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Anchoring of membrane proteins via phosphatidylinositol is deficient in two classes of Thy-1 negative mutant lymphoma cells

Andreas Conzelmann, Annalisa Spiazzi[1], Robert Hyman[2] and Claude Bron

Institute of Biochemistry, University of Lausanne, 1066 Epalinges, Switzerland, and [2]Department of Cancer Biology, The Salk Institute for Biological Studies, San Diego, CA 92138, USA

[1]Present address: Istituto di Scienze Immunologiche, Università di Verona, Verona, Italy

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Recent evidence shows that mature Thy-1 glycoprotein lacks amino acids 113-143 predicted from the cDNA sequence and is anchored to the plasma membrane by a phosphatidylinositol-containing glycolipid attached to amino acid 112. Previously characterized Thy-1-deficient mutant lymphoma lines of complementation classes A and E were analysed. They make detergent binding Thy-1 precursors but, in contrast to wild-type, the detergent binding moiety cannot be removed by phospholipase C. Moreover, tryptophan which only occurs at position 124 is incorporated into mutant but not parental Thy-1. This suggests that the mutants make a Thy-1 precursor of 143 amino acids but fail to replace its C-terminal end by a glycolipid anchor.

Key words: Thy-1/glycoprotein/plasma membrane attachment

Introduction

The Thy-1 glycoproteins are major cell surface constituents of rodent thymocytes and neurons (Reif and Allen, 1964; Williams and Gagnon, 1982). Thy-1 has both structural and sequence homologies with immunoglobulins which has led to the suggestion that Thy-1 may be derived from a primitive cell recognition molecule and may itself mediate cell-cell interactions (Williams and Gagnon, 1982). Antibodies against Thy-1 can be mitogenic to mouse T lymphocytes (Maino *et al.*, 1981; Gunter *et al.*, 1984; MacDonald *et al.*, 1985).

According to a recent analysis by Tse *et al.* (1985), the mature Thy-1 molecule is anchored in the lipid bilayer via a glycolipid which contains ethanolamine, glucosamine, galactosamine, mannose, myoinositol, phosphate, fatty acids and glycerol. This glycolipid is attached to the carboxyl group of cysteine 112 which in turn is linked by a disulfide bond to cysteine 9. The cDNA sequence of Thy-1 predicts a protein of 143 amino acids, but residues 113 to 143 could not be found in the mature Thy-1 from rat thymus and brain (Tse *et al.*, 1985). Multiple genes, tissue/species differences or post-translational mRNA processing do not appear to be responsible for this discrepancy (Seki *et al.*, 1985a, b). The molecule can be released from whole cells by phosphatidylinositol-specific phospholipase C (PLC) of *Staphylococcus aureus* (Low and Kincade, 1985). Thy-1 seems therefore to belong to the family of proteins comprising alkaline phosphatase, the membrane form of acetylcholinesterase, 5'-nucleotidase, the variant surface glycoproteins (VSGs) of trypanosomes and probably several more glycoproteins which can be released from plasma membranes by phosphatidylinositol-specific PLC (reviewed by Low *et al.*, 1986). The best characterized species of this group are the VSGs. They contain ethanolamine, glucosamine, mannose and phosphatidylinositol in their tail (Holder, 1985). Like Thy-1, mature VSGs lack a C-terminal hydrophobic peptide extension of 17-23 amino acids predicted from cDNA sequences (Boothroyd *et al.*, 1980, 1981; Cross, 1984). During biosynthesis, VSG becomes linked to the glycolipid tail within one minute after its translation suggesting that this modification occurs in the rough endoplasmic reticulum (RER) (Bangs *et al.*, 1985; Ferguson *et al.*, 1986).

The lymphoma lines analysed in this study were produced by selection with anti-Thy-1 antibodies plus complement (Hyman, 1973; Hyman and Stallings, 1974; Hyman and Trowbridge, 1978; Hyman *et al.*, 1980). Mutants were put into eight complementation classes (Hyman and Trowbridge, 1978; Hyman, 1985) but only in one class, D, was the Thy-1 structural gene affected (Evans *et al.*, 1984). They were found to express Thy-1 at $<1\%$ (most $<0.2\%$) of the amount found on wild-type cells whereas the expression of other surface glycoproteins was normal (Trowbridge *et al.*, 1978a, b). Biochemical analysis of the mutants in complementation classes A and E showed that they make normal amounts of Thy-1 but turn it over at a 5- to 10-fold faster rate than wild-type cells; this, however, cannot explain the almost complete lack of Thy-1 at the surface, and it seems therefore that the mutant Thy-1 molecules are blocked in their transport to the cell surface. The Thy-1$^-$e mutants have been characterized thoroughly and found to be deficient in dolichol-P-mannose synthetase (Chapman *et al.*, 1980). Dolichol-P-mannose is required for the addition of four of the nine mannose residues of the dolichol-PP-$GlcNAc_2$-Man_9Glc_3 high mannose oligosaccharide precursor. The class E mutant cells transfer $GlcNAc_2Man_5Glc_3$ high mannose oligosaccharides to protein and this species can be processed to form typical complex oligosaccharides. Thy-1 contains three N-linked oligosaccharides two of which become complex during normal maturation (Luescher and Bron, 1985; Carlsson, 1985). The original investigations led to the suggestion that the Thy-1 glycoprotein was not properly folded when glycosylated with $GlcNAc_2Man_5Glc_3$ oligosaccharides and might become susceptible to proteolytic degradation soon after its biosynthesis (Chapman *et al.*, 1980). The discovery of mannose in the glycolipid tail (Tse *et al.*, 1985) raised the possibility that the class E mutation affected the biosynthesis of glycolipid tails in addition to the biosynthesis of N-linked high-mannose oligosaccharides. This study was undertaken to investigate this possibility.

