A Glycophospholipid Tail at the Carboxyl Terminus of the Thy-1 Glycoprotein of Neurons and Thymocytes

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Abstract. *Cell surface molecules of eukaryotic cells have been considered to be integrated into the membrane bilayer by a transmembrane protein sequence. The Thy-1 antigen of rodent thymocytes and brain was the first eukaryotic membrane molecule for which biochemical data clearly suggested membrane integration via a nonprotein tail. Direct evidence is now presented showing that a glycophospholipid structure is attached to the carboxyl-terminal cysteine residue and that 31 carboxyl-terminal amino acids predicted from the Thy-1 complementary DNA sequence are not present in the mature glycoprotein. These experimental results raise questions concerning signaling across a cell membrane since antibodies to Thy-1 can stimulate T lymphocytes to release lymphokines and undergo cell division.*

The Thy-1 glycoproteins are major cell surface constituents of rodent thymocytes and neurons (*1–3*) and seem likely to be involved in cell interactions. The Thy-1 protein sequence is related to a single immunoglobulin variable domain (*2, 4*), and antibodies to Thy-1 can be mitogenic for mouse T lymphocytes (*5*) and can facilitate the outgrowth of neuron processes in tissue culture (*6*). Protein sequences of 111 or 112 amino acids accounted for the amino acid composition of pure rat or mouse Thy-1 (*2, 7*), but a hydrophobic sequence suitable for membrane integration was not identified. Instead, the known hydrophobicity of the molecules (*8*) appeared to be due to a nonprotein hydrophobic tail attached at the COOH-terminus (*7*). This interpretation was challenged when an extra 31 amino acids were predicted, from complementary DNA (cDNA) sequences, to be at the carboxyl terminus (*9*), and it was argued that this sequence was responsible for membrane integration. We now show that Cys^{111} is the COOH-terminal amino acid of mature rat Thy-1 from brain and thymus and that a glycophospholipid structure is attached to this residue.

Purification of COOH-terminal peptides. A number of COOH-terminal peptides, produced by different proteolytic enzymes, have been analyzed and these are shown along with the peptide nomenclature in Fig. 1a. The small COOH-terminal peptides are unusual in that they are lost in conventional isolation procedures, and for a considerable period they could only be isolated by allowing the peptides to bind to Brij 96 detergent micelles. A typical result in which peptide T(C) was found at the gel front of a Biogel P10 column along with the Brij 96 detergent micelles while all the other peptides were retarded by the column is shown in Fig. 1b. In an amino acid analysis of the unretarded peak *S*-carboxymethylcysteine (SCMC) was the only amino acid detected (Fig. 1c). The presence of other material is indicated because peptide T(C) has apparently bound to the detergent micelles and also because ethanolamine is seen in the amino acid analysis (Fig. 1c). The SCMC in peptide T(C) is Cys^{111} since the same method was used to purify peptides T(DKLVKC) and P(VKC) which are definitely Cys^{111} peptides on the basis of their sequence (*7*).

The problem with peptides isolated as in Fig. 1b is that the preparations contain Brij 96 detergent and this causes difficulties in analysis by gas chromatography (GC). Thus thin-layer chromatography (TLC) was used to purify peptide T(C) (Fig. 2). With the TLC method, peptide T(C) isolated with Brij 96 (Fig. 1b) could then be separated from the detergent and the results for brain and thymus preparations are shown in Fig. 2, a and b. The same method could be used to purify peptide T(C) after trypsin digestion of peptide SP6 or total Thy-1 without detergent, and results are shown for brain T(C) in Fig. 2, c and d.

Figure 1d shows amino acid analysis for brain T(C) purified from SP6 as in Fig. 2c. The presence of SCMC and ethanolamine is clear and in this case glucosamine and galactosamine residues are also seen (these residues are often destroyed with the standard conditions used for peptide hydrolysis (for example, as in Fig. 1a). Peptide T(C) purified from thymus Thy-1 migrated faster on TLC than brain T(C) (Fig. 2b) and was more heterogeneous, but the analysis (Fig. 1e) of spot 1 in Fig. 2b indicated that it was similar to brain T(C) except that only a small amount of galactosamine was detected; spot 2 from Fig. 2b was the same except that galactosamine was not detected at all.

Components at the COOH-terminus. The components detected with the various COOH-terminal peptide fractions are summarized in Table 1. The identification of the following components has been verified by mass fragmentograms after gas chromatography (GC/MS) of trimethylsilyl (TMS) derivatives: ethanolamine, myoinositol, stearic acid, phosphate, and glycerol. The identification of mannose is based on the elution positions of TMS derivatives of α-methylmannoside and mannitol in GC and that of amino sugars on their elution positions in the amino acid analyzer. With the possible exception of glycerol (see below) none of the components in Table 1 could be due to free contaminants since they were not seen with peptides other than those shown in Fig. 1 and no data could be obtained unless samples were hydrolyzed prior to analysis.

The main hydrophobic component detected at the Thy-1 COOH-terminus is stearic acid. This was seen in a previous analysis of total Thy-1 (*10*) (Table 2) and in our present studies was first confirmed by transesterification by means of base-catalyzed methanolysis and subsequent GC (Fig. 3, a and b). For derivatization by this procedure, the fatty acid

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must be in an ester linkage and free fatty acid or fatty acid in an amide linkage would not be detected.

For the quantitation of stearic acid (Table 1) samples were subjected to total hydrolysis and then converted to methyl or TMS derivatives. The anlaysis of SP6 peptide compared with a mixture of all other V-8 protease peptides is shown in Fig 3, c and d, and it can be seen that stearic acid is seen only with the SP6 peptide. Analysis of the T(C) peptides was first carried out in the presence of Brij 96 detergent, which gave rise to many background peaks in the gas chromatograph. But there was no background in the stearic acid position and the presence of stearic acid was clearly seen in samples containing peptide T(C) (Table 1). With brain or thymus T(C) purified by TLC there were no background problems; a stearic acid peak is clearly seen for brain peptide T(C) in the GC/MS trace shown in Fig. 3e and the GC trace in Fig. 3h and for thymus peptide T(C) in the GC trace shown in Fig. 3i. Quantitation of stearic acid gave values that varied in the range 1 to 2 moles per mole.

