not cross link βPP to alter its trafficking pathway, as was suggested by the radiolabeled antibody experiments in the companion study (Koo et al., 1996). Control specimens incubated with mouse anti-rabbit IgG or cold DPBS instead of the primary antibody were entirely negative. Treatment with saponin resulted in no detectable loss of signal derived from internalized βPP antibodies from 15-60 minutes (data not shown). Immuno-reactivity of non-immune mouse IgG was effectively lost after saponin treatment, suggesting that the antibodies internalized by fluid phase endocytosis had diffused out of the cells (data not shown). Therefore, these results further suggested that the immunoreactivity represented intact antibody-βPP complexes within the cells after internalization.

**RESULTS**

**Internalization of βPP visualized by immunofluorescence microscopy**

To directly demonstrate the trafficking pathway of βPP after internalization, CHO cells were surface-labeled with monoclonal antibodies 5A3/1G7 for 1 hour at 4°C and then returned to 37°C for varying intervals. Subsequently, the cells were fixed and permeabilized, and secondary antibodies were added to visualize the location of the primary antibodies. The staining pattern was essentially the same in wild-type βPP751 and V717F (familial AD 717V→F mutant) transfected cells, with the exception that the signal was somewhat more intense in the latter due to their higher βPP expression. At time 0, βPP showed a fine punctate staining pattern over the cell surface (Fig. 1a,b). After a 3-5 minute incubation at 37°C, fine granular staining appeared inside the cells (Fig. 1c). After 15 minutes, this staining appeared more diffusely within the cytoplasm (Fig. 1d) and in some cells, immunoreaction accumulated around nuclei (Fig. 1e). By 30-60 minutes, the vesicles appeared larger and more coarse, and their distribution was distinctly perinuclear and generally less abundant than at earlier time points (Fig. 1f). After 30 minutes, almost no cell surface staining was observed in wild-type βPP transfected cells (Fig. 1g). In the ΔC transfected cells, more robust cell surface staining with little to no intracellular staining was observed at all time points after rearming to 37°C as compared to wild-type transfecants. At 30 minutes, there was generally no staining remaining on the cell surface (Fig. 1g), although a few rare cells in the ΔC cell line showed diminished but still detectable surface immunoreactivity (Fig. 1h). In untransfected cells, the staining was virtually negative, with rare scattered cells showing immunoreactivity most likely representing endogenous βPP (data not shown).

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Internalization of βPP visualized by immunoelectron microscopy

We initially attempted to conjugate βPP monoclonal antibodies directly to colloidal gold particles without success (Geoghegan and Ackerman, 1977). As an alternative, EM studies were undertaken using HRP-conjugated monovalent antibodies. As in the immunofluorescence studies, monovalent antibodies were used to avoid the possibility that βPP might be crosslinked by whole IgG. Subsequent studies were carried out using whole IgG followed by secondary antibodies conjugated to colloidal gold when it became apparent that the patterns of βPP immunochemistry obtained with the two methods were identical. However, the reactivity using the former (HRP) method was weaker, and the ultrastructural detail without uranyl acetate counterstaining was suboptimal. Therefore, the following description relates primarily to observations obtained with whole IgG and colloidal gold-conjugated secondary antibodies.

At time 0, scattered gold particles were observed at the cell surface. These particles were arranged in small clusters rather than diffusely on the cell membrane or at any apparent specific structural specializations (Fig. 3a). The apparent lack of surface staining over the area of the nucleus is because this area is out of the plane of focus. V717F transfected cells (b) demonstrated more intense staining than wild-type transfected cells (a) due to a higher level of βPP expression. After 5 minutes incubation at 37°C, a granular pattern appeared in the cytoplasm in wild-type cells (c). After 15 minutes, the intracellular granular pattern of the wild-type transfectants was distributed throughout the cytoplasm (d) including some βPP-positive granules in the perinuclear region (e). After 30-60 minutes, βPP was localized mainly in the perinuclear region and showed a coarse vesicular pattern in V717F (f) cells. A very few isolated AC cells showed the cell surface staining pattern after a 30 minute incubation (h), although less than at time 0, a result not obtained in the wild-type (g) and V717F transfectants. Note that most of the other cells in the microscopic field in (h) are not immunostained (arrows). Numbers at the right upper corners indicate the time in minutes after rewarming. Cell surface βPP were labeled by 5A3/1G7 antibodies. Bars, 10 μm.

