Glycolipid anchors are attached to Thy-1 glycoprotein rapidly after translation

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The attachment of glycolipid anchors to the Thy-1 glycoprotein during biosynthesis was followed by the change of detergent-binding properties of biosynthetically labelled Thy-1 precursors upon phospholipase C treatment in the murine thymoma lines BW5147 and S1A. In S1A, 80% of the Thy-1 molecules were phospholipase-C-sensitive after a 2 min pulse with [³⁵S]methionine, indicating that these molecules were already anchored via a glycolipid tail. In BW5147, 47% of the Thy-1 molecules had phospholipase-C-sensitive anchors attached after a 1.5 min labelling and, with longer pulses, this percentage rose to 76%. Tunicamycin did not block the addition of glycolipid anchors, and glycolipid attachment also occurred at 21 °C. The findings suggest that the attachment of glycolipid anchors occurs in the rough endoplasmic reticulum.

INTRODUCTION

Integral membrane proteins are usually anchored in the lipid bilayer by stretches of hydrophobic amino acids [1–3]. A few externally exposed plasma-membrane proteins from protozoan and mammalian origin, however, are anchored via a covalently attached glycolipid which contains phosphatidylinositol (PI), and these proteins can be released from the membranes by phospholipases C (PLC) [4,5]. The growing list of glycoproteins of this type comprises the variable surface glycoproteins (VSG) of trypanosomes [6,7], the p63 surface glycoprotein of *Leishmania* [8], the membrane forms of alkaline phosphatase [9], acetylcholinesterase [10], 5'-nucleotidase [11,12], the 120 kDa form of rodent neural-cell adhesion molecule ('NCAM') [13], and several immunologically relevant surface proteins such as decay-accelerating factor ('DAF') [14], the rat lymphocyte alloantigen RT-6.2 [15], the mouse Ly-6 antigen [16] and the Thy-1 antigen of lymphocytes and brain cells [17,18]. Considerable chemical information exists about the membrane-anchoring glycolipid of VSG and Thy-1; amino-acid-sequencing studies revealed in both cases that the mature molecules lack a C-terminal stretch of hydrophobic amino acids predicted from the cDNA sequences [17,19,20]. Instead, the mature molecules contain a novel glycolipid attached to the α -carboxy group of the C-terminal amino acid [17,21]. Glycolipid anchors of VSGs as well as of Thy-1 are similar, since they both contain PI, ethanolamine, mannose and glucosamine. However, they differ in their content of other sugars (Thy-1 contains galactosamine; most VSGs contain galactose) and the kind of fatty acids in the PI [6,7,17]. The replacement of the C-terminal peptide by the glycolipid during the biosynthesis of VSG has been found to occur very rapidly (within 1–2 min), and it was therefore proposed that this process involves the 'en bloc' transfer of a preformed glycolipid [23,24]. A likely candidate for such a preformed glycolipid has been identified in trypanosomes [25]. In the present study, we

chose the Thy-1 antigen in order to investigate the kinetics of addition of glycolipid anchors to surface glycoproteins in a mammalian system. Thy-1 is a major surface glycoprotein on brain cells, thymocytes and T-lymphomas, and the presence of glycolipid anchors can be monitored using the change of the partitioning of the molecule in Triton X-114 (TX-114) solutions upon PLC treatment [26,27]. We find that, by this criterion, the attachment of glycolipid anchors occurs with similar kinetics as in trypanosomes. We will discuss the implications of this finding for the understanding of the phenotype of mutant thymoma cells which fail to replace the C-terminal hydrophobic amino acids of Thy-1 by a glycolipid anchor [27,28].

MATERIALS AND METHODS

Cells

Cell lines BW5147 and S1A.TB.4.8.2 thymoma lines were obtained through Dr. R. Hyman, Department of Cancer Biology, the Salk Institute, San Diego, CA 92138, U.S.A., and were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal-calf serum, 1.5 mM glutamine and gentamycin (10 μ g/ml).