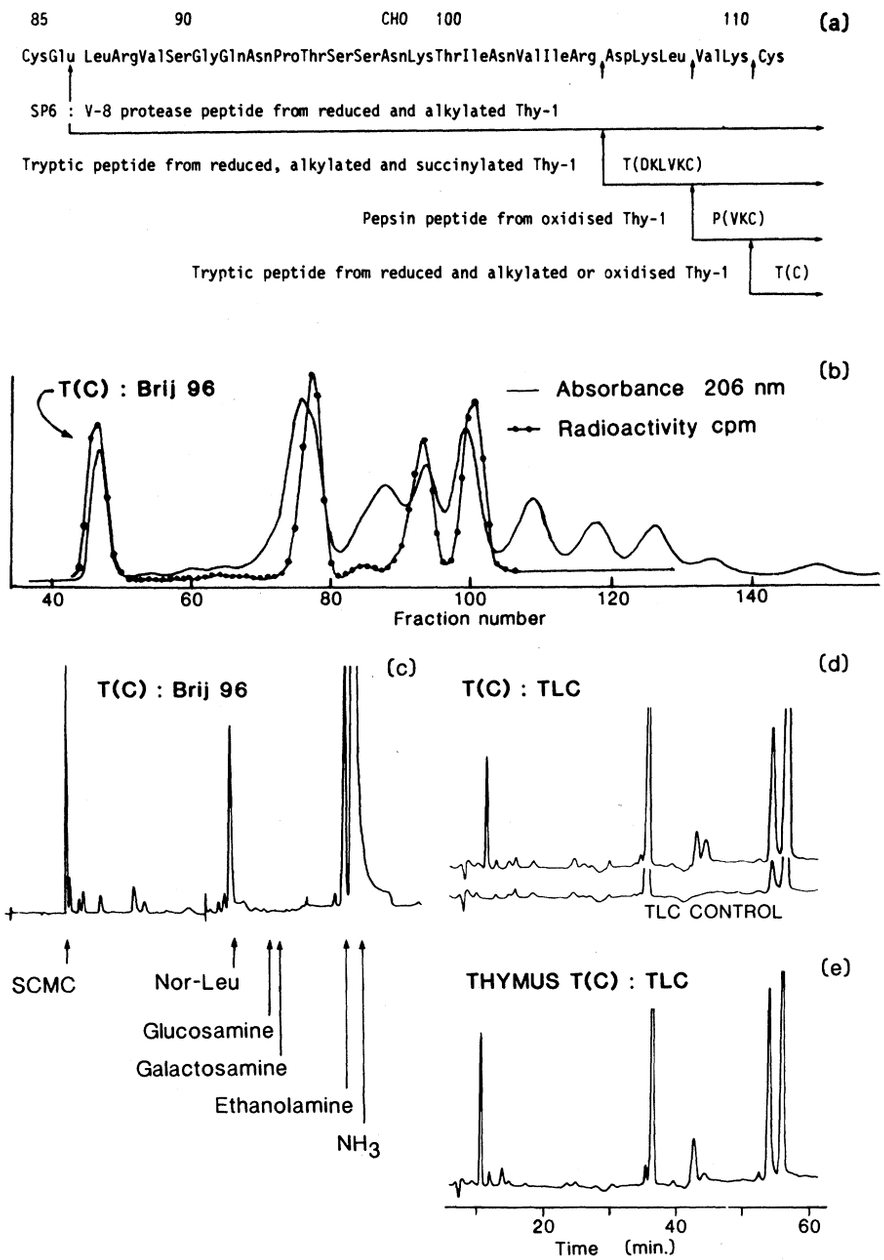

Fig. 1. Purification and analysis of Thy-1, COOH-terminal peptides. (a) Carboxyl-terminal peptides used in the analysis. SP6, T(DKLVKC), and P(VKC) were prepared as in (*7*) and T(C) was prepared as shown in panel b or in Fig. 2. (b) Preparation of brain T(C) by gel filtration of tryptic peptides with Brij 96. Brain Thy-1 (5 mg) was reduced and alkylated with [^{14}C]iodoacetic acid (*7*) and was digested for 24 hours at 37°C with a total of 0.2 mg of trypsin added in two portions at 0 and 7 hours in 0.1*M* NH_4HCO_3 plus 50 μl of 10 percent Brij 96 detergent. The digest was fractionated on a Biogel P10 column (1.4 by 100 cm) developed in 0.1*M* NH_4HCO_3. The yield of T(C) peptide was 58 percent. (c) Amino acid analysis of brain T(C) prepared as in (b). (d) Amino acid analysis of brain T(C) prepared as in Fig. 2c. (e) Amino acid analysis of thymocyte T(C) spot 1 as in Fig. 2b, except that T(C) was derived from a tryptic digest of SP6 peptide. Hydrolysis conditions are described in Table 1. Nor-leu indicates the elution position of norleucine, the internal standard.

Two further specific peaks that may be fatty acids were seen in Fig. 3c and these are labeled as $?C_{20}$ and $?C_{22}$ on the basis that they eluted in the same regions as C_{20} and C_{22} fatty acids on the gas chromatograph. The $?C_{22}$ peak was seen in every analysis that included the Thy-1 COOH-terminal peptides, but not in any other fractions and was always considerably larger than the $?C_{20}$ peak. The quantitation of $?C_{22}$ with the stearic acid calibration varied from 0.1 to 0.8 mole per mole (Table 1). The mass fragmentogram of $?C_{22}$ was the same as that for $?C_{20}$ and did not correspond to any compound in the data bank of the Oxford Botany Department Finnigan 1020 GC/MS system. The partial spectrum obtained is shown in Fig. 3g. The largest ion mass per charge seen in this spectrum is 167, which could not correspond to a fatty acid with a 22-hydrocarbon chain length. However, because the amount of the compound present was small, larger ions will have gone undetected. For the present, the argument that $?C_{20}$ and $?C_{22}$ may be long-chain fatty acids rests on their elution positions in gas chromatography.

