Alzheimer’s disease, a progressive neurodegenerative disorder of the elderly, is characterized by the presence of parenchymal deposits of Aβ, a 39- to 43-amino acid peptide derived from APP (1–4). APP are integral membrane glycoproteins that mature through the secretory pathway (5). A fraction of newly synthesized APP appears on the cell surface (6–9) and some of these molecules are cleaved by α-secretase (7, 8) within the Aβ sequence (10–13), resulting in the release of the APP ectodomain (APP/>. In a cell-type specific manner, APP/> is generated in the trans-Golgi network or other late compartments of the constitutive secretory pathway (14–17). A fraction of APP are directly sorted, or reintegrated from the cell surface, to endosomal/lysosomal compartments (7), where a complex set of Aβ-containing membrane-bound fragments accumulate (7, 18). Finally, it is fully established that APP (4–8 kDa) and a truncated form of APP (~3 kDa) are released constitutively in vitro and in vivo (19–23).

The biochemical mechanism(s) and cellular compartments involved in APP production have not been fully elucidated. Despite earlier excitement created by the discovery of potential amyloidogenic fragments generated in endosomal-lysosomal pathways, several lines of evidence now suggest that lysosomal degradation of APP is unlikely to contribute to the production of Aβ (reviewed in Ref. 24). However, agents that interfere with pH gradients (i.e., ammonium chloride and chloroquine) inhibit the production of Aβ (19, 21), suggesting that Aβ may be generated in acidic compartments (i.e., endosomes or late Golgi). Indeed, biochemical studies by Koo and Squazzo (23) confirmed that APP production and release involves the endocytosis of full-length APP from the cell surface and subsequent recycling. In this model, β-secretase cleavage occurs within endocytic compartments while γ-secretase cleavage of the residual ~100-amino acid membrane fragment occurs virtually simultaneously with Aβ formation and release. However, the stoichiometry of Aβ contributed by reintegrated APP to APP generated in the biosynthetic pathway is not known.

To identify potential intracellular compartments involved in APP production, we examined the metabolism of APP with a double mutation at codons 670 and 671 (of APP-770) described in two large Swedish pedigrees with familial Alzheimer’s disease. Cells expressing this mutant APP secrete ~6–8-fold higher levels of Aβ relative to cells expressing wild-type APP (25, 26). In preceding efforts, we examined the trafficking and metabolism of APPwt and APPsw in polarized epithelial cells (MDCK) (27). We demonstrated that APPsw was cleaved at the β-secretase site and that the resulting soluble derivative, termed APPsw, was detectable in cell lysates. These results lead to the suggestion that “β-secretase” cleavage occurs in an intracellular compartment. In the present report, we extend the observations in MDCK cells by characterizing the maturation of APPsw in transiently and stably transfected N2a cells, a mouse peripheral neuroblastoma line. We demonstrate that
N2a cells expressing APP harboring the Swedish mutations secrete high levels of Aβ peptides and APP\(^{\text{sw}}\) derivatives relative to cells expressing APPwt. Interestingly, we observed a concomitant diminution in the levels of secreted APP\(^{\text{sw}}\). One scenario to explain this intriguing result is that normal α-secretase substrates are disabled by the prior action of β-secretase. This model predicts that β-secretase must cleave APP\(^{\text{sw}}\) in an intracellular compartment proximal to the cellular site(s) of α-secretase activity. We now confirm that β-secretase cleavage occurs within the Golgi apparatus, as early as the medial compartment.

**MATERIALS AND METHODS**

Plasmid Construction and Expression—The construction of expression plasmids pAPPwt and APP\(^{\text{sw}}\) were described previously (27). Briefly, to generate pAPPwt, a DNA fragment encoding wild-type human APP-695 with an carboxy-terminal epitope tag of 12 amino acids from the c-Myc oncogene (MEQKLIISEDLN), at the COOH terminus of APP, was subcloned downstream of a cytomegalovirus promoter in plasmid pCB6. Plasmid pAPP\(^{\text{sw}}\) (27) encodes Myc epitope-tagged human APP-695 that harbors the Swedish FAD-specific amino acid substitutions (K595N and M596L). To generate plasmid pEF-VSVG, a 1.7-kilobase XhoI fragment encoding the VSVG protein from plasmid pJC/G (28) was subcloned downstream of the elongation factor 2\(\alpha\) promoter in plasmid pEF-Bos (29).

