The Alzheimer's amyloid protein (Aβ) is released from the larger amyloid β-protein precursor (APP) by unidentified enzymes referred to as β- and γ-secretase. β-Secretase cleaves APP on the amino side of Aβ producing a large secreted derivative (sAPPβ) and an Aβ-bearing C-terminal piece that is subsequently cleaved by γ-secretase to release Aβ. Alternative cleavage of the APP by α-secretase at Aβ16/17 releases the secreted derivative sAPPα. In yeast, α-secretase activity has been attributed to glycosylphosphatidylinositol (GPI)-anchored aspartyl proteases. To examine the role of GPI-anchored proteins, we specifically removed these proteins from the surface of mammalian cells using phosphatidylinositol-specific phospholipase C (PI-PLC). PI-PLC treatment of fetal guinea pig brain cultures substantially reduced the amount of Aβ40 and Aβ42 in the medium but had no effect on sAPPα. A mutant CHO cell line (gpi85), which lacks GPI-anchored proteins, secreted lower levels of Aβ40, Aβ42, and sAPPβ than its parental line (GPI+). When this parental line was treated with PI-PLC, Aβ40, Aβ42, and sAPPβ decreased to levels similar to those observed in the mutant line, and the mutant line was resistant to these effects of PI-PLC. These findings provide strong evidence that one or more GPI-anchored proteins play an important role in β-secretase activity and Aβ secretion in mammalian cells. The cell-surface GPI-anchored protein(s) involved in Aβ biogenesis may be excellent therapeutic target(s) in Alzheimer's disease.

The amyloid that is invariably deposited in Alzheimer's disease (AD) is composed of an approximately 4-kDa peptide (amylloid β-peptide, Aβ) that is derived from a larger protein referred to as the amyloid β-protein precursor (APP) (1, 2). APP is a type I integral membrane glycoprotein with a large N-terminal extracellular domain, a single transmembrane domain, and a short cytoplasmic tail. The Aβ peptide begins 99 amino acids from the C terminus of APP, and it extends from the extracellular region to a point half-way through the APP membrane-spanning domain (1). Aβ is released from APP by cleavage on its N- and C-terminal ends by β- and γ-secretase, respectively. β-Secretase cleavage before residue 1 of Aβ (672 of APP770) also releases the secreted derivative sAPPβ, whereas an alternative cleavage before residue 17 by α-secretase releases sAPPα. In most culture systems tested, the predominant cleavage product is sAPPα, and this may serve to prevent the production of Aβ (1). The proteolytic processing of APP to sAPP and Aβ is regulated by protein kinase C (3), protein tyrosine kinase (4), muscarinic receptors (5), and estrogens (6). The regulatory pathways involved are cell type-dependent, have little or no effect in some cell types, and normally stimulate the secretion of sAPPα while simultaneously reducing the secretion of Aβ (2). A metalloproteinase related to the tumor necrosis factor-α converting enzyme (7, 8) can cleave APP to sAPPα upon activation of PKC by phorbol esters (9, 10).

Strong evidence that Aβ plays an important role in AD pathogenesis has come from the study of mutations in the APP (11–14), presenilin 1 (15), and presenilin 2 (16) genes that are known to cause early onset familial AD (FAD) (17). A fundamental effect of all the FAD-linked mutations is to increase the concentration of Aβ42 or of both Aβ40 and Aβ42 (18–22). Aβ42 is deposited early and selectively in the senile plaques that are observed in all forms of AD, so these findings provide strong evidence that the FAD-linked mutations all act to cause AD by increasing the extracellular concentration of Aβ42.