Labelling with [³⁵S]methionine

Exponentially growing cells were washed twice in Hanks balanced salt solution and once in methionine-free culture medium. They then were preincubated at 3×10^6 cells/ml at 37 °C for 30–45 min in labelling medium, which was Eagle's modified Dulbecco's medium supplemented with 3% (v/v) dialysed fetal-calf serum, leupeptin (30 μ g/ml; Fluka AG, Buchs, Switzerland), which had its methionine content decreased to 0.25%. Thereafter, portions of cells (3×10^7) were centrifuged at 250 *g* for 5 min and resuspended at $(1.5\text{--}3) \times 10^7$ cells/ml in prewarmed labelling medium supplemented with 1–2 mCi of [³⁵S]methionine (Amersham; 800 Ci/mmol)/ml. After incubation at 37 °C, cells

were removed by centrifugation at 5000 *g* for 10 s, tubes were immediately placed on ice, the labelling medium was removed and cells lysed in 1% TX-114 as described previously [27]. Spent labelling medium was briefly sonicated to destroy remaining cells and then used for a further round of labelling. In this way the same medium was used, for instance, to label sequentially for 1.5, 3, 5 min and after a 1:1 dilution with fresh medium, for a further 10 min labelling (Fig. 2 below). After lysis for 30–60 min on ice, soluble and membrane proteins were separated and re-extracted as described previously [27]. In some experiments (Fig. 4 below) the lysate was centrifuged at 10000 *g* for 5 min to remove aggregates formed during phase-separation procedures before the subsequent pre-clearing and immunoprecipitations.

PLC treatment of detergent phase

Detergent phases from labelled cells were re-extracted with 1 ml of 0.1 M-Tris/HCl (pH 7.4)/0.05 M-NaCl/0.25 M- α -methyl mannoside/1 mM-EDTA and proteinase inhibitors as described previously [27] and treated in the same buffer with PLC (*Bacillus cereus*, Type III, Sigma). In some cases, PLC was inactivated by boiling in 10 mM-Tris/150 mM-NaCl for 15 min before being added to cell lysates. The enzyme was still water-soluble after the heat treatment. The PLC preparation contains large amounts of phosphatidylcholine-specific PLC, but is contaminated with the PI-specific PLC that is present in the same organism [29]. EDTA is included to block the phosphatidylcholine-specific enzyme [29]. PLC treatments of detergent phases were for 60 min at 30 °C with addition of 5 and 2.5 units of PLC at 0 and 30 min (final concn. 20 units/ml). Then phases were separated, pre-cleared and immunoprecipitated.

Immunoprecipitations

The Thy-1.2 antigen of S1A was immunoprecipitated by using the monoclonal III-5 antibody [rat anti-(mouse Thy-1)] [30]. We found that this antibody does not immunoprecipitate the Thy-1.1 antigen of BW5147, although it reacts with it when tested by fluorescence flow cytometry. Therefore we used a polyclonal rabbit anti-(mouse Thy-1) antiserum [31] to immunoprecipitate the Thy-1 of BW5147. Immunoprecipitations were done overnight at 7 °C after two consecutive pre-clearings with non-immune rabbit serum plus protein A-Sepharose (Pharmacia) or bovine IgG coupled to Sepharose for 6 and 2 h at 7 °C. The second pre-clearings and immunoprecipitates were analysed by SDS/polyacrylamide-gel electrophoresis [32] in 11 or 12.5% (w/v) polyacrylamide gels; the gels were soaked in ENLIGHTNING or EN³HANCE (New England Nuclear) and exposed to Kodak X-Omat S films at –70 °C. Occasionally the bands corresponding to Thy-1 were cut from the gels, incubated at 75 °C for 2 h in 30% (v/v) H₂O₂ and counted for radioactivity by liquid-scintillation counting in Aquasol (New England Nuclear). When immunoprecipitating Thy-1 with polyclonal antibody from cells labelled with [³⁵S]methionine, we observed significantly more non-specific bands than with the previously used [³H]leucine labellings [27]. This might partly be explained by the fact that mature Thy-1 contains only one methionine, but 11 leucine, residues. We therefore ran the second pre-clearings along with the immunoprecipitates in most gels.

