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No Glycolipid Anchors Are Added to Thy-1 Glycoprotein in Thy-1-Negative Mutant Thymoma Cells of Four Different Complementation Classes

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Recent evidence shows that the mature Thy-1 surface glycoprotein lacks the C-terminal amino acids 113 to 143 predicted from the cDNA sequence and is anchored in the plasma membrane by a complex, phosphatidylinositol-containing glycolipid attached to the α-carboxyl group of amino acid 112. Here we studied the biosynthesis of Thy-1 in two previously described and two newly isolated Thy-1-deficient mutant cell lines. Somatic cell hybridization indicated that their mutations affected some processing step rather than the Thy-1 structural gene. The Thy-1 made by mutants of classes C, F, and H bound detergent but, in contrast to wild-type Thy-1, their detergent-binding moieties could not be removed by phospholipase C. In addition, tryptophan, which only occurs in position 124, was incorporated into Thy-1 of these mutants but not of wild-type cells. Last, the Thy-1 of wild-type but not mutant cells could be radiolabeled with [^{3}H]palmitic acid. Together, these findings strongly suggest that mutants of classes C, F, and H accumulate a biosynthetic intermediate of Thy-1 which retains at least part of the hydrophobic C-terminal peptide. The Thy-1 of these mutants remained endoglycosidase H sensitive, suggesting that it accumulated in the rough endoplasmic reticulum or the Cis-Golgi. A different Thy-1 intermediate was found in a class B mutant cell line: the Thy-1 of this mutant was 2 kilodaltons smaller than the Thy-1 of other cell lines, did not bind detergent, and was rapidly secreted via a normal secretory pathway.

A number of protozoan and mammalian surface glycoproteins are anchored in the lipid bilayer by a phosphatidylinositol-containing glycolipid (reviewed in references 7 and 18). Best studied are the glycolipid anchors of the variable surface glycoproteins (VSG) of trypanosomes (11–13) and of the mammalian Thy-1 antigen (19, 27) which both contain phosphatidylinositol, ethanolamine, mannose, and glucosamine. The mature forms of VSG and Thy-1 are linked through the α-carboxyl group of their C-terminal amino acid to the glycolipid anchor (13, 27) but they lack a C-terminal stretch of hydrophobic amino acids which is predicted from the cDNA sequences (6, 23, 24, 27). It has therefore been proposed that this C-terminal sequence is present on the nascent VSG and Thy-1 polypeptides but is subsequently removed by a specific endopeptidase and replaced by the glycolipid, a process termed glypiation (7).

One of us isolated mutants of T-cell lymphomas that did not express Thy-1 glycoprotein on the cell surface (15, 17, 25, 26). The Thy-1$^-$ mutants originally described fell into five complementation classes when analyzed by somatic cell hybridization. Four of them were recessive and classified as processing mutants, as they made normal amounts of intracellular Thy-1 precursors but turned these over much more rapidly than wild-type cells (26). The processing mutants expressed normal levels of all other surface glycoproteins examined except the Ly-6 antigen (14, 26).

MATERIALS AND METHODS

Cell lines. The class F Thy-1-deficient mutant EL4$^-$f was derived from EL4.G1 by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (0.3 μg/ml) for 30 min and immunoselection with monoclonal anti-Thy-1.2 antibody 30-H-12 and rabbit complement, as described (15). The class H Thy-1$^-$ mutant S49$^-$h was derived from S49.1 by treatment with mitomycin C (0.25 μg/ml) for 26 h and immunoselection with the monoclonal anti-Thy-1 antibody C22/22.7.1.1 and complement as described (16). Quantitative cytotoxic absorption analysis (15) indicated that EL4$^-$f and S49$^-$h expressed <0.5% and <1% of the wild-type level of Thy-1.2 on their cell surfaces, respectively. Somatic cell hybridization and serological analysis of hybrids were carried out as described (15). Other Thy-1$^-$ mutants (classes B and C) have been described (17). Cells were grown in RPMI 1640 medium with 10% heat-inactivated horse serum.