The evidence implicating Aβ in AD pathogenesis has made both β- and γ-secretase important therapeutic targets, but neither has yet been identified. It was recently demonstrated, however, that APP is proteolytically processed by an α-secretase-like pathway in the yeast Saccharomyces cerevisiae (23, 24). This processing pathway was shown to involve the glycosylphosphatidylinositol (GPI)-anchored aspartyl proteases YAP3 and MKC7 (25, 26). In mammals, GPI-anchored proteins are a relatively small class of membrane proteins that are anchored to the outer leaflet of the cell membrane by a glycolipid anchor consisting of ethanolamine, mannos, glycolamine, and phosphatidylinositol (27). These proteins can be removed from the cell surface by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC), which cleaves the acid; ELISA, enzyme-linked immunosorbent assay; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; PKC, protein kinase C; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)glycine. 2 L. M. Refolo and K. Sambamurti, manuscript in preparation.
phosphatidylinositol in GPI-anchored proteins to release the protein. In this study we used PI-PLC to evaluate the role of GPI-anchored proteins in mammalian secretase activity and Aβ biogenesis. Our data indicate that at least one form of β-secretase or one of its essential, constitutively active regulators is GPI-anchored. It appears, however, that α-secretase is not a GPI-anchored protease in mammalian cells.

**EXPERIMENTAL PROCEDURES**

**Purification of PI-PLC—**PI-PLC was expressed in Bacillus subtilis cultures transformed with a plasmid containing the Bacillus thuringiensis gene and was purified from 8 liters of the culture mixture. Briefly, the secreted PI-PLC was concentrated by filtration through a Millipore pellicon filter, adjusted to 0.2 M NaCl, and loaded onto a 50-ml octyl-Sepharose column. The column was washed with 100 ml of 20 mM Na2PO4, 0.2 M NaCl, pH 7, and eluted with 20 mM Na2PO4, pH 7. Fractions containing active PI-PLC were adjusted to pH 8.5 and 0.055% Triton X-100 and loaded onto an 80-ml DEAE column. The column was washed with 70 ml of Tris acetate, pH 8.5, 0.55% Triton X-100 and eluted with 20 mM Tris acetate, 0.55% Triton X-100, pH 5.0. The activity of the fractions was assayed by the measurement of its capacity to hydrolyze 1H-labeled phosphatidylinositol (PI) in 50 mM Tris acetate, pH 5.5, containing 2 mM 1,2-diheptanoyl-sn-glycero-3-phosphatidycholine (Avanti Polar Lipids, Inc.). The free inositol released by PI-PLC was measured in the aqueous fraction after stopping the reaction in 10:5:0.1 chloroform:methanol:HCl. The protein concentration was determined by a BCA assay (Pierce). At this stage the PI-PLC is very pure as judged by the detection of a single band on a Coomassie Blue-stained SDS-polyacrylamide gel that was subsequently stained with silver. For most of our treatments, we used 2 µg of this purified PI-PLC.

**Mixed Brain Cultures—**Mixed brain cultures from guinea pig fetuses were prepared from 30-day embryos using a method developed by Dr. Yasumasa Ohyagi (see Ref. 29). Briefly, a pregnant guinea pig was sacrificed with carbon dioxide, and six embryos were recovered from the uterus. The brains were dissected and minced into fine pieces. These pieces were treated with 10 volumes of 0.03% trypsin and 0.02% EDTA in HEPES-buffered saline, pH 6.4, for 3 min, and the reaction was stopped by the addition of an equal volume of 5% calf serum in Opti-MEM (Life Technologies, Inc.). The cells were collected by centrifugation, and the pellet was resuspended in 20 ml of Opti-MEM containing 5% calf serum and filtered through a 67-µm polyester mesh (Spectrum). The resulting cells were plated at a high density (107 cells per dish) to maximize neurite outgrowth. These cultures can be maintained in the same medium for over a month without loss of viability. In addition to being a good cell culture model for events that occur in the brain, this system benefits from the secretion of easily detectable levels of the Aβ40 and Aβ42 peptides. Furthermore, unlike rodents, the guinea pig Aβ sequence is identical to that in man, thereby allowing the use of the human-specific antibody against Aβ1–16 (BAN50) for the analysis of sAPPα (30).

**RESULTS**

**PI-PLC Treatment Reduces the Secretion of Aβ but Not sAPPα in Guinea Pig Mixed Brain Cultures—**Guinea pig mixed brain cultures were treated with PI-PLC for 3 days in triplicate. Over 3 days of PI-PLC treatment, sAPPα was not reduced in amount (Fig. 1), but both Aβ40 and Aβ42 were substantially reduced (Table I). This large reduction was readily observed after 1 day of treatment and was sustained for at least 3 days. Since PI-PLC treatment is known to release GPI-anchored proteins from the cell surface, these data suggest that one or more GPI-anchored proteins are involved in Aβ biogenesis. The lack of an effect on sAPPα indicates that cleavage by α-secretase is not affected by the removal of cell-surface GPI-anchored proteins and that PI-PLC treatment has no appreciable effect on APP synthesis.