Results

The detergent-binding domain of solubilized mature Thy-1 can be released by phospholipase C

Partitioning of proteins into the detergent phase of Triton X-114 (TX114) solutions can be used as an indicator of the proteins capacity to bind detergent (Bordier, 1981) and was used to measure the release of the detergent-binding domain of a Leishmania surface glycoprotein by PLC (Etges *et al.*, 1986).

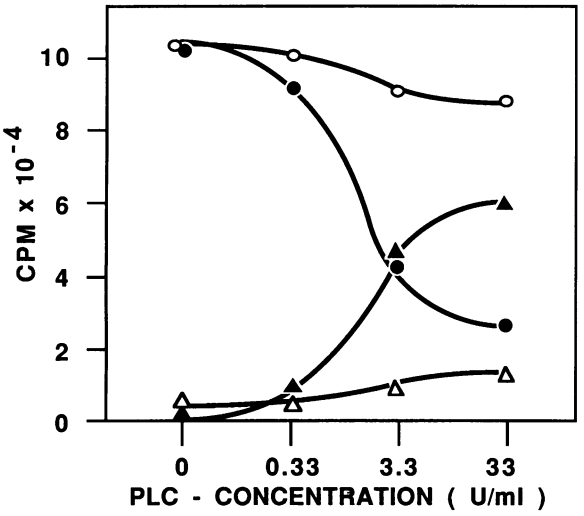

Fig. 1. Membrane proteins from surface radioiodinated thymocytes were cleared of soluble proteins through six phase separations; aliquots corresponding to 10^7 cells and 2.8×10^6 c.p.m. were incubated with variable amounts of PLC for 150 min at 13°C. Phases were separated, re-extracted and Thy-1 was immunoprecipitated in both phases. The aqueous phase contained between 1% (no PLC) and 4.8% (33 U/ml of PLC) of total counts. Of counts in the aqueous phases after PLC treatment with 33 or 3.3 U/ml 46% were precipitated as Thy-1. Immunoprecipitates contained only Thy-1 when analysed in SDS−PAGE. ●, Thy-1 in detergent phase; ▲, Thy-1 in aqueous phase; ○, sum of Thy-1 in both phases; △ trichloroacetic acid-precipitable surface proteins remaining in the aqueous phase after immunoprecipitation of Thy-1.

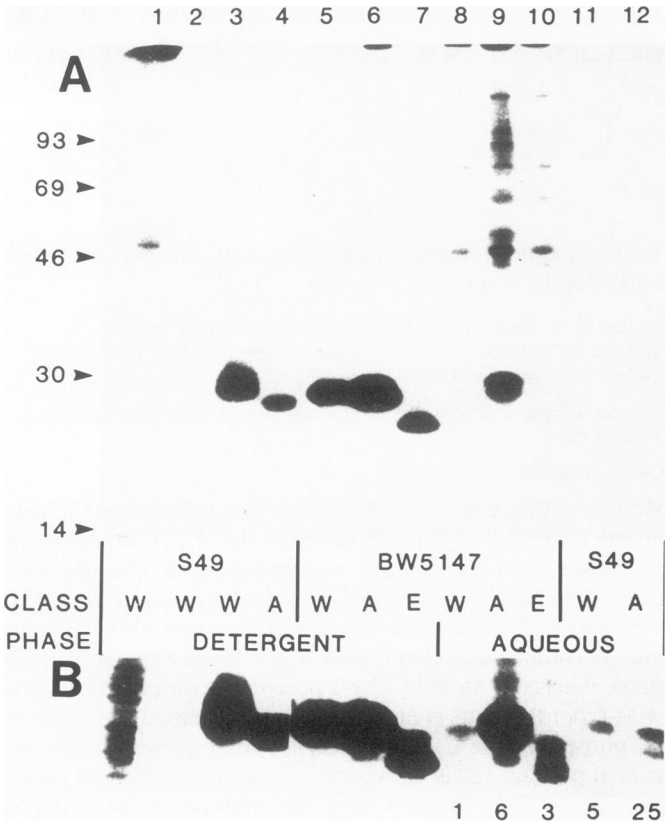

Fig. 2. Wild-type (W) or mutant cells of classes A and E (A,E) were labeled with [^{3}H]leucine for 3 h and extracted with TX114. Detergent and aqueous phases were re-extracted once before immunoprecipitation with polyclonal anti-Thy-1 antibody. **Lanes 1** and **2** represent first and second preclearings, **lanes 3−12** are immunoprecipitates. **Lanes 1,2,3** and **11**, S49; **lanes 4** and **12**, S49 (Thy-1$^-$a); **lanes 5** and **8**, BW5147; **lanes 6** and **9**, BW5147(Thy-1$^-$a); **lanes 7** and **10**, BW5147(Thy-1$^-$e).236. **A**, 5 days exposure, **B**, 36 days exposure. The numbers below panel B refer to the percentage of Thy-1 found in the aqueous phase determined by liquid scintillation counting of Thy-1 bands cut out from the gel. Mol. wts of standards are indicated as kilodaltons.

