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Trafficking of cell-surface amyloid β-protein precursor

I. Secretion, endocytosis and recycling as detected by labeled monoclonal antibody

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SUMMARY

Amyloid β-protein, the principal constituent of amyloid fibrils found in senile plaques and blood vessels in Alzheimer's disease, is constitutively produced and released into medium of cultured cells. Amyloid β-protein is derived by proteolysis of the β-amyloid precursor protein by unclear mechanisms. β-amyloid precursor protein is a transmembrane protein which can be processed to release a large secretory product or processed in the endosomal/lysosomal pathway without secretion. Previous studies have shown that from the cell surface, β-amyloid precursor protein may be released after cleavage or internalized without cleavage, the latter in a pathway that both produces amyloid β-protein and also targets some molecules to the lysosomal compartment. Analysis of β-amyloid precursor protein trafficking is confounded by the concomitant secretion and internalization of molecules from the cell surface. To address this issue, we developed an assay, based on the binding of a radioiodinated monoclonal antibody, to measure the release and internalization of cell surface β-amyloid precursor protein in transfected cells. With this approach, we showed that surface β-amyloid precursor protein is either rapidly released or internalized, such that the duration at the cell surface is very short. Approximately 30% of cell surface β-amyloid precursor protein molecules are released. Following internalization, a fraction of molecules are recycled while the majority of molecules are rapidly sorted to the lysosomal compartment for degradation. When the C terminus of β-amyloid precursor protein is deleted, secretion is increased by approximately 2.5-fold as compared to wild-type molecules. There is a concomitant decrease in internalization in these mutant molecules as well as prolongation of the resident time on the cell surface. This observation is consistent with recent evidence that signals within the cytoplasmic domain mediate β-amyloid precursor protein internalization.

Key words: Alzheimer's disease, Amyloid β-protein precursor (βPP), Endocytosis, Trafficking

INTRODUCTION

Alzheimer's disease is a neurodegenerative disorder characterized by the progressive deposition of amyloid β-protein (Aβ) in senile plaques and in the walls of cerebral blood vessels. Aβ is a 40-43 amino acid polypeptide derived by proteolytic cleavage of the β-amyloid precursor protein (βPP), an integral membrane protein encoded by a gene on chromosome 21 (Goldgaber et al., 1987; Kang et al., 1987). Alternative splicing leads to three major isoforms containing 695 (βPP695), 751 (βPP751), and 770 (βPP770) amino acid residues (Müller-Hill and Beyreuther, 1989). The longer isoforms contain a Kunitz protease inhibitor (KPI) domain and are ubiquituously expressed while the βPP695 isoform is selectively enriched in brain. Within βPP, the Aβ fragment is derived from portions of both the extracellular and transmembrane domains. A population of βPP is secreted following cleavage within the Aβ domain by an enzyme activity, termed 'α-secretase', to generate C-terminally truncated secreted βPP (βPP_S) (Esch et al., 1990). Both intracellular and cell surface βPP are substrates for this as yet unidentified protease. Because this cleavage occurs within the Aβ domain, β-secretase cleaved βPP precludes the formation of an intact Aβ polypeptide (Esch et al., 1990; Sisodia et al., 1990).

In addition to the secretory pathway, βPP can be processed through the endosomal/lysosomal system (Golde et al., 1992). This alternative pathway was first identified from full-length cell surface molecules that were subsquently internalized without secretion and targeted to the lysosomal compartment to generate an array of C-terminal βPP fragments (Haass et al., 1992). The identification of βPP within isolated clathrin coated vesicles from PC12 cells is consistent with utilization of this endocytic pathway (Nordstedt et al., 1993). It is unclear whether a direct targeting pathway to the endosomal/lysosomal system without trafficking via the cell surface exists (Sinha and Lieberburg, 1992).

The cellular mechanism that results in the generation of Aβ is only partially understood. Recent studies showed that the endocytic pathway contributes to Aβ that is released into medium of cultured cells (Koo and Squazzo, 1994). This was demonstrated by the recovery of radiolabeled Aβ in medium after selective cell surface iodination. In addition, inhibition of endocytosis by depletion of extracellular potassium to disrupt clathrin coated vesicles impaired Aβ production. Furthermore, deletion of the βPP C-terminal cytoplasmic domain or a six residue sequence (YENPTY) resulted in a dramatic reduction in Aβ release. The latter approach was undertaken because of the presence of a tetrapeptide sequence, -NPTY-, at residues 759-762 (βPP770 numbering) of the cytoplasmic domain which conforms to a signal for coated pit mediated endocytosis of the low density lipoprotein receptor (Chen et al., 1990). Taken together, these findings demonstrated the contribution of the endocytic processing to Aβ production and that inhibition of Aβ production by deleting the YENPTY motif is likely to be due to impaired internalization.

To date, analysis of βPP processing in the endocytic pathway has been difficult because of the simultaneous secretion and internalization of cell surface molecules. As a result, a number of studies that examined the cytoplasmic signals for βPP processing assessed changes in βPP_S secretion as representative of corresponding alterations in βPP internalization (De Strooper et al., 1993; Haass et al., 1993; Jacobsen et al., 1994). As such, the approach is indirect at best and may be misleading because potential alterations in α-secretase cleavage intracellularly as well as alterations in protein sorting may offset changes in endocytosis of cell surface molecules (Haass et al., 1995). Therefore, the levels of βPP_S secretion may not accurately reflect the utilization of the internalization pathway. In order to examine simultaneously both βPP_S release and βPP internalization from the cell surface, a method that directly measures the rate of βPP trafficking into either compartment would be highly desirable. Recently, a βPP-transferrin receptor hybrid molecule was successfully used to examine the internalization signals in the βPP cytoplasmic domain, although βPP_S secretion could not be analyzed in this chimeric molecule (Lai et al., 1995).