The values for ethanolamine in Table 1 were obtained from the amino acid analyzer. To avoid minor amounts of contaminants found in the same position as ethanolamine under standard conditions (Fig. 1d) two programs for analysis of collagen hydrolyzates were used. In these conditions the ethanolamine peaks were moved away from the unknown contaminants to positions after the ammonia peak (not shown). The values for ethanolamine were consistently higher than 1 mole per mole of COOH-terminal peptide.

Roughly equal amounts of glucosamine and galactosamine were associated with the COOH-terminus of brain Thy-1 while with thymus Thy-1 glucosamine was present at higher levels than galactosamine. Similar results were previously obtained for P(VKC) peptides (*7*). Mannose was the only neutral saccharide found to be associated with the COOH-terminal peptides.

Myo-inositol was unexpectedly found by GC/MS analysis of hydrolyzed Thy-1 samples derivatized with TMS reagent. It has been found at about 1 mole per

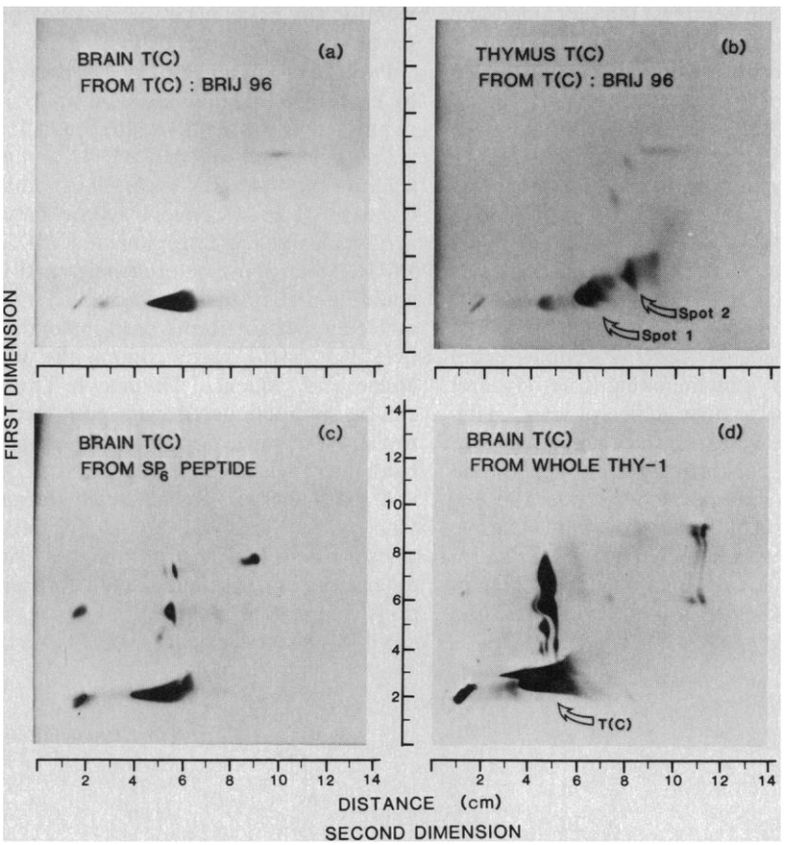

Fig. 2. Autoradiography of T(C) peptides purified by two-dimensional thin-layer chromatography. For plates (a) and (b) T(C) peptides in Brij 96 were prepared from reduced and alkylated (^{14}C-labeled) Thy-1 from brain (70 nmol) and thymus (70 nmol), repectively, as in Fig. 1b; and the T(C) was then separated from detergent by TLC. For plate (c), 40 nmol of brain SP6 peptide was digested with trypsin prior to TLC and for (d) 150 nmol of whole brain Thy-1 was trypsin digested. For TLC, samples were spotted on the bottom left corner of TLC plates (Silica G60; from Merck; 20 by 18 cm; thickness 0.25 mm). The plates were developed in dimension one with the following solvent systems (*25*), which in combination moved most of the peptides except peptide T(C); with drying under vacuum after each solvent. (i) Chloroform, acetone, methanol, acetic acid, water (6:8:2:2:1); (ii) butan-1-ol, acetic acid, water (3:1:1); and (iii) propan-1-ol, H_2O, 25 percent NH_3 (7:2:1). Then in the second dimension the solvent propan-1-ol, water (7:3) was used twice. The plates were exposed to x-ray film at room temperature for 6 to 12 hours. The position of peptide T(C) is obvious in (a) and (c); it is marked by arrows in (b) and (d). The T(C) peptides were recovered by extracting the scrapings with 400 μl of a mixture of propan-1-ol and water (7:3) three times. The recoveries with respect to material applied to the plates as based on ^{14}C labeling and amino acid analysis were (a) 61 percent; (b) 32 percent for spot 1 and 16 percent for spot 2; (c) 40 percent; and (d) 60 percent.

Table 1. Components at the COOH-terminus of brain and thymocyte Thy-1. Values are expressed as moles of component per mole of Thy-1 peptide. The number of determinations, where greater than 1, is indicated next to the value. The plus sign indicates that the material was present but not quantitated; ND indicates not determined; and NR, not relevant because of presence of carbohydrate at positions other than COOH-terminus.

