Enhanced Release of Amyloid β-Protein from Codon 670/671 “Swedish” Mutant β-Amyloid Precursor Protein Occurs in Both Secretory and Endocytic Pathways*

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The mutation at codons 670/671 of β-amyloid precursor protein (βPP) dramatically elevates amyloid β-protein (Aβ) production. Since increased Aβ may be responsible for the disease phenotype identified from a Swedish kindred with familial Alzheimer’s disease, evaluation of the cellular mechanism(s) responsible for the enhanced Aβ release may suggest potential therapies for Alzheimer’s disease. In this study, we analyzed Chinese hamster ovary cells stably transfected with either wild type βPP (βPP-wt) or “Swedish” mutant βPP (βPP-sw) for potential differences in βPP processing. We confirmed that increased amounts of Aβ are produced in the βPP-sw cells and that increased Aβ production is increased by β-secretase. Interestingly, more Aβ was present in medium from βPP-sw cells than βPP-wt cells after either cell surface iodination or [35S]methionine labeling, indicating that βPP-sw cells have enhanced Aβ release in both the endocytic and secretory pathways. Furthermore, a variety of drug treatments known to affect protein processing similarly reduced Aβ release from both βPP-wt and βPP-sw cells. Taken together, the data suggest that the processing pathway for βPP is similar for both βPP-wt and βPP-sw cells and that increased Aβ production by βPP-sw cells arises from enhanced cleavage of mutant βPP by β-secretase, the as-yet unidentified enzyme(s) that cleaves at the NH2 terminus of Aβ.

In Alzheimer’s disease a characteristic pathological finding in the brains of affected individuals is the deposition of amyloid β-protein (Aβ)1 in senile plaques (1). Aβ is the 39- to 43-amino acid proteolytic cleavage product of the type I integral mem-

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The abbreviations used are: Aβ, amyloid β-protein; βPP, β-amyloid precursor protein; βPPsw, soluble βPP; βPP-sw, “Swedish” mutant βPP; βPP-wt, wild type βPP; CHO, Chinese hamster ovary; DMEM, Dulbecco’s modified Eagle’s medium; MAb, monoclonal antibodies; 5A3 and 167T used together; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; DPBS, Dulbecco’s phosphate-buffered saline.

2 During constitutive secretion some full-length βPP molecules are proteolytically cleaved between lysine and leucine residues at positions 16 and 17 of Aβ (Fig. 1) by an enzyme termed α-secretase (3, 4). Cleavage of βPP at this position creates a soluble –100–120-kDa NH2-terminal fragment (5) and a COOH-terminal membrane-retained fragment of –10 kDa (6). Generation of these fragments by α-secretase proceeds formation of an intact Aβ sequence from full-length βPP.

Aβ, however, is known to be released during normal cellular metabolism both in vivo (7, 8) and in a number of cell culture systems (9, 10). Cleavage of βPP at the NH2 terminus of the Aβ sequence by an enzyme designated β-secretase creates a short-ened form of βPPsw and the –12-kDa COOH-terminal fragment (11, 12). An additional enzymatic cleavage at the COOH terminus of the Aβ sequence by the as yet unidentified enzyme designated γ-secretase generates the 4-kDa Aβpeptide. The γ-secretase enzyme is also hypothesized to generate p3, the 3-kDa NH2-terminal piece of the membrane-retained –10-kDa COOH-terminal fragment of βPP produced by α-secretase cleavage (7–9, 13). In addition to the secretory cleavage, βPP can also be processed in an endosomal/lysosomal pathway (14–17). Although Aβ-containing COOH-terminal fragments are generated in lysosomes, evidence suggests that these are not an important source of Aβ (18). Recently, it was shown that cell surface βPP molecules can be processed in the endocytic pathway and may be the direct precursors of Aβ, presumably by recycling internalized molecules from the cell surface (19).

Evidence that Aβ and βPP contribute to the pathogenesis of Alzheimer’s disease comes from the findings of missense mutations within and adjacent to the Aβ region of the βPP gene in families with autosomal dominant forms of Alzheimer’s disease (20). The concurrence of the mutations with the disease phenotype suggests that altered βPP function or processing may be pathogenic. A double mutation at amino acids 670 and 671 (βPP670,671 numbering) changing Lys670 to Asn670 and Met671 to Leu671 (K670N/M671L) was identified in a Swedish pedigree with familial Alzheimer’s disease (21). In vitro analyses of transfected cells expressing the Swedish form of βPP (12, 22) and primary cell cultures of fibroblasts obtained from affected individuals (23) reveal a dramatic increase in Aβ production. However, the mechanism by which Aβ generation is increased has not been elucidated. Furthermore, a detailed analysis of cellular processing of βPP with this mutation has not been
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**Fig. 1.** AβPP structure, enzymatic cleavage sites, COOH-terminal fragments, and antibody epitopes. Schematic diagram of AβPP. The vertical cross-hatched box represents the plasma membrane. The white box labeled Aβ represents the Aβ peptide (also shown enlarged with the amino acid sequence listed). The horizontally striped box labeled KPI represents the Kunitz protease inhibitor domain along the adjacent exon indicated by the small open box. The NH2-terminal black box represents the signal sequence. α, β, and γ mark the sites of the enzymatic cleavages by α-, β-, and γ-secretases, respectively. Also indicated are the ~10-kDa fragment (including the p3 region, transmembrane region, and COOH terminus) and the ~12-kDa fragment (including the Aβ region, transmembrane region, and COOH terminus). -NPTY- indicates the putative dathrin internalization signal. Horizontal black bars indicate the approximate epitopes of antibodies B5, C7, 6E10, MMAb, R1280 and R1282, and R1736.

