Biosynthesis of glycosylphosphatidylinositol-anchored human placental alkaline phosphatase: evidence for a phospholipase C-sensitive precursor and its post-attachment conversion into a phospholipase C-resistant form

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Previous studies have shown that some cells (e.g. SKG3a) express human placental alkaline phosphatase (AP) in a form which can be released from the membrane by bacterial PtdIns-specific phospholipase C (PI-PLC) while others (e.g. HeLa) are relatively resistant to this enzyme. Chemical and enzymic degradation studies have suggested that the PI-PLC resistance of AP is due to inositol acylation of its glycosylphosphatidylinositol (GPI) anchor. In order to identify the biosynthetic origin of PI-PLC resistance we determined the PI-PLC sensitivity of AP in ^{35}S-labelled cells (10 min pulse; 0–60 min chase) by Triton X-114 phase separation. At the beginning of the chase period, the majority of the AP synthesized was hydrophilic, indicating that it had not acquired a GPI anchor. The concentration of hydrophilic AP species decreased with a $t_{\frac{1}{2}}$ of 30–60 min but was not processed to an endoglycosidase H-resistant species or secreted into the medium. In both SKG3a and HeLa cells all of the hydrophobic, GPI-anchored AP detectable at the beginning of the chase was PI-PLC sensitive. PI-PLC-resistant species of AP were only observed in HeLa cells and these only appeared after about 30 min. The delayed appearance of PI-PLC resistance was unexpected as previous studies have suggested that candidate GPI-anchor precursors are PI-PLC-resistant as a result of inositol acylation. This work reveals unanticipated complexities in the biosynthesis of AP and its GPI anchor.

INTRODUCTION

Over 100 membrane proteins are known to be anchored on the cell surface via a glycosylphosphatidylinositol (GPI) moiety (reviewed in [1–5]) Although all of the GPIs utilized by eukaryotes for protein anchoring appear to contain a conserved glycan core structure linked to the protein there is considerable variation in the side chains attached to the glycan, as well as in the hydrophobic lipid part of the molecule which inserts into the lipid bilayer. The latter group of variations include the number, type (i.e. acyl or alkyl) and length of the hydrocarbon groups giving rise to a large and varied collection of hydrophobic moieties. The biochemical significance of these structural variations in the hydrophobic part of the GPI molecule is unknown. However, GPI anchors can contain an additional hydrophobic modification, inositol acylation, which has a profound influence on its biochemical properties, i.e. sensitivity to phospholipase-mediated degradation. A characteristic feature of GPI anchors is their ability to be cleaved by the bacterial enzyme PtdIns-specific phospholipase C (PI-PLC) and the ensuing release of membrane proteins has become a basic criterion for the identification of GPI anchors. There are several instances where proteins, known to be GPI-anchored on the basis of other criteria, exhibit some degree of resistance to PI-PLC cleavage (see [1,6,7] for references). The most striking example is the acetylcholinesterase (AChE) on human erythrocytes or erythroleukaemia cells, where 90–95% is resistant due to inositol acylation (probably of the 2-hydroxy group) of its GPI anchor [8–10]. Although this degree of PI-PLC resistance is not found with AChE on the erythrocytes of most other mammalian species [8] it is associated with other GPI-anchored proteins on human erythrocytes, suggesting that the occurrence of GPI-anchor inositol acylation is cell-specific rather than protein-specific [11]. Inositol acylation has also been reported in the PI-PLC-resistant anchor of *Trypanosoma brucei* procyclic acidic repetitive protein [12].

PI-PLC resistance of human placental alkaline phosphatase (AP) has been observed in several studies [13–17] but until recently its mechanism was unknown. We have recently analysed a group of human cell lines that express human placental AP for their resistance towards PI-PLC cleavage using both intact cells as well as butanol-extracted cell lysate [18]. In some cell lines (e.g. HeLa R) only 20–30% of the AP was sensitive to PI-PLC compared with 90% in another cell line (SKG3a). Although the relatively low-mass abundance of the PI-PLC-resistant AP hampered investigation of the structural basis for PI-PLC resistance, non-denaturing electrophoretic analyses of AP after a variety of enzymic and chemical degradation procedures indicated that the PI-PLC resistance was probably due to inositol acylation [18].