Component	Brain					Thymus T(C) (TLC)*
	Total	SP6	P(VKC) (Brij)	T(C) (Brij)	T(C) (TLC)	
Ethanolamine†	1.6 (n = 3)	1.5 (n = 6)	1.8	1.8	1.8 (n = 7)	1.6 (n = 4)
Glucosamine‡	NR	NR	+	+	0.7 (n = 3)	0.8 (n = 4)
Galactosamine‡	+	+	+	+	0.9 (n = 3)	0.3 (n = 4)
Mannose§	NR	NR	ND	ND	2.7 (n = 2)	2.0 (n = 3)
Myo-Inositol‖	0.9	1.3	ND	0.6	0.8 (n = 2)	0.7
Stearic acid¶	1.7 (n = 6)	1.6	ND		1.2 (n = 2)	1.1 (n = 2)
Stearic acid‖		1.0		1.3	0.9	0.9
?C_{20} fatty acid‖	+	+	ND			
?C_{22} fatty acid‖	+	0.5	ND	0.8	0.2	0.1
Phosphate‖		2.0 (n = 2)		2.6	2.6 (n = 2)	2.8
Phosphate#	3–4 (n = 3)		2–3			
Glycerol‖		0.2	ND	0.5		

*For thymus T(C) all values were the same for spot 1 and 2 as in Fig. 2b, except for galactosamine where spot 1 gave a value of 0.4 (n = 2) while spot 2 gave 0.0 (n = 2). The value shown above is mean-adjusted for the amount of material in each spot. †Amino acid analysis: 1- to 10-nmol samples were hydrolyzed with 5.7*M* HCl, 5 m*M* phenol, and 7.2 m*M* mercaptoethanol at 110°C for 24 hours. The residues were analyzed on an LKB 4400 or Durrum D500 amino acid analyzer. One anomaly was observed in that the yield of SCMC varied greatly with T(C) peptides prepared with Brij 96 even after TLC. The presence of SCMC was indicated by ^{14}C radioactivity and problems were not observed with T(C) peptides prepared from SP6 peptide or whole Thy-1. Also cysteic acid was recovered in good yield from P(VKC). ‡Amino sugar analysis: 1- to 5-nmol samples were hydrolyzed with 3*M* *p*-toluenesulfonic acid at 110°C for 24 hours, and the hydrolyzates were neutralized with 2*M* NaOH (*27*) before analysis on an LKB 4400 amino acid analyzer. §Preparation and derivatization of alditols: 5- to 15-nmol samples were hydrolyzed with 2.5*M* trifluoroacetic acid under nitrogen at 100°C for 7 hours. The residue was reduced with 1 percent $NaBH_4$, and the reaction was stopped with a mixture of concentrated HCl and methanol (1:23); the excess borohydride was removed as methyl borate by repeated evaporation with methanol (*28*). The residue was treated with acetic anhydride (*29*), and the reacetylated product was treated with *N-O*-bis-(trimethylsilyl)-trifluoroacetamide containing 1 percent trimethylchlorosilane in pyridine (*30*). The derivatives were analyzed on a BP.1 capillary column (Scientific Glass Engineering Limited) by GC with a temperature program up to 260°C as in (*29*), except that the rate of increase was 4°C per minute rather than 8°C per minute. Mannose was also detected as methylglycosides after methanolysis and trimethylsilylation (*29*). ‖Trimethylsilyl (TMS) derivatization: samples (10 to 50 nmol) were hydrolyzed with 5.7*M* HCl at 100°C for 24 hours (Fig. 3, c to f) or with 5.7*M* HCl at 100°C for 8 hours (Fig. 3, h to j) (yields of myo-inositol were higher with the latter condition). The residues were then treated with 2 percent methoxyamine in pyridine at 60°C for 30 minutes and then with *N-O*-bis-(trimethylsilyl)-trifluoroacetamide with 1 percent trimethylchlorosilane at 100°C for 1 hour (*30*). For quantitative analyses, methyl nonadecanoate was added as an internal standard before the derivatives were prepared. Fractions of the samples (1/10 or 1/30) were analyzed either on a Hewlett-Packard OV-1 capillary column (0.25 mm by 50 m) with a Finnigan gas chromatography–mass spectrometer using a one-gradient temperature program to 300°C (80°C for 2 minutes then 3°C per minute to 300°C where it was held for 5 minutes), or on a Scientific Glass Engineering Limited BP.1 capillary column (0.32 mm by 25 m) with a Hewlett-Packard gas chromatography system having a four-step temperature program to 260°C (80°C for 5 minutes, 5°C per minute to 100°C; 2.5°C per minute to 140°C; 15°C per minute to 230°C; 230°C until 50 minutes; 15°C per minute to 260°C and 260°C until 70 minutes). ¶Methanolysis: in the base-catalyzed method, samples (10 to 30 nmol) were treated with 1*M* sodium methoxide in a mixture of methanol and benzene (3:2) at room temperature for 4 minutes (*31*); they were then neutralized with methanolic-HCl (*31*) before extracting with hexane. The hexane extracts were analyzed on a 10 percent SP-2330 fatty acid column (4 mm by 3 m) isothermally at 200°C by GC (Fig. 3, a and b). In acid-catalyzed methanolysis, the same amounts of sample were treated with 1.5*M* methanolic HCl after hydrolysis with 5.7*M* HCl or directly with a mixture of BF_3 and methanol (*32*). #Phosphate was analyzed by the Triton X-100 turbidity method involving formation of a phosphate-molybdate complex (*11*).

mole in all peptides that included the COOH-terminus from brain and thymus Thy-1 but not in other peptides (Fig. 3 and Table 1).

Phosphate was specifically detected with COOH-terminal peptides at 2 to 3 moles per mole either by use of the gas chromatograph (Fig. 3, h and i) or by a quantitative assay that involved phosphate-dependent formation of aggregates by solutions of Triton X-100 (*11*).

The presence of glycerol remains uncertain because control samples often contained contaminating free glycerol and also because glycerol can easily evaporate when samples are dried after hydrolysis or derivatization (*12*). In one analysis of peptide T(C) prepared in Brij 96, glycerol at the level of about 0.5 mole per mole was detected against a control sample that contained no glycerol, but in other cases background levels were too high for any confidence to be placed on the detection of glycerol (Fig. 3, f and j).