reported. Because recent studies have implicated the endocytic pathway in Aβ production (19), we speculated that Aβ production may be similarly enhanced in this pathway in cells expressing the K670M/M671L AβPP751 mutation.

In this report, biosynthetic analyses confirmed the increase in Aβ production and the abundant secretion of a shorter βPP species by Chinese hamster ovary (CHO) cells stably transfected with the AβPP751 K670M/M671L mutation. Furthermore, Aβ generation was increased in both the secretory and endocytic pathways. We postulate that this increase in Aβ production is the result of enhanced proteolytic cleavage of the mutant AβPP by the β-secretase enzyme.

### Experimental Procedures

**Cell Culture**—Stably transfected CHO cell lines were generated with wild-type AβPP (19) or with 670/671 AβPP (25). "Swedish" mutation cloned into pcDNA3 (Invitrogen, San Diego) by CapOPO transfection and selection by G418 resistance. The mutant AβPP construct was obtained by subcloning the 500-base pair BamHI-EcoRI fragment containing the mutation from AβPPes, K670/671M (22), generously provided by Dr. Martin Citron) into the wild-type AβPP expression vector. Cells were grown in Dulbecco's modified Eagle's medium (DMEM, BioWhitaker, Walkersville, MD) with 10% Fetal Clone II (HyClone Laboratories, Logan, UT) at 37°C, with 5% CO2.

**Antibodies**—Several anti-AβPP antibodies were used (see Fig. 1). The monoclonal antibodies 5A3 and 1G7 (referred to as MMAb when used together), recognize a midregion extracellular AβPP domain (19), AβPP monoclonal antibody 6E10 (from K. S. Kim and H. Wisniewski) recognizes Aβ (1–17) and α-secretase-cleaved βPP (24). Five previously described polyclonal antibodies were also used: R1280 and R1282 raised against synthetic Aβ (1–40) precipitate Aβ and p3 fragments (25, 26). R1282 (1:100) gave consistent recovery of both Aβ and the p3 fragment during immunoprecipitation. C7 was raised against AβPP (751–770) and recognizes full-length AβPP and COOH-terminal fragments of AβPP (27). B5 raised against the midregion of AβPP (519–667) precipitates both α-secretase- and β-secretase-cleaved βPP (28), and R1736 raised against AβPP (670–868) recognizes α-secretase-cleaved βPP (15).

**Metabolic Labeling**—Confluent cultures of AβPP-transfected CHO cells were incubated in methionine-free DMEM for 15 min followed by incubation with methionine-free DMEM supplemented with 200 μCi/ml [35S]methionine for 10 min (pulse labeling) or for 2–4 h with 50–100 μCi/ml [35S]methionine (long labeling). Cells were lysed immediately or incubated with 2-fold unlabeled methionine (chase) in DMEM from 10 min to 4 h. For some experiments, single dishes of confluent cells were pulse labeled and chased at multiple time points with repeated collection of medium to evaluate the incremental secretion of AβPP products at each time point of the chase period. AβPPs were immunoprecipitated using βPP-specific antibodies and separated by SDS-polyacrylamide gel electrophoresis (using 6–10% Tris-glycine gels for high molecular weight proteins and 16.5% Tris-Tricine gels for low molecular weight proteins). Gels were either fluorographed enhanced and exposed to x-ray film or dried and exposed on a Phosphor screen (Molecular Dynamics). All experiments reported herein were performed two to six times, and a representative example of each is shown. Where applicable, average values ± S.E. are given.

**Assessment of Total AβPP and AβPP, β/AαβPP, and γ/AβPP quantification were determined using parallel triplicate cultures of stably transfected CHO cells expressing wild-type AβPP or "Swedish" AβPP. One set of cultures was lysed immediately after a 10-min pulse labeling and immunoprecipitated with C7 to determine total AβPP. The other set was chased for 4 h, and media were collected and immunoprecipitated with MMAb to determine AβPP, secretion. Samples were separated by SDS-polyacrylamide gel electrophoresis and analyzed by Phosphorimaging. Comparison of AβPP levels was made after normalization for total AβPP expression.