Fig. 1. Localization of βPP in CHO cell lines during internalization by immunofluorescence microscopy using monoclonal antibodies 5A3/1G7. At time 0, βPP located on the cell surface showed a fine granular punctate pattern (a, b). The apparent lack of surface staining over the area of the nucleus is because this area is out of the plane of focus. V717F transfected cells (b) demonstrated more intense staining than wild-type transfected cells (a) due to a higher level of βPP expression. After 5 minutes incubation at 37°C, a granular pattern appeared in the cytoplasm in wild-type cells (c). After 15 minutes, the intracellular granular pattern of the wild-type transfectants was distributed throughout the cytoplasm (d) including some βPP-positive granules in the perinuclear region (e). After 30-60 minutes, βPP was localized mainly in the perinuclear region and showed a coarse vesicular pattern in V717F (f) cells. A very few isolated AC cells showed the cell surface staining pattern after a 30 minute incubation (h), although less than at time 0, a result not obtained in the wild-type (g) and V717F transfectants. Note that most of the other cells in the microscopic field in (h) are not immunostained (arrows). Numbers at the right upper corners indicate the time in minutes after rewarming. Cell surface βPP were labeled by 5A3/1G7 antibodies. Bars, 10 μm.
Immunolocalization of internalized βPP

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ally tubular structures consistent with tubular endosomes or the so-called compartments of uncoupling of receptor and ligand (CURL) (Geuze et al., 1983) were immunolabeled (Fig. 4d). Within CURL compartments and endosomes, βPP reactivity was localized in the periphery, just beneath the membrane (Fig. 4d,e,f). After 15 minutes of internalization, some βPP immunoreactivity (arrows) located around the nucleus (e) was colocalized with LAMP2 positive granules (f), but not the βPP reaction distributed more peripherally (e). After 30 minutes incubation, immunoreaction of βPP (g) and LAMP2 (h) were colocalized (arrows). Cells are wild-type βPP751 transfectants and cell surface βPP were labeled by 5A3/1G7 antibodies. Bars, 10 μm.

was located within the latter compartments (not shown). When these ultrastructural observations are considered in light of the immunofluorescence studies, it is likely that these organelles are prelysosomes/lysosomes. In these sites, the immunogold particles were found diffusely within the structure rather than at the periphery, as was noted at earlier time points (data not shown).

The organelle localizations of βPP detected by the HRP reaction and the immunogold particles corresponded well (see Fig. 4). Therefore, the use of gold-labeled secondary antibodies did not appear to substantially alter the βPP trafficking pathway. As expected, the immunoreactivity was most intense in the high expressing V717F cells, although an identical subcellular pattern of immunofluorescence was seen in wild-type transfectants and was barely detectable in ΔC transfectants. In these CHO cell lines, no apparent reaction due to endogenous HRP was observed; control specimens with the secondary antibody alone or after competition with excess unlabeled primary antibodies were entirely negative.

Recycling of cell-surface βPP

To assess recycling of endocytosed βPP, cells in which βPP antibodies had first been internalized were then incubated with secondary antibody at 37°C. The rationale is that secondary antibodies will bind only to primary antibody/βPP complexes that are re-exposed at the cell surface following recycling (Matteoli et al., 1992). This approach requires that residual primary antibody on the cell surface be effectively removed before incubation with the secondary antibody.