In this study, we asked whether a monoclonal antibody recognizing the extracellular domain of βPP can be used to follow the trafficking of cell surface molecules. This method has been successfully used in the past to analyze the endocytosis of a number of cell surface receptors, such as low density lipoprotein, transferrin, CD4, or macrophage Fc receptors (Beisiegel et al., 1981; Hopkins and Trowbridge, 1983; Mellman et al., 1983; Pelchen-Matthews et al., 1989). Our results show that the binding of a βPP monoclonal antibody can be used to analyze the trafficking of cell surface molecules. As determined by the release of radiolabeled antibody from the cell surface, we showed that about 30% of a cohort of surface βPP molecules are released after proteolytic cleavage from cells expressing wild-type βPP. Both the secretion, internalization, and recycling of cell surface βPP molecules occurred rapidly. In the absence of the cytoplasmic domain, the level of βPP_S secretion is increased and βPP internalization is correspondingly decreased, as compared to wild-type molecules. These results are consistent with the concept that cytoplasmic sequences contain functional internalization signal(s) and that this method can be used to assess quantitatively the internalization and secretion of cell surface βPP molecules.

MATERIALS AND METHODS

Cell culture

Chinese hamster ovary (CHO) cells were grown in Dulbecco's modified Eagle's medium containing 10% Fetal Clone II serum (HyClone Laboratories, Logan, UT). Stably transfected CHO cell lines expressing wild-type βPP 751 (WT) were generated with expression vector pCMV751 using the Lipofectin (Life Technologies, Bethesda, MD) mediated transfection and selected by G418 resistence (Koo and Squazzo, 1994). Two WT clones (D4 and D10) with different expression levels were chosen for analysis. A C-terminally truncated mutant βPP construct (ΔC), with deletion of residues 729-770 (βPP770 numbering), was generated by PCR and stably transfected into CHO cells (Haass et al., 1993). The putative coated pit-mediated internalization signal (NPTY) at residues 757-762 is absent in this construct.

Antibodies

The monoclonal antibodies 5A3 and 1G7 were raised to human βPP_S purified from βPP-transfected CHO cells and recognize non-overlapping epitopes in the extracellular domain of βPP between residues 380-665, as defined by their reactivity against a bacterial fusion protein with this sequence (data not shown), thereby excluding both the KPI and Aβ domains. The specificity of these antibodies for βPP has previously been demonstrated by immunoprecipitation and immunofluoresence studies (Koo and Squazzo, 1994; Yamazaki et al., 1995). In the binding studies, 1G7 IgG and Fab antibodies were radioiodinated by IODO-GEN (Pierce, Rockford, IL) according to manufacturer's instructions to approximately 3-6 μCi/μg and purified by G25 Sepharose chromatography. 5A3 and 1G7 were used together to immunoprecipitate βPP_S from medium, and a polyclonal antibody against the C-terminal 15 amino acid residues of βPP (CT15) (Sisodia et al., 1993) was used to immunoprecipitate full length βPP from lysates in surface biotinylation experiments. Monoclonal βPP antibody 22C11 (Boehringer Mannheim) was used for immunoblotting studies.

Cell-surface βPP binding

To determine the kinetics of βPP trafficking from the cell surface in βPP transfected CHO cells, radioiodinated 1G7 whole antibody or Fab fragment were added to confluent CHO cells plated 48 hours previously in 12-well tissue culture plates. In preliminary studies, competition experiments with increasing amounts of unlabeled antibody as well as saturation experiments with increasing amounts of labeled antibody were carried out with both Fab fragments and whole IgG. Both antibody preparations produced similar results with regard to antibody affinity and specificity. In subsequent experiments described herein, only whole 1G7 IgG was used, and this antibody was added at approximately 7 nM to the cultures such that the antibody concentration was at least fivefold the concentration required for half-maximal saturation.

In all experiments, the antibodies were incubated in binding medium (BM) consisting of RPMI containing 20 mM Hepes supplemented with 0.2% BSA. The antibody binding to cell surface βPP was carried out at 4°C for one hour, followed by two washes with BM and two washes with Dulbecco's phosphate buffered saline (DPBS). The cells were then placed in prewarmed medium and incubated at 37°C. At various time points from 5 minutes to 2 hours, medium was collected and cells were rapidly chilled with ice-cold DPBS at pH 2.8. After an additional five minute wash with acidic buffer to detach residual surface-bound antibody, the cells were lysed in 0.2 M NaOH. In control experiments, the acid wash consistently detached 90-95%

of cell-surface radioactivity. The resultant medium, acid wash, and lysate samples therefore represent secreted, cell-surface, and intracellular βPP pools, respectively. The results are expressed as the percentage of the total radioactivity in the three fractions, thereby showing the relative amounts of βPP in each of the three compartments. After the radioactivity was determined in a gamma counter, TCA precipitation was carried out on medium and lysate samples by adding 50% TCA to a final concentration of 10%. After centrifugation, the supernatant was collected and radioactivity was measured. To calculate specific binding, radioactivity from untransfected CHO cells performed in parallel was subtracted from the counts obtained from the transfected cells in each condition. All experiments were performed in triplicate and repeated two to four times. Either representative results from one experiment or the average results of all repetitions (± s.e.m.) are shown.