Pure Thy-1 glycoprotein is not derived by proteolysis. The amino acid analysis of purified Thy-1 fits the sequence of 111 amino acids and not that of 142 amino acids (Fig. 4A). However, Thy-1 analyzed by sodium dodecyl sulfate–polyacrilamide gel electrophoresis (SDS-PAGE) does show heterogeneity and it could be argued that the higher relative molecular weight forms seen in thymocyte Thy-1 (*10*) might contain the 142 amino acid sequence. Thymocyte Thy-1 can be subfractionated with lentil lectin to yield a major lower molecular weight band that binds to the lectin (Thy-1 L+), and the higher molecular weight forms that do not (Thy-1 L−) (*10*) (Fig. 4B, tracks 2 and 3). These forms show no differences in their amino acid compositions (Fig. 4A).

To look for the possibility that the cell surface form of Thy-1 might differ from the purified form, we labeled the thymocyte surface with [^{3}H]borohydride and made a detergent extract in conditions aimed to minimize possible proteolysis (legend to Fig. 4). On SDS–PAGE one of the major ^{3}H-labeled bands from the extract is Thy-1 as shown by comparison of total extract (Fig. 4C, track 1) with extract depleted of Thy-1 with antibody coupled to Sepharose 4B beads (Fig. 4C, track 3) and with material eluted from the beads (Fig. 4C, track 4). The mobility of the surface-labeled Thy-1 band was identical to that of pure Thy-1 (as in Fig. 4B) that had been labeled with ^{3}H after purification (Fig. 4C, track 1, compared with track 2). The experiment was also done by adding unlabeled pure Thy-1 to the ^{3}H-labeled cell extract such that a Thy-1 Coomassie blue band could be