The physiological role of inositol acylation of GPIs is currently a matter of speculation. The presence of an additional hydrophobic group might reduce the ability of a GPI anchor to exit the bilayer and thereby prevent GPI-anchored proteins being shed from the cell surface or from engaging in a potentially deleterious process of intercellular transfer. Studies with decay-accelerating factor suggest that even though inositol acylation has little effect on its functional properties [19] it may help to retain it in the membrane [20]. Inositol acylation could also have an additional effect on membrane anchoring, either by blocking phospholipase-mediated GPI cleavage completely or by leaving a hydrophobic substituent after cleavage. However, this possibility is difficult to evaluate because cleavage of GPI anchors, mediated by endogenous phospholipases, has not yet been observed under physiological conditions [6,21]. Another possibility is that inositol

Abbreviations used: AP, alkaline phosphatase; AChE, acetylcholinesterase; DMEM, Dulbecco's modified Eagle's medium; EBSS, Earle's balanced salts solution; endo H, endoglycosidase H; FCS, fetal calf serum; GPI, glycosylphosphatidylinositol; NP-40, Nonidet P-40; PI-PLC, PtdIns-specific phospholipase C.

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acylation has a role in the process of GPI anchoring but is redundant in the mature protein after GPI attachment is completed. In mammalian cells inositol acylation has been detected on an early intermediate, GlcN-PtdIns, as well as all of the candidate GPI-anchor precursors [3,22–24], supporting the view that this modification might play an important role in biosynthesis. For example, inositol acylation of a GPI precursor might facilitate its membrane translocation or the correct orientation of the inositol ring so that it is accessible to the glycosyltransferases responsible for elongation of the glycan chain. It is presumed that the acyl group on the inositol ring is removed from most of the GPI anchors subsequent to attachment to the protein as the majority of GPI-anchored protein molecules expressed on the surface of mammalian cells are PI-PLC-sensitive. The observation that PI-PLC sensitivity is a dominant trait [17,25] is consistent with an inositol deacylase being located in the endoplasmic reticulum or Golgi but there is, as yet, no direct experimental evidence for the existence of such an enzyme. Presumably, in some cells (e.g. human reticulocytes and HeLa R cells) removal of the acyl group by the inositol deacylase must be relatively slow, allowing the expression of a large proportion of PI-PLC-resistant AChE or AP at the cell surface.

In the present study we have investigated the biosynthetic origin of PI-PLC-resistant AP by [^{35}S]methionine pulse–chase studies in PI-PLC-sensitive and PI-PLC-resistant cell lines. On the basis of current information regarding inositol acylation we would predict that newly synthesized species of AP (i.e. those detected after short chase periods) would all contain PI-PLC-resistant GPI anchors regardless of the cell line used. Subsequently, in a sensitive cell line, AP would become less resistant as the chase progressed and the newly synthesized species was deacylated. By contrast in a resistant cell line there would be little or no decrease in the PI-PLC sensitivity during the chase. Unexpectedly, the results we obtained differed in two important respects from this scheme. First, in both cell lines the newly synthesized AP was PI-PLC-sensitive. Secondly, in the resistant cell line there was a slow increase in PI-PLC resistance during maturation of AP.

EXPERIMENTAL

Materials

Dulbecco's modified Eagle's medium (DMEM), Earle's balanced salts solution (EBSS), methionine-free RPMI 1640, fetal calf serum (FCS), L-glutamine and penicillin–streptomycin were obtained from Gibco Life Technologies (Grand Island, NY, U.S.A.). Ham's F-12 culture medium was purchased from Meditech (Washington, DC, U.S.A.). Tran^{35}S-Label (85% methionine, 15% cysteine; specific radioactivity 1025 mCi/mol) was from ICN Radiochemicals (Irvine, CA, U.S.A.). Rabbit anti-(human placental AP) and normal rabbit immunoglobulin fraction were from Dako Corp. (Carpentaria, CA, U.S.A.). Endoglycosidase H (endo H) was from Oxford GlycoSystems (Rosedale, NY, U.S.A.). Affigel Protein A–agarose, electrophoretic grade Tris and polyacrylamide mix were from Bio-Rad Laboratory (Richmond, CA, U.S.A.).

PI-PLC was purified from culture supernatants of *Bacillus subtilis* BG2320 transformed with a high copy number plasmid encoding the gene for *Bacillus thuringiensis* PI-PLC as described previously [7,26].