In some experiments, after initial antibody binding and chase periods of 10 and 30 minutes, cells were lysed with 1% NP40 and incubated with anti-mouse agarose beads to recover 1G7 antibody. Radioactivity of intact IgG was quantitated by phosphorimager after immunoprecipitation and fractionation by non-reducing SDS-PAGE of duplicate samples. This method provided a more accurate estimation of the amount of residual radiolabeled antibody than the TCA precipitation described above.

Cell surface biotinylation

To confirm the reliability of the antibody binding results, surface biotinylation and immunoprecipitation of WT and ΔC cell lines were performed at one time point to obtain independent estimation of the time course of βPP secretion. Biotinylation was carried out on confluent CHO cells with hydroxysulfosuccinimide-biotin (Pierce, Rockford, IL). The reagent was dissolved in PBS (pH 8) at 1 mg/ml and added to the cultures for 30 minutes at 4°C. After thorough washing with DPBS supplemented with 50 mM glycine, the cells were either lysed immediately (time 0) with 1% NP40 or returned to the 37°C incubator with prewarmed CHO medium. After 10 minutes, medium was collected and βPP_S immunoprecipitated with 5A3/1G7 monoclonal antibodies. Cell surface full length βPP at time 0 was also immunoprecipitated with 5A3/1G7 to estimate the relative amounts of cell surface βPP in WT and ΔC cell lines. In addition, full length βPP was immunoprecipitated from the lysates of surface biotinylated cells with C-terminal βPP antibody (CT15) at time 0, 10, and 30 minutes after rewarming to estimate the amount of βPP left in the cell (surface plus internalized). The immunoprecipitated βPP species were fractionated on SDS-PAGE, transferred to nitrocellulose, incubated with anti-biotin monoclonal antibody (Jackson ImmunoResearch, West Grove, PA), and then detected with ^{125}I-labeled anti-mouse secondary antibody. After the autoradiograms were exposed and developed, the nitrocellulose blots were exposed to phosphoimager for quantitation.

Recycling studies

To determine βPP recycling by radioiodinated antibody, WT CHO cell line was incubated with 1G7 IgG for 15 minutes at 37°C to allow binding and internalization of the antibody. The cells were rapidly chilled to 4°C on ice and thoroughly washed to remove unbound antibodies. Surface bound antibodies were then detached with two acid washes (three minutes each). Following two more washes with BM, the cells were incubated with prewarmed CHO medium and returned to the 37°C incubator. The medium was then collected from the cells after 5 to 30 minutes and radioactivity determined from both medium and cell lysates. The results are expressed as radioactivity from medium (TCA precipitable) as a percentage of the total radioactivity (medium plus lysate). To show that the acid washes did not perturb βPP trafficking, a control experiment was carried out in which WT CHO cells were first rinsed with two 3 minute acid washes. Subsequently, treated and untreated control cells were incubated with radioiodinated 1G7 IgG at 4°C and a time course study of βPP secretion and internalization was carried out as described above.

βPP recycling was also determined biochemically by reversible surface biotinylation as described (Bretscher and Lutter, 1988). In brief, WT CHO cell line was incubated with sulfosuccinimidyl-2-ethyl-1,3-dithiopropionate [NHS-SS-biotin] (Pierce, Rockford, IL) in TEA buffer (10 mM triethanolamine, 125 mM NaCl, 2 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 9.0) (Schoenenberger et al., 1994). After two 20 minute incubations, the cells were washed with DPBS supplemented with 50 mM glycine. The cells were incubated for 10 minutes at 37°C, rapidly chilled, and conditioned medium saved. The culture was then treated with glutathione and iodoacetamide sequentially to remove the biotin moieties from cell surface proteins exactly as described (Zurzolo et al., 1994). Treated cells were then returned to the 37°C incubator for 45 minutes and the medium was collected. Biotinylated proteins from both medium samples were recovered after incubation with streptavidin-agarose (Pierce). The samples were then fractionated by SDS-PAGE, transferred to nitrocellulose, immunoblotted with a βPP monoclonal antibody (22C11), and visualized by chemiluminescence. The conditioned medium from the same dish of cells was collected prior to biotinylation and treated identically as a negative control.

RESULTS

Binding of 1G7 to CHO transfected cells

The goal of this study was to determine the kinetics of βPP secretion and internalization using the approach of binding of radioiodinated monoclonal antibody 1G7 to cell surface βPP. Initially, competition experiments using unlabeled antibody with labeled antibody were carried out on WT (D4) CHO cell line. This antibody (1G7) was chosen from a panel of monoclonal antibodies developed against βPP_S because its binding to cell surface βPP did not appear to be temperature labile at 37°C (see below). The binding of 1G7 to cell surface APP was specific because radiolabeled IgG can be displaced by increasing amounts of cold antibody (Fig. 1) but not by 100-fold excess of unlabeled non-immune mouse IgG (data not shown). Moreover, the affinity of radioiodinated IgG to both WT (D10) and ΔC cell lines was essentially identical, with a half-maximal saturation of approximately 1.3 nM (Fig. 1). These two cell lines were chosen because the amounts of βPP on the cell surface amount were within 5% of each other whereas surface βPP in D4 WT cell line was approximately one third of these values. Further preliminary studies showed that there was more rapid appearance of antibody in the medium (i.e. βPP_S release) and less internalization of βPP when measured by the Fab fragment than by whole IgG at early time points. As a result, whole IgG was used in all subsequent experiments.