**Intracellular AβPP**—Separate dishes of stably transfected CHO cells, one each for each chase time point, were pulse labeled with 10 nM antibody for either 10, 30, or 60 min. Media were collected, and cells were washed with ice-cold DPBS and rapidly chilled to 4°C. After washing, DPBS was replaced with DPBS containing 0.1% saponin plus protease inhibitors (leupeptin and Pefabloc, Boehringer Mannheim). Cells were treated with saponin buffer for 40 min at 4°C as described previously (29), to allow the release of intracellular AβPP. Saponin buffer was then collected, and both the chase media and saponin buffers were immunoprecipitated using antibody B5 for total AβPP, and antibody C7 for full-length AβPP.

**Cell Surface Iodination—Derivatized Bolton-Hunter reagent, sulfosuccinimidyl 3-(4-hydroxyphenyl)propionate (sulfos-HPDP, Pierce) was labeled with Na2251 in the presence of IODO-GEN (Pierce) at room temperature essentially as described (30). After 5 min, the iodination reaction was quenched with p-hydroxyphenylacetic acid (Sigma), diluted with DPBS, and immediately added to chilled washed cells for 40 min at 4°C. After iodination, the cells were extensively washed in DPBS containing 1 mg/ml lysine followed by incubation in prewarmed CHO medium at 37°C. Three independent experiments were performed.

**Surface Antibody Binding**—To determine the amount of cell surface and total AβPP in stably transfected CHO cells, 5A3 monodonal antibody Fab fragments were radiiodinated with IODO-GEN to 2–4 μCi/μg (19). Confluent cell cultures were chilled and washed. One set of cultures was treated with 0.1% saponin in DPBS for 30 min at 4°C to permeabilize cells gently and permit the labeling of both cell surface and intracellular AβPP. Parallel cultures for each cell line were treated for 30 min at 4°C with DPBS for evaluation of cell surface AβPP. Both sets were incubated with radiiodinated antibody at 10 nM in binding medium (RPMI 1640 medium supplemented with 0.2% bovine serum albumin) at 4°C for 1 h, followed by two washes with binding medium and two washes with DPBS. The cells were then lysed with 0.2 M NaOH, and radioactivity was determined by γ-counting. To calculate specific binding, background levels of radioactivity were determined from parallel cultures of untransfected CHO cells to subtract from the counts obtained from AβPP-transfected CHO cells. Four separate experiments were performed using triplicate cultures. Drug Studies—Confluent CHO cells were metabolically labeled with 50–100 μCi/ml [35S]methionine for 2 h and then chased for 2 h in media containing either chloroquine (100 μM), Wako BioProducts, Richmond, VA), or brefeldin A (35 μM, Epicentre Technologies, Madison, WI). These drugs are known to affect protein processing as described below. Some bafilomycin experiments were performed using a 10-min pulse with [35S]methionine followed by a 20-min chase with buffer containing bafilomycin A1. AβPP products were immunoprecipitated and visualized as described previously. Six separate experiments were performed.

**RESULTS**

βPP Processing by β-Secretase Is Enhanced in CHO Cells Stably Expressing "Swedish" Mutant AβPP751—Multiple stably transfected AβPP-sw CHO cell lines were selected based on their equivalent AβPP expression levels to AβPP-wt cell lines. Consis-
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**Fig. 2.** βPP turnover, βPP, species, and precursor product relationship of ~12-kDa fragments and Aβ in CHO cells stably transfected with βPPsw. Panel A, turnover of full-length βPP immunoprecipitated with antibody C7 from βPP-wt and βPP-sw cells pulse-labeled for 10 min with [35S]methionine and chased for 0, 1, 2, or 4 h. Panel B, immunoprecipitation of βPPs from conditioned medium with antibodies B5, R1736, and 6E10 from βPP-wt and βPP-sw cells labeled for 4 h with [35S]methionine. R1736 and 6E10 are specific for α-secretase-cleaved βPPs. Panel C, COOH-terminal fragments and Aβ release from βPP-sw cells following a 10-min pulse with [35S]methionine and 10- or 20-min chase. The COOH-terminal fragments were immunoprecipitated with antibody C7 from cell lysates after the 10- or 20-min chase. The ~12-kDa fragments (at arrowhead), clearly apparent by 10 min, increased by 20 min. Media from parallel cultures immunoprecipitated with antibody R1282 show an Aβ (at arrowhead) signal by 20 min. No Aβ signal is observed at 10 min even when the signal is intentionally amplified as in the lanes on the right. For comparison, the unamplified Aβ image is presented to the left of the darkened image. Molecular weights determined from prestained standards are indicated. wt = βPP-wt cells; sw = βPP-sw cells.