**Effects of PI-PLC Treatment on Aβ Secretion from CHO Cultures—**To investigate further the role of GPI-anchored proteins in Aβ biogenesis, we investigated the mutant CHO cell line, gpi85. This line was derived (31) from a recombinant CHO cell line (GPI-), which expresses human PLAP as a GPI-anchored reporter protein. The gpi85 line is deficient in N-acetylgalcosaminylphosphatidylinositol deacetylase (4), which is responsible for the second step in the biosynthesis of the GPI anchor (32). The loss of GPI anchor synthesis is neither lethal nor detrimental to the growth of mammalian cells. However, in cells (e.g. gpi85) that do not synthesize the GPI anchor, proteins that are normally GPI-anchored are degraded in the...
endoplasmic reticulum (36, 37) and are not, therefore, found at the cell surface.

To determine the concentration of PI-PLC needed to remove GPI-anchored proteins from the cell surface, we treated GPI+ cultures with two doses of PI-PLC and measured the release of PLAP (Fig. 2). More than 80% of total cellular PLAP was released in 1 h by treatment with 20 μg/ml PI-PLC. The small amount of PLAP remaining after this treatment appears to be inaccessible to PI-PLC and is presumably mostly intracellular because no additional PLAP was released by treating cells for 3 h with 20 μg/ml PI-PLC (Fig. 2). The amount of PLAP released by treatment with 2 μg/ml PI-PLC for 1 h was 75% of that released by 20 μg/ml, but essentially the same amount was released when treatment with 2 μg/ml PI-PLC was extended to 3 h (Fig. 2). Thus treatment with 2 μg/ml PI-PLC for 3 h appears to be adequate to remove virtually all of the GPI-anchored protein at the cell surface, and subsequent experiments were conducted using 2 μg/ml PI-PLC.

In 10 independent experiments, secreted Aβ was compared in gpi85 and GPI+ cells cultured in parallel. In 9 of these experiments, the effect of PI-PLC treatment (2 μg/ml for 3 h) was analyzed in both cell types. The results of this study, which were analyzed statistically using the Wilcoxon signed rank test, are shown in Table II. In untreated gpi85 cells, which lack GPI-anchored proteins, there was a consistent, highly significant reduction in Aβ40 (p < 0.0008) and Aβ42 (p < 0.03) relative to untreated GPI+ cells. In gpi85 cells, PI-PLC had no significant effect on Aβ40 or Aβ42, as expected. In GPI+ cells, as in fetal guinea pig brain cultures, removal of cell-surface GPI-anchored proteins with PI-PLC caused a consistent, highly significant reduction in Aβ40 (p < 0.008) and Aβ42 (p < 0.03). As shown in Table II, GPI+ cells treated with PI-PLC and mutant gpi85 cells produced remarkably similar amounts of Aβ40 and Aβ42 that were consistently ~40% less than the amount produced by normal GPI+ cells. Thus, the removal of GPI-anchored proteins by PI-PLC and by mutation causes a similar, highly significant reduction in Aβ40 and Aβ42. Confirming these findings, we have also observed that total secreted Aβ (4-kDa band), immunoprecipitated from [35S]methionine-labeled CHO cells expressing high levels of human APP695, is reduced upon PI-PLC treatment (p < 0.05) to approximately the same extent as shown in Table II without significantly changing sAPPα (data not shown).

Activation of PKC by PI-PLC-Mediated Hydrolysis of PI Does Not Account for the Reduction in Secreted Aβ Levels in GPI+ Cells—In many cell types, PKC activation results in a substantial stimulation of sAPPα release coupled with a reduction in Aβ secretion (3). By hydrolyzing PI in membranes, PI-PLC treatment could, in principle, activate PKC by releasing diacylglycerol (DAG). It is, however, highly unlikely that PKC activation contributes significantly to the effect of PI-PLC on guinea pig brain cultures, because PI-PLC decreased Aβ but did not increase sAPPα in this preparation (Fig. 1). That Aβ was reduced similarly in PI-PLC-treated GPI+ cells (where DAG may be released) and in untreated gpi85 cells (where no DAG is released) also makes it highly unlikely that DAG reduction contributes importantly to the Aβ reduction observed in PI-PLC-treated CHO cells.