Kinetics of βPP trafficking

The kinetics of βPP internalization and βPP_S release were studied in ΔC and WT (D10) CHO cell lines using radioiodinated 1G7 IgG. These studies examined a population of surface βPP molecules that were initially bound to radiolabeled antibody at 4°C and then allowed to transit when rewarmed to 37°C. Using this paradigm, cell surface βPP from WT cell line was rapidly released into the medium, with $t_{1/2}$ around 10 minutes (Fig. 2A). At the same time, cell-surface βPP, as measured by the acid washes, declined rapidly and remained at low levels for the duration of the experiment (Fig. 2B). As

detected in cell lysate (acid resistant) fractions, the remaining cell-surface molecules (over 50%) were internalized within 10 minutes of rewarming to 37°C (Fig. 2C). The internalized pool subsequently declined concurrently with an increase in the fraction of secreted βPP. Since the cell-surface pool remained low, we interpreted these data to suggest that a fraction of internalized βPP was subsequently recycled to the surface and rapidly released between 10 and 30 minutes after rewarming (see below). From 30 to 120 minutes, there was essentially no change in the amount of TCA precipitable radioactivity in medium, suggesting that there was no further release of βPP_S from surface labeled βPP (Fig. 2A). This can be better appreciated when the radioactivity in the medium is expressed as a percentage of the total radioactivity obtained at time 0 (Fig. 2D). In cell lysates, TCA precipitable radioactivity showed that approximately 50% and 30% of original radioactivity at time 0 remained at 30 and 60 minutes, respectively (Fig. 2D).

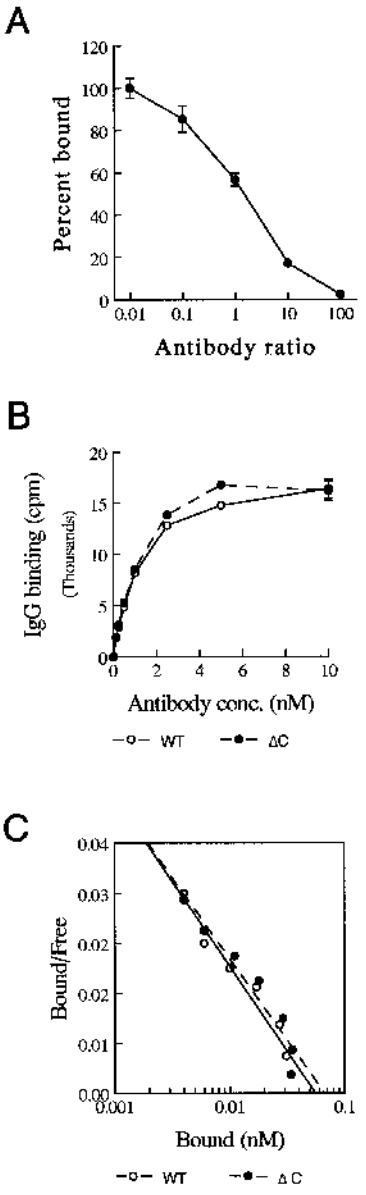

Fig. 1. Competition and saturation assays of radioiodinated 1G7 monoclonal antibody binding. (A) In a competition assay, increasing amounts of unlabeled Fab fragment or whole IgG were added to a constant amount (10 nM) of radiolabeled Fab fragment or IgG, respectively. The amount of cold antibody added is expressed as a ratio of unlabeled to labeled antibody. The results are expressed as the percentage radioactivity of control (untreated) cells analyzed in parallel. (B and C) Saturation binding studies of 1G7 binding to ΔC and WT cell lines. In B, specific binding, obtained by subtracting the radioactivity of untransfected from transfected CHO cells, of 1G7 IgG to ΔC and WT cell lines is shown as a function of radiolabeled antibody concentration. In C, a Scatchard plot of the data from B showed virtually identical saturation characteristics. The calculated K_d is 1.23±0.15 nM and 1.3±0.12 nM for WT and ΔC cell lines, respectively.

In contrast to wild-type βPP751, CHO cells transfected with the C-terminal deleted construct showed a significantly altered profile of βPP trafficking. Specifically, substantially more βPP_S was secreted from the cell surface of ΔC than from WT cell line (Fig. 2A). Interestingly, the acid releasable fraction showed more residual surface βPP in ΔC cell line up to 30 minutes, suggesting increased residence time of βPP on the cell surface (Fig. 2B). This observation indicated that although cell surface βPP is cleared by both secretory and internalization pathways, endocytosis may normally be more rapid than the cleavage process. Consistent with the preceding observations, internalization of ΔC βPP was correspondingly diminished at every time point, as much as a sixfold reduction between 5 and 10 minutes (Fig. 2C). Expressed as TCA precipitable radioactivity as a percentage of original radioactivity at time 0, the secreted and internalized pools at 60 minutes were approximately 80% and 15%, respectively. Therefore, these results showed that the absence of the cytoplasmic domain of βPP resulted in a marked reduction in internalization of cell surface molecules. It should be pointed out that our studies could not determine the actual rates of secretion and internalization because of the complexity of the βPP trafficking pathways, i.e. concomitant movement into two different compartments. Suffice it to say that by 10 minutes, the majority of surface molecules have been either internalized or released.