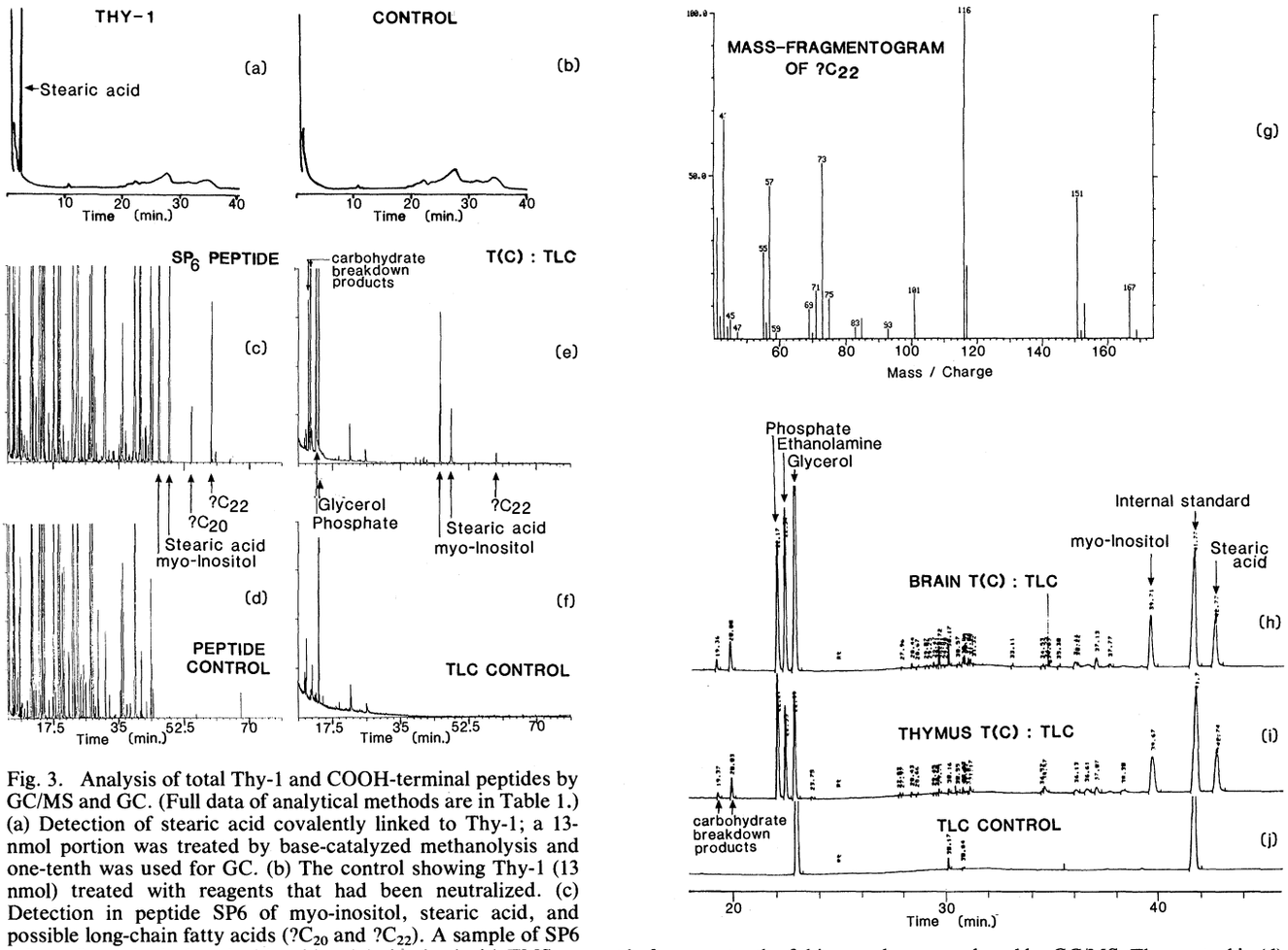

Fig. 3. Analysis of total Thy-1 and COOH-terminal peptides by GC/MS and GC. (Full data of analytical methods are in Table 1.) (a) Detection of stearic acid covalently linked to Thy-1; a 13-nmol portion was treated by base-catalyzed methanolysis and one-tenth was used for GC. (b) The control showing Thy-1 (13 nmol) treated with reagents that had been neutralized. (c) Detection in peptide SP6 of myo-inositol, stearic acid, and possible long-chain fatty acids (?C_{20} and ?C_{22}). A sample of SP6 (45 nmol) was hydrolyzed with acid and derivatized with TMS reagent before one-tenth of this sample was analyzed by GC/MS. The control in (d) shows the rest of the peptides pooled and treated in the same way. The unnamed peaks are due to amino acids, carbohydrates, and other components mentioned in Table 1. (e) Analysis of peptide T(C) purified by TLC as in Fig. 2, by GC/MS (30 nmol hydrolyzed and TMS derivatized, 1/30 loaded on the columns). (f) Control consisting of analysis of the extract from an adjacent equal area of TLC plate to that scraped to yield the T(C) peptide. Glycerol and phosphate are marked, on (e), in addition to the other components; ethanolamine was poorly derivatized in this trace, but see (h). (g) The fragmentogram of the unknown compound ?C_{22} detected in (c) and (g). The fragmentogram for ?C_{20} was identical to this. (h, i, and j) Tracings from the GC of TMS derivatized material from brain T(C), thymus T(C), and a control area from one of the thin-layer plates on which the peptides were prepared. The T(C) peptides (25 nmol) were hydrolyzed and derivatized and 1/30 of this was analyzed. Glycerol is the only component that does not show clear specificity between samples and control.

seen against the other bands in the membrane extract (Fig. 4D; Thy-1 is marked by an arrow in track 1 as compared to 2). The gel was then subjected to fluorography, and the labeled bands (Fig. 4D, tracks 3 and 4) coincided exactly with the Coomassie blue band for pure Thy-1 (Fig. 4D, track 1).

Glycophospholipid component established. The data in Table 1 clearly establish the existence of glycolipid-like components in association with the COOH-terminal Cys of purified Thy-1 from both the brain and thymus. The molecule is now shown to be a glycolipophosphoprotein with the well-characterized antigenic determinants being protein based (*2, 10, 13*). The exact structure at the COOH-terminus remains to be resolved, but the recent finding that Thy-1 can be released from the cell surface by a phospholipase C specific for phosphatidylinositol (*14*) suggests that this lipid is part of the structure. This possibility is consistent with the analyses shown in Table 1 provided that the uncertainty with regard to glycerol is ultimately resolved to give a positive result.

Our data show that the extra 31 amino acids predicted from the cDNA sequence (*9*) cannot be present in anything but a very small fraction of the mature glycoprotein. The composition of the purified glycoproteins is inconsistent with the 142-residue sequence and this is also true of Thy-1 purified by others (*15*). Furthermore, we could find no evidence that pure Thy-1 could have been derived by proteolysis. Seki *et al.* (*9*) suggested that tryptophan was present in mouse Thy-1 on the basis of biosynthetic labeling; this would indicate the presence of the sequence after Cys^{112} since tryptophan is present only at predicted residue 124 in mouse Thy-1. However, their studies seem preliminary in that the labeled bands were extremely weak, and no controls were shown even though biosynthetic labeling often results in background bands. In two other studies on biosynthesis (*16*), it has been shown

A

Amino acid	Composition: From analyses per 111 residues		From sequence of residue number	
	Thy-1 L+	Thy-1 L−	111	142
Cys	3.7	3.6	4	4
Asx	14.8	14.8	15	17
Thr	9.5	9.5	10	12
Ser	8.3	7.9	8	13
Glx	11.3	11.3	10	12
Pro	2.6	2.6	3	3
Gly	5	5.2	4	6
Ala	2	2.1	2	3
Val	7.6	7.6	9	10
Met	.9	.9	1	1
Ile	4	3.9	5	7
Leu	13	13	13	24
Tyr	2.3	2.3	2	2
Phe	4.4	4.2	4	6
His	4.2	4.2	4	4
Lys	8.2	8.1	8	8
Arg	9.4	9.3	9	9
Trp	0	0	0	1

