Purification and characterization of the mouse thymocyte Thy-1 glycoprotein

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The thymocyte Thy-1 glycoprotein was purified from mouse strain NMRI (Thy 1.2) thymus. Crude membranes were prepared in Tween 20 and solubilized in deoxycholate. The glycoproteins were isolated by affinity chromatography on concanavalin A–Sephacrose, and Thy-1 was further purified by two gel-filtration cycles on Sephacryl S-200. The concentration of Thy-1 in fractions obtained during purification was measured by a solid-phase radioimmunoassay with pure mouse brain Thy-1 as standard. Analysis of the purified preparation by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis revealed at least five distinct bands in the apparent- M_r region 25 000–30 000, the polymorphism probably being due to carbohydrate heterogeneity. Amino-acid-analysis data were compatible with the previously published sequence of mouse brain Thy-1. Sugar content was determined at 31% (w/w), and the carbohydrate composition indicates the presence of ‘complex-type’ oligosaccharide chains. The mean M_r of mouse thymocyte Thy-1 was calculated to be 18 100.

Besides transplantation antigens and immunoglobulins, few membrane-bound glycoproteins on lymphocytes have been more extensively studied than the Thy-1 antigen. This antigen was first found in brain and thymus of the mouse (Reif & Allen, 1964) and was shown to exist as two alloantigens, Thy 1.1 and Thy 1.2. Later it was found that in peripheral blood and in secondary lymphoid organs the expression of Thy-1 was restricted to a subpopulation of lymphocytes, namely the T-lymphocytes (Raff, 1969). Other cells of the mouse have also been shown to possess this antigen, such as fibroblasts (Stern, 1973), epidermal cells (Scheid *et al.*, 1972) and mammary cells (Gillette, 1977). For many years, mouse bone-marrow cells were considered as Thy-1-negative, but recent studies have revealed small but significant amounts present on as much as 25–30% of the cells, including the pluripotential stem cells (Basch & Berman, 1982; Schrader *et al.*, 1982).

Most of the biochemical studies on the Thy-1 molecule have been made on material isolated from the rat. The tissue distribution of Thy-1 is somewhat different in the rat compared with the mouse. Peripheral rat T-lymphocytes lack the antigen (Acton *et al.*, 1974), which instead is found on cells belonging to the B-cell lineage (Hunt *et al.*, 1977;

Ritter *et al.*, 1978). Both the rat and the mouse, however, express large amounts of Thy-1 on thymocytes and on neuronal cells (reviewed by Williams *et al.*, 1977). The molecule has been purified from brains of both species (Barclay *et al.*, 1975; McClain *et al.*, 1978) and from rat thymocytes (Letarte-Muirhead *et al.*, 1975). Also, the human and the dog brain analogue has been purified and characterized (Hamann *et al.*, 1980; Cotmore *et al.*, 1981; McKenzie *et al.*, 1981).

The primary structures of the peptide chains of rat and mouse brain Thy-1 are known and consist of 111 and 112 amino acids respectively (Campbell *et al.*, 1981; Williams & Gagnon, 1982). Besides the difference in total number of amino acids, the sequences differ in 19 positions. Thy 1.1 and Thy 1.2 allotypes of the mouse were shown to differ at only one position (Arg/Glu interchange at residue 89).

Although the Thy-1 polypeptide of different tissues seems to be identical within the species, the carbohydrate composition differs considerably (Barclay *et al.*, 1976). This fact raises the question of a possible biological function of the oligosaccharide chains of the molecule, since it seems unlikely that the same polypeptide, restricted almost exclusively to nervous and lymphoid tissues, would have the same function in two such differently specialized organ systems. The Thy-1 polypeptide would then rather provide a backbone for the display of different carbohydrate structures. In order to evaluate the

Abbreviations used: IgG, immunoglobulin G; CmCys, carboxymethylcysteine; SDS, sodium dodecyl sulphate.

function of the Thy-1 molecule, more biochemical data are needed.