**Fig. 3.** Incremental release of βPPs, Aβ, and p3 from CHO cells transfected with wild type or K670N/M671L mutant βPPsw. Immunoprecipitations of βPPs, Aβ, and p3 from conditioned chase media from single cultures of βPP-wt and βPP-sw cells following a 10-min [35S]methionine pulse label were collected at 10-min intervals. The level of βPP holoprotein expression was somewhat lower in βPP-sw cells in this experiment. On low percentage polyacrylamide gels, βPPs, from CHO cells migrates as a doublet. Panel A, total βPPs was immunoprecipitated with antibody B5. Note the lower molecular weight species of βPPsw indicated by the arrowhead, from βPP-sw cell media. Panel B, the presence of βPPsw at 10 min is confirmed by this long exposure of the gel shown in panel A. The shortened βPPs form, at the arrowhead, is apparent at the earliest time point. Panel C, Aβ and p3 immunoprecipitated by R1282 from the same media as panels A and B. Positions of Aβ and p3 are indicated. Molecular weights determined from prestained standards are indicated on the right. wt = βPP-wt cells; sw = βPP-sw cells.

Though both cell lines secreted comparable levels of total βPPs by B5 antibody immunoprecipitation (Fig. 2B), βPP-sw cells had dramatically reduced levels of α-secretase-cleaved βPPsw (6 ± 1.3-fold less) than βPP-wt cells using antibodies R1736 and 6E10 (Fig. 2B). Consistent with this finding, and as reported by others (12, 31–33), βPP-sw cells also had correspondingly higher levels of ~12-kDa COOH-terminal βPP fragments (see below).

The onset of secretion of total βPPsw was first detectable at 10 min as determined with B5 immunoprecipitation (Fig. 3B). However, at this first time interval only minute amounts of βPPsw were secreted from both βPP-wt and βPP-sw cells because the signal could be seen in the 10-min lane only after prolonged autoradiographic exposures (Fig. 3B). βPPsw became pronounced at 20 min for both cell lines with peak secretion at approximately 30 min (Figs. 3A and 4). The profile of βPPsw secretion as a function of time was essentially identical for the two cell lines (Fig. 4). In the experiment shown, although βPPsw secretion by βPP-sw cells was lower because of diminished expression of full-length βPP, the profile of secretion is essentially identical to that of βPP-wt cells. This profile of βPPsw secretion did not depend on the level of βPP expression because other wild type and Swedish cell lines exhibited the same patterns of release (not shown). Furthermore, comparison of βPP-wt and βPP-sw cells that expressed equivalent levels of βPP confirmed that βPPsw secretion by both cell lines was essentially identical in βPP-wt and βPP-sw cells—Since the turnover rate of full-length βPP was essentially the same in the βPP-wt and βPP-sw cells, we next examined the biosynthetic rate for the generation of βPP-secreted products (βPPsw, Aβ, and p3). Following a 10-min pulse labeling, media were collected from a single dish each from βPP-wt and βPP-sw cells and reapplied at 10-min intervals to define the incremental release of βPP secretion products during the 1st h of the chase period.

The onset of secretion of total βPPsw was first detectable at 10 min as determined with B5 immunoprecipitation (Fig. 3B). However, at this first time interval only minute amounts of βPPsw were secreted from both βPP-wt and βPP-sw cells because the signal could be seen in the 10-min lane only after prolonged autoradiographic exposures (Fig. 3B). βPPsw became pronounced at 20 min for both cell lines with peak secretion at approximately 30 min (Figs. 3A and 4). The profile of βPPsw secretion as a function of time was essentially identical for the two cell lines (Fig. 4). In the experiment shown, although βPPsw secretion by βPP-sw cells was lower because of diminished expression of full-length βPP, the profile of secretion is essentially identical to that of βPP-wt cells. This profile of βPPsw secretion did not depend on the level of βPP expression because other wild type and Swedish cell lines exhibited the same patterns of release (not shown). Furthermore, comparison of βPP-wt and βPP-sw cells that expressed equivalent levels of βPP confirmed that βPPsw secretion by both cell lines was essentially identical.

### Footnotes

1. Effect of “Swedish” Mutation on Aβ Processing

### References

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### Figures

**Figure 2.** βPP turnover, βPP, species, and precursor product relationship of ~12-kDa fragments and Aβ in CHO cells stably transfected with βPPsw. Panel A, turnover of full-length βPP immunoprecipitated with antibody C7 from βPP-wt and βPP-sw cells pulse-labeled for 10 min with [35S]methionine and chased for 0, 1, 2, or 4 h. Panel B, immunoprecipitation of βPPs from conditioned medium with antibodies B5, R1736, and 6E10 from βPP-wt and βPP-sw cells labeled for 4 h with [35S]methionine. R1736 and 6E10 are specific for α-secretase-cleaved βPPs. Panel C, COOH-terminal fragments and Aβ release from βPP-sw cells following a 10-min pulse with [35S]methionine and 10- or 20-min chase. The COOH-terminal fragments were immunoprecipitated with antibody C7 from cell lysates after the 10- or 20-min chase. The ~12-kDa fragments (at arrowhead), clearly apparent by 10 min, increased by 20 min. Media from parallel cultures immunoprecipitated with antibody R1282 show an Aβ (at arrowhead) signal by 20 min. No Aβ signal is observed at 10 min even when the signal is intentionally amplified as in the lanes on the right. For comparison, the unamplified Aβ image is presented to the left of the darkened image. Molecular weights determined from prestained standards are indicated. wt = βPP-wt cells; sw = βPP-sw cells.