Thymocytes were first surface-labeled by the lactoperoxidase method and then extracted with TX114. During the subsequent phase separations 98% of Thy-1 partitioned into the detergent phase. When the detergent phase was subsequently treated with PLC, the cleaved Thy-1 molecules partitioned into the aqueous phase during a subsequent phase separation, whereas the bulk of membrane proteins stayed in the detergent phase (Figure 1). When analysed by SDS−PAGE the release of the detergent-binding moiety by PLC treatment did not change the mobility of Thy-1, however, a minor difference might have escaped detection because of the heterogeneity of Thy-1. PLC treatment at 13 and 30°C gave the same result.

Although pure phosphatidylinositol-specific PLC was not used, it is almost certainly this enzyme which cleaves off the detergent-binding domain. Firstly, the phosphatidylcholine-specific PLC is completely blocked in the presence of EDTA (Ikezawa *et al.*, 1976). Secondly, proteases most likely are not involved since PLC treatments were done in the presence of protease inhibitors and also because the analysis of Thy-1 by SDS−PAGE after PLC treatment did not indicate any signs of degradation. Even though PLC treatment caused an apparent 12% loss of total immunoprecipitable Thy-1 (Figure 1), this loss can be largely accounted for by the loss of released Thy-1 during the phase separation and re-extractions after PLC treatment (see Materials and methods). We conclude that a large proportion of mature Thy-1 can be shifted from an amphipathic to a water-soluble state by PLC.

Most of Thy-1 in Thy-1-deficient mutants is amphipathic

By analogy with the VSGs of trypanosomes it has been proposed that Thy-1 is made as a precursor of 143 amino acids which subsequently gets cleaved at position 112 to allow the attachment of the phospholipid tail (Low and Kincade, 1985). The precursor of 143 amino acids could potentially behave as an integral membrane protein since it contains a stretch of hydrophobic amino acids between positions 115 and 138. However, molecules without detergent-binding domains might be expected if a mutant cell line were competent to cleave the peptide chain but were unable to add the glycolipid tail. After a 3-h labelling with [^{3}H]leucine, the bulk of Thy-1 molecules was found in the detergent phase in both parental and Thy-1$^-$ mutant cells (Figure 2). This shows that the Thy-1 molecules of parental and mutant cells are mostly amphipathic although a small percentage of Thy-1 was found in the aqueous phase (Figure 2B, bottom figures). The water-soluble Thy-1 molecules had the same mobility in SDS−PAGE as the detergent-binding ones. The BW5147 (Thy-1$^-$a) line produced a significant amount of water-soluble Thy-1 in all experiments. The incorporation of label into Thy-1 varied from experiment to experiment and was always highest in BW5147(Thy-1$^-$a) but the total incorporation into membrane proteins was quite comparable in all lines. Similar findings have been previously reported (Trowbridge and Hyman, 1978a). In this 3-h labelling we found trace amounts of Thy-1 to be secreted into the culture medium only by the class E mutant. The higher mobility of the Thy-1 molecule of the class E mutant results from the presence of truncated glycans as previously described (Trowbridge *et al.*, 1978a).