Although much effort has been made to study the expression and the tissue distribution of Thy-1 in mouse tissues, the only source from which mouse Thy-1 has been isolated so far, besides brain, is a lymphoblastoid cell line (BW 5147) (Zwerner *et al.*, 1977a). In the present paper we describe the purification and characterization of Thy-1 from normal mouse thymocytes, and discuss the carbohydrate content and composition in relation to that of Thy-1 obtained from other sources.

Materials and methods

Unless otherwise stated all procedures were performed at 0–4°C.

Chemicals

CNBr-activated Sepharose 4B, concanavalin A–Sepharose 4B, Sephacryl S-200 and reference substances for gel filtration and gel electrophoresis were from Pharmacia Fine Chemicals, Uppsala, Sweden; Tween 20 was from Sigma Chemical Co., St. Louis, MO, U.S.A.; sodium deoxycholate was from Riedel–de Haen AG, Hannover, Germany, and was recrystallized twice before use; rabbit anti-(concanavalin A) serum was from P-L Biochemicals, Milwaukee, WI, U.S.A. IgG was isolated by affinity chromatography on protein A–Sepharose 4B (Pharmacia).

Animals

Male and female mice, 3–4 weeks of age, of the outbred NMRI strain (obtained from Anticimex, Stockholm, Sweden) were used. This strain was tested for Thy-1 allotype by complement-mediated cytotoxic reaction with anti-(Thy 1.1) and anti-(Thy 1.2) alloantisera on thymocytes as described by Carlsson *et al.* (1981). The strain was shown to express the Thy 1.2 alloantigen.

Radioimmunoassay for Thy-1

The details of the radioimmunoassay for Thy-1 are presented elsewhere (Carlsson & Stigbrand, 1983). Thy-1 was purified from mouse brains essentially as described by McClain *et al.* (1978), and was used for production of rabbit anti-(Thy-1) antibodies and as tracer and standard in the assay. The antibodies were shown not to distinguish between the two allotypes of Thy-1.

The solid-phase principle originally described by Wide (1969) was used for the radioimmunoassay. A 100 µl portion of suspension (1:100, v/v) of microcellulose particles to which was coupled rabbit anti-(Thy-1) IgG (1–2 mg/ml of wet cellulose) was mixed with 50 µl of ¹²⁵I-labelled Thy-1 (2 ng/ml) and 50 µl of standard (2–2000 ng/ml) or sample. The

mixture was incubated for 16 h at room temperature by slow vertical rotation. After three washings the radioactivity bound to the cellulose particles was measured and the Thy-1 concentration of the samples was obtained from the standard curve. Standards and samples were measured in duplicate and were always diluted in the same buffer. The Thy-1 concentration of the standards was based on protein content as measured by amino acid analysis. The sensitivity of the assay was 5 ng/ml.

Membrane preparation and solubilization

For preparation of crude membranes, a modification of the detergent method described by Standing & Williams (1978) was used. Thymi were dissected, freed from capsule and blood vessels and weighed. A detergent buffer consisting of 2% Tween 20, 25 mM-Tris/HCl, 150 mM-NaCl, 1 mM-MgSO₄, 2 mM-CaCl₂ and 0.02% NaN₃, pH 7.4, was added to give 5 g of tissue per 100 ml of buffer. The thymi were homogenized in a motor-driven Potter–Elvehjem homogenizer and the mixture was stirred for 1 h. After a few additional strokes in the homogenizer, samples were taken for analysis, and the homogenate was centrifuged at 3000 g for 30 min. The supernatant was further centrifuged at 70 000 g for 1 h and the membranes were collected in the pellet fraction. The membranes were solubilized in a volume of 2% deoxycholate/1 mM-phenylmethane sulphonyl fluoride in Tris buffer (10 mM-Tris/HCl/0.02% NaN₃, pH 8.0) equal to the volume of the thymus homogenate. The mixture was stirred for 1 h, centrifuged at 70 000 g for 1 h and the supernatant was collected.