Cell surface biotinylation

The aforementioned results are generally consistent with recent studies describing the rapid release of membrane bound βPP and the presence of a cytosolic internalizaton signal. Nevertheless, although a substantial difference in βPP trafficking was seen in the absence of the βPP cytoplasmic domain, we could not unequivocally ascertain that the radiolabeled antibody was stable throughout the 37°C incubation period. That is, a certain percentage of the antibody may detach prematurely from bound βPP, thereby providing misleading results. To obtain independent estimation of the difference in βPP_S secretion between WT and ΔC cell lines, surface biotinylation and immunoprecipitation for βPP and βPP_S were performed. In the first experiment, medium was collected from ΔC and WT CHO cells after surface biotinylation and a chase period of 10 minutes at 37°C. Immunoprecipitation of βPP from cell lysates immediately after labeling showed that the levels of cell surface molecules were almost identical between the two cell lines (Fig. 3B). After 10 minutes, the amount of βPP_S in the medium of WT cells was approximately 33% of βPP_S in the medium of ΔC cells (Fig. 3A,B), a value that is extremely close to that obtained by antibody binding studies (32%) (Figs 2A and 3A).

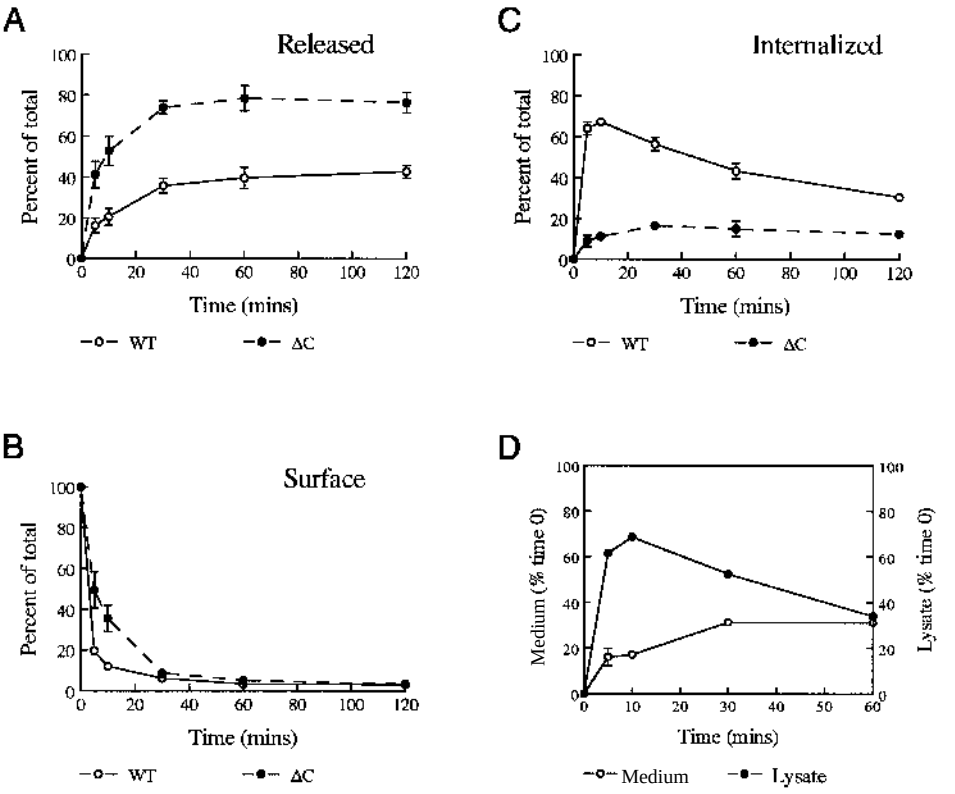

Fig. 2. Time course study of cell surface βPP trafficking in CHO cells transfected with βPP751 WT or ΔC construct. Radioiodinated 1G7 IgG was used to determine the rate of βPP release and internalization in the two cell lines. The radioactivity from medium (A), acid labile wash (B), and acid resistant lysates (C) were taken to represent secreted, cell surface, and internalized cell surface βPP, respectively. Within each of the three fractions, the result obtained at each time point is expressed as a percentage of total radioactivity from the three fractions (medium + acid wash + cell lysate) obtained at this same time point. To simplify the graphs, only TCA precipitable radioactivity from medium samples are represented in this experiment. Beyond 30 minutes, the value from the three fractions is less than 100%, with the remainder being TCA soluble radioactivity from medium. The data are the averages ± s.e.m. of three independent experiments. In D, the time course data from one representative experiment from βPP751 WT cells are replotted to show radioactivity (TCA precipitable) in medium and lysate as a percentage of total radioactivity obtained at time 0.