Mouse N2a neuroblastoma cells were transfected using a high-efficiency CaPO4 co-precipitation procedure (30). To generate cell lines that express human APPwt and APP\(^{\text{sw}}\), N2a cells were transfected with pAPPwt or pAPP\(^{\text{sw}}\) and stable transfectants were selected in medium containing 0.4 mg/ml G418 (Life Technologies, Inc.) containing 0.4 mg/ml G418 (Life Technologies, Inc.) (the pCB6 vector contains a gene encoding resistance to the neomycin analog, G418). Expression of c-Myc-modified human APP was assayed by immunoblotting and immunoprecipitation with a polyclonal antiserum Myc-I raised against the synthetic peptide MEQKLIISEDLN (31).

Metabolic Labeling and Immunoprecipitation Analysis—For metabolic labeling, N2a cells were starved for 20 min in methionine-free Dulbecco's modified Eagle's medium (Life Technologies, Inc.) and then labeled with \(1 \text{mCi/mL}[\text{35S}]\text{methionine (DuPont NEN)}\) in methionine-free Dulbecco's modified Eagle's medium supplemented with 1% dia-
fected into N2a cells and recipient cells were labeled con-

RESULTS

Expression and Metabolism of Human APP and the “Swedish” Variant in N2a Cells—Expression constructs encoding c-Myc tagged APPwt or APP\(^{\text{sw}}\) (27) were transiently trans-

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ble derivatives derived from APPswe which are detected by antibody P2-1 (Fig. 1A, lane 6) are likely generated following cleavage upstream of the $\alpha$-secretase site, and most likely at the $\beta$-secretase site (i.e. at the amino terminus of A$\beta$). These latter observations are not the result of overloading of trafficking pathways in transient transfection assays as similar results have been obtained following transfection of as little as 250 ng of expression plasmids (not shown) and by analysis of stably transfected N2a cells that constitutively express APPwt or APPswe (see below). Finally, we demonstrate that cells expressing APPswe secrete 4–5-fold higher levels of A$\beta$ (Fig. 1A, lane 11) than cells expressing APPwt (Fig. 1A, lane 10), a result consistent with earlier studies of APPswe in transfected HEK293 (26) and M17 (25) cells.

Finally, and to confirm that endoproteolytic cleavage by $\beta$-secretase occurs between Leu596 and Asp587 of APPswe, we characterized the carboxyl terminus of the secreted soluble derivative and the amino terminus of A$\beta$. To confirm the authenticity of the $\beta$-secretase-generated soluble derivative, we subjected parallel aliquots of conditioned medium shown on
To analyze the amino terminus of a membrane-bound fragment generated by precipitation of APP-related species using APP NH2-terminal antibody S54, we radiolabeled cells with [3H]phenylalanine and immunoprecipitated APP from cells expressing APPwt and APPsw, respectively. To determine if the membrane-bound fragment is ultimately cleaved by β-secretase, we radiolabeled APPwt and APPsw at the NH2-terminal with [3H]phenylalanine and immunoprecipitated APPwt, APPsw, and stably transfected N2a cells, the levels of soluble derivatives (Fig. 1A, lanes 1 and 2, respectively). We radiolabeled cells with [3H]phenylalanine and immunoprecipitated APP from the conditioned medium; the isolated 4-kDa band was subjected to radiosequencing. Our findings indicate that the total level of soluble derivatives at the NH2-terminal of APPwt and APPsw, respectively, is essentially indistinguishable from medium of cells expressing APPsw (Fig. 1B, lane 3); this result is in sharp contrast to the isolation of nonspecific polypeptides of ~85 and ~95 kDa from medium of cells expressing APPsw (Fig. 1B, lane 3). We radiolabeled cells with [3H]phenylalanine and immunoprecipitated APP from the conditioned medium; the isolated 4-kDa band was subjected to radiosequencing. Our findings indicate that the total level of soluble derivatives at the NH2-terminal of APPwt and APPsw, respectively, is essentially indistinguishable from medium of cells expressing APPsw (Fig. 1B, lane 3); this result is in sharp contrast to the isolation of nonspecific polypeptides of ~85 and ~95 kDa from medium of cells expressing APPsw (Fig. 1B, lane 3).