Affinity chromatography on concanavalin A–Sepharose

A column (12 ml) of concanavalin A–Sepharose 4B (10 mg of concanavalin A/ml of gel) was washed with elution buffer (see below) and with a large volume of 0.5% deoxycholate in Tris buffer (see above). The solubilized membranes (400 ml) were applied at a flow rate of 15 ml/h and the column was washed until $A_{280}^{1\text{cm}}$ was less than 0.025 (approx. 7 column volumes). Fractions (10 ml) were collected. The bound material was eluted with 0.5 M-methyl α-D-mannoside/1 M-ethylene glycol/0.5% deoxycholate in Tris buffer, at a flow rate of 3 ml/h. Before elution, the outlet from the concanavalin A column was connected to a column (2.5 ml) of Sepharose 4B to which was coupled anti-(concanavalin A) IgG (5 mg/ml of gel). Fractions (5 ml) were collected.

Gel filtration on Sephacryl S-200

Samples were concentrated to 3 ml and applied to a column (1.6 cm × 95 cm) of Sephacryl S-200 equilibrated with 0.5% deoxycholate in Tris buffer. The flow rate was 15 ml/h and 3 ml fractions were

collected. The column was previously calibrated with the following reference substances: Blue Dextran (void-volume marker), bovine serum albumin, ovalbumin, chymotrypsinogen A and ribonuclease A. Between two consecutive runs the gel was extensively washed with 0.5 M-methyl α -D-mannoside.

SDS/polyacrylamide-gel electrophoresis

Electrophoresis was performed on slab gels using the system described by Laemmli (1970). Stacking and separation gels consisted of 3 and 12.5% (w/v) total acrylamide respectively, with 2.5% (w/w) bis-acrylamide. The samples were boiled for 3 min in 1% SDS/1% 2-mercaptoethanol/10% glycerol/0.001% Bromophenol Blue/50 mM-Tris/HCl, pH 6.8. M_r markers were: phosphorylase *b* (M_r 94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soya-bean trypsin inhibitor (20 100) and α -lactalbumin (14 400). The gels were stained with Coomassie Blue.

Concentration

Concentration of samples was performed by ultrafiltration in an Amicon cell using a PM10 filter.

Protein determination

The protein content of the fractions was determined by the method described by Markwell *et al.* (1978), with bovine serum albumin as standard, or by amino acid analysis.

Amino acid analysis

Samples were hydrolysed for 24 or 48 h in 5.7 M-HCl at 110°C and analysed on a modified Beckman 120C amino acid analyser. Cysteine residues were measured as CmCys after reduction and carboxymethylation of the samples (Scoffone & Fontana, 1975). Degradation of serine and threonine was corrected for by extrapolating the values from 24 and 48 h hydrolysis to zero time.

Carbohydrate analysis

Samples were treated with 90% formic acid (100°C, 5 h) and hydrolysed with 0.25 M-H₂SO₄ at 100°C for 18 h. After reduction and acetylation the samples were analysed by g.l.c./mass spectrometry (Sawardeker *et al.*, 1965; Chizhov *et al.*, 1966). Sialic acid was determined by the colorimetric method described by Jourdian *et al.* (1971). Small amounts of glucose and arabinose were detected. These sugars were assumed to be contaminants and were not accounted for when calculating the carbohydrate content of Thy-1.

Results and discussion

Purification of Thy-1

The strategy for purification of Thy-1 from mouse thymus is shown in Scheme 1. The procedure

Scheme 1. Purification of Thy-1

Table 1. Purification of Thy-1 from mouse thymus. The values correspond to material from 23.8 g (wet weight) of mouse thymus. The protein content of the fraction obtained from the second gel filtration was measured by amino acid analysis; otherwise the method of Markwell *et al.* (1978) was used. Thy-1 content was determined by radioimmunoassay with pure mouse brain Thy-1 as standard.

Fraction	Total protein (mg)	Thy-1 (mg)	Yield (%)	Purification (fold)
Thymus homogenate	2600	5.2	100	1
3000 g supernatant	1400	5.0	96	1.8
Deoxycholate supernatant	160	3.4	65	11
Concanavalin A eluate	6.6	2.8	54	210
Gel filtration 1	3.1	2.6	50	420
Gel filtration 2	2.1	2.1	40	500

involved preparation of crude membranes by use of a weak detergent (Tween 20), which keeps the Thy-1 molecule membrane-bound (Strandberg & Williams, 1978), and subsequent solubilization of the membranes in deoxycholate. Glycoproteins with affinity for concanavalin A were isolated, and the Thy-1 molecule was purified by two gel-filtration cycles. The purification data are shown in Table 1.