RESULTS

Kinetics of replacement of the C-terminal peptide of Thy-1 by glycolipid anchors in BW5147

PLC from *B. cereus* releases the detergent-binding moiety from mature Thy-1 molecules and causes them to partition into the aqueous phase during subsequent phase separation in a TX-114 solution [27]. This effect was exploited to monitor the presence of glycolipid anchors on Thy-1 molecules labelled for short periods with [³⁵S]methionine. In a preliminary experiment we labelled the BW5147 lymphoma line with [³⁵S]-methionine for short periods of time, lysed cells in TX-114, separated the phases and immunoprecipitated Thy-1. Thy-1 could already be detected after 1.5 min of labelling as a band with an apparent molecular mass of 24.5 kDa and was found mainly in the detergent phase, with only a small fraction partitioning to the aqueous phase. In addition, the antibody also immunoprecipitated a band of 21.5 kDa that might represent an underglycosylated form of Thy-1. Fig. 1 shows that newly synthesized Thy-1 of the detergent phase was released to a considerable extent into the aqueous phase by PLC (lanes 4 and 5), whereas after control incubations with no (not shown) or heat-inactivated PLC, all the Thy-1 was found in the detergent phase (lanes 2 and 3). This result indicates that glycolipid attachment can occur very rapidly after completion of biosynthesis. As a control, we used the cell line BW5147(Thy-1^{-a}), a Thy-1-deficient mutant cell line which accumulates Thy-1 intermediates which still contain at least parts of the original C-terminal peptide [27]. As expected, the Thy-1 intermediates from this line are not released into the aqueous phase by PLC (lanes 6 and 7). To rule out the possibility that the replacement of the C-terminal peptide by the glycolipid anchor took place after cell lysis and during the incubation with PLC, we added 3 × 10⁷ unlabelled BW5147 cells to labelled BW5147(Thy-1^{-a}) cells at the

Fig. 1. Release of Thy-1 from detergent phases by PLC

BW5147 cells (lanes 1–5) or BW5147(Thy-1^{-a}) cells (lanes 6–7) were labelled with [³⁵S]methionine for 1.5 min (lanes 1–5) or 3 min (lanes 6–7) and lysed in 1% TX-114. Phases were separated, the detergent phases were re-extracted once and then treated with PLC (+) or with heat-inactivated PLC (i). After the treatment, the samples were warmed to separate the aqueous (A) from the detergent (D) phase and Thy-1 was immunoprecipitated from both phases. The fluorograph shows the immunoprecipitates, except for lane 1, which is a second pre-clearing. Thy-1 appears as a distinct band at 24.5 kDa.

moment of cell lysis. Even under these conditions the mutant Thy-1 intermediate remained PLC-resistant (not shown). This makes it likely that the addition of glycolipid anchors to Thy-1 precursors observed in BW5147 cells occurs before cell lysis.

The percentage of Thy-1 that could be released by PLC increased when cells were labelled for longer periods, as shown in Figs. 2 and 3. Even after labelling for 10 min and a chase of 1 h, only 76% of the radioactivity in the bands corresponding to Thy-1 partitioned into the aqueous phase after PLC treatment (results not shown). Curves very similar to those shown in Fig. 3 were also obtained when the amounts of Thy-1 were determined by liquid-scintillation counting of radioactivity in the corresponding gel pieces. Murine Thy-1 only contains two methionine residues (positions 96 and 125); since the removal of the C-terminal peptide (positions 113–143) decreases its specific radioactivity by a factor of 2, the actual percentage of processed Thy-1 in Fig. 3 might be higher than indicated. The amount of Thy-1 that had already partitioned into the aqueous phase before PLC treatment remained relatively constant (< 5%), irrespective of the chase period. This minor fraction of Thy-1 might represent a short-lived biosynthetic intermediate, but the fact that the relative amount of this species does not decrease suggests that it is not an intermediate but rather is the result of an unsuccessful glycolipid-transfer reaction or that it is generated by an endogenous PLC. The 21.5 kDa band which we consider to be an underglycosylated form of Thy-1 is also released by PLC, as shown in Fig. 2, lane 4.