In transiently transfected N2a cells, a corresponding diminution in electrophoretic mobility. Moreover, the levels of APPsw derivatives in medium of cells expressing APPsw are higher relative to cells expressing APPwt and that over 80% of these species exhibited accelerated electrophoretic mobility. Moreover, concomitant diminution in APPsw levels in medium of the stable cell line expressing APPsw strongly supports our view that potential α-secretase substrates are disabled by the prior action of β-secretase.

In the aggregate, we have demonstrated that in transient and stably transfected N2a cells, the levels of soluble derivatives and Aβ in medium of cells expressing APPsw are elevated relative to respective species secreted by cells expressing APPwt. Moreover, the vast majority of soluble derivatives in medium of cells expressing APPsw are precursors of APP, molecules generated following cleavage at the β-secretase site. The production of elevated levels of APPsw from cells expressing APPsw is essentially identical to results obtained by expression of chimeric human placental alkaline phosphatase-APP molecules harboring the Swedish substitutions in H4 globastoma cells. These studies demonstrated that human placental alkaline phosphatase-APPsw were sensitive to endoproteolytic cleavage at the β-secretase site and that high levels of chimeric human placental alkaline phosphatase-APPsw species were secreted into the conditioned medium (40). More importantly, we document that the total level of APPsw derivatives is diminished in medium of cells expressing APPsw. This result can be accommodated by a model in which a significant population of substrates which might normally be subject to cleavage by α-secretase are disabled by the prior action of β-secretase. Implicit in this model is that β-secretase must cleave APPsw substrates in an intracellular compartment proximal to the cellular site(s) of α-secretase activity. Thus, we argue that APPsw is obligatorily cleaved by β-secretase in an intracellular compartment wherein α-secretase is either inactive or absent.

Kinetics of α- and β-Secretase Processing of APPwt and APPsw APP in N2a Cells—If our interpretation that β-secretase cleavage of APPsw occurred during transit through the secretory pathway is correct, then we anticipated recovery of a specific membrane-bound, carboxyl-terminal fragment containing Aβ1-42 sequences in cell lysates prior to appearance of an α-secretase-generated carboxyl-terminal fragment. We prepared detergent lysates from N2a cells transiently transfected with cDNA encoding Myc-tagged APPwt or APPsw and metabolically labeled with [35S]methionine for 3 h. Lysates were subjected to immunoprecipitation with Myc-1 and 369 antibodies, specific for epitopes in the carboxyl terminus of transgene-derived polypeptides. Both antibodies recovered similar steady-state levels of a common ~12-kDa peptide from either APPwt (Fig. 2A, lanes 2 and 5) or APPsw (Fig. 2A, lanes 3 and 6) cell lysates and a specific fragment of ~3.5 kDa in lysates of cells expressing APPsw (Fig. 2, lanes 3 and 6). Since the half-life of the α-secretase generated carboxyl-terminal fragment of ~12
kDa has not been determined, it is not possible to interpret the significance of the finding that cells expressing APPwt and APPswe accumulate similar levels of this fragment. Thus, the accumulation of this fragment is silent with respect to its rate of production. Moreover, this fragment does not necessarily derive from \( \alpha \)-secretase processing of the precursor; for example, high levels of peptides that migrate with the bona fide \( \alpha \)-secretase-generated carboxyl-terminal fragment are apparent in lysates of cells which express APP with a lysine to valine mutation at position 612 (8). In this case, APPswe secretion is diminished by over 80%. Hence, we argue that proteolysis of APP in endosomal/lysosomal compartments may also contribute to accumulated \( \sim \)12-kDa species. On the other hand, the \( \sim \)13.5-kDa fragment is likely generated following cleavage at the \( \beta \)-secretase site, and is analogous to the carboxyl-terminal 100-amino acid fragment that is enriched in human M17 cells expressing APP harboring the Swedish mutations (39).