Cell lines

Human cervical epithelia carcinoma SKG3a (originally a gift from Dr. Jose L. Millan, La Jolla Cancer Research Foundation, La Jolla, CA, U.S.A.) and HeLa R were as described in our previous studies on PI-PLC resistance of AP [18]. HeLa R was maintained in DMEM medium with 10% (v/v) FCS and SKG3a was cultured in Ham's F-12 medium with 10% (v/v) FCS. All culture media were supplemented with 2 mM L-glutamine, 5 μg/ml streptomycin and 50 i.u./ml penicillin.

Biosynthetic labelling

Cells were cultured in 100 mm diam. culture dishes at 4×10^6 cells/dish at 37 °C for 2 days. The dish was rinsed twice with EBSS and preincubated with 5 ml of methionine-free RPMI 1640 medium at 37 °C for 1 h. The cells were then pulse-labelled with 100 μCi/dish Tran^{35}S-Label in 3 ml of the same medium for 10 min. The dish was washed twice with EBSS and chased for the desired period in 10 ml of medium containing 10% (v/v) FCS. Cells were rinsed twice and then scraped from the dish with 1 ml of ice-cold buffer A (10 mM Tris/HCl, pH 8.2, 140 mM NaCl, 5 mM EDTA, 1 mM phenylmethanesulphonyl fluoride, 1% Triton X-114, 1 μg/ml pepstatin, 0.5 μg/ml leupeptin). The extract was kept on ice for 30 min, centrifuged at 13000 *g* for 30 min and the supernatant harvested.

PLC digestion and Triton X-114 phase separation

The supernatant (0.5 ml) from the lysed cells was incubated with or without PI-PLC (1 unit/ml) at 37 °C for 1 h. An equal volume of buffer A was added and incubation continued at 37 °C for another 10 min. The lysate was centrifuged at 13000 *g* for 5 min to separate into Triton X-114-poor and -rich phases. The upper, aqueous (i.e. Triton X-114-poor), phase was harvested and the lower, Triton-enriched, phase was rinsed with 1 ml of buffer B (same as buffer A without 1% Triton X-114) and adjusted to 1 ml with buffer B. Both phases were supplemented with 0.1 ml of 5% (v/v) Nonidet P-40 (NP-40), 5% (w/v) sodium deoxycholate, 5% (w/v) SDS in buffer B before immunoprecipitation.

Immunoprecipitation of AP

The AP in each phase was first precleared by incubating with 5 μl of normal rabbit IgG on ice for 1 h. An aliquot of 100 μl of a 20% (v/v) slurry of Affigel Protein A–agarose was then added and the mixture incubated at 4 °C for 30 min with rotary mixing. The supernatant was harvested after centrifugation at 4000 *g* for 5 min. Another 100 μl of Affigel was added and the above procedure was repeated. The precleared supernatant was then incubated with 5 μl of rabbit anti-(human AP) antibody for 2 h on ice. A 50 μl aliquot of Affigel was added and the mixture was left overnight at 4 °C with rotary mixing. The Affigel was sedimented and washed sequentially with 1 ml of (i) 50 mM Tris/HCl (pH 8.2)/500 mM NaCl/0.5% NP-40/0.05% SDS, (ii) 50 mM Tris/HCl (pH 8.2)/150 mM NaCl/0.5% NP-40/0.05% SDS/0.5% sodium deoxycholate and (iii) 50 mM Tris/HCl/0.05% SDS. The AP was eluted from the Affigel in 15 μl of SDS/PAGE sample buffer at 100 °C for 5 min, centrifuged for 1 min at 13000 *g* and the supernatant subjected to SDS/PAGE in a 10% gel. ^{35}S-labelled proteins were visualized by soaking the gel in En^{3}Hance followed by fluorography for 4–9 days at −80 °C.

Endo H digestion

After immunoprecipitation, as described above, AP was eluted from the Affigel beads by boiling at 100 °C for 5 min in 15 μl of 10 mM Tris/HCl (pH 7.0)/30 mM dithiothreitol. The supernatant was incubated with or without 2 μl of endo H (0.4 unit/ml) at 37 °C overnight. Samples were then mixed with

SDS/PAGE sample buffer and boiled for 5 min before electrophoresis.