To address further the question of DAG-induced PKC activation, the PKC inhibitor BIS (G109203X) was added to GPI+ cultures prior to treatment with PI-PLC. This compound has previously been shown to block the changes in APP metabolism (4) that occur when PDBu is used to activate PKC (4). Our control experiments showed that PDBu reduces Aβ secretion in GPI+ cells, and this reduction was completely blocked by 2.5 μM BIS. In addition, as previously reported (4), PDBu treatment elevated total sAPP secretion by about 15-fold, and this was also blocked by the BIS treatment (data not shown). Treatment of GPI+ cultures with BIS did not, however, block the reduction in Aβ40 and Aβ42 caused by PI-PLC (Table III). Thus, our results clearly show that the effect of PI-PLC on Aβ biogenesis is not due to DAG-induced PKC activation.

β-Secratease Processing of APP Is Reduced in PI-PLC-treated CHO Cultures—The PI-PLC-induced reduction in Aβ could be caused by a reduction in either β- or γ-secretase activity. To evaluate whether β-secretase activity is reduced when GPI-anchored proteins are removed, several experiments were performed in which we treated GPI+ and gpi85 cultures in triplicate with PI-PLC for 24 h and then evaluated sAPPβ and total sAPP (sAPPα + sAPPβ) by immunoblotting (Fig. 3). For spe-
specific detection of sAPPβ, we used antibody 192, which efficiently binds APP cleaved between residues −1 and +1 of the Aβ sequence but shows a low affinity for full-length APP, sAPPα, and other APP fragments (34). We could not specifically analyze the sAPPα secreted from the GPI+- and gpi85 cell lines, because antibodies to human Aβ1–16 do not detect the hamster APP sequence (GenBank accession number AF030413), which is the same as the sequence in other rodents. For this reason, we analyzed total sAPP (sAPPα + sAPPβ) by immunoblotting with monoclonal antibody 22C11, which recognizes both human and rodent sAPP.

Densitometric analysis showed that sAPPβ was reduced by approximately 90% upon PI-PLC treatment of GPI+ cultures (Fig. 3). In contrast, total sAPP (sAPPβ + sAPPα) was only slightly reduced (−16%). Since sAPPα is the major secreted species in GPI+ cells, the small reduction in total sAPP indicates that PI-PLC has little effect on sAPPα levels. Levels of sAPPβ were also highly (71%) reduced in untreated gpi85 cells as compared with GPI+ cells, but again total sAPP was only slightly affected. As expected, PI-PLC treatment did not consistently affect sAPPβ or the total sAPP secreted by gpi85 cells. These results provide strong evidence that GPI-anchored cell-surface proteins play an important role in β-secretase activity.

**DISCUSSION**

The proteins involved in Aβ biogenesis are important therapeutic targets in Alzheimer's disease. To date, none of these proteins has been unequivocally identified. In this study, we investigated the role of GPI-anchored cell-surface proteins in Aβ biogenesis. Our results provide strong evidence that, in mammalian cells, one or more cell-surface GPI-anchored proteins play an important role in β-secretase activity and Aβ secretion. In addition, they indicate that cell-surface GPI-anchored proteins do not play a major role in α-secretase activity. These conclusions are based on the following observations. (i) PI-PLC, which removes GPI-anchored proteins from the cell surface, substantially reduces the Aβ40 and Aβ42 secreted from fetal guinea pig brain cultures and the wild type GPI+ CHO cell cultures. (ii) The mutant gpi85 line, which lacks cell-surface GPI-anchored proteins, shows reductions in secreted Aβ40 and Aβ42 similar to those observed when the parental GPI+ line is treated with PI-PLC. (iii) The PI-PLC treatment that lowers the Aβ40 and Aβ42 secreted from GPI+ cells has no detectable effect on the residual Aβ secretion that occurs in gpi85 cells. (iv) sAPPβ is substantially reduced in gpi85 cells and in PI-PLC-treated GPI+ cells but shows no additional reduction in PI-PLC-treated gpi85 cells. (v) PI-PLC treatment does not affect the levels of sAPPα in fetal guinea pig brain cultures and only slightly reduces total sAPP (sAPPα + sAPPβ) in GPI+ and gpi85 cultures.