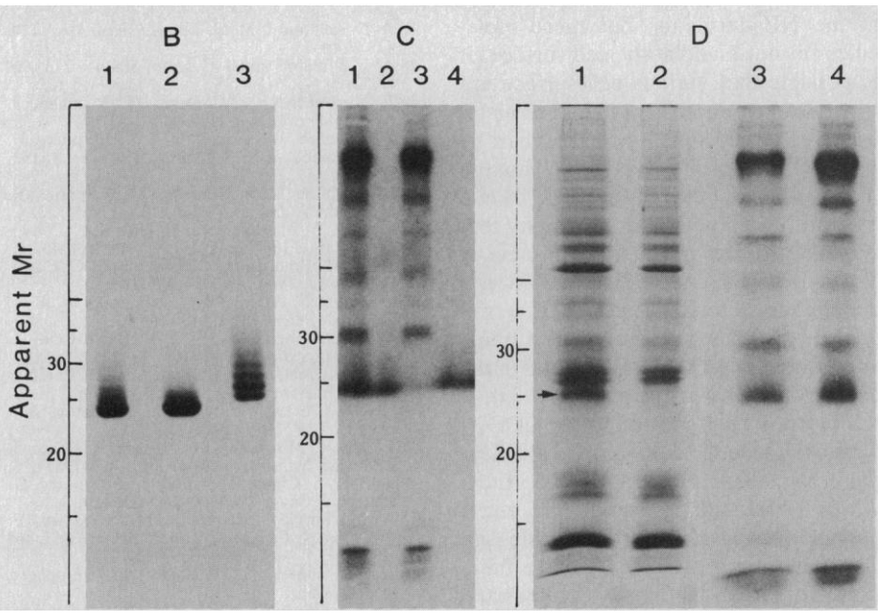

Fig. 4. Rat Thy-1 glycoprotein consists of 111 amino acids. (A) Amino acid analysis of thymocyte Thy-1 samples. Thy-1 L+ was purified from rat thymocytes after solubilization of membrane in deoxycholate and subsequent affinity chromatography with lentil lectin and then gel filtration (*10*). The unbound Thy-1 antigen (Thy-1 L−) was then purified by an antibody affinity column containing Sepharose 4E beads with MRC OX-7 anti-Thy-1.1 monoclonal antibody attached (*7*). Values for amino acid composition were from six analyses (see legend to Table 1) on oxidized Thy-1. Values for Ser and Thr were corrected for degradation by extrapolation from analyses after hydrolysis for 24, 48, and 72 hours. The absence of Trp in thymocyte Thy-1 samples is indicated by the ultraviolet spectra, which are identical to that for brain Thy-1 (*7*). (B) Shows 5 μg each of total thymocyte Thy-1 (track 1) and Thy-1 L+ (track 2) and Thy-1 L− (track 3) analyzed by SDS–PAGE on 12 percent gels (*26*) and stained with Coomassie blue. (C and D) SDS–PAGE of detergent extracts from rat thymocytes that had been surface labeled with NaB^3H_4. All tracks in (C) are fluorographs; track 1 shows extract from labeled cells; track 2 shows Thy-1 that had been ^3H-labeled after purification and subjected to electrophoresis with extract from unlabeled cells; track 3 shows an extract from labeled cells with Thy-1 depleted by anti-Thy-1.1 Sepharose 4B beads; track 4 shows material eluted from the anti-Thy-1.1 beads and subjected to electrophoresis with extracts from unlabeled cells. (D) Tracks 1 and 3 show extract from labeled cells with 5 μg of pure Thy-1 added (indicated by arrow), track 1 shows staining with Coomassie blue, and track 3 is the fluorograph of the same material as in track 1. Tracks 2 and 4 show the extract without added Thy-1. Thymocytes (5×10^8) were labeled with 5 mCi of NaB^3H_4 (Amersham, 6.5 Ci/mmol) after periodate oxidation (*26*). After being labeled, cells were washed and suspended in 4.4 ml of 1 percent Lubrol PX detergent in 10 m*M* tris-HCl, *p*H 7.4, and 0.14*M* NaCl, 2 m*M* EDTA, 4 m*M* benzamidine, 5 m*M* iodoacetamide, 1 m*M* phenyl methyl sulfonyl fluoride (PMSF); after suspension PMSF was again added to 1 m*M*. The mixture was kept on ice for 10 minutes and then centrifuged at 1500*g* for 5 minutes to remove nuclei. Half of the supernatant was immediately frozen in 0.25-ml portions, while the rest was added to 1 ml of packed anti-Thy-1.1 Sepharose 4B beads and incubated for 120 minutes on ice. The beads were then sedimented and the supernatant was stored frozen in 0.325-ml portions. The beads were washed, and bound material was eluted with 2 ml of diethylamine HCl (*p*H 11.5), 0.5 percent deoxycholate buffer (*7*) which was then neutralized and stored in 0.325-ml portions. Unlabeled extract was prepared from thymocytes (5×10^8) as above and stored in 0.25-ml portions, which were added (for SDS–PAGE) to the ^3H-labeled Thy-1 eluted from the beads or [^3H]Thy-1 which was prepared by labeling pure Thy-1 as in (B), track 1. Pure Thy-1 (50 μg) was oxidized with 1 m*M* periodate in 0.5 ml of Dulbecco's A + B buffer (DAB) for 5 minutes on ice. To stop the reaction, 50 μl of 0.1*M* glycerol was added and the Thy-1 was separated from reactants by gel filtration on a 5-ml Sephadex G25 column in DAB. The Thy-1 was collected (1.5 ml), and NaB^3H_4 (5 mCi) in 0.125 ml of 0.01*M* NaOH was added. After 30 minutes on ice, the [^3H]Thy-1 was separated from reactants by gel filtration on a 10-ml Sephadex G25 column. The [^3H]Thy-1 was recovered (2.5×10^6 count/min) and a portion (1.1×10^4 count/min) was subjected to electrophoresis (C, track 2). For the SDS gels, portions as above were thawed into 15 percent trichloroacetic acid on ice and, after 60 minutes, centrifuged. The sediment from each portion was washed once with acetone at −20°C and solubilized in 100 μl of sample buffer; 40 μl of each was subjected to electrophoresis on 12 percent acrylamide gels and gels were stained with Coomassie blue and examined by fluorography (*26*). Mr, molecular weight.

that deglycosylated Thy-1 labeled with [^{35}S]methionine could be detected only at a molecular weight of 13,500 and this is not compatible with a sequence of 142 residues.

It has long been known that bacterial proteins could be attached to the membrane via lipid groups attached to either NH_2-terminal (*17*) or COOH-terminal residues (*18*), but hydrophobicity due to lipids alone seems much less common for cell surface molecules of higher organisms. Often a viral or mammalian cell surface molecule has fatty acid covalently bound via thioester linkages (*19*), but this is usually in addition to the presence of a transmembrane hydrophobic protein segment. Transforming proteins of retroviruses can be found with fatty acid (*20*) at the NH_2-terminus, but these molecules are not found at the cell surface. It is possible that various cell surface enzymes of vertebrates are linked to membrane solely by lipid since they can be released by phospholipase C (*21*), but the only well-characterized molecule from a eukaryotic cell with hydrophobicity that is similar to Thy-1 is the coat protein of *Trypanosoma brucei* (*22*). This has glycolipid-like material at the COOH-terminus and also has protein sequence translated from cDNA that is not found in the cell surface form of the molecule (*23*). It has recently been shown that the COOH-terminal glycophospholipid is added to the *Trypanosoma brucei* glycoprotein within 1 minute of translation (*24*), and it may be that in both this molecule and Thy-1 the extra protein sequence functions to target the newly synthesized polypeptide to the biosynthetic machinery responsible for cleaving the protein and attaching the nonprotein tail.

Why should Thy-1 be attached to the cell surface via a glycophospholipid component? It might be to facilitate release of the structure by phospholipases in certain circumstances, but the requirement for this is not obvious. Alternatively, it may be that lipid anchorage is the most primitive form of membrane attachment and that Thy-1 is a eukaryotic example in which this mechanism has been retained. Such possibilities cannot be evaluated at present, but it is of general interest that cross-linking by antibody of a molecule that apparently cannot traverse the lipid bilayer can cause T lymphocytes to release lymphokines and undergo cell division (*5*).