In initial experiments, the radioimmunoassay was used to check the yield of Thy-1 during preparation and washings of thymocytes, and it was found that considerable amounts of the antigen were lost to the washing medium (up to 25%). This antigenic activity could be pelleted by centrifugation at 100 000 *g* for 1 h, which showed that it was present as aggregates, probably membrane-bound. Thy-1 has previously been shown to be rapidly shed into the incubation medium by culture of murine thymocytes *in vitro* (Vitetta *et al.*, 1974). This phenomenon could be a likely explanation for the losses during processing of the cells, even though the washings were performed in the cold. In order to avoid these losses, and to increase the recovery of Thy-1, whole thymi were used as starting material for purification. It can, however, be argued that Thy-1 may be present not only on thymocytes but also on other cells in the thymus and that the purified preparation would not be representative of the thymocyte molecule. In fact, one study has been presented demonstrating that cultured mouse thymus epithelial cells were able to absorb anti-(Thy-1) antibodies (Raedler *et al.*, 1978). It was, however, not shown in that study that the antigen was synthesized by the epithelial cells, and the presence of Thy-1 in these cultures can as well be explained by the presence of remnants from the dying thymocytes. If cells other than thymocytes are expressing Thy-1 in the thymus, this only contributes to a small extent, since the major part of Thy-1 in the thymus homogenate can be found on isolated thymocytes. Also, the biochemical characteristics of the preparation described in the present paper (see below) agree well with results of previous experiments in which isolated thymocytes were used (Carlsson & Stigbrand, 1982).

The reason for using Tween 20 in the membrane preparation instead of Tween 40 as originally described (Standring & Williams, 1978) was that concentrated Tween 40 solutions have a tendency to precipitate in the cold, and that the yield was somewhat increased, in our hands, by use of the former detergent.

The recovery of Thy-1 after membrane preparation and solubilization was 65% and an 11-fold purification was obtained.

Since concanavalin A was shown in a previous study to be the only lectin of several tested that was able to absorb all Thy-1 molecules (Carlsson & Stigbrand, 1982), a column of this lectin immobilized on Sepharose was used for further purification of Thy-1. The result is shown in Fig. 1. In order to recover the bound material in good yield from the column it was found necessary to include ethylene glycol in the elution buffer (beside a high concentration of competing sugar). By lowering the flow rate during elution ($0.7 \text{ ml} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$) the Thy-1 activity was eluted as a sharp peak. Some con-

Fig. 1. Affinity chromatography of deoxycholate solubilized thymus membranes on concanavalin A-Sepharose

Deoxycholate solubilized membranes (400 ml) were passed through a column of concanavalin A-Sepharose. After washing, the bound material was eluted with 0.5 M-methyl α -D-mannoside/1 M-ethylene glycol in 0.5% deoxycholate/Tris buffer and passed through a small column of anti-(concanavalin A) IgG-Sepharose. ●, $A_{280}^{1 \text{ cm}}$; △, Thy-1 concentration measured by radioimmunoassay. The absorbance of the elution buffer was 0.1.

canavalin A was leaking from the column even after extensive washing, and most of this contamination was removed by passing the eluate through a column of anti-(concanavalin A) IgG-Sepharose. Small amounts of concanavalin A still remaining in the eluate were subsequently effectively adsorbed on the gel-filtration matrix. The yield of Thy-1 in the concanavalin A-chromatography step was 82% and a further 19-fold purification was achieved.

Gel filtrations of the concanavalin A eluate were performed on a column of Sephacryl S-200 (Fig. 2). Most of the protein was eluted in or near the void volume of the column, whereas the Thy-1-detergent complexes eluted approximately in the same position as ovalbumin. Fractions with Thy-1 activity were pooled, concentrated and applied to the same column for a second gel filtration. Samples of all fractions with Thy-1 activity were analysed by SDS/polyacrylamide-gel electrophoresis, and those where no contaminants could be detected were pooled (fractions between 90 and 105 ml of eluate). The yield in each gel filtration was about 90%. The overall purification was 500-fold.