Accordingly, we determined whether bound primary antibodies can be removed from cell-surface βPP by incubation in ice-cold acidified medium. To establish the conditions for this process, CHO cells were incubated with primary antibodies for 1 hour at 4°C, after which the washed cells were incubated with ice-cold medium at pH 2.8. The cells were then fixed and incubated with fluorescent secondary antibodies. Treatment with acidic medium for 2 minutes efficiently removed all detectable primary antibody (Fig. 5a,b). To confirm that the cells were not injured by the acidic wash, the cells were pretreated with pH 2.8 buffer for 2 minutes at 4°C, washed, labeled with primary antibodies at 4°C for 1 hour and then incubated at 37°C for various times. Following fixation, the cells were labeled with secondary fluorescent antibodies. The results of this internalization study (Fig. 5c) were identical to those described above under standard conditions (i.e. without acid exposure), indicating that cell-surface primary antibodies can be effectively detached without detectable adverse morphological effects to βPP internalization upon return to normal culture conditions. An

Fig. 2. Double label fluorescence immunocytochemistry shows that after 5 minutes of internalization, βPP (a,c) was colocalized in the same compartment as transferrin receptor (b) and transferrin (d). (a and b) Digital images obtained from confocal laser scanning microscopy. Identical images were seen with either antibody alone. After 15 minutes incubation, some βPP immunoreaction (arrows) located around the nucleus (e) was colocalized with LAMP2 positive granules (f), but not the βPP reaction distributed more peripherally (e). After 30 minutes incubation, immunoreaction of βPP (g) and LAMP2 (h) were colocalized (arrows). Cells are wild-type βPP751 transfectants and cell surface βPP were labeled by 5A3/1G7 antibodies. Bars, 10 μm.
additional control experiment was performed to rule out the possibility that the signal was derived from the minor amount of antibodies that were not removed by the acid wash. Here, cultures were labeled by βPP antibodies at 4°C, treated with acidic buffer and then warmed to 37°C for either 1 hour in the presence of secondary antibodies or for 15 minutes without secondary antibody. In the latter case, the cells were then fixed, permeabilized, and incubated with secondary antibodies. In either case, no immunoreactivity could be detected (data not shown), suggesting that the acid treatment effectively removed cell surface bound βPP antibodies and the signal in the experiments described below was not derived from residual surface antibody.

Using this paradigm, CHO cells were incubated with either polyclonal (B5) or mixed monoclonal (5A3/1G7) antibodies at 4°C for one hour, allowed to internalize for 15 minutes at 37°C, then rapidly chilled in acidic medium to detach primary antibodies remaining on the cell surface. The cells were then returned to 37°C for 30 to 60 minutes in the presence of either FITC-conjugated secondary antibodies for fluorescence microscopy or colloidal gold-conjugated secondary antibodies for EM. By fluorescence microscopy, various sized βPP-positive granular structures with a pattern suggestive of both endosomal and lysosomal localization were present throughout the cytoplasm of cells undergoing this treatment (Fig. 5d). This experiment strongly suggested that internalized βPP is recycled to the cell surface whereupon the cycle can be repeated. This staining pattern was intense in cell lines with full length βPP (wild-type and V717F transfected cells) and virtually undetectable in the ΔC cell line, as expected. By a time course study, we were able to detect recycling of internalized βPP antibodies to the cell surface as early as 5 minutes, albeit faintly, after incubation at 37°C (Fig. 5g). This finding suggested that βPP was internalized and recycled to the cell surface rapidly, apparently within 10 minutes.

By immunoelectron microscopy, a few scattered gold particles were seen on the cell surface, but most were clearly localized within small vesicular structures (Fig. 5e). Gold-labeled perinuclear structures were also observed (Fig. 5f) and were similar to the prelysosomal/lysosomal structures seen above in the internalization study. Therefore, not surprisingly some of the recycled βPP molecules had been targeted to lysosomes. Compared to the primary internalization studies described above, the βPP labeling in these recycling studies was less intense, both at the light microscopic and ultrastructural levels. As controls, specimens labeled with secondary antibodies alone with or without serum were uniformly negative.