With the BW5147 line we did not observe the increase with time of the mobility of Thy-1 in SDS/polyacrylamide-gel electrophoresis, due to the removal of mannose residues, which was previously noted with thymocytes [33].

Most of the Thy-1 precursors get glycolipid anchors within 2 min after translation in S1A

Although in BW5147 we observed only incomplete release of Thy-1 molecules from the detergent phase by PLC after 1 h of chase (Fig. 3) and also after a 3 h labelling period [27], we incidentally found that, in the S1A thymoma line labelled for 3 h with [³⁵S]methionine, this release is near to complete. Therefore we performed an experiment similar to the one described in Fig. 1 with S1A. As shown in Fig. 4, most of Thy-1 labelled during a 2 min pulse is released from the detergent phase by PLC. By liquid-scintillation counting of bands cut from gels, we found that 80 and 88% of Thy-1 have glycolipid anchors after pulses of 2 and 11 min respectively. In S1A, the monoclonal anti-(Thy 1) antibody also precipitates a doublet of 21 and 22 kDa which might represent an underglycosylated form of Thy-1 lacking one N-linked glycan. When cells were labelled at 21 °C for 30 min, 89% of Thy-1 was released from the detergent phase by PLC (Fig. 4) and no Thy-1 was detectable in the aqueous phase before PLC treatment (result not shown).

Tunicamycin does not inhibit the addition of glycolipid anchors

An experiment analogous to the one illustrated in Fig. 2 was performed with cells preincubated and labelled in the presence of tunicamycin. As shown in Fig. 5, Thy-1 synthesized in the presence of the inhibitor partitioned

Fig. 2. PLC-sensitivity of Thy-1 in BW5147 increases with time

Using a protocol identical with the one described in Fig. 1, we determined the proportion of Thy-1 that is sensitive to PLC with increasing labelling periods (indicated at the bottom). (a) Thy-1 released into aqueous phase by PLC; (b) Thy-1 remaining in the detergent phase after PLC treatment; (c) Thy-1 already partitioning into the aqueous phase before PLC treatment. The arrow on the right indicates the position of Thy-1. Lane 1, second pre-clearings of the 10 min labelling; lanes 2–5, immunoprecipitates.

Fig. 3. PLC-sensitivity of Thy-1 increases with time

The fluorographs from Fig. 2 were analysed by densitometry. The 70 min point represents a 10 min-labelling, 60 min-chase-time point performed in the same experiment (not shown in Fig. 2). Densitometric tracings of Thy-1 bands appeared as symmetrical peaks and the areas below the peaks were calculated from manual measurements. The bases of peaks for Thy-1 in Figs. 2(b) and 2(c) were set at the level of the adjacent background in the area ranging from 22 to 28 kDa. The ordinate indicates the percentage of immunoprecipitated Thy-1 partitioning into the aqueous phase before PLC treatment (■) or after (▲) PLC treatment of the detergent phase.

Fig. 4. Attachment of glycolipid anchors to Thy-1 in the S1A line

S1A cells were pulse-labelled for 2 or 11 min at 37 °C (lanes 1–8) or 21 °C (lanes 9 and 10). Thereafter cells were processed as described in the legend to Fig. 1. Lanes 1 and 2, second preclearings; lanes 3–10, Thy-1 partitioning into detergent (D) or aqueous (A) phases after incubation of detergent phase with (+) or without (–) PLC.