Analytical procedures. For biosynthetic labeling, exponentially growing cells were grown at 2.5 × 10^6 per ml in medium with the methionine content reduced to 0.25% and with 5% dialyzed fetal calf serum, leupeptin (30 μg/ml), and 30 to 60 μCi of [^{35}S]methionine (800 Ci/mmol) per ml or in tryptophan-deficient medium supplemented with the same additions plus 15 mM glucose and 80 μCi of [^{3}H]tryptophan (60 Ci/mmol) per ml or in medium with gentamicin (20 μg/ml), 0.5% serum, and 65 μCi of [^{3}H]palmitic acid (55 Ci/mmol) per ml. Labeled cells were lysed in 1% Triton X-114 (TX-114) and separated into soluble and membrane proteins, and the phases were reextracted further as described (2, 5). Detergent phases of labeled cells were treated with phospholipase C (PLC) (*Bacillus cereus*, type III; Sigma Chemical Co.) at 25 U/ml as described (5). The reasons for the incomplete release of Thy-1 into the aqueous phase by PLC in some cell lines as well as the evidence that this release was not due to proteases contaminating the PLC preparation were discussed recently (4). Immunoprecipitation procedures and sodium dodecyl sulfate-polyacrylamide

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gel electrophoresis (SDS-PAGE) were performed as described (5). Before immunoprecipitation of secreted Thy-1, the labeling medium was ultracentrifuged at 100,000 × *g* for 90 min to remove cell debris. Quantitation of radioactivity in bands corresponding to Thy-1 was done as described (4). Treatment of immunoprecipitates with endo-β-*N*-acetylglucosaminidase H (endo H) from *Streptomyces plicatus* (New England Nuclear Corp.) were performed essentially as described (21).

RESULTS

Derivation of Thy-1⁻ mutant classes F and H. Thy-1⁻ mutant cell lines isolated from the wild-type T-cell lymphomas EL4.G1 and S49.1 were typed by somatic cell hybridization with mutants of all known Thy-1⁻ complementation classes (A to E) and with each other. This analysis defined two new complementation classes of Thy-1⁻ mutants (classes F and H). Both class F and class H mutants expressed the mutant (Thy-1.2) allele when hybridized with Thy-1.1-expressing wild-type cell lines. The recessive behavior of these mutants was identical to that of those mutant classes described previously and is consistent with the interpretation that these mutations define processing defects. The levels of T200, Lyt-2, Pgp-1, and LFA-1 surface glycoproteins and of transferrin receptor were similar in the wild-type and mutant cell lines.

Phospholipase C does not remove the detergent-binding moiety of Thy-1 in mutants of classes C, F, and H. Biosynthetic labeling of cells with [^{35}S]methionine showed that Thy-1⁻ mutants TIMI⁻c, EL4⁻f, and S49⁻h made intracellular forms of Thy-1 which, on the basis of their mobilities in SDS-PAGE, seemed to be about 1,000 daltons (Da) smaller than the corresponding wild-type forms (Fig. 1). (Mutant Thy-1 appears as a doublet because it comigrates with the light chain from the monoclonal anti-Thy-1 antibody used for immunoprecipitation.) During separation of membrane proteins from soluble proteins in TX-114 (2), the Thy-1 of wild-type cells partitioned completely into the detergent phase. In the mutants, however, some Thy-1 (less than 5% of the total) was immunoprecipitated in the aqueous phase. When the TX-114 detergent phases were incubated with PLC prior to subsequent phase separation and immunoprecipitation, the Thy-1 from all wild-type cells was released to a large extent into the aqueous phase, whereas the Thy-1 of all the mutant cells stayed in the detergent phase (Fig. 1, Table 1). This suggests that the detergent-binding domain of mutant cells is different from that of wild-type cells. Immunoprecipitation of Thy-1 from the labeling medium showed that the mutant lines T1M1⁻c, EL4⁻f, and S49⁻h secreted small amounts of Thy-1 (<5%) of 23, 25, and 25 kDa, respectively. A quantitative evaluation of the data is given in Table 1.