The coordinate reduction of Aβ40 and Aβ42, the profound reduction of sAPPβ, and the lack of change in sAPPα that occurs when GPI-anchored proteins are removed from the cell surface clearly implicate one or more cell-surface GPI-anchored proteins in β-secretase activity and Aβ biogenesis. This finding fits well with recent reports that APP and Aβ are found in detergent-insoluble membrane domains enriched in GPI-anchored proteins (38, 39). These domains are rich in cholesterol, which has been reported to be important for Aβ production (40, 41).

Although reduced, secretion of sAPPβ, Aβ40, and Aβ42 continues both in PI-PLC-treated cells and in mutant gpi85 cells deficient in GPI-anchored proteins. This suggests that normal β-secretase activity may involve both GPI-dependent and -independent proteins. One should, however, be cautious in drawing this conclusion. A small percentage of the GPI-anchored proteins may be resistant to PI-PLC because of fatty acylation of the GPI anchor (28). PI-PLC treatment removes cell-surface GPI-anchored proteins but may not remove intracellular GPI-anchored proteins that could continue to produce Aβ and sAPPβ. In gpi85 cells, GPI-anchored proteins continue to be synthesized. These proteins are rapidly degraded when they fail to be GPI-anchored, and they do not accumulate at the cell surface, but some residual protein precursor capable of processing intracellular APP to sAPPβ and Aβ may remain in the secretory pathway. Thus our findings are consistent with separate GPI-anchored protein-dependent and -independent β-secretase pathways, but they do not eliminate the possibility.

**TABLE III**

Addition of the PKC inhibitor BIS blocks PDBu-mediated but not PI-PLC-mediated Aβ reduction in GPI+ cultures

The GPI+ and gpi85 cultures were treated for 3 h with PDBu, PI-PLC, and/or BIS. Media were collected and analyzed for AβsAPPoccurring when GPI-anchored proteins are removed from the cell

| Culture | sAPPβ | sAPPα |
|---------|-------|-------|
| GPI+    | 1     | 3     |
| PDBu 1  | 2     | 4     |
| PDBu 2  | 3     | 4     |

* NT, not tested.

**FIG. 3.** PI-PLC substantially reduces mammalian sAPPβ. The proteins in media from gpi85 cells (1 untreated; 2–4 PI-PLC-treated) and GPI+ cells (5–7 untreated; 8–10 PI-PLC-treated) were separated on 10–20% Tris-Tricine gels. A, immunoblotted with antibody 192, which specifically detects sAPPβ (solid arrow). B, immunoblotted with antibody 22C11, which detects total sAPP (sAPPα + sAPPβ, open arrow). Densitometric analysis of lanes from two independent blots showed that sAPPβ was reduced by 90% when GPI+ cultures were treated with PI-PLC. sAPPβ was 71% lower in gpi85 than in GPI+ cultures, and PI-PLC treatment had no consistent effect on the amount of sAPPβ secreted by gpi85 cells. Total sAPP was slightly reduced (−16%) when GPI+ cultures were treated with PI-PLC. Total sAPP was also slightly lower in gpi85 than in GPI+ cultures. PI-PLC treatment had no consistent effect on the amount of total sAPP secreted by gpi85 cells. The relative levels of sAPP are shown in the graphs in C. Since the differences in total sAPP were small, we repeated this study, and the data in C are summarized from the two experiments.
that normal β-secretase cleavage is carried out entirely by one or several GPI-anchored proteins.