We could not accurately determine the amount of βPP_S at 10 minutes relative to full length βPP at the cell surface at time 0, i.e. estimate the percentage of βPP that is released as βPP_S. This is because the antibodies (5A3/1G7) showed higher affinity for βPP_S than full length βPP molecules and consequently gave higher recovery of βPP_S from medium. Therefore, in the second experiment, we quantitated the amount of full-length βPP left in cell lysates 10 and 30 minute after surface biotinylation in WT cells (Fig. 3C and D). The percentage of initial βPP remaining at 10 and 30 minutes in WT cell lysates was approximately 74% and 32% (average of 5 experiments), respectively. These values are essentially identical to the 73% and 29% (average of 4 experiments) obtained at the same respective time points by immunoprecipitation of radioiodinated 1G7 from cell lysates (Fig. 3C and D). Assuming therefore that 30-40% of the original surface pool of βPP was released as βPP_S, the half-life of full length βPP after internalization using either method was approximately 30 minutes. Note that the values obtained by immunoprecipitating radiolabeled 1G7 from cell lysates were different from those obtained in the time course experiments shown earlier using TCA precipitation (Fig. 2). In the kinetic studies, approximately 78% and 50% of initial radioactivity were estimated at the same time points, suggesting that TCA precipitation overestimated the amount of intact radiolabeled antibody within the cells.

Recycling of cell-surface βPP

Results from the above binding experiments suggested that between 10 and 30 minutes, there was recycling and secretion of the initial pool of internalized βPP. To confirm this trafficking pathway, WT (D10) CHO cells were briefly incubated with radiolabeled 1G7 IgG at 37°C to allow for antibody internalization. Residual surface bound antibody was detached from chilled cells by acid treatment and the amount of TCA precipitable radioactivity reappearing in medium subsequently determined at various time points after rewarming. With this experimental paradigm, the radioactivity in medium gradually increased for 30 minutes, suggesting that internalized antibody and βPP were recycled and released over that period of time (Fig. 4A). In the ΔC cell line, there was essentially no measurable increase in radioactivity in the medium during this same time period (data not shown). For this paradigm to be valid, it is essential that the transient acidification did not alter the subsequent trafficking of cell surface βPP. In control experiments, neither the amount of cell surface βPP nor the internalization fraction was significantly altered by this treatment (data not shown).

The recycling of cell surface βPP and subsequent release after proteolytic cleavage was also demonstrated by biochemical means. WT cells were biotinylated by a reversible agent (NHS-SS-biotin) and cell surface molecules allowed to internalize or released for 10 minutes at 37°C. Residual biotinylated proteins on the cell surface were stripped by glutathione, following which the cells were incubated at 37°C for an additional 45 minutes. Biotinylated βPPs was precipitated by streptavidin-agarose from the medium of both incubation periods and detected by immunoblotting. Qualitatively comparable amounts of biotinylated βPP_S were recovered in both time periods (Fig. 4B). Assuming that most of the βPPs released during the first incubation period were derived directly from the cell surface without internalization, then approximately half of the total βPP_S was derived from the recycling pool, a value similar to that extrapolated from the radioiodinated 1G7