**DISCUSSION**

βPP is constitutively processed in both the secretory (exocytic) and endosomal/lysosomal (endocytic) pathways. The major product of the former pathway is βPPα, derived from cleavage of mature βPP by α-secretase, thus precluding the formation of an intact Aβ peptide (Esch et al., 1990). From the cell surface, βPP is sorted to lysosomes where subsequent degradation results in an array of C-terminal fragments, including those that contain the entire Aβ region.
Immunolocalization of internalized βPP (Golde et al., 1992; Haass et al., 1992a). Whether these fragments are the immediate precursors for soluble Aβ is unclear, especially in light of the fact that Aβ cannot be detected in purified lysosomes (Haass et al., 1993). Recent studies, however, showed that cell surface βPP molecules are one source of precursors to Aβ subsequently released into the medium and in this pathway appear to require endocytic processing (Koo and Squazzo, 1994). To date, the pathway that βPP takes following internalization has not been defined. We therefore undertook a direct morphological characterization of the βPP trafficking pathways from the cell surface using βPP monoclonal antibodies as a probe to localize βPP. In this study, we demonstrate that βPP on the cell surface is rapidly internalized via the clathrin mediated endocytic pathway. The bulk of internalized βPP appears to be destined for lysosomal degradation after transiting through the endosomal compartment, although a population is rapidly recycled to the cell surface for subsequent internalization and presumably secretion as well. Finally, our results comparing the trafficking of wild-type and C-terminal truncated βPP molecules showed considerably less internalization of surface molecules in the latter cell line.

In this study, monoclonal antibodies were used to following the trafficking pathway of βPP from the cell surface. Central to our approach is the assumption that the antibodies did not perturb normal protein trafficking and that the antibodies remained attached to βPP as a complex, at least until degradation has begun. Although this method of using monoclonal antibodies as a probe has been used previously to successfully track the internalization of a variety of cell surface receptors, we do not have the benefit of a natural ligand for βPP to confirm the findings. Nevertheless, a number of control experiments from this and the companion study (Koo et al., 1996) argue that the results are reliable and meaningful. Results comparing monovalent to bivalent antibodies were essentially identical, although the signal was, not unexpectedly, lower with the former antibodies. This suggested that βPP is not cross-linked by whole IgG. As pointed out in the companion

Fig. 4. Immuno EM localization of internalized βPP in endosomes and lysosomes. After 3-10 minutes of internalization of 5A3/1G7 antibodies at 37°C, vesicular and tubular structures suggestive of early endosomes were strongly labeled in V717F cells (a,b) and wild-type transfectants (c)(a, gold method; b and c, HRP method). Occasionally, the tubular portions of CURL were positive in wild-type transfectants (d)(HRP method). In large vesicular endosomes, labeled βPP was localized to their perimeter membrane (e, gold method; f, HRP method). After 15-30 minutes of internalization of 5A3/1G7 at 37°C, gold particles were located within multivesicular structures (g) and prelysosomes/lysosomes (h). In these sites, the immunogold particles were found throughout the structures, not just at the periphery (as in e,f). (e-h, V717F cells). Bars, 0.1 μm.
report (Koo et al., 1996), internalization did not appear to be induced by the antibodies because both the secretion rate and the half-life of membrane bound βPP as measured by antibody binding and biotinylation studies are virtually identical. The rapid sorting to the degradative compartments (within 15-30 minutes) seen ultrastructurally in this study is consistent with the preceding biochemical observations. The antibody-antigen complex also appeared to be stable in the early stages after internalization. Saponin permeabilization showed that the antibodies were not diffusable and were thus presumably attached to membrane bound βPP. Moreover, in the ultrastructural studies, βPP immunostaining as visualized by both colloidal gold and HRP reactivity was located only at the periphery of coated vesicles and endosomes, i.e. presumably still membrane associated. In contrast, at later time points after endocytosis, βPP immunoreactivity present in prelysosomal organelles, such as multivesicular structures, and in lysomes per se was distributed centrally rather than at the perimeter. This suggested that either the monoclonal antibodies had detached from βPP or that βPP was in the process of degradation and no longer membrane bound. The observation of apparent βPP degradation in prelysosomal and lysosomal compartments is in accord with previous biochemical recovery of βPP proteolytic fragments within lysosomes (Golde et al., 1992; Haass et al., 1992a). Taken together, these observations argue that the antibodies remained attached to βPP throughout the initial phases of internalization. Thus, in the mildly acidic early endosomal compartment, the antibodies are not labile, a conclusion consistent with our own observation that the βPP antibodies can be incubated in acidic medium to pH 4 without significant detachment (data not shown).