To evaluate the kinetics of cleavage of APPwt and APPswe at the \( \alpha \)- or \( \beta \)-secretase sites specifically, we performed pulse-chase analyses. Parallel dishes of N2a cells were transiently transfected with cDNAs expressing APPwt or APPswe. Cell monolayers were pulse-labeled with \(^{35}\)S-methionine for 10 min, then chased at 37 °C for varying periods of time up to 45 min. At the end of each chase period, medium was saved and detergent soluble lysates were prepared from cell monolayers. Lysates or conditioned medium were subjected to immunoprecipitation with either MAb P2-1 or 369 antiserum. The \( \sim \)12-kDa band (marked by an arrowhead) likely represents the carboxyl-terminal fragment generated after \( \alpha \)-secretase cleavage of APP.

2 S. Sisodia, T. Golde, and S. Younkin, unpublished observations.

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**Fig. 2. Kinetics of APP processing in N2a cells.** A, analysis of APP carboxy-terminal fragments generated by proteolytic processing. Detergent lysates of transiently transfected N2a cells (Fig. 1A) were analyzed by immunoprecipitation with either Myc-I or 369 antiserum. B and C, kinetics of cleavage and secretion of APP molecules. Parallel dishes of N2a cells transfected with plasmids encoding APPwt (Wt) or APPswe (Swe) were pulse-labeled for 10 min with \(^{35}\)S-methionine and chased for the times indicated. At each time point, medium was collected and lysates were prepared from cell monolayer. Carboxyl-terminal APP fragments were immunoprecipitated from lysates with 369 antiserum. Secrected APP* molecules were immunoprecipitated from conditioned medium using mAb P2-1. The \( \sim \)13.5-kDa fragment whose levels were elevated in cells expressing APPswe is marked by an arrow. The \( \sim \)12-kDa band (marked by an arrowhead) likely represents the carboxyl-terminal fragment generated after \( \alpha \)-secretase cleavage of APP.

**Fig. 3. Cell surface biotinylation and release assay.** Transiently transfected N2a cells were labeled with \(^{35}\)S-methionine and cell surface biotinylated. One set of cells were incubated at 37 °C for 10 min, while the other set was kept at 4 °C. Conditioned medium and cell lysates were analyzed as described under "Materials and Methods." Lanes 1 and 2 represent secreted APP* molecules immunoprecipitated with mAb P2-1 from conditioned medium collected during the 3-h \(^{35}\)S-methionine labeling period. Lanes 3–6 represent cell-surface biotinylated APP molecules immunoprecipitated with 369 antiserum from lysates of cells incubated at 37 °C for 0 or 10 min. Lanes 7–10 represent mAb 22C11 or 6E10 immunoprecipitates of biotinylated APP* molecules secreted during 10 min incubation at 37 °C.
APPswe contain a prominent ~13.5-kDa fragment which first appears within 10 min into the chase period (Fig. 2B, right panel). Notably, a ~12-kDa carboxyl-terminal fragment is also present, albeit at somewhat lower levels, in cells expressing APPswe, and the kinetics of appearance of this species fragment is indistinguishable to that observed in cells expressing APPwt. Interestingly, APP* first appear in the conditioned medium within 20 min of chase (Fig. 2B, right panel), a rate considerably accelerated relative to the secretion of APP* species from cells expressing APPwt. Moreover, the soluble derivatives in medium of cells expressing APPswe clearly exhibit accelerated migration relative to APP* species. Our interpretation of this result is that these APP* species which are produced early in the secretory pathway are liberated from specific intracellular sorting/retention signals contained within the core protein and hence rapidly transit through the secretory pathway prior to secretion. Thus, the early production of the ~13.5-kDa carboxyl-terminal fragment and accelerated appearance of secreted APP* supports our view that β-secretase cleavage of APPswe is initiated early in the secretory pathway. Interestingly, we have failed to recover APP carboxyl-terminal fragment(s) that encompasses sequences between Ala637 (in APPwt) and the myc-epitope tag, a species which would be generated by the action of γ-secretase (data not shown). The reason for this discrepancy is unclear but suggests that γ-secretase-generated 56–59 amino acid fragment is subject to rapid degradation.