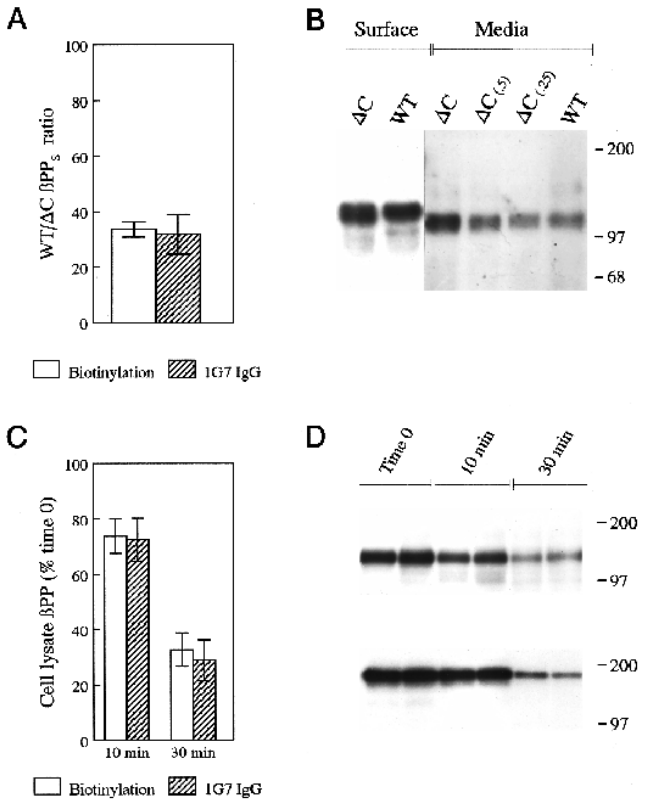

Fig. 3. Release of βPP_S from WT and ΔC cells determined by surface biotinylation. In A, the difference in βPP_S secretion between ΔC and WT (D10) cell lines is expressed as a ratio of the amount of labeled βPP_S in the medium of the respective cell lines after surface biotinylation and chased for 10 minutes at 37°C. This value is compared to that obtained from 1G7 antibody binding at the same time point from Fig. 2A. The ratio from the biotinylation and antibody studies is an average of three independent experiments (± s.e.m.). In B, βPP and βPP_S from ΔC and WT of a representative autoradiogram after biotinylation is shown. The ΔC sample from medium was loaded into three separate lanes using twofold dilutions (1, 0.5 and 0.25) to demonstrate the magnitude of βPP_S reduction in WT cells. In C, full length βPP in lysate from WT cell line was determined by immunoprecipitation with CT15 antibody immediately after biotinylation (time 0) and at 10 and 30 minutes after 37°C chase. The data are expressed as a ratio of the amount of βPP at 10 or 30 minutes and at time 0 (average of five experiments ± s.e.m.). The amount of full length radiolabeled 1G7 IgG is determined by immunoprecipitation with anti-mouse secondary antibody immediately after surface binding by 1G7 (time 0) and at 10 and 30 minutes after 37°C chase. The data is expressed as a ratio of the amount of 1G7 antibody at 10 or 30 minutes and at time 0 (average of four experiments ± s.e.m.). In D, autoradiograms from a representative biotinylation (upper panel) and radiolabeled 1G7 (lower panel) experiments using WT cell line described in C are shown. Duplicate samples were analyzed from each of three time points (0, 10, and 30 minutes). Molecular masses (in kDa) are indicated on the right in B and D as determined by prestained markers. Note the anomalous mobility of the IgG in the lower panel in D because the samples were not reduced prior to fractionation.

results (Fig. 2A). As expected, βPP_S was not detected in conditioned medium derived from the same dish of cells collected prior to biotinylation.

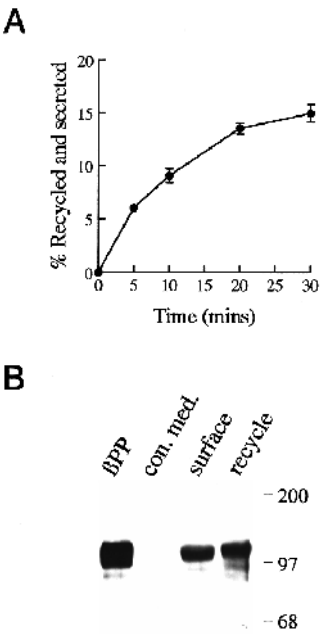

Fig. 4. Recycling and release of βPP from the cell surface. In A, WT CHO cells were incubated with radioiodinated 1G7 IgG at 37°C for 15 minutes. After removing bound antibodies from the cell surface with acid washes at 4°C, the cells were returned to the 37°C incubator. TCA precipitable radioactivity was subsequently determined in medium at the indicated time points. The results are shown as percentage radioactivity from medium over total (medium + lysate). Note that these cells were initially labeled at 37°C and not 4°C. In B WT CHO cells were surface biotinylated with NHS-SS-biotin. Biotinylated βPP_S was precipitated from the medium by streptavidin-agarose from the first 10 minute incubation period after biotinylation (surface) and from the second 45 minute incubation period that followed glutathione stripping (recycle) as described in Materials and Methods. The precipitated samples were immunoblotted with a βPP monoclonal antibody (22C11) after fractionation and transfer to nitrocellulose membrane. There is no signal in conditioned medium collected from the same cells prior to biotinylation (con. med.). As a positive control (βPP, left lane), unlabeled βPP_S was immunoprecipitated from conditioned medium of WT CHO cells with βPP monoclonal antibodies (5A3/1G7) and immunoblotted in parallel with the above samples.

DISCUSSION

In cultured cells, βPP can be processed in the secretory pathway or sorted to the cell surface where the molecules can either be internalized without cleavage or cleaved and released as additional βPP_S. The ability to accurately determine the secretion and endocytosis of cell surface molecules would facilitate the analysis of cytoplasmic signals that regulate processing in these pathways (Trowbridge et al., 1993). Moreover, we have recently shown that processing of cell surface βPP in the endocytic pathway is one source of Aβ that is produced and subsequently released from cultured cells (Koo and Squazzo, 1994). Therefore, because the relative utilization of the secretory versus internalization pathways affects Aβ generation, a rapid and reproducible method to examine the trafficking of cell surface βPP would be advantageous. Moreover, such a method would allow one to

determine the relative utilization of these pathways under normal and perturbed conditions, and the signals that may regulate the processing and trafficking of cell surface βPP.

At present, the ability to simultaneously assay the transit of βPP into the secretory or endocytic pools has been difficult in the absence of a natural ligand that binds to the extracellular region of βPP. Although βPP has been hypothesized to be a cell surface receptor, there is at present little direct evidence to support this notion and a ligand has yet to be identified. Radiolabeled monoclonal antibody has been successfully used to localize and follow the trafficking of many cell surface receptors (Beisiegel et al., 1981; Hopkins and Trowbridge, 1983; Mellman et al., 1983; Pelchen-Matthews et al., 1989). Therefore, we asked whether this method can be used to follow kinetically the trafficking of cell surface βPP. In initial studies, the specificity of binding of 1G7 monoclonal antibody to cell surface βPP expressed in CHO cells was determined by competition and saturation experiments. In the early experiments, both radiolabeled Fab fragment and whole IgG were used because we could not be certain a priori that this approach accurately reflected the trafficking of cell surface βPP in light of studies showing that bivalent antibodies can alter the normal endocytic trafficking of receptors (Anderson et al., 1982; von Figura et al., 1984; Ukkonen et al., 1986). However, the striking concordance of results obtained by antibody binding and surface biotinylation studies argues that whole IgG by itself does not alter secretion or induce internalization. Until a natural ligand is demonstrated for cell surface βPP, the present approach appears to be fundamentally sound and represents the only one possible at this time for both morphological localization and kinetic analysis of βPP trafficking from the cell surface.