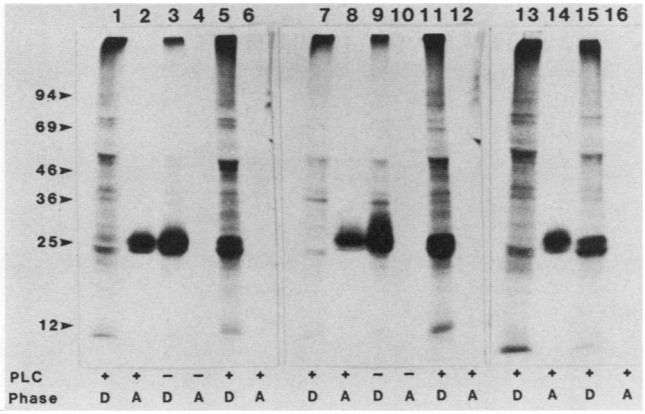

FIG. 1. PLC treatment of Thy-1 in mutants of classes C, F, and H. Cells (3 × 10^7) of each line were labeled for 2.5 h with [^{35}S]methionine and lysed in TX-114. The detergent phases were incubated with (+) or without (−) PLC and separated again. The Thy-1 was immunoprecipitated first with monoclonal and then with polyclonal antibody in both the detergent (D) and the aqueous (A) phases, and the immunoprecipitates from the two antibodies were combined and analyzed by SDS-PAGE. Wild-type cell lines are in the following lanes: T1M1 (1 to 4), EL4 (7 to 10), S49 (13 and 14); Thy-1-deficient mutants are T1M1⁻c (lanes 5 and 6), EL4⁻f (lanes 11 and 12), S49⁻h (lanes 15 and 16). Molecular mass standards (in kilodaltons) are indicated by arrows. Exposure: 8 days.

TABLE 1. Relative amounts of various Thy-1 forms[a]

Cell line	Relative ^{35}S incorporation into Thy-1[b]	Thy-1 secreted into medium (%)[c]	Thy-1 released from detergent phase by PLC[d] (%)
T1M1	5.7 × 10⁻⁴	1.6	70 (0)
T1M1⁻c	3.1 × 10⁻⁴	5	6
EL4	10.1 × 10⁻⁴	1.7	91 (4)
EL4⁻f	4.8 × 10⁻⁴	2.5	8
S49	5.5 × 10⁻⁴	1.9	71
S49⁻h	3.9 × 10⁻⁴	2.5	9
S1A	15.9 × 10⁻⁴	0.8	93 (0)
S1A⁻b	8.4 × 10⁻⁴	30	8

[a] Incorporation of [^{35}S]methionine into Thy-1 was determined in the experiments shown in Fig. 1 and 3A.
[b] Ratio of the sum of counts in Thy-1 immunoprecipitated from the medium and aqueous and detergent phases over total counts in the reextracted detergent phase.
[c] Thy-1 immunoprecipitated from medium over sum of Thy-1 in all fractions, multiplied by 100.
[d] Ratio of Thy-1 released from detergent phase by PLC over sum of the same plus the Thy-1 remaining in the detergent phase, multiplied by 100. Numbers in parentheses indicate the percentage of Thy-1 that became water soluble during a control incubation in the absence of PLC.

Incorporation of tryptophan and palmitic acid into Thy-1 of mutant classes C, F, and H. Tryptophan is found in Thy-1 at position 124 only and is therefore expected to be absent from mature Thy-1 molecules. When labeled with [^{3}H]tryptophan followed by immunoprecipitation, mutants of classes C, F, and H but not the corresponding wild-type cells contained a radiolabeled band of 25 kDa, corresponding to Thy-1 (Fig. 2A). The other bands on the gel were precipitated nonspecifically, since they were also present in the preclearing (not shown). The total incorporation of [^{3}H]tryptophan into the mutant cells was between 35 and 62% of incorporation into the wild type. Moreover, no radiolabeled Thy-1 was immunoprecipitated in the aqueous phase (not shown). These results suggest that mutants of classes C, F, and H accumulate Thy-1 molecules which still contain at least parts of the C-terminal peptide (amino acids 113 to 143) predicted by the cDNA sequence.