RESULTS

Validation of extraction procedure for ^{35}S-labelled AP

The protocol for the determination of PI-PLC sensitivity required an initial extraction of the labelled cells in ice-cold Triton X-114 whereas our previous studies on PI-PLC resistance of mature AP used either intact cells or aqueous butanol extracts [18]. Since it has been shown that AP and other GPI-anchored proteins in some cell types are relatively insoluble in cold Triton X-114 and X-100 [27–29] it was possible that Triton X-114 might extract selectively a sub-population of AP molecules differing in PI-PLC sensitivity. This possibility was assessed by two independent methods.

(i) The HeLa R and SKG3a cells were extracted either with aqueous butanol or with cold Triton X-114 and the PI-PLC sensitivity determined by monitoring the distribution of AP enzymic activity in a Triton X-114 phase separation. These experiments showed no difference in PI-PLC sensitivity between the Triton- or butanol-extracted AP and were comparable with our previous results [18] with HeLa R (mainly PI-PLC-resistant) and SKG3a cells (mainly PI-PLC-sensitive).

(ii) HeLa R and SKG3a cells were labelled overnight with [^{35}S]methionine/cysteine and then analysed for PI-PLC sensitivity. These experiments demonstrated that essentially all of the ^{35}S-labelled AP extractable from SKG3a cells by cold Triton X-114 was PI-PLC-sensitive, whereas a substantial proportion of the HeLa R AP was resistant and remained in the Triton-enriched phase after PI-PLC treatment (Figure 1). This result confirms that Triton X-114 is able to extract both PI-PLC-sensitive and -resistant forms of AP. Essentially the same result was obtained if the labelled cells were chased for 1 h before extraction, indicating that the bands are representative of mature AP (data not shown).

Most newly synthesized AP does not acquire a GPI anchor

SKG3a (Figure 2a) and HeLa R (Figure 2b) were pulse-labelled for 10 min, chased for 0–60 min and cell extracts analysed by Triton X-114 phase separation. Unexpectedly, even after chase times as long as 30 min, the majority of the AP-associated radioactivity was found in the aqueous phase instead of the Triton-enriched phase of the Triton X-114 phase separation (compare upper and middle panels in Figures 2a and 2b). At the start of the chase period three bands were evident in the aqueous phase. These bands had approximate molecular masses of 69, 67 and 65 kDa and were designated, in order of increasing mobility, A_1, A_2 and A_3 (Figures 2a and 2b, upper panels). Levels of A_2 and A_3 changed relatively little during the first 20–30 min of the chase and A_2 was the predominant species for most of the chase period. By contrast the concentration of A_1 decreased markedly during the early part of the chase ($t_{\frac{1}{2}}$ 5–10 min) and it was barely detectable after 20 min. The data suggest either that A_2 and A_3 are relatively stable species or that they are unstable but being continually replenished from A_1. The precise relationship between these three hydrophilic species was not determined. Attempts to demonstrate proteolytic processing at the C-terminus during the conversion of A_1 into A_2, using a polyclonal antibody against the C-terminal peptide, were unsuccessful. With both cell lines the amount of A_2 decreased substantially in the period after 30 min of chase but this was not accompanied by a corresponding increase in T_2 (Figures 2a and 2b, upper panels).

In the Triton-enriched phase two species with approximate

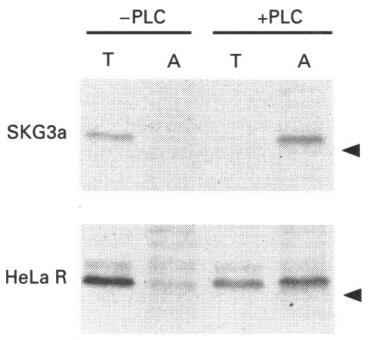

Figure 1 PI-PLC sensitivity of [^{35}S]methionine-labelled AP

Triton X-114 extracts of SKG3a (upper panel) and HeLa R (lower panel) cells, labelled for 24 h with [^{35}S]methionine/cysteine, were incubated in the presence (+PLC) or absence of PI-PLC (−PLC). The distribution of AP between the Triton-enriched (T) and aqueous phases (A) was determined by immunoprecipitation, SDS/PAGE and fluorography as described in the Experimental section. Arrowheads indicate the migration position of the 67 kDa-molecular-mass standard. The major species has a mobility similar to T_1 (see Figure 2). The 67 kDa species corresponding to A_2 and T_2 were not visible. The additional, higher-molecular-mass species in HeLa R cells is probably due to heterogeneity of AP glycosylation as suggested by previous studies [18].