β-Secretase Cleavage Does Not Occur on the Plasma Membrane—To assess whether β-secretase could exert its activity at the plasma membrane, we performed a cell surface biotinylation and release assay (9, 27). N2a cells transiently transfected with cDNA encoding APPwt or APPswe APP were labeled continuously for 3 h with [35S]methionine and chased for the times indicated. Carboxyl-terminal fragments of APP were immunoprecipitated with 369 antisera from cell lysates prepared at each time point. B, kinetics of acquisition of endoglycosidase H resistance by APP molecules. Full-length APP molecules were immunoprecipitated from cell lysates of selected time points with Myc-I antiserum. One-half of the sample was incubated with endoglycosidase H (Endo H). Arrowhead indicates the endoglycosidase H-resistant APP species. C, kinetics of maturation of VSVG. VSVG protein was immunoprecipitated from cell lysates with rabbit anti-VSV serum. Arrowhead indicates the mature ~67-kDa species which is resistant to endoglycosidase H digestion (data not shown; 41).

Fig. 4. Kinetics of β-secretase cleavage of APPswe molecules. A, kinetic analysis of carboxyl-terminal APP fragments in Swe.24, an N2a line expressing APPswe. Parallel dishes of Swe.24 cells transiently transfected with plasmid encoding VSVG protein were pulse labeled for 5 min with [35S]methionine and chased for the times indicated. Carboxyl-terminal fragments of APP were immunoprecipitated with 369 antisera from cell lysates prepared at each time point. B, kinetics of acquisition of endoglycosidase H resistance by APP molecules. Full-length APP molecules were immunoprecipitated from cell lysates of selected time points with Myc-I antiserum. One-half of the sample was incubated with endoglycosidase H (Endo H). Arrowhead indicates the endoglycosidase H-resistant APP species. C, kinetics of maturation of VSVG. VSVG protein was immunoprecipitated from cell lysates with rabbit anti-VSV serum. Arrowhead indicates the mature ~67-kDa species which is resistant to endoglycosidase H digestion (data not shown; 41).
surface of cells expressing APPswe are generated following endoproteolytic cleavage by α-secretase, not β-secretase.

**β-Secretase Cleavage of APPswe Is Initiated in the Medial Golgi Compartment**—In order to identify potential intracellular compartments in which β-secretase exerts its activity, we examined the rate of appearance of specific APP carboxyl-terminal fragments and the extent of oligosaccharide modification of APP or other glycoproteins. For these analyses, we transiently transfected cDNA encoding the G protein of the vesicular stomatitis virus (VSVG) (28) into parallel dishes of N2a cells stably transfected with cDNA encoding APPswe (line Swe.24). Cells were pulse-labeled for 5 min, then chased for various periods of time at 37 °C. Detergent soluble lysates prepared at each time point were subjected to immunoprecipitation analysis with APP-specific COOH-terminal antibody Ab369 (Fig. 3A). Clearly, and consistent with our data from transiently transfected N2a cells (Fig. 2B), a ~13.5-kDa fragment appeared within 12.5 min into the chase period in Swe.24 cells. In parallel, we assessed the rate of appearance of endoglycosidase H-resistant, N-linked mixed oligosaccharide-modified forms of APP. Full-length APPswe were immunoprecipitated from lysates prepared at selected time points, and one-half of the recovered material was reacted with endoglycosidase H. Studies of a variety of glycoproteins in a host of mammalian cells have established that the generation of endoglycosidase H-resistant complex-type N-linked oligosaccharides occurs in the medial Golgi compartment (41). As expected, newly synthesized ~105-kDa APP was sensitive to digestion by endoglycosidase H (Fig. 4C, t = 0 min), similar to observations in transfected CHO cells (42); the residual ~100-kDa species represents APPswe lacking high mannose oligosaccharides. However, within 7.5–12.5 min into the chase period, an ~115-kDa APP form appeared that was resistant to digestion with endoglycosidase H. The ~115-kDa form likely represents APPswe with complex-type N-linked oligosaccharides. Thus, in view of the similar rates of appearance of the ~13.5-kDa carboxyl-terminal fragment and ~115-kDa complex-type oligosaccharide modified forms of APP, we suggest that β-secretase cleavage is initiated in the Golgi apparatus, perhaps as early as the medial compartment. However, since the bulk of immature ~105-kDa APP is not converted to the mature ~115-kDa species in N2a cells, it is not presently certain that the ~13.5-kDa carboxyl-terminal fragment is uniquely generated from the ~115-kDa precursor. In any event, we derived additional support for our view that β-secretase cleavage of APPswe occurs in the Golgi compartment by examining the maturation of the VSVG protein using the same detergent-soluble lysates used for the analysis shown in Fig. 4, A and B. Newly synthesized VSVG of ~65 kDa (0 min) matures to ~67 kDa within 12.5–15 min (Fig. 4C), resulting from the enzymatic conversion of high mannose oligosaccharides to complex-type oligosaccharides (43, 44); we have confirmed that the ~67-kDa species is resistant to digestion by endoglycosidase H (data not shown). While it is highly conceivable that the rates of maturation and/or trafficking of VSVG protein and APP in N2a cells are different, the nearly concomitant appearance of the ~13.5-kDa carboxyl-terminal fragment (Fig. 4A), the mature ~115-kDa APP species (Fig. 4B), and mature ~67-kDa VSVG protein (Fig. 4C) provides strong support for our view that β-secretase cleavage of APPswe APP is initiated in the Golgi apparatus.