To confirm the absence of glycolipid anchors from the Thy-1 of mutant classes C, F, and H, we labeled cells with [^{3}H]palmitic acid. As shown in Fig. 2B, only the wild-type forms of Thy-1 became labeled. This finding cannot be explained merely by more rapid turnover of Thy-1 in the mutants, since the labeling period was short (2.5 h) and because the incorporation of [^{35}S]methionine into Thy-1 during a 3-h labeling period was comparable in wild-type and mutant cells (Table 1).

Phenotype of the S1A⁻b mutant. Unlike the mutants of classes C, F, and H, the S1A⁻b mutant made a Thy-1

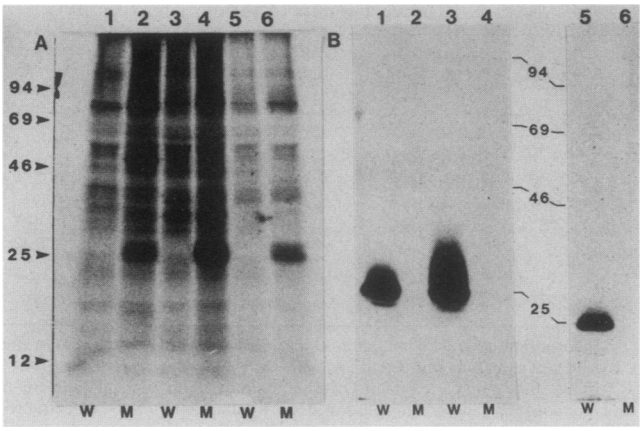

FIG. 2. Tryptophan and palmitic acid incorporation into Thy-1. (A) Cells (2×10^7) of wild-type (W) or mutant (M) lines were labeled with [^{3}H]tryptophan for 3 h. (B) Cells (5×10^7) of each line were labeled with 1.2 mCi of [^{3}H]palmitic acid for 2.5 h. After lysis in TX-114, the phases were separated and Thy-1 was immunoprecipitated consecutively by polyclonal and monoclonal antibody. The two consecutive immunoprecipitates from the detergent phase were pooled and run in SDS-PAGE. The fluorographs contain T1M1 (lanes 1), T1M1$^-$c (lanes 2), EL4 (lanes 3), EL4$^-$f (lanes 4), S49 (lanes 5), and S49$^-$h (lanes 6).

molecule which partitioned to a large extent into the aqueous phase of the TX-114 solution and had an apparent molecular mass of 21 to 23 kDa, i.e., ~3 kDa smaller than wild-type Thy-1 (23 to 27 kDa) and ~2 kDa smaller than the Thy-1 from the other Thy-1$^-$ mutants (Fig. 3A). In addition, the mutant secreted a significant fraction of Thy-1 of slightly higher molecular weight into the culture medium in a form which could not be sedimented by ultracentrifugation (100,000 × *g*, 1.5 h) (Table 1, Fig. 3B).

Two of the three N-linked glycans of Thy-1 become complex-type during normal maturation (20). Complex oligosaccharides are resistant to endo H, and during biosynthesis this resistance is acquired as glycoproteins pass through the mid-Golgi region (8). As expected, endo H treatment of Thy-1 from wild-type cells labeled for 3 h with [^{35}S]methionine showed that most of the Thy-1 ran as a 21.5- to 23-kDa band, indicating that only one of the three glycans could be removed and that most of Thy-1 had reached or passed the Golgi apparatus (Fig. 3B, lane 13). A minor fraction (~17%) ran at 14.5 kDa, which is the molecular mass of completely deglycosylated Thy-1 (20). This lower band probably represents intracellular Thy-1 which has not yet reached the mid-Golgi region. In S1A$^-$b cells, however, the majority of cell-associated Thy-1 (contained in the aqueous phase) was reduced by endo H from 22 to 12.5 kDa (Fig. 3B, lanes 14 and 15). This indicated that in S1A$^-$b, most of the cell-associated Thy-1 contained only immature high-mannose oligosaccharides. The completely deglycosylated Thy-1 from S1A and S1A$^-$b cells differed in molecular mass by about 2 kDa (Fig. 3B, lanes 13 and 14). Thy-1 was secreted into the medium only by S1A$^-$b (Fig. 3B, lanes 10, 11, 16, and 17). This secreted material contained two endo H-resistant oligosaccharides (Fig. 3B, lane 16).