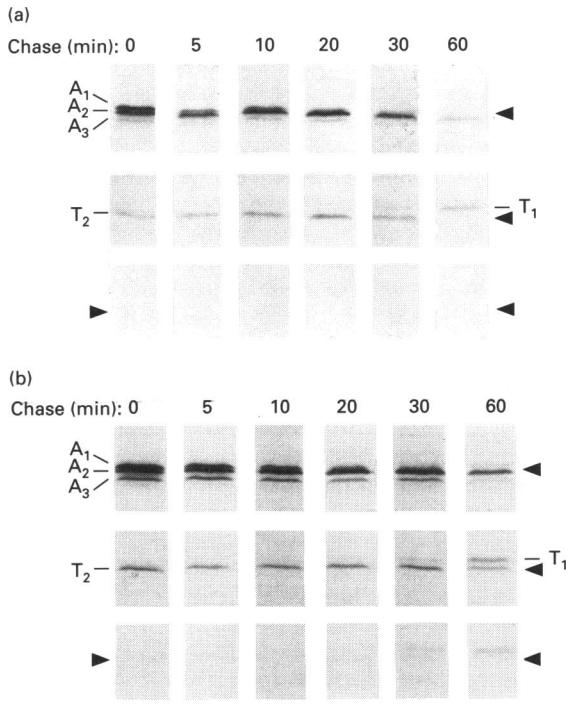

Figure 2 PI-PLC sensitivity of newly synthesized AP

(**a**) SKG3a and (**b**) HeLa R cells were labelled for 10 min with [^{35}S]methionine/cysteine and chased in unlabelled medium for 0–60 min as indicated along the top of the figure. Cells were extracted, incubated in the presence or absence of PI-PLC, and analysed as described in the legend to Figure 1. Upper panels: aqueous phase of Triton X-114 phase separation, not PI-PLC-treated. Since the products of PI-PLC action on AP are not readily visible (see text) the aqueous phases after PI-PLC treatment are not shown. Middle panels: Triton-enriched phase, not PI-PLC-treated. Lower panels: aqueous phase, PI-PLC-treated. Arrowheads indicate the migration position of the 67 kDa-molecular-mass standard. A_1–A_3 and T_1 and T_2 indicate the migration positions of AP species described in the text.

molecular masses of 71 and 67 kDa were evident (designated T_1 and T_2 in order of mobility) and as remarked above were collectively much less abundant than the species which partition into the aqueous phase (Figures 2a and 2b). T_2 was present at the start of the chase in HeLa R cells and remained relatively stable for the first 20–30 min. In some experiments with SKG3a (Figure 2a) T_2 was also present at the beginning of the chase but increased in concentration slightly during the first 20 min. T_1 only became visible after 10–20 min (HeLa R) or 20–30 min (SKG3a) and then increased during the remainder of the chase period. The relative intensities of these two bands suggested that T_2 was converted into T_1 during the chase, a process that was particularly obvious between 30 and 60 min and is probably due to processing of the N-linked glycans to the complex form (see below). The relative abundance of A_2 and T_2 was quite variable between experiments and in other experiments with SKG3a cells T_2 was not visible at all until 10–20 min and then increased progressively until it was converted into T_1 (data not shown).

Although the molecular identities of the hydrophilic AP species were not determined, several experiments provided additional information about the origin and fate of the A_2 species. (i) After overnight labelling of HeLa R and SKG3a cells the A_2 species was not visible and T_1 was the major labelled species (Figure 1). (ii) In control experiments which did not include the 60 min preincubation of the cell lysate the relative amounts of the A_2 and T_2 species were not altered, suggesting that degradation by endogenous phospholipases and proteases does not produce A_2 from T_2 during analysis. Furthermore, anchor degradation following cell lysis would have prevented detection of the T_1 and T_2 species, which accumulate after 30 min of chase (Figure 2) or overnight labelling (Figure 1). (iii) HeLa R cells were labelled for 10 min, chased for 30 and 60 min (i.e. the period over which cell-associated A_2 shows a marked decline) and the medium concentrated and analysed. No accumulation of secreted A_2 in the medium was observed (results not shown). (iv) The lack of significant A_2 accumulation in either the cells or the medium suggested that it was retained in the cell and then degraded after about 30–60 min. In order to identify the site of retention more closely the immunoprecipitates prepared from HeLa R cells (labelled for 40 min without chase) were digested with endo H before electrophoretic analysis. As shown in Figure 3, the molecular mass of A_2 decreased from approx. 67 to 60 kDa after endo H treatment (Figure 3). A_3 decreased by a similar amount from approx. 65 to 58 kDa. These results demonstrated that the N-linked glycans were not processed to the complex type and suggested that the hydrophilic species of AP were retained in the endoplasmic reticulum or the *cis*-Golgi until they were degraded