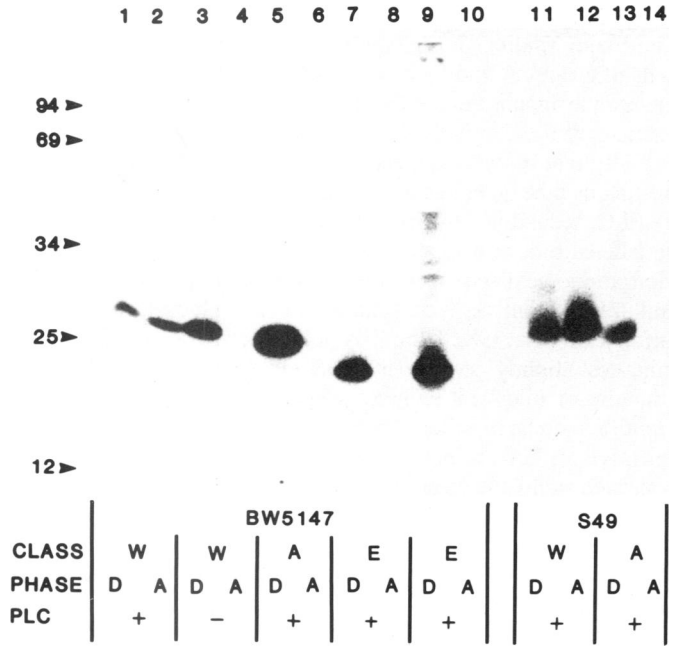

Fig. 3. Wild-type (W) or mutant (A,E) cells were labeled with [^{3}H]leucine for 3 h and lysed in TX114. The detergent phase was re-extracted three times and then incubated with (+) or without (−) PLC for 60 min at 30°C. Phases were separated, re-extracted, precleared and Thy-1 was immunoprecipitated from the detergent (D) or the aqueous (A) phase by polyclonal antibody. A second immunoprecipitation with monoclonal anti-Thy-1 yielded an average of 16% of additional material. First and second immunoprecipitates were pooled before loading onto a gel. This experiment is reported as Exp 2 in Table I. **Lanes 1–4**, BW5147; **lanes 5** and **6**, BW5147(Thy-1$^-$a); **lanes 7** and **8**, BW5147(Thy-1$^-$e).10; **lanes 9** and **10**, BW5147(Thy-1$^-$e).236; **lanes 11** and **12**, S49; **lanes 13** and **14**, S49(Thy-1$^-$a).

Table I. Release of the detergent binding domain of Thy-1 by phospholipase C

Cell line	PLC	Percent of Thy-1 released by PLC	
		Exp 1	Exp 2
BW5147(Thy-1$^+$)	+	53	61
BW5147(Thy-1$^+$)	−	14	4
BW5147(Thy-1$^-$a)	+	2.5	4
BW5147(Thy-1$^-$e).10	+	5	2
BW5147(Thy-1$^-$e).236	+	5	7
S49(Thy-1$^+$)	+	54	73
S49(Thy-1$^-$a)	+	11	6

Figures were obtained from experiments of the type described in legend to Figure 3. In experiments 1 and 2 the membrane proteins of 2×10^7 and 2.4×10^7 cells were incubated for 60 min (Exp 1) or 90 min (Exp 2) at 30°C in the presence of 1.5 or 2.3 units of PLC respectively. For Exp 1, a 2 μl aliquot of the immunoprecipitate was counted; for Exp 2, the region of the gel corresponding to Thy-1 according to the fluorograph (see Figure 3) was cut out and counted by liquid scintillation counting after dissolution in 30% H_2O_2 for 2 h at 80°C.

Thy-1 from mutant cell lines cannot be cleaved by phospholipase C

Treatment of integral membrane proteins by PLC followed by phase separation showed that parental Thy-1 could be released to a significant extent into the aqueous phase whereas the Thy-1 from both mutant classes, A and E, remained with the detergent phase (Figure 3, Table I). The release of the detergent-binding site was dependent on the addition of PLC in BW5147, but the spontaneous release was higher than the spontaneous release of Thy-1 from surface-labeled thymocytes (Figure 1). This discrepancy might result from the presence of endogenous PLCs in BW5147.

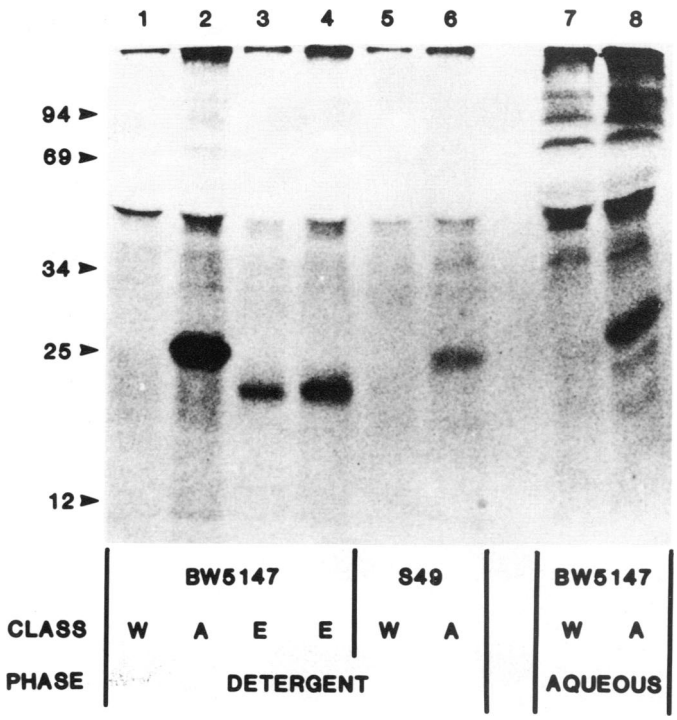

Fig. 4. 1.5×10^6 cells from each line were labeled with [^{3}H]tryptophan for 3 h. After lysis in TX114, phases were separated, re-extracted twice, precleared and Thy-1 was immunoprecipitated twice with polyclonal antibody. The total incorporation of [^{3}H]tryptophan into detergent-binding proteins was between 3.2×10^6 and 4.7×10^6 c.p.m. for all lines. The fluorograph was exposed for 1 month. **Lanes 1** and **7**, BW5147; **lanes 2** and **8**, BW5147(Thy-1$^-$a); **lane 3**, BW5147(Thy-1$^-$e).10; **lane 4**, BW5147(Thy-1$^-$e).236; **lane 5**, S49; **lane 6**, S49(Thy-1$^-$a).