References and Notes

1. A. E. Reif and J. M. V. Allen, *J. Exp. Med.* **120**, 413 (1964).
2. A. F. Williams and J. Gagnon, *Science* **216**, 696 (1982).
3. J. N. Beech, R. J. Morris, G. Raisman, *J. Neurochem.* **41**, 411 (1983).
4. F. E. Cohen, J. Novotny, M. J. E. Sternberg, D. G. Campbell, A. F. Williams, *Biochem. J.* **195**, 31 (1981).
5. V. C. Maino, M. A. Norcross, M. S. Perkins, R. T. Smith, *J. Immunol.* **126**, 1829 (1981); K. C. Gunter, T. R. Malek, E. M. Shevach, *J. Exp. Med.* **159**, 716 (1984); H. R. MacDonald, C. Bron, M. Rousseaux, C. Horvath, J. C. Cerottini, *Eur. J. Immunol.* **15**, 495 (1985).
6. D. Leifer, S. A. Lipton, C. J. Barnstable, R. H. Masland, *Science* **224**, 303 (1984).
7. D. G. Campbell, J. Gagnon, K. B. M. Reid, A. F. Williams *Biochem. J.* **195**, 15 (1981).
8. P. W. Kuchel, D. G. Campbell, A. N. Barclay, A. F. Williams, *ibid.* **169**, 411 (1978); M. J. Owen, J. C. A. Knott, M. J. Crumpton, *Biochemistry* **19**, 3092 (1980).
9. T. Seki, T. Moriuchi, H.-C. Chang, R. Denome, J. Silver, *Nature* (*London*) **313**, 485 (1985); T. Seki, H.-C. Chang, T. Moriuchi, R. Denome, H. Ploegh, J. Silver, *Science* **227**, 649 (1985).
10. A. N. Barclay, M. Letarte-Muirhead, A. F. Williams, R. Faulkes, *Nature* (*London*) **263**, 563 (1976).
11. H. Eibl and W. E. M. Lands, *Anal. Biochem.* **30**, 51 (1969).
12. F. W. LeBaron, J. Folch, E. E. Rothleder, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **16**, 209 (1957).
13. H. Alexander, D. A. Johnson, J. Rosen, L. Jerabek, N. Green, I. L. Weissman, R. A. Lerner, *Nature* (*London*) **306**, 697 (1983).
14. M. G. Low and P. W. Kincade, *ibid.*, in press.
15. L. D. McClain, M. Tomana, R. T. Acton, *Brain Res.* **159**, 161 (1978); M. Letarte and G. Meghji, *J. Immunol.* **121**, 1718 (1978); S. F. Cotmore, S. A. Crowhurst, M. D. Waterfield, *Eur. J. Immunol.* **11**, 597 (1981); S. R. Carlsson and T. I. Stigbrand, *Biochem. J.* **211**, 641 (1983).
16. B. Luescher and C. Bron, *J. Immunol.* **134**, 1084 (1985); S. Pont, A. Van Agthoven, P. Naquet, A. Pierres, A.-M. Schmitt-Verhulst, M. Pierres, *Immunogenetics* **21**, 459 (1985).
17. K. Hantke and V. Braun, *Eur. J. Biochem.* **34**, 284 (1973); J. S. Lai, M. Sarvas, W. J. Brammar, K. Neugebauer, H. C. Wu, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 3506 (1981).
18. Y. Kamiya, A. Sakurai, S. Tamura, N. Takahashi, E. Tsuchiya, K. Abe, S. Fukui, *Agric. Biol. Chem.* **43**, 363 (1979)
19. M. F. G. Schmidt and M. J. Schlesinger, *Cell* **17**, 813 (1979); M. B. Omary and I. S. Trowbridge, *J. Biol. Chem.* **256**, 4715 (1981); J. F. Kaufman, M. S. Krangel J. L. Strominger, *ibid.* **259**, 7230 (1984).
20. L. E. Henderson, H. C. Krutsch, S. Oroszlan, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 339 (1983); D. Pellman, E. A. Garber, F. R. Cross, H. Hanafusa, *Nature* (*London*) **314**, 374 (1985).
21. M. G. Low and D. B. Zilversmit, *Biochemistry* **19**, 3913 (1980); A. H. Futerman, R.-M. Riorin, E. Roth, M. G. Low, I. Silman, *Biochem. J.* **226**, 369 (1985).
22. M. A. J. Ferguson, K. Haldar, G. A.M. Cross, *J. Biol. Chem.* **260**, 4963 (1985); M. A. J. Ferguson, M. G. Low, G. A. M. Cross, *ibid.*, in press.
23. J. C. Boothroyd, C. A. Paynter, G. A. M. Cross, A. Bernards, P. Borst, *Nucleic Acids Res.* **9**, 4735 (1981).
24. J. D. Bangs, D. Hereld, J. L. Krakow, G. W. Hart, P. T. Englund, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3207 (1985).
25. G. Rouser, *J. Chrom. Sci.* **11**, 60 (1973).
26. W. R. A. Brown and A. F. Williams, *Immunology* **46**, 713 (1982).
27. A. K. Allen and A. Neuberger, *FEBS Lett.* **60**, 76 (1975).
28. Y. Arakawa, T. Imanari, Z. Tamura, *Chem. Pharm. Bull.* **24**, 2032 (1976).
29. M. F. Chaplin, *Anal. Biochem.* **123**, 336 (1982).
30. M. G. Horning, E. A. Boucher, A. M. Moss, E. C. Horning, *Anal. Lett.* **11**, 713 (1968).
31. R. L. Glass, *Lipid* **6**, 919 (1971).
32. L. D. Metcalfe and A. A. Schmitz, *Anal. Chem.* **33**, 363 (1961).

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