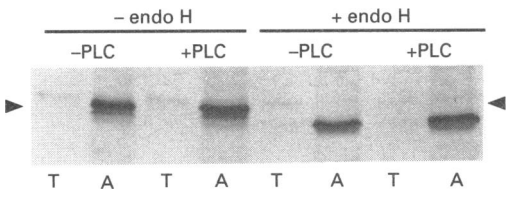

Figure 3 Endo H sensitivity of newly synthesized AP

HeLa R cells were labelled for 40 min with [^{35}S]methionine/cysteine, extracted and incubated in the presence or absence of PI-PLC. The distribution of AP between the Triton-enriched (T) and aqueous (A) phases was determined as described in the legend to Figure 1. Before loading on the gel the samples were incubated in the presence or absence of endo H as described in the Experimental section. Arrowheads indicate the migration position of the 67 kDa-molecular-mass standard.

[30]. The observation that T_1 is relatively insensitive to endo H digestion suggests that it is produced from T_2 by processing of its N-linked glycans to the complex type (Figure 3). However, the T_2 band was not intense enough to determine whether it was sensitive to endo H or not.

PI-PLC sensitivity of newly synthesized GPI-anchored AP

In HeLa R cells the majority of T_2 was sensitive to PI-PLC at the beginning of the chase (Figure 2b; compare middle and lower panels). However, after 20 min a residual T_2 band becomes increasingly obvious in the Triton-enriched phase after PI-PLC treatment, suggesting that T_2 acquires resistance soon after attachment of the GPI anchor. T_1 seems to be PI-PLC-resistant as soon as it is detected (i.e. 20 min). This is consistent with the conversion of T_2 into T_1 during the chase period as proposed above and suggests that acquisition of PI-PLC resistance begins at about the same time that T_2 is converted into T_1.

In SKG3a cells, however, both T_2 and T_1 were completely sensitive to PI-PLC as soon as they could be detected (Figure 2a; compare middle and lower panels). Of particular interest was the fact that in one experiment there was sufficient T_2 visible at the beginning of the chase to show that it was PI-PLC-sensitive (Figure 2a). In another experiment with SKG3a cells the same end result was obtained but it was only after 20 min that sufficient intensity had accumulated in the T_2 band to make an assessment of PI-PLC sensitivity feasible (results not shown).

DISCUSSION

The observation that most newly synthesized AP does not acquire a GPI anchor was unexpected. The reason was not determined precisely although the experimental evidence seems to preclude the trivial possibility that AP first acquired a GPI anchor which was then removed by endogenous degradative enzymes during analysis of cell lysates. Previous studies of human placental AP biosynthesis in JEG-3 cells by Takami et al. [31] have indicated that removal of the C-terminal signal peptide (and presumably the simultaneous addition of GPI) occurs with a $t_{\frac{1}{2}}$ of approx. 5–10 min. In those studies abortive C-terminal processing would not have been detected because the products were not separated by Triton X-114 phase separation and the presence or absence of the GPI anchor on AP cannot be distinguished from its SDS/PAGE mobility (i.e. A_2 and T_2 have essentially the same molecular mass). However, in a subsequent study by the same group a 5 min [^{35}S]methionine-labelling period followed by a 60 min chase indicated that essentially all of the newly synthesized AP was converted into the mature form as shown by further processing of its N-linked glycans in the Golgi [32]. In contrast our results indicate that most of the newly synthesized AP (i.e. A_2) does not get converted into the mature, endo H-resistant form and is retained in the endoplasmic reticulum or *cis*-Golgi for eventual degradation after about 30–60 min [30].