As shown by our morphological studies, βPP from the cell surface is rapidly internalized into the cell via the receptor-mediated endocytic pathway. Shortly after rewarming, only a
small fraction of molecules is left on the cell surface. The majority of internalized molecules appeared to be targeted to lysosomes. Interestingly, our morphological studies further demonstrated that internalized βPP is rapidly recycled to the cell surface in CHO cells, a conclusion also reached by biotinylation and radiolabeled antibody studies described in the companion report (Koo et al., 1996). Because βPP is both simultaneously released and internalized from the cell surface, the number of times βPP recycles to the cell surface cannot be estimated. The short recycling time we documented (~10 minutes) suggests that internalized βPP recycled back to the surface is likely to involve the endosomal compartment. The presence of βPP immunoreactivity in the tubular portion of CURL, in which receptors destined for recycling after ligand detachment are known to accumulate (Geuze et al., 1983), is consistent with this interpretation. Thus, a number of possible trafficking routes are available for cell surface βPP molecules: secretion, internalization, recycling with subsequent secretion or internalization, and targeting to lysosomes, either directly from the cell surface or presumably following recycling. Our findings of endosomal recycling are directly relevant to the observation that one route for Aβ generation involves endocytosis of cell surface βPP molecules (Koo and Squazzo, 1994). However, which of the compartments is central to Aβ production remains unclear.

The consensus sequence, -NPXY-, known to mediate the internalization of certain cell surface receptors, such as LDL and EGF receptors and LRP, via coated pits is present in the βPP cytoplasmic tail (Chen et al., 1990). Our data thus directly demonstrate that cell surface βPP is internalized via the receptor-mediated endocytic pathway. This result is consistent with the recent report of the ultrastructural localization of βPP in purified clathrin coated vesicles (Nordstedt et al., 1993). Although our study was not designed to define the precise internalization signal, the results from the βPP cytoplasmic deletion construct (ΔC) are consistent with the concept that the -GYENPTY- sequence at residues 756-762 serves as a functional signal for endocytosis (Lai et al., 1995). In this cell line, we observed a significant reduction in βPP internalization and recycling, concomitant with a prolongation of the residence time of cell surface molecules (Fig. 1h). Interestingly, more βPP was present on the surface of cells expressing this deletion construct, suggesting that more mature βPP may be directed to the surface. Studies are underway to define which of the three tyrosine residues in the βPP cytoplasmic domain may be critical for internalization.

Finally, missense mutations within and flanking the Aβ region of the βPP gene have been identified in a few families with early-onset familial AD. Although rare, these mutations have provided mechanistically important insights into the role of βPP and Aβ in the pathogenesis of the disease. It was recently shown that the percentage of longer Aβ peptides (ending at residue 42 of Aβ) was increased from cells expressing the βPP codon 717 mutations (Suzuki et al., 1994; Tamaoka et al., 1994). Because such longer Aβ peptides aggregate more avidly in vitro studies (Jarrett et al., 1993; Jarrett and Lansbury, 1993), this finding provides a route by which mutations at codon 717 may enhance amyloid deposition. Our study includes a morphological analysis of trafficking of βPP molecules bearing one of the familial AD mutations (V7I7F). In this cell line, the pattern of immunoreactivity by both light and electron microscopy was essentially identical to cells overexpressing wild-type βPP. Although modest qualitative or quantitative changes would not be detected by our analyses, our results suggest that codon 717 βPP mutations do not lead to gross alterations in the trafficking of cell surface molecules.

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