Using the approach of radiolabeled 1G7 antibody binding, we showed that cell surface βPP is either rapidly internalized or released as βPPs into the medium. Consequently, the residence time of βPP on the cell surface appeared to be quite short. The rapid release of βPP from the cell surface is consistent with results obtained after surface radioiodination or from isolated cell membranes (Sisodia, 1992; Roberts et al., 1994; Koo and Squazzo, 1994). All these studies demonstrated that the majority of βPP_S was released within 10-20 minutes. In addition, a recent report showed that by surface biotinylation, approximately 20% of cell surface βPP is released into the medium after 10 minutes (Lai et al., 1995), a value that is virtually identical to our antibody binding result at the same time point from WT cells (20.6±4.1). Therefore, results from these studies are all consistent with each other and provide additional confirmation of the validity of our antibody binding paradigm. The correspondingly rapid internalization of βPP is within the time course reported for several cell surface receptors after ligand binding (Brown and Goldstein, 1979; Haigler et al., 1980; Schwartz et al., 1982; Ciechanover et al., 1983). Interestingly, the scrapie prion protein, whose disease-related protease resistant isoform is produced after endocytosis, is internalized from the cell surface with a $t_{1/2}$ of approximately 20 minutes (Shyng et al., 1993), a rate that approaches that seen in βPP. After internalization, a fraction of βPP molecules recycles back to the cell surface and is subsequently released. The number of cycles βPP normally undertakes in this recycling pathway is unclear but may contribute up to one-half of the βPPs released from the surface. The $t_{1/2}$ of full-length βPP after internalization is approximately 30 minutes as determined by surface biotinylation and by measuring the degradation of labeled antibody. Nevertheless, the short time βPP resides on the cell surface, coupled with the relatively rapid degradation after internalization, is consistent with a half-life of total cellular βPP of approximately one hour as determined by [^{35}S]methionine labeling pulse chase studies (Weidemann et al., 1989).

The tetrapeptide sequence, -NPXY-, known to mediate the internalization of certain cell surface receptors such as low density lipoprotein receptor via coated pits is present in the βPP cytoplasmic tail (Chen et al., 1990). 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 there is a functional signal for endocytosis within this domain. Specifically, at all time points sampled, significantly more βPP was released from the surface in the cell line expressing ΔC as compared to wild-type βPP. During this period, βPP_S secretion from ΔC cell line was approximately 2- to 2.5-fold higher than from WT cell line, a result similar to that seen by biotinylation studies. This difference is slightly higher than the 1.6-fold increase in βPP_S secretion reported with a βPP construct lacking the six amino acid sequence YENPTY (residues 757-762) as compared to wild-type βPP (Lai et al., 1995). It is possible that, as suggested, sequences between 729 and 757 provide additional signals for internalization (Lai et al., 1995). At five to ten minutes, internalization of mutant (ΔC) βPP decreased by over 80% compared to controls. This reduction is comparable to that seen in LDL and transferrin receptors in the absence of the internalization signals (Trowbridge et al., 1993). Consistent with our finding, a 'tail-less' mutant in a βPP-transferrin receptor chimera, somewhat analogous to our ΔC construct, resulted in a 75% reduction in the efficiency of internalization (Lai et al., 1995). In our studies, the reduction in endocytosis of the mutant βPP also resulted in a prolongation of the residence time on the cell surface. In short, these observations are consistent with the interpretation that impairment of Aβ production in the absence of the cytoplasmic tail or the seven amino acid sequence -GYENPTY- is due to an inhibition of internalization (Koo and Squazzo, 1994; Lai, et al., 1995).

The cellular localization of α-secretase cleavage has been a subject of some debate. C-terminally truncated βPP cleaved at the α-secretase site is detected intracellularly and, by metabolic studies, these species are produced before the appearance of secreted βPP_S in medium (Sambamurti et al., 1992; De Strooper et al., 1993; Kuntzel et al., 1993). At the same time, βPP_S is also derived from full length cell surface βPP (Haass et al., 1992; Sisodia 1992), although it has not been established whether the cleavage requires endocytosis and recycling or occurs entirely on the cell surface. Our studies reported here strongly suggest that the latter conjecture is likely to be true. This is because βPP_S secretion is both rapid and, more importantly, increased in the absence of the cytoplasmic domain, which reduces βPP internalization.

In summary, using radiolabeled monoclonal antibody, we have approximated the rate of βPP trafficking from the cell surface. This method appears to provide reliable and accurate estimates for both βPP_S release and βPP endocytosis. As such, this approach can now be used to determine whether mutations within βPP, as identified in a few families with hereditary Alzheimer's

disease, affect the trafficking of βPP. Moreover, we can now determine the internalization and sorting signals in native βPP molecules. Finally, this approach has been adapted to visualize directly the βPP trafficking pathway after internalization at the light and ultrastructural levels, the subject of the companion study (see accompanying paper, Yamazaki et al., 1996).

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