**Figure 3.** Incremental release of βPPsw, Aβ, and p3 from CHO cells transfected with wild type or K670N/M671L mutant βPPsw. Immunoprecipitations of βPPsw, Aβ, and p3 from conditioned chase media from single cultures of βPP-wt and βPP-sw cells following a 10-min [35S]methionine pulse label were collected at 10-min intervals. The level of βPP holoprotein expression was somewhat lower in βPP-sw cells in this experiment. On low percentage polyacrylamide gels, βPPsw, from CHO cells migrates as a doublet. Panel A, total βPPsw was immunoprecipitated with antibody B5. Note the lower molecular weight species of βPPsw indicated by the arrowhead, from βPP-sw cell media. Panel B, the presence of βPPsw at 10 min is confirmed by this long exposure of the gel shown in panel A. The shortened βPPsw form, at the arrowhead, is apparent at the earliest time point. Panel C, Aβ and p3 immunoprecipitated by R1282 from the same media as panels A and B. Positions of Aβ and p3 are indicated. Molecular weights determined from prestained standards are indicated on the right. wt = βPP-wt cells; sw = βPP-sw cells.
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βPP<sub>s</sub>

![Graph 1: Profiles of βPP<sub>s</sub>, Aβ, and p3 release from CHO cells transfected with wild type or K670N/M671L mutant βPP<sub>wt</sub>](image)

Data from Phosphorimage analysis of gels in Fig. 3 represent the percent secretion for each time point relative to the cumulative (100%) secretion during the entire 60-min chase. The top panel shows the βPP<sub>s</sub> release from βPP-wt (designated by the solid line and circles in all graphs) and βPP-sw cells (designated by the dotted line and triangles in all graphs) from antibody B5 immunoprecipitation. Aβ (middle panel) and p3 (bottom panel) release from βPP-wt and βPP-sw cells, immunoprecipitated with R1282 antibody, are also shown. wt = βPP-wt cells; sw = βPP-sw cells.

sentially the same (within ~10% of each other as determined by Phosphorimage analysis of media from triplicate cultures from each cell line, Student’s t test, p = 0.49).

Regarding Aβ release, the timing of Aβ secretion during the 1st h from βPP-wt and βPP-sw cells was also identical (Figs. 3C and 4). The Aβ signal was first apparent at the 20-min collection time by autoradiography (Fig. 3C) and reached a peak at 30–40 min. Although no discernible Aβ signal was ever seen on either autoradiograms or Phosphorimages at the 10-min chase time, after long exposures a few Phosphorimage counts higher than background were detected in the 10-min lane (Fig. 4). At each chase time, βPP-sw cells consistently released more Aβ than βPP-wt cells. The timing of p3 secretion mirrored that of Aβ in both βPP-wt and βPP-sw cells throughout the chase period (Fig. 3C), although βPP-sw cells consistently released more p3 relative to Aβ than did βPP-sw cells. Authentication of Aβ (beginning at Asp<sup>1</sup>) and p3 (beginning at Lys<sup>17</sup>) was obtained by radiosequencing (not shown), as reported previously (15, 19). Thus, a difference in the ratios of α-secretase- and β-secretase-generated molecules was also reflected by the levels of p3 and Aβ released by these cell lines. Finally, the formation of the β-secretase-generated –12-kDa βPP COOH-terminal fragments preceded the release of Aβ from pulse-labeled βPP-sw cells (Fig. 2C). After a 10-min labeling with <sup>35</sup>S) methionine, the –12-kDa fragment was apparent by the 10-min chase time in βPP-sw cells and increased at 20 min (Fig. 2C). Consistent with the above results, Aβ was not apparent in the corresponding media until 20 min of the chase period (Fig. 2C). This earlier generation of the –12-kDa fragment prior to Aβ release, consistently seen in three experiments, indicates a precursor-product relationship between the two molecules.