Labeling of Thy-1 with [^{3}*H*]tryptophan

Since the only tryptophan residue of Thy-1 is found at position 124, only forms of Thy-1 which retain parts of the C-terminal peptide but not the ones which are attached via a glycolipid tail should be labeled by [^{3}H]tryptophan. When this amino acid was used in a 3-h labeling we could detect labeled Thy-1 in all mutants but not in parental cells, although the overall incorporation into proteins was the same in all cell lines (Figure 4). The relative intensities of Thy-1 bands among mutants was comparable to the one observed with leucine labelings (Figure 2). The important finding is that the parental:mutant ratio of incorporation into Thy-1 of the [^{3}H]tryptophan labeling is drastically lower than that found in [^{3}H]leucine labeling experiments. To our surprise we found that the water-soluble form of Thy-1 of BW5147(Thy-1$^-$a) was also labeled by [^{3}H]tryptophan.

Some other integral membrane proteins are released by phospholipase C into the aqueous phase in S49 but not S49(Thy-1$^-$a)

In various lymphocyte populations from rat and mouse we found in addition to Thy-1 other, distinct surface glycoproteins which could be released into the aqueous phase by treatment with PLC. Such surface glycoproteins could also be detected in S49 but not in BW5147 (Figure 5, lane 2). Their approximate mol. wts, as measured by their mobility in SDS−PAGE was 97, 87, 62, 54, 44, 39 and 11kd. These molecules seem to be minor surface

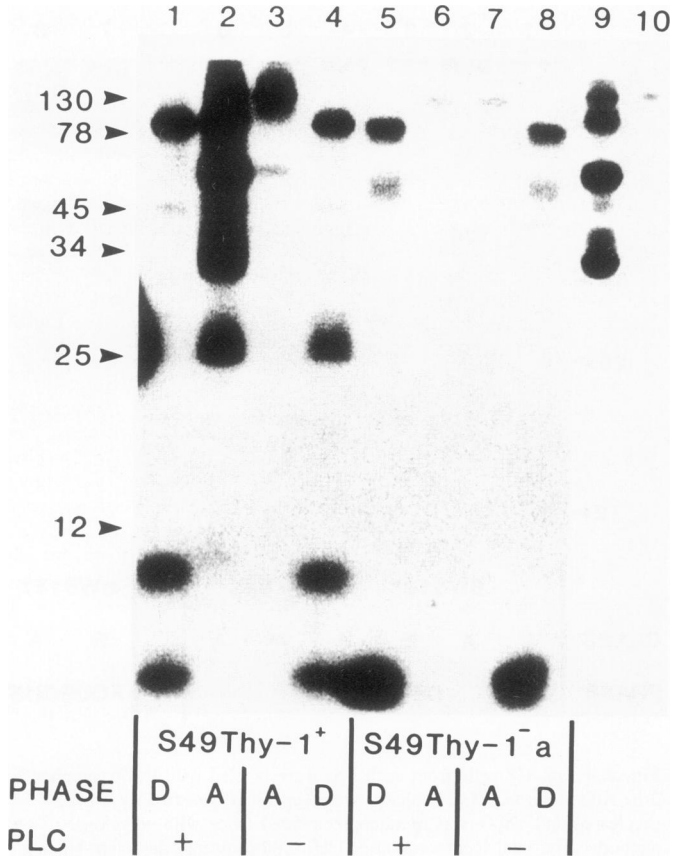

Fig. 5. 2 × 10^7 cells from each line were labeled with 0.5 mCi of Na[^{125}I] using the lactoperoxidase technique. After lysis in TX114, the detergent phases were re-extracted three times and divided into two aliquots which were incubated with (+) or without (−) 2.5 units of PLC for 1 h at 30°C. After phase separation we removed the bulk of Thy-1 from the aqueous phase of PLC-treated S49 (**lane 2**) by immunoprecipitation with monoclonal anti-Thy-1 (1.5 h, 4°C), since the Thy-1 band could have obscured the presence of minor bands in this sample. Subsequently, aqueous phases (A) were precipitated with trichloroacetic acid and analysed in SDS−PAGE along with a small fraction (2%) of the corresponding detergent phase (D). Exposure: 3 days. **Lanes 1−4**, S49; **lanes 5−8**, S49(Thy-1⁻a); **lanes 9** and **10** are a shorter exposure of lanes 2 and 3. The Thy-1 band can be seen in the region of 26−29 kd. Note that PLC removed most of Thy-1 from the detergent phase (lane 1 versus 4). A small fraction of PLC-released Thy-1 was still present after immunoprecipitation (lane 2), whereas no Thy-1 appeared in the aqueous phase after a control incubation (lane 3).