For comparison, the cell-associated Thy-1 of T1M1$^-$c and EL4$^-$f was also probed with endo H. All their deglycosylated Thy-1 ran at 14.5 kDa, indicating intracellular accumulation of Thy-1 (Fig. 3B, lanes 19 and 20). The fact that only wild-type Thy-1 contained complex oligosaccharides explains why the glycosylated Thy-1 forms of T1M1$^-$c and EL4$^-$f were slightly smaller than the wild-type molecules (Fig. 1). Since the deglycosylated forms of mutant and wild-type Thy-1 ran at 14.5 kDa, it appears that the aberrant membrane anchor of mutants of classes C and F does not change the mobility of Thy-1 to a significant extent.

DISCUSSION

Four Thy-1$^-$ mutants failed to add normal glycolipid anchors to Thy-1. Three of these mutants (classes C, F, and H) had the same phenotype as two other Thy-1$^-$ mutants

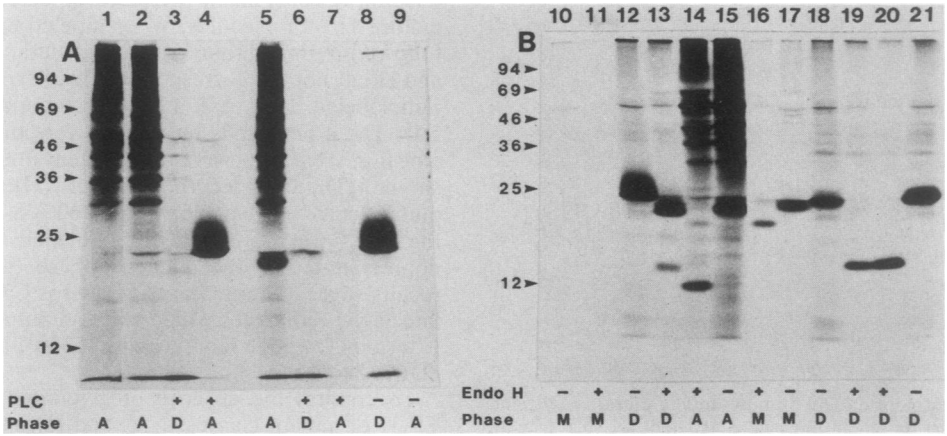

FIG. 3. Phenotype of Thy-1 of S1A$^-$b. (A) S1A and S1A$^-$b cells were labeled with [^{35}S]methionine for 3 h and lysed in TX-114, and the phases were separated. Thy-1 immunoprecipitated from the aqueous phases (A) after this first separation is shown in lanes 2 (S1A) and 5 (S1A$^-$b). The preclearing of the aqueous phase of S1A is shown in lane 1. The detergent phases were incubated with (+) or without (−) PLC. Then, the detergent (D) and aqueous (A) phases were separated again and Thy-1 was immunoprecipitated: S1A is in lanes 1 to 4, 8, and 9; S1A$^-$b is in lanes 5 to 7. A further immunoprecipitation with polyclonal antibody did not bring down any more Thy-1. (B) Cells (3×10^7) were labeled and lysed as for panel A. After separation of the aqueous (A) and detergent (D) phases, Thy-1 was immunoprecipitated with monoclonal antibody. Thy-1 was also immunoprecipitated from the labeling medium (M) of S1A and S1A$^-$b. The immunoprecipitates were then split into two (uneven) portions, one of which was treated with endo H (+). No additional Thy-1 was precipitated by further immunoprecipitation with polyclonal anti-Thy-1. The lanes contained S1A (10 to 13), S1A$^-$b (14 to 17), TIMI$^-$c (18 and 19), or EL4$^-$f (20 and 21).