Fig. 5. Glycolipid attachment of non-glycosylated Thy-1

BW5147 cells were preincubated in methionine-free medium and labelled with [³⁵S]methionine for 60 min in the presence of tunicamycin (10 µg/ml). They were then processed as described in the legend to Fig. 1. Lanes 1–4, Thy-1 immunoprecipitated from the aqueous (A) or detergent (D) phases after treatment with PLC (+) or a control incubation (–); lane 5, immunoprecipitate of the Thy-1 already partitioning into the aqueous phase before the PLC treatment.

Thy-1 labelled during a 60 min pulse was released by PLC. The Thy-1 molecule produced under these conditions has an apparent molecular mass of 13.5 kDa, a value that is in close agreement with the estimated molecular mass for Thy-1 from thymocytes and produced in the presence of tunicamycin or treated by endoglycosidase F [33]. Thus *N*-glycosylation is not required for the attachment of glycolipid anchors.

DISCUSSION

We chose the Thy-1 antigen in order to study the kinetics of addition of glycolipid anchors to surface glycoproteins in a mammalian system. This study was possible because Thy-1 is a major surface glycoprotein on thymocytes and T-lymphomas and because the presence of glycolipid anchors can be monitored by using the change of the partitioning of the molecule in TX-114 solutions upon PLC treatment. Although this method is indirect and hence subject to certain limitations, there are more direct approaches which have been employed in the case of the VSG of trypanosomes; it was shown convincingly that the cross-reacting determinant ('CRD'), an antigen carried by the glycolipid anchor of VSG, was present on virtually all VSGs synthesized during a 2 min pulse with [³⁵S]methionine [23,24]. However, a preliminary attempt to demonstrate the CRD determinant on Thy-1 was unsuccessful (C. Bordier and C. Bron, unpublished work). This might be because the glycolipid of Thy-1 does not contain galactose, since the CRD determinant was also absent in a galactose-dependent glycolipid anchor from trypanosomes [17,21]. Incorporation of [³H]myristate into VSG during a 2 min labelling period was also demonstrated [23]. However, VSG is a major cell protein accounting for ~ 7 % of total [³⁵S]methionine incorporation [23], whereas the corresponding value for Thy-1 in BW5147 is 0.003 %. It therefore appeared unlikely that the incorporation of radiolabelled fatty acids or other components of the glycolipid anchor into Thy-1 would be detectable within minutes. Nevertheless, we are confident that the PLC-dependent release of Thy-1 into the aqueous phase used in the present study is indicative of the presence of glycolipid anchors and is not caused by a contaminating proteinase for several reasons: (1) there are no signs of degradation of Thy-1 by the PLC treatment, since the recovery of Thy-1 is near to complete [27]; (2) the released Thy-1, which is immunoprecipitated, appears as a sharp band with the same mobility as the untreated species; (3) the Thy-1 from BW5147 (Thy-1^a) cells is not cleaved.

Our results indicate that the glycolipid anchor can be added very rapidly after completion of translation in mammalian cell lines. It remains, however, unclear whether *all* newly synthesized Thy-1 molecules are processed within seconds or minutes after the biosynthesis, as seems to be the case for the VSG of trypanosomes, or whether some molecules are processed at a later stage or escape this post-translational modification altogether, as might be concluded from the fact that, in BW5147, after a 1 h chase, there still remains a substantial amount of Thy-1 in the detergent phase. Since in the present study we did not titrate the amounts of PLC necessary to obtain maximal release of Thy-1, we cannot be sure that the molecules remaining in the detergent phase are PLC-resistant. Our previous study

into the detergent phase and was released to a considerable extent into the aqueous phase by PLC. Densitometric analysis of the autoradiograph showed that 50 % of

on thymocytes had shown that, even with very high enzyme concentrations, only about 70% of mature Thy-1 could be released by PLC [27]. To us it seems unlikely that the Thy-1 remaining in the detergent phase after PLC treatment still contains the original C-terminal peptide (113–143), because we previously did not find any [³H]tryptophan-labelled Thy-1 in BW5147 [27] (tryptophan only occurs at position 124 in the molecule). It seems more likely that this incomplete release is due to a structural heterogeneity of the glycolipid anchors, part of which might be resistant to PLC from *B. cereus*. Alternatively, it could be that Thy-1 is associated, even after cell lysis, with other detergent-binding membrane proteins or that a partial denaturation of Thy-1 occurring during lysis or during the treatment with PLC renders the molecule amphipathic, even after the removal of the glycolipid anchor.