Given the complexity of the cell surface and intracellular events that are involved in Aβ biogenesis, it is likely that some secreted Aβ is produced through alternative proteolytic pathways that do not involve cell-surface GPI-anchored proteins. Thus there is likely to be an upper limit to the reduction in Aβ secretion that can be achieved by removing GPI-anchored proteins. In the CHO cell lines that we examined, removal of cell-surface GPI-anchored proteins caused a coordinate ~50% reduction of Aβ40 and Aβ42 at 24 h that was smaller than the 80–90% reduction in sAPPβ. This sizeable difference suggests that, in CHO cells, most sAPPβ is produced by a GPI-anchored protein-dependent pathway, whereas a substantial percentage of Aβ production is mediated by alternative pathways that do not result in the secretion of sAPPβ. This finding agrees well with the published evidence for the presence of secondary β-secretase activities, which cleave APP at alternative locations close to the N-terminal end of the Aβ sequence. The sequence of the secreted Aβ begins at all known residues ranging from 1 to 11 indicating that the cleavage can occur at any one of these residues (42, 43). The sAPPβ-specific antibody, 192, will not detect sAPP cleaved at these secondary sites, but the sandwich ELISA will detect the Aβ generated by these cleavages which may be GPI anchor-independent.

The number of cell-surface GPI-anchored proteins that are involved in β-secretase activity and the precise nature of these proteins remain to be determined. It is entirely possible that the effects we have observed are all due to the removal of a single catalytically active β-secretase that is GPI-anchored to the cell surface. Alternatively, a GPI-anchored protein could (i) be an essential non-catalytic subunit in a multi-subunit β-secretase; (ii) perform a post-translational modification required to sustain β-secretase activity; (iii) chaperone to bring together APP and β-secretase into one compartment; or (iv) activate another factor that performs one of the functions listed above. Whatever its precise nature, any cell-surface GPI-anchored protein involved in β-secretase activity is of great interest as a therapeutic target in AD. GPI-anchored proteins can be substantially enriched in cell lysates on the basis of their detergent insolubility, and they can be released specifically from intact cells or cell lysates by PI-PLC. In addition, these proteins can be incorporated from the medium into live cultured cells in a functionally active form. These unique properties and the relatively small number of mammalian GPI-anchored proteins should be highly advantageous in the effort to isolate and characterize the specific GPI-anchored protein(s) that are involved in β-secretase activity.

Acknowledgments—We thank Dr. Victoria Stevens for providing us with the gpi+ and gpi85 strains, Dr. Ivan Lieberburg and Dr. John Anderson of Elan Corp. for the 192 antibody, and Dr. Nobu Suzuki of Genentech, Inc. for提供 192 antibody, and Dr. Nobu Suzuki of Genentech, Inc. for providing us with the GPI-anchored protein gpi85. We thank Dr. Victoria Stevens for providing us with the gpi+ and gpi85 strains, Dr. Ivan Lieberburg and Dr. John Anderson of Elan Corp. for the 192 antibody, and Dr. Nobu Suzuki of Genentech, Inc. for the 192 antibody, and Dr. Nobu Suzuki of Genentech, Inc. for providing us with the GPI-anchored protein gpi85. We thank Dr. Victoria Stevens for providing us with the gpi+ and gpi85 strains, Dr. Ivan Lieberburg and Dr. John Anderson of Elan Corp. for the 192 antibody, and Dr. Nobu Suzuki of Genentech, Inc. for providing us with the GPI-anchored protein gpi85. We thank Dr. Victoria Stevens for providing us with the gpi+ and gpi85 strains, Dr. Ivan Lieberburg and Dr. John Anderson of Elan Corp. for the 192 antibody, and Dr. Nobu Suzuki of Genentech, Inc. for providing us with the GPI-anchored protein gpi85. We thank Dr. Victoria Stevens for providing us with the gpi+ and gpi85 strains, Dr. Ivan Lieberburg and Dr. John Anderson of Elan Corp. for the 192 antibody, and Dr. Nobu Suzuki of Genentech, Inc. for providing us with the GPI-anchored protein gpi85. We thank Dr. Victoria Stevens for providing us with the gpi+ and gpi85 strains, Dr. Ivan Lieberburg and Dr. John Anderson of Elan Corp. for the 192 antibody, and Dr. Nobu Suzuki of Genentech, Inc. for providing us with the GPI-anchored protein gpi85.

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