The apparent inefficiency of GPI anchoring has limited our ability to determine the PI-PLC sensitivity of the newly synthesized GPI-anchored species which was the major purpose of this study. In spite of this limitation it is clear that the earliest GPI-anchored species detected in both SKG3a and HeLa R cells are not PI-PLC-resistant. Even in the HeLa R cells (which express mainly resistant AP on their cell surfaces) acquisition of PI-PLC resistance is a relatively late event. This result was contrary to our expectation because the candidate GPI-anchor precursor molecules that have been identified in mammalian cells all appear to be PI-PLC-resistant as a result of inositol acylation at an early

stage in their biosynthesis [3,22–24]. Consequently we had predicted that newly synthesized AP would also be resistant to PI-PLC regardless of the PI-PLC sensitivity of cell-surface AP. However, the present results do not support this simple interpretation. It is conceivable that an inositol deacylase [17,25] is activated by detergent during the extraction procedure and renders the GPI sensitive to subsequent PI-PLC treatment. Although this possibility cannot be excluded in SKG3a cells it seems unlikely in HeLa R cells because deacylation should also prevent the detection of the PI-PLC-resistant species which begin to appear after the 20 min chase and are predominant by 60 min.

As far as we are aware there has been only one previous study where Triton X-114 phase separation was used to monitor PI-PLC sensitivity of a newly synthesized GPI-anchored protein in intact mammalian cells [33]. Although the previous work (done with Thy-1 in the murine thymoma cell lines BW5147 and S1A) was designed to demonstrate attachment of the GPI anchor it revealed that, even after a labelling period as short as 2 min, 45–80% of the Thy-1-associated radioactivity was PI-PLC-sensitive. PI-PLC sensitivity has also been observed with GPI anchors attached to polypeptides generated in cell-free translation systems where the GPI precursors were derived from the rough microsomes added to the system [34,35]. This result would suggest either that the GPI precursors were PI-PLC-sensitive to start with or that the rough microsomes contained an inositol deacylating activity necessary to convert them into a sensitive form. In our view, the earlier work provides independent confirmation of the conclusion that newly attached GPI anchors are PI-PLC-sensitive.

The observation of a PI-PLC-sensitive GPI anchor on newly synthesized AP and Thy-1 was not predicted by previous studies of mammalian GPI biosynthesis. As mentioned above, in all mammalian cell types examined so far (i.e. fibroblasts, HeLa and thymoma cells) the candidate GPI-precursor molecules (i.e. those containing three mannose residues and a terminal phosphoethanolamine) are inositol-acylated and PI-PLC-resistant [3,22–24]. Assuming that the major features of GPI biosynthesis are conserved among mammalian cell lines there are two likely explanations for this discrepancy. First, the PI-PLC-resistant GPIs that have been observed previously might not be used for attachment to proteins. Instead this latter function could be served by a small pool of non-acylated, PI-PLC-sensitive GPI molecules which do not normally accumulate and therefore escaped detection in most studies of GPI biosynthesis. PI-PLC-sensitive GPI molecules might be generated from the acylated species immediately before attachment to protein as well as by stepwise assembly from GlcN-PtdIns. A second possibility is that PI-PLC-resistant GPIs may be attached to the protein but are immediately rendered PI-PLC-sensitive by an inositol deacylase. This sequence of events is not excluded by our experimental results because the inefficiency of GPI anchoring necessitated relatively long (i.e. 10 min) labelling periods. If deacylation occurred with a $t_{\frac{1}{2}}$ of $\leqslant 2$ min the deacylated, PI-PLC-sensitive species would have become dominant ($> 90\%$) during a 10 min labelling period and consequently it is unlikely that the PI-PLC-resistant species would have been detected. Further experimentation using different cell lines, or GPI-anchored proteins exhibiting higher efficiency of GPI-anchoring, will be required to resolve this technical problem.

Whichever of these two explanations accounts for the unexpected PI-PLC sensitivity of newly synthesized AP, they do not address the origin of the PI-PLC resistance of mature AP expressed on the surface of HeLa cells. Our results show that PI-PLC resistance develops at a relatively late stage in the maturation of AP, possibly after transport into the *cis*-Golgi, and is therefore separated, both spatially and temporally, from the GPI-attachment process. The apparent occurrence of inositol acylation at two distinct points in the lifetime of a GPI anchor is puzzling given that the role of this unusual modification has not yet been determined. However, our observation of post-attachment inositol acylation of AP in some cell types and its persistence on mature AP strongly suggests that modification of GPI anchors by this mechanism serves a useful purpose at the cell surface.

This work was supported, in part, by NIH grants GM 40083 and GM 35873, a Grant-in-Aid from the American Heart Association, and Samuel W. Rover.

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Received 16 August 1993/20 December 1993; accepted 3 February 1994