βPP<sub>s</sub> and Aβ Appear to Be Present Intracellularly in βPP-wt and βPP-sw Cells—The release of βPP<sub>s</sub> at very early chase times suggested that βPP may be cleaved by β-secretase in the secretory pathway. To determine if soluble βPP<sub>s</sub> was present intracellularly, metabolically labeled cells were treated with 0.1% saponin in buffer. Saponin is a mild detergent that permeabilizes cells but does not solubilize the lipid bilayer (29) and therefore allowed the intracellular βPP<sub>s</sub> to diffuse into the buffer. Essentially no full-length βPP was detected in the saponin buffer of treated cells. However, soluble intracellular βPP<sub>s</sub> was recovered from the saponin buffer from both βPP-wt and βPP-sw cells (Fig. 5, A and B). Intracellular βPP<sub>s</sub> species from βPP-wt cells (a finding previously reported by others; see Refs. 34–36) migrated with an M<sub>r</sub> consistent with α-secretase-cleaved molecules (Fig. 5A). A shorter βPP<sub>s</sub> species with an M<sub>r</sub> identical to secreted βPP<sub>s</sub> and consistent with β-secretase-cleaved molecules was observed from βPP-sw cells (Fig. 5A; Ref. 32). Furthermore, in pulse-chase experiments a precursor-product relationship could be demonstrated between intracellular βPP<sub>s</sub> from the saponin-treated cells and βPP<sub>s</sub> secreted into the medium (Fig. 5B).

These results suggested that Aβ can be formed within the secretory pathway. Indeed, intracellular Aβ appeared to be present in both βPP-wt and βPP-sw cell lysates labeled for 4 h (Fig. 5C). Preabsorption of R1280 antibody with the Aβ 1–40 peptide totally eliminated immunoprecipitation of Aβ from the cytosolic lysates by R1280 antibody (Fig. 5C), and no 4-kDa band was observed from the same lysate using antibody C7. Treatment with trypsin prior to immunoprecipitation did not diminish the Aβ signal (not shown), indicating that Aβ was present inside the cells. In addition, cells pulse labeled with <sup>35</sup>S)methionine followed by a 20-min or 30-min chase had both Aβ and p3 isolated from cell lysates (not shown). Thus, the immunoprecipitated Aβ had not been derived from secreted molecules present on the extracellular plasma membrane at the time of cell lysis. Furthermore, the appearance of these intracellular Aβ and p3 molecules after short pulse-chase intervals provides indirect evidence of their production in the secretory and not the endosomal/lysosomal pathway. Nevertheless, Aβ and p3 bands were visualized only after 8–10 weeks of autoradiographic exposure, suggesting that only very low levels of Aβ were ever present intracellularly. The minute amounts of intracellular Aβ precluded definitive identification by amino acid radiosequencing.

βPP from the Cell Surface Contributes to Aβ Production in Both βPP-wt and βPP-sw Cells—To determine if the endocytic
pathway contributed to Aβ production in βPP-sw cells, release of Aβ was analyzed after selective cell surface radioiodination. Consistent with an earlier report (19), little radiolabeled Aβ was secreted within the first 10 min by either cell line, but considerable Aβ was released by 2 h from both βPP-wt and βPP-sw cells (Fig. 6A), with βPP-sw cells releasing greater than 2-fold more Aβ than βPP-wt cells at the 2-h collection time (2.4 ± 0.4). The timing of Aβ release following labeling of cell surface βPP was essentially the same in the two cell lines (Fig. 6A). However, in sharp contrast to the timing of Aβ secretion, the majority of the iodinated βPPs was released within the first 5 min of incubation at 37°C from both βPP-wt and βPP-sw cells (Fig. 6B). In addition, these profiles of both Aβ and βPPs release are distinctly different from the timing observed for Aβ and βPPs, release observed using [35S]methionine labeling (Figs. 3 and 4).

Two additional observations are noteworthy from these experiments. First, βPPs derived from cell surface βPP by βPP-sw cells had an Mw compatible with α-secretase-cleaved βPPs (Fig. 6B). A lower Mw, β-secretase-cleaved βPPs species was not readily apparent after surface labeling. However, resolution of the labeled bands is significantly less distinct from an iodine signal because of radiographic intensification, and minor differences may be undetectable. Second, we consistently observed more full-length βPP on the surface of βPP-wt cells than βPP-sw cells (Fig. 6C) expressing the same amount of AβPP. To confirm and quantify this difference, the levels of cell surface and total βPP were measured by an antibody binding assay using iodinated antibody 5A3 Fab fragments, which bind to an extracellular βPP epitope (19). Treatment with 0.1% saponin permitted labeling of both cell surface and intracellular βPP. Multiple repetitions of this experiment showed that βPP-sw cells had approximately 50% less cell surface βPP than βPP-wt cells (49.8% ± 0.7, p < 0.0001). Interestingly, βPP-sw cells showed more of the COOH-terminal ~12-kDa fragment and less of the ~10-kDa fragment than βPP-wt cells (Fig. 6C) present on the cell surface. Drug Treatments Affect βPP-wt and βPP-sw Cells Similar-
...and wild type or K670N/M671L mutant \( \beta PP \) gene. Panel A, immunoprecipitation of \( \beta PP \)-wt and \( \beta PP \)-sw conditioned media with antibody R1282, and cell lysates with antibody C7 from a 2-h \[^{35}S\]methionine label followed by a 2-h chase containing either no drug (Cont), brefeldin A (Bref), chloroquine (CQ), or bafilomycin A1 (Bafio). Less \( \alpha JI \) was released in the presence of drugs compared with the control condition for both cell lines. Panel B, antibody C7 immunoprecipitation of \( \beta PP \)-sw cell lysates after a 10-min \[^{35}S\]methionine pulse followed by a 20-min chase in the absence (0) or presence (25) of bafilomycin A1. Note the presence of the ~12-kDa COOH-terminal fragment generated by \( \beta \)-secretase cleavage at 20 min in the control lane (0) and its near absence after bafilomycin A1 treatment. wt = \( \beta PP \)-wt cells; sw = \( \beta PP \)-sw.