**DISCUSSION**

Aβ, the principal component of parenchymal amyloid deposits in Alzheimer’s disease, is derived from integral membrane glycoproteins, APP. Although Aβ is normally secreted by a variety of cultured cells, the molecular mechanisms involved in Aβ production have not been fully clarified. The present report provides several insights into the cellular compartments involved in β-secretase cleavage and the production of Aβ. We have assessed the metabolism of a APP (APPswe) harboring a double mutation at codons 670 and 671 (of APP-770) in N2a cells. Using surface biotinylation and release approaches, we demonstrate that relative to the steady-state levels of surface-bound wild-type APP, the levels of APPswe are diminished.
Moreover, surface-bound APPsw and APPwt molecules are released from the plasma membrane after cleavage by \( \beta \)-secretase, but not by \( \gamma \)-secretase. Finally, using kinetic approaches, we provide compelling evidence that \( \beta \)-secretase cleavage of APPsw occurs early in the secretory pathway and unequivocally demonstrates that a population of APPsw molecules are cleaved at the \( \beta \)-secretase site within the Golgi apparatus, as early as the medial compartment. Moreover, and consistent with the cell surface labeling and release studies, cleavage of both the APPwt and APPsw substrates occurs several minutes later, concomitant with the appearance of soluble APP\( \alpha \)-derivatives in the conditioned medium. The principal conclusions of this work are summarized in Fig. 5.

Our finding that \( \beta \)-secretase cleavage of APP harboring the Swedish mutations is initiated in the Golgi apparatus provides a conceptual framework for developing a model to explain the increase in A\( \beta \) secretion by APPsw cells. We propose that the production of the membrane-bound –13.5-kDa A\( \beta \)-containing carboxyl-terminal fragment early in the secretory pathway liberates the fragment from potential sorting/retention signals in the APP luminal domain. Support for a role for APP luminal sequences in intracellular trafficking has recently emerged from studies of soluble APP molecules expressed in MDCK cells (45, 46). Thus, in sharp contrast to APP or APP with a deletion of the cytoplasmic sequence, YENPTY, in which only 20 or 60%, respectively, of newly synthesized molecules reach the cell surface and are secreted (9), we suggest that the –13.5-kDa fragment is efficiently shunted through the secretory pathway. These molecules would subsequently encounter \( \gamma \)-secretase near, or at, the plasma membrane immediately prior to A\( \beta \) release.

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Metabolism of the Swedish Amyloid Precursor Protein Variant in Neuro2a (N2a) Cells: EVIDENCE THAT CLEAVAGE AT THE "β-SECRETASE" SITE OCCURS IN THE GOLGI APPARATUS

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