(classes A and E) reported earlier (5, 9, 10). All five mutant classes (A, C, E, F, and H) synthesize Thy-1 precursors which have PLC-resistant detergent-binding domains and accumulate tryptophan-containing Thy-1 intermediates which can be assumed to contain parts of the C-terminal hydrophobic peptide. These abnormalities strongly suggest that mutants A, C, E, F, and H fail to replace a C-terminal hydrophobic peptide predicted from the DNA sequence (24). However, we cannot exclude the alternative possibility, that they replace it by a PLC-resistant glycolipid which is not labeled by [^{3}H]palmitic acid and that this replacement is slow so that they accumulate an intermediate containing the C-terminal hydrophobic peptide.

The defect in the class B mutant differed from that in all other classes, since S1A⁻b made Thy-1 which partitioned to a large extent into the aqueous phase of TX-114 solutions and was rapidly secreted. It is conceivable that in this cell line the hydrophobic C-terminal peptide of Thy-1 gets cleaved by the endopeptidase but that no glycolipid anchor is attached. If so, Thy-1 would be expected to be smaller than in the wild type, and indeed, the Thy-1 of S1A⁻b cells was smaller in both its glycosylated and its deglycosylated form (Fig. 3).

It was previously demonstrated that in Thy-1⁻ mutants (classes A, B, C, and E) the half-life of Thy-1 was reduced 4- to 15-fold, but this could not account for the almost complete absence of Thy-1 at the cell surface (26). We found that the intracellular Thy-1 of T1M1⁻c and EL4⁻f was completely endo H sensitive after a 3-h pulse, whereas in normal cells Thy-1 was mostly endo H resistant after 45 min (20) (Fig. 3B, lanes 12 and 13). This supports the notion that Thy-1 accumulates proximal to the mid-Golgi in these mutants (26) and raises the possibility that the persistence of the C-terminal hydrophobic peptide interferes with the normal exit from the rough endoplasmic reticulum or Cis-Golgi, whereas anchorless Thy-1 molecules made by S1A⁻b do seem to be secreted along a normal pathway.

The biochemical defects in all but class E (3) Thy-1⁻ mutants are unknown. First, the mutations might affect a special transport mechanism required for Thy-1 to reach the compartment where glypiation occurs. This appears unlikely, since most Thy-1 precursors are PLC sensitive 2 min after translation, suggesting that glypiation occurs in the rough endoplasmic reticulum (4). Second, the mutations might affect some posttranslational modification of Thy-1 which has to be recognized by the enzymes which attach glycolipid anchors. Again, this is rendered unlikely by the rapid kinetics of glycolipid attachment and the fact that unglycosylated Thy-1 precursors get glypiated (4). To us, the most likely explanation would be that the mutations affect the enzymatic machinery involved in the biosynthesis and attachment of glycolipid anchors. This hypothesis might seem difficult to reconcile with the existence of five mutation classes which fail to cleave the C-terminal hydrophobic peptide, since the removal of this peptide should be carried out by a single enzyme. The hypothesis can however be maintained by assuming, e.g., an allosteric effect of a preformed glycolipid anchor on the glypiation endopeptidase. Thus, defects in the biosynthesis of the glycolipid anchor precursor might lead to a failure to remove the C-terminal peptide. It is conceivable that the hypothetical endopeptidase exchanges the peptide for a preformed glycolipid anchor in a single step, as already proposed by others to explain the extremely rapid attachment of glycolipid anchors to VSG (1).

Preliminary evidence indicates that there is a general deficiency in glycolipid attachment to membrane proteins in several Thy-1⁻ mutant classes. This is also supported by the fact that Ly-6, an antigen which was found to be lost in Thy-1⁻ mutants, is also anchored via a PLC-sensitive anchor (14, 22).

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