**DISCUSSION**

A double mutation in the \( \beta PP \) gene from a Swedish kindred with familial Alzheimer’s disease is invariably linked with Alzheimer’s disease (21). All cell systems reported to date which express the \( \beta PP \) mutation produce dramatically more \( \beta A \) peptide than do cells expressing wild type \( \beta PP \) (12, 22, 23, 31–33). Since excess \( \beta A \) production may be causally related to the Alzheimer’s phenotype in individuals affected with the “Swedish” mutation (21), it is important to evaluate the mechanisms by which \( \beta A \) is produced from \( \beta PP \) with this alteration. In this study, we performed a detailed analysis of the biosynthetic processing of \( \beta PP \) in \( \beta PP \)-wt and \( \beta PP \)-sw CHO cells.

Our results showed that, as anticipated, \( \beta PP \)-sw cells released substantially more \( \beta A \) than \( \beta PP \)-wt cells. Interestingly, the timing of onset and the duration of \( \beta A \) secretion during the 1st h following a short pulse labeling were coincident with \( p3 \) release for both cell lines. Only the amounts of \( \alpha JI \) and \( p3 \) varied between \( \beta PP \)-wt and \( \beta PP \)-sw cells. Furthermore, treatments known to decrease \( \alpha JI \) in \( \beta PP \)-wt cells (8, 13, 38) also affected \( \beta PP \)-sw cells. We interpret our data to suggest that the pathway of \( \beta A \) production is similar for \( \beta PP \)-wt and \( \beta PP \)-sw cells. In contrast, however, the timing of \( \beta A \) release differed substantially depending on whether cells were \[^{35}S\]methionine-labeled or surface iodinated. In both cell lines \( \beta A \) was released with a shorter time course from \[^{35}S\]methionine-labeled cells than from cells that were surface iodinated. This difference in the timing of \( \beta A \) secretion leads us to propose that \( \beta A \) is generated in both the secretory and endocytic pathways from both \( \beta PP \)-wt and \( \beta PP \)-sw cells. A number of observations suggest that \( \alpha JI \) is generated in the secretory pathway (10, 39). First, the timing of secretion of \( \beta PP \), \( \beta A \), and \( p3 \) was essentially identical at early chase times in both cell lines. Specifically, within the first 30 min in a short pulse-chase experiment, the profiles of \( \beta PP \), \( \beta A \), and \( p3 \) secretion were remarkably similar. This chase paradigm was chosen specifically to reveal the incremental release of these early secretory products. Second, permeabilization of \[^{35}S\]methionine-labeled cells followed by immunoprecipitation with a \( \beta PP \) midregion antibody (B5) showed that intracellular soluble \( \beta PP \) was present in both \( \beta PP \)-wt and \( \beta PP \)-sw cells as reported previously (32). The major intracellular species of soluble \( \beta PP \), from \( \beta PP \)-sw cells had a lower M, than \( \beta PP \), from \( \beta PP \)-wt cells, consistent with production by \( \beta \)-secretase cleavage. Significantly, intracellular \( \beta PP \) was present before abundant \( \beta PP \) was secreted into the culture medium, thus demonstrating a precursor-product relationship. Third, the ~12-kDa COOH-terminal fragment of \( \beta PP \) and \( \beta A \) showed a precursor-product relationship, with the ~12-kDa molecules apparent 10 min prior to the appearance of \( \beta A \). Moreover, consistent with a recent report (12), this COOH-terminal ~12-kDa fragment was specifically increased in \( \beta PP \)-wt cells compared with \( \beta PP \)-wt cells. Fourth, our data suggest that intracellular \( \beta A \) is present in both \( \beta PP \)-wt and \( \beta PP \)-sw cells. Based on results from trypsin digestion using a short pulse-chase paradigm, \( \beta PP \) in cell lysates did not appear to represent extracellular \( \beta A \) attached to the cell surface or to be derived from the lysosomal pathway. However, the exceedingly small amount of intracellular \( \beta PP \) suggests that \( \beta A \) turnover and secretion are rapid. This is consistent with the earlier postulation that \( \beta A \) is released from cells soon after it is formed and suggests that \( \gamma \)-secretase cleavage occurs at or near the cell surface (19). Previously, intracellular \( \beta PP \) has only been detected in neurons (40). Thus our preliminary findings suggest that the pathways of \( \beta A \) production in neurons and non-
Regarding the endocytic processing of pPP, cells labeled by selective cell surface iodination confirmed that pPP-sw cells produced more Aβ from cell surface precursors than did pPP-wt cells. However, the timing of Aβ release after surface labeling was essentially identical for both pPP-wt and pPP-sw cells. As shown previously for cells expressing wild type pPP (19), Aβ generated from surface-labeled molecules was released more slowly than pPP by pPP-sw cells. These profiles of Aβ and pPP, release from surface-labeled molecules are dramatically different from the [35S]methionine labeling experiments in which Aβ and pPP, were released simultaneously. Interestingly, pPP and Aβ release from [35S]methionine pulse-chase experiments showed that pPP secretion peaked at ~30 min followed by a sharp decrease, whereas Aβ release continued at the same level until later chase times. We interpret the sustained Aβ release into the medium at a time when pPP secretion decreased (40–50 min) to represent the addition of newly generated Aβ, derived from the endocytic pool, after the contribution of the secretory pool of Aβ has peaked. Therefore, our data indicate that Aβ can be derived from both the secretory and endocytic pathways and that more Aβ is formed within each pathway by pPP-sw cells.