glycoproteins since they cannot be detected in the detergent phase of the untreated sample and the total amount of counts in these bands is much smaller than the counts in Thy-1. [This was also found with thymocytes (Figure 1).] The 87 and 54 kd proteins could also easily be detected as [^{3}H]leucine labeled bands (not shown). Interestingly, no such bands could be released by PLC in the S49(Thy-1⁻a) mutant (Figure 5, lane 6). Since we have not yet identified these proteins, we cannot rule out the possibility that the mutant does not make them, although this does not seem likely. The result might be better explained by assuming that S49(Thy-1⁻a) cells cannot transport these molecules to the surface and/or that their mode of anchoring in the membrane is different than in the wild-type, or indeed that they have already partitioned into the aqueous phase before treatment with PLC. It should be noted that there are several differences between parental and mutant cell lines with regard to the major cell surface proteins, for instance a 10 kd protein is not found in the mutant and this protein does not seem to be released readily by PLC. It is unclear whether these differences are related to the class A mutation or are due to clonal variation in this line.

Discussion

The major finding in this study is that Thy-1-deficient mutant cells of classes A and E do not contain the C-terminal glycolipid membrane anchor but retain their original C-terminal peptide sequence predicted by the cDNA sequence. The evidence for this is firstly that in two independent isolates of each mutation class we find that the detergent binding domain of Thy-1 is not removed by PLC; secondly, Thy-1 molecules of both mutation classes can be labeled biosynthetically with [^{3}H]tryptophan, indicating that their amino acid sequence continues at least up to position 124; and thirdly, after deglycosylation with endoglycosidase F (Elder and Alexander, 1982), the Thy-1 from class A and E mutants migrates slightly slower in SDS−PAGE than its wild-type counterpart (data not shown). The fact that some other, non-identified surface proteins which are also attached via a PLC-sensitive anchor are not found in the S49(Thy-1⁻a) mutant, is consistent with the hypothesis that other surface glycoproteins which normally undergo the same post-translational modification as Thy-1 are not correctly processed in this line. However, it will be necessary to identify these proteins, to show that they are synthesized by the S49(Thy-1⁻a) line and that they contain C-terminal peptide tails similar to Thy-1 in order to corroborate this interpretation.

Our inability to detect [^{3}H]tryptophan-labeled Thy-1 in parental cells is at variance with the study of Seki *et al.* (1985a) who found some incorporation of [^{3}H]tryptophan into the Thy-1 of BW5147(Thy-1+) which persisted during 45 min of chase. It might well be that the sensitivity of detection in our experiment is lower, but also different labeling conditions, differences between subclones of the line or differences in the purity of the reagent might be responsible for the discrepancy.

At this stage we feel that the failure to add glycolipid tails to Thy-1 in the mutants can be explained in three alternative ways. Firstly, it might be that the mutations of class A and E directly or indirectly affect the enzymatic machinery which removes C-terminal peptide sequences and adds the constituents of the glycolipid tail. For the class E mutants this would mean that dolichol-P-mannose is the donor for the mannoses contained in the glycolipid tail of wild-type cells. (Since the class E mutants not only fail to attach the glycolipid tail but also are unable to cleave off the C-terminal peptide, we would have to postulate that a single enzyme cleaves the peptide the attaches at least parts of the glycolipid tail.) It also could mean that some enzymes involved in the biosynthesis and the attachment of glycolipid tails are inactive or missorted when they have incomplete high mannose oligosaccharides. A second way of explaining our results would be to assume that the persistence of protein tails in the mutants is a consequence of the failure of Thy-1 to reach the organelle in which these tails are normally removed. Since the mutants make glycosylated Thy-1 molecules (Trowbridge *et al.*, 1978a) it is clear that they translocate Thy-1 into the RER. We have evidence that Thy-1, like the VSGs of trypanosomes, becomes attached to glycoplipid anchors within one minute after translation (data not shown). A third explanation of our results would be that the mutants are deficient in some post-translational modifications of Thy-1 which are necessary for this molecule to be recognized by the enzymes which cleave the peptide and add the glycolipid components. If the first interpretation is correct, then this would imply that the biosynthetic pathway used for the addition of glycolipid tails is not essential for the survival of cells in tissue culture and also, that the attachment of glycolipid tails might be important for the transport of certain glycoproteins to the cell surface.

Partitioning in TX114 detergent solutions before and after digestion with PLC was used to determine the amphipathic nature of Thy-1. Even with high amounts of PLC only 50–70% of wild-type Thy-1 can be shifted from the detergent to the aqueous phase (Figure 1, Table I). At least for the lymphoma cells this cannot be due to the persistence of amino acids 113–143 since no tryptophan is present in the Thy-1 of these cells. It is therefore conceivable that some molecules are resistant to PLC because their phospholipid tails are somewhat modified. Indeed phospholipase C form *Staphylococcus aureus* released no more than 50% of surface Thy-1 from whole cells (Low and Kincade, 1985). However, the incomplete release observed by us could well be artefactual; for instance, partial degradation of Thy-1 during the incubation might expose hydrophobic residues which render the molecule amphipathic even when the phospholipid tail is cleaved off. We have not performed any experiments to look for conditions in which PLC can release the Thy-1 glycolipid more completely.