Characteristics of the purified preparation

The purified preparation was analysed by SDS/polyacrylamide-gel electrophoresis, together with other fractions obtained during purification (Fig. 3). From this gel it is seen that the isolated Thy-1 is very heterogeneous with regard to size. This phenomenon has been shown before, both for rat and

Fig. 2. Gel filtration on Sephacryl S-200

The eluate from the concanavalin A chromatography was concentrated and applied to a column (1.6 cm $\times$ 95 cm) of Sephacryl S-200, equilibrated with 0.5% deoxycholate/Tris buffer (a). The fractions between 87 ml and 105 ml of eluate were pooled, concentrated and rechromatographed on the same column (b). $\bullet$, $A_{280}^{1\text{cm}}$; Δ , Thy-1 concentration measured by radioimmunoassay. The arrows denote the elution of reference substances which were (from left to right): Blue Dextran, bovine serum albumin, ovalbumin, chymotrypsinogen A and ribonuclease A.

mouse thymocyte Thy-1 (Barclay *et al.*, 1976; Carlsson & Stigbrand, 1982). What can also be seen, however, is that this heterogeneous zone is divided into at least five distinct bands. The apparent M_r difference between the bands is approx. 1200. The difference in migration of the components on the gel reflected true size differences and was not due to charge effects, since SDS/polyacrylamide-gel electrophoresis of different fractions of the Thy-1 peak from the second gel filtration showed different staining patterns. The front fractions of Thy-1 showed an enrichment of bands in the 'high- M_r ' region, whereas in the later eluted fraction the 'low- M_r ' components were enriched (results not shown). The size heterogeneity is probably due to glycosylation differences, since after separation of Thy-1 on lentil-lectin-Sepharose, which binds only 50% of the molecules (Carlsson & Stigbrand, 1982), the unbound material showed a higher mean apparent M_r than the bound and eluted fraction (result not shown). The same observation has also

Fig. 3. SDS/polyacrylamide-gel electrophoresis of fractions obtained during purification

The following were electrophoresed on a 12.5% polyacrylamide slab gel: (a) 65 μg of whole thymus homogenate; (b) 75 μg of deoxycholate-solubilized membranes; (c) 30 μg of material eluted from concanavalin A; (d) 20 μg of pooled fractions from the first gel filtration; and (e) 15 μg of pooled fractions from the second gel filtration. The gel was stained with Coomassie Blue.

been reported for rat thymocyte Thy-1 (Barclay *et al.*, 1976). The structural basis for the differential binding to lentil lectin is not known and requires investigation.

M_r values of Thy-1 determined by SDS/polyacrylamide-gel electrophoresis have been shown to be overestimated by 35–40% (Kuchel *et al.*, 1978). The preparation described in the present paper migrated with an apparent M_r of 25 000–30 000, which is in accordance with previous results (Trowbridge *et al.*, 1975; Barclay *et al.*, 1976), but the true M_r is probably below 20 000. Since it is reasonable to suggest that the protein part of brain and thymocyte Thy-1 are identical (see below), this would account for 12 500 Da (Williams & Gagnon, 1982), and since the mean carbohydrate content was found to be 31% (see below), the mean M_r of mouse thymocyte Thy-1 would be 18 100. This is in agreement with the M_r for rat thymocyte Thy-1, which was reported to be 18 700 (Kuchel *et al.*, 1978). Taking the size heterogeneity into account, the molecules would probably range in molecular size from approx. 16 000 to 20 000 Da.