Our studies have defined a number of similarities in pPP processing between pPP-wt and pPP-sw cells. First, the timing of secretion of pPPs, Aβ, and p3 is essentially the same for both cell lines within the 1st hour following a 10-min pulse label. Second, various drug treatments decrease Aβ in both pPP-wt and pPP-sw CHO cells. Third, intracellular pPP, species and Aβ appear to be present in both cell lines. Fourth, both secretory and endocytic pathways appear to contribute to Aβ generation and release. Fifth, both pPP-wt and pPP-sw cells secrete primarily α-secretase-cleaved pPPs from surface-labeled pPP. Thus, within the limits and sensitivity of our experimental system, the timing and the pathway of Aβ secretion appear to be identical in pPP-wt and pPP-sw cells. Only the amounts of Aβ and α-secretase-cleaved precursors differ between pPP-wt and pPP-sw cells. Our data and interpretation are therefore consistent with the results of previous investigators who have suggested that the "Swedish" mutation at the NH2 terminus of Aβ enhances secretase cleavage (12, 22, 23). This altered β-secretase cleavage produces abundant β-secretase-cleaved pPPs in the secretory pathway in pPP-sw cells, leading to excess Aβ production. However, it remains unclear at present which pathway, secretory or endocytic, plays the greater role in Aβ production.

pPP-sw cells did show some differences in pPP processing from pPP-wt cells. In addition to the increase in β-secretase-cleaved products described above, there was a 50% reduction in the amount of cell surface pPP in pPP-sw cells. Concomitantly, there was an increase in the ~12-kDa membrane-retain pPP fragments present on the cell surface of pPP-sw cells. Whether this increase in ~12-kDa fragments is sufficient to account for the decrease in full-length pPP molecules at the cell surface of pPP-sw cells is unclear. Because secreted pPP, levels are similar between pPP-wt and pPP-sw cells, the reduction in full-length pPP at the surface of pPP-sw cells suggests that the amount of β-secretase targeted to the cell surface may represent a minor fraction of the total pPP processed in the secretory pathway. Otherwise, one would expect to see a substantial increase in pPP, released into the medium from pPP-sw cells, which was not detected. Furthermore, this interpretation is also consistent with reports of other cell types that express little or no pPP on the cell surface (34–36).

In summary, our data suggest that there is a similar mechanism for Aβ generation in both pPP-wt and pPP-sw cells. The increased Aβ production from pPP-sw cells appears to result from enhanced β-secretase cleavage of the mutant pPP in both the secretory and endocytic pathways. A recent report has demonstrated altered pPP processing in mutant pPP molecules with natural or designed mutations in codon 692 (41), whereas another report demonstrated an increased percentage of longer Aβ peptides from pPP with codon 717 mutations (42). Taken together, it appears that FAD pPP mutations lead to pleiotropic effects on pPP and Aβ metabolism. The Alzheimer phenotype associated with these dominant mutations may therefore result from different cellular perturbations that specifically modify pPP processing.

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Note Added in Proof—Similar findings of intracellular Aβ recently have been reported by Martin et al. (Martin, B. L., Schrader-Fischer, G., Bussigilojoy, D., Duke, M., PaganiERI, and Yankner, B. A. (1995) J. Biol. Chem. 270, 26727–26730) in cells transfected with Swedish mutant pPP.
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