It is unclear whether the mutant forms of Thy-1 are membrane bound or not. The cDNA sequence does not predict any positively charged residues at the end of the 23 amino acid long hydrophobic stretch (115–138), but such charged residues do not seem to be absolutely necessary for proper membrane anchoring (Davis and Model, 1985; Cutler and Garoff, 1986). Our data show that some Thy-1 precursor from the class A mutants partitions to a significant extent into the aqueous phase of TX114 solutions and that this material contains tryptophan. This suggests that either these molecules do not contain the complete C-terminal hydrophobic peptide sequence or that this sequences does not bind detergent as efficiently as the glycolipid tail. The Thy-1 from class E mutants might be less prone to partition into the aqueous phase because it contains smaller N-linked oligosaccharides. Further biochemical analysis of the C-terminal parts of the mutant Thy-1 should resolve this issue.

Materials and methods

Cells and culture

The following cell lines were analysed: BW5147(Thy-1$^+$); BW5147(Thy-1$^-$a); BW5147(Thy-1$^-$e).10; BW5147(Thy-1$^-$e).236; S49(Thy-1$^+$); S49(Thy-1$^-$a). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1.5 mM glutamine, 10 μg/ml gentamycin and 10% FCS. Thymocytes were from 4-week-old female C57BL/6 mice.

Radioisotopic labeling procedures

Cells were labeled using the glucose oxidase-coupled lactoperoxidase technique (Hubbard and Cohn, 1975): 3×10^7 cells were labeled in 1 ml of PBS containing 5×10^{-6} mol of glucose, 10^{-9} mol KI, three units of lactoperoxidase (Sigma) and 0.5–1 mCi of Na[^{125}I] on ice for 30 min. Glucose oxidase (Sigma), 0.12 unit, was added every 10 min. Cells were washed three times in Eagle's medium supplemented with 5 mM cysteine. For biosynthetic labeling with leucine, cells were incubated in DMEM with the leucine content reduced to 2.5% of normal levels and containing 30 μg/ml leupeptin, 10% dialysed FCS, 25 μCi/ml of L-[^{3}H]leucine (67 Ci/mmol) and $1.5–1.8 \times 10^6$ cells/ml for 3 h at 37°C. For biosynthetic labeling with tryptophan, washed cells were incubated in tryptophan-deficient F12 medium, containing 5% of dialysed FCS, 30 μg/ml leupeptin, 8 μCi/ml of L-[^{3}H]tryptophan (3.7 Ci/mmol) at 1.5×10^6 cells/ml for 3 h at 37°C. Washed cells were lysed in Tris buffered saline (TBS) (0.15 M NaCl, 0.01 M Tris, pH 7.4) containing 1% (w/v) TX114 (Serva), 1 mM EDTA, 20 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml antipain at $2–5 \times 10^7$ cells/ml for 30 min on ice. Nuclei were removed at 10 000 *g* for 5 min at 4°C. The lysate was warmed to 32°C for 3 min and centrifuged at 10 000 *g* for 20 s at room temperature to separate phases. The upper, aqueous phase was removed and re-extracted by the addition of 100 μl of 11% TX114 ml followed by an additional phase separation to remove residual amphipathic proteins; similarly, detergent phase was re-extracted by the addition of a 10-fold volume of TBS with 1 mM EDTA, protease inhibitors and 0.06% TX114 followed by a phase separation to remove remaining water-soluble molecules. All re-extractions were discarded. Since sucrose cushions were not used to separate aqueous and detergent phases (Bordier, 1981), only ~90% of the aqueous phase was usually recovered after phase separation and further losses occurred in the aqueous phase during subsequent re-extractions.

Phospholipase C treatment

Washed detergent phases were made to 0.1 M Tris pH 7.4, 0.05 M NaCl, 0.25 M α-methylmannoside, 1 mM EDTA, protease inhibitors as in lysis buffer and diluted to 3–4% of TX114. PLC from *Bacillus cereus* (Sigma, Type III) was added for treatments as specified in the text. This PLC preparation, although purified using phosphatidylcholine as a substrate, is contaminated with the phosphatidylinositol-specific PLC which has been purified from the same organism (Ikezawa *et al.*, 1976). All our studies were carried out with a single batch of enzyme and the amount of enzyme used is indicated as units of phosphatidylcholine-specific PLC. After incubation, 0.5 ml of 2% TX114 in TBS plus 1 mM EDTA and protease inhibitors was added and the phases separated. Each phase was then re-extracted by three more phase separations as described.

Immunoprecipitation procedures

For immunoprecipitation of Thy-1 we used either the monoclonal rat anti-mouse antibody III-5 (MacDonald *et al.*, 1985) coupled directly to Sepharose or a polyclonal rabbit anti-mouse Thy-1 antibody (Bron *et al.*, 1976) followed by protein A-Sepharose (Pharmacia). Preclearings were carried out with bovine-IgG–Sepharose or non-immune rabbit serum plus protein A–Sepharose respectively, and did not precipitate any Thy-1. All immunoprecipitations were carried out at 6°C overnight and were near to quantitative after a single round. No Thy-1 could be detected in the supernatants after two consecutive immunoprecipitations. Precipitates were washed six times with 1% TX-100, 0.5 M NaCl, 0.1 M Tris pH 7.4 (III-5) or pH 8.0 (polyclonal anti-Thy-1).