The same type of events could be responsible for the increase of the percentage of Thy-1 that can be released by PLC with longer labelling periods observed in BW5147. It could well be that, in this line, all Thy-1 molecules receive glycolipid anchors within seconds or within a minute after translation, but that some of the transferred glycolipids are PLC-resistant, and that, during further maturation, some of these glycolipid moieties become PLC-sensitive.

Tunicamycin seems not to interfere with the glycolipid anchor attachment, suggesting that dolichol diphosphate *N*-acetylglucosamine is not involved in the biosynthesis of glycolipid tails. The same finding was reported for the biosynthesis of VSG [24]. In an attempt to accumulate biosynthetic precursors of Thy-1 containing the original peptide (positions 113–143), we performed a labelling at 21 °C. It seems, however, that the attachment of glycolipid anchors occurs normally at this temperature.

Recently it was proposed that paroxysmal nocturnal haemoglobinuria might be due to a deficiency in the pathway involved in the biosynthesis and attachment of glycolipid anchors, since several surface glycoproteins with this anchor (e.g. acetylcholinesterase, decay-accelerating factor) are not expressed by the red blood cells of paroxysmal-nocturnal-haemoglobinuria patients [5,34]. We recently analysed some thymoma mutants that might represent a model for such a deficiency. These mutants had been selected to be deficient in the surface expression of Thy-1 and had been shown to rapidly degrade this molecule intracellularly, but to express most other surface glycoproteins tested for at normal levels [35,36]. Mutants belonging to two different complementation classes (A and E) accumulated a form of Thy-1 which contained at least parts of the C-terminal hydrophobic peptide predicted by the DNA sequence and consequently did not attach glycolipid anchors [27]. One of these mutants (class E) had previously been found to be deficient in the dolichol-phosphate-mannose synthetase, a defect which resulted in the transfer of GlcNAc₂Man₅Glc₃ instead of GlcNAc₂Man₉Glc₃ oligosaccharides to nascent glycoproteins [37]. As possible reasons for the incomplete processing of Thy-1 by these two mutant classes we considered several hypotheses: (1) the mutants might fail to transport Thy-1 from the rough endoplasmic reticulum to the compartment where this post-translational modification normally takes place; (2) the mutants might be deficient in one of the enzymes involved in the biosynthesis and attachment of the glycolipid anchors or in enzymes which regulate their activity; (3) the mutants might fail to carry out some post-translational processing

of Thy-1 which affects its ability to be recognized as a substrate by the enzymes which attach glycolipid anchors.

The rapid addition of glycolipid tails within a few minutes to at least a majority of the Thy-1 molecules suggests that this processing occurs in the rough endoplasmic reticulum and seems to rule out the first explanation. Moreover, it suggests that post-translational modifications occurring outside the rough endoplasmic reticulum are not necessary to tag Thy-1 for glycolipid attachment. In addition, the tunicamycin experiment shows that non-glycosylated Thy-1 can also be recognized by the key enzymes of the glycolipid-attaching pathway, and this is evidence against the hypothesis that the Thy-1 precursors of the mutants of class E (deficient in dolichol-phosphate-mannose synthetase) fail to be properly processed because of their altered *N*-linked oligosaccharides. Thus the data obtained here are most compatible with the second hypothesis, namely that class-A and -E mutants lack enzyme activities that are involved in the biosynthesis and attachment of glycolipid anchors.

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