Gel electrophoresis and detection of labeled proteins

Gel electrophoresis was carried out according to Laemmli (1970) using 5% stacking and 12.5% separating gels. Gels were soaked in ENLIGHTNING (NEN), dried and exposed at −70°C using Kodak X-Omat XAR-5.

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References

Bangs,J.D., Hereld,D., Krakow,J.L., Hart,G.W. and Eglund,P.T. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 3207–3211.
Boothroyd,J.C., Cross,G.A.M., Hoeijmakers,J.H.J. and Borst,P. (1980) *Nature*, **288**, 624–626.
Boothroyd,J.C., Paynter,C.A., Cross,G.A.M., Bernards,A. and Borst,P. (1981) *Nucleic Acids Res.*, **9**, 4735–4743.
Bordier,C. (1981) *J. Biol. Chem.*, **256**, 1604–1607.
Bron,C., Ankers,C. and Sauser,D. (1976) *Eur. J. Immunol.*, **6**, 594–598.
Carlsson,S.R. (1985) *Biochem. J.*, **226**, 519–525.
Chapman,A., Fujimoto,K. and Kornfeld,S. (1980) *J. Biol. Chem.*, **255**, 4441–4446.
Cross,G.A.M. (1984) *Phil. Trans. R. Soc. Lond. Ser. B.*, **307**, 3–12.
Cutler,D.F. and Garoff,H. (1986) *J. Cell Biol.*, **102**, 889–901.
Davis,N.G. and Model,P. (1985) *Cell*, **41**, 607–614.
Elder,J.H. and Alexander,S. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 4540–4544.
Etges,R., Bouvier,J. and Bordier,C. (1986) *EMBO J.*, **5**, 597–601.
Evans,G., Ingraham,H., Lewis,K., Cunningham,K., Siki,T., Moriuchi,T., Chang,H., Silver,J. and Hyman,R. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 5532–5536.
Ferguson,M.A.J., Duszenko,M., Lamont,G.S., Overath,P. and Cross,G.A.M. (1986) *J. Biol. Chem.*, **261**, 356–362.
Gunter,K.C., Malek,T.R. and Stevach,E.M. (1984) *J. Exp. Med.*, **159**, 716–730.
Holder,A.A. (1985) *Curr. Top. Microbiol. Immunol.*, **117**, 57–74.
Hubbard,A.L. and Cohn,A.Z. (1975) *J. Cell Biol.*, **64**, 438–460.
Hyman,R. (1973) *J. Natl. Cancer Inst.*, **50**, 415–422.
Hyman,R. (1985) *Biochem. J.*, **255**, 27–40.
Hyman,R. and Stallings,V. (1974) *J. Natl. Cancer Inst.*, **52**, 429–436.
Hyman,R. and Trowbridge,I. (1978) *Cold Spring Harbor Conf. Cell Proliferation*, **5**, 741–754.
Hyman,R., Cunningham,K. and Stallings,V. (1980) *Immunogenetics*, **10**, 261–271.
Ikezawa,J., Yamanegi,M., Taguchi,R., Miyashita,T. and Ohyabu,T. (1976) *Biochim. Biophys. Acta*, **450**, 154–164.
Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
Low,M.G. and Kincade,P.W. (1985) *Nature*, **318**, 62–64.
Low,M.G., Ferguson,A.M.J., Futerman,A.H. and Silman,I. (1986) *Trends Biochem. Sci.*, **11**, 212–215.

Luescher,B. and Bron,C. (1985) *J. Immunol.*, **134**, 1084–1089.
MacDonald,H.R., Brown,C., Rousseaux,M., Horvath,C. and Cerottini,J.C. (1985) *Eur. J. Immunol.*, **15**, 495–501.
Maino,V.C., Norcross,M.A., Perkins,M.S. and Smith,R.T. (1981) *J. Immunol.*, **126**, 1829–1836.
Reif,A.E. and Allen,J.M.V. (1964) *J. Exp. Med.*, **120**, 413–433.
Seki,T., Chang,H., Moriuchi,T., Denome,R., Ploegh,H. and Silver,J. (1985a) *Science*, **227**, 649–651.
Seki,T., Moriuchi,T., Chang,H.C., Denome,R. and Silver,J. (1985b) *Nature*, **313**, 485–487.
Trowbridge,I., Hyman,R. and Mazauskas,C. (1978a) *Cell*, **14**, 21–32.
Trowbridge,I., Hyman,R., Ferson,T. and Mazauskas,C. (1978b) *Eur. J. Immunol.*, **8**, 716–723.
Tse,A.G.D., Barclay,A.N., Watts,A. and Williams,A.F. (1985) *Science*, **230**, 1003–1008.
Williams,A.F., and Gagnon,J. (1982) *Science*, **216**, 696–703.

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Since we submitted this paper the absence of glycolipid anchors from Thy-1 of class E mutant has been reported by Fatemi,S.H. and Tartakoff,A. (1986) *Cell*, **46**, 653–657.