Amino acid and carbohydrate composition

The amino acid composition of the purified preparation is presented in Table 2, where the number of residues per molecule from the sequence data of mouse brain Thy-1 (Williams & Gagnon,

Table 2. *Amino acid composition of mouse thymocyte Thy-1, compared with published data for mouse brain Thy-1*

The value for each amino acid, except for CmCys, is the mean from a total of five analyses of three different preparations. One analysis was made after reduction and carboxymethylation for determination of CmCys. The samples were hydrolysed in 5.7M-HCl at 110°C for 24 h. Corrections for the destruction of threonine and serine were calculated after hydrolysis for 24 and 48 h by extrapolation to zero time. Data for mouse brain Thy-1.2 were taken from Williams & Gagnon (1982).

Amino acid	Composition		
	Mouse thymocyte Thy-1.2		Mouse brain Thy-1.2 (from sequence data) (residues/molecule)
	(mol %)	(residues/112 amino acids)	
Asx	12.5	14.0	14
Thr	8.8	9.8	10
Ser	10.0	11.2	11
Glx	10.4	11.7	11
Pro	3.7	4.1	3
Gly	4.6	5.2	4
Ala	3.5	3.9	3
CmCys	3.3	3.7	4
Val	6.4	7.2	8
Met	0.7	0.8	1
Ile	5.3	5.9	4
Leu	9.9	11.1	12
Tyr	2.9	3.3	4
Phe	2.5	2.8	3
Lys	7.0	7.8	9
His	2.7	3.0	4
Arg	5.8	6.5	7

Table 3. *Carbohydrate composition of mouse thymocyte Thy-1*

Sugar	Carbohydrate composition (residues per 112 amino acids)
Fucose	1.6
Mannose	9.2
Galactose	6.3
N-Acetylglucosamine	9.5
N-Acetylgalactosamine	0
Sialic acid	3.1
Total	29.7
Carbohydrate by weight (%)	31

1982) is also shown. The composition of thymocyte Thy-1 agrees well with that of brain Thy-1, and it is reasonable to suggest that the peptides are identical. Tryptophan was not measured, but since the u.v. spectrum of mouse thymocyte Thy-1 (results not

shown) was almost identical with that of the rat brain Thy-1 (Campbell *et al.*, 1981), which was shown to lack this amino acid, it is likely that the same is true for the mouse thymocyte analogue.

Carbohydrate-analysis data are shown in Table 3, where values are expressed as the number of sugar residues per 112 amino acids. The composition is consistent with the presence of *N*-linked 'complex-type' oligosaccharide chains. The high mannose value indicates that 'high-mannose' chains may also be present.

Carbohydrate-composition data for mouse Thy-1 isolated from brain (McClain *et al.*, 1978) and that of a lymphoblastoid cell line (BW 5147) (Zwerner *et al.*, 1977b) were published previously. Meaningful comparison with those data is, however, not possible, owing to inconsistencies in the published compositions. Although the calculations of the carbohydrate values were not stated in those studies, it seems that both brain and BW 5147 Thy-1 contain less sugar than thymocyte Thy-1. The ratio between the sugar residue values indicates differences in the carbohydrate structures between mouse brain and thymocyte Thy-1. A similar result was also obtained with rat Thy-1 (Barclay *et al.*, 1976). Both rat and mouse brain Thy-1 contain approximately one residue of *N*-acetylgalactosamine, which indicates the presence of an *O*-linked chain. This sugar is absent from thymocyte Thy-1 of both species. The sugar content and composition of rat thymocyte Thy-1 is about the same as that of the preparation described in the present paper.

Thy-1 isolated from the BW 5147 cell line has often been thought of as being representative of that occurring in mouse lymphocytes, but several studies have shown that Thy-1 expressed by the tumour cell is different from normal thymocyte and peripheral lymphocyte Thy-1 (Zwerner *et al.*, 1977a; Carlsson & Stigbrand, 1982, 1983). Besides an apparent difference in carbohydrate content, the appearance on SDS/polyacrylamide gels, isoelectric point and lectin-binding characteristics are different. The latter phenomena strongly indicate differences in the structure of the oligosaccharide chains.

The total M_r of the carbohydrate moiety of thymocyte Thy-1 can be calculated to be approx. 5600. This would account for either two or three *N*-linked oligosaccharide chains (Kornfeld & Kornfeld, 1980). From the sequence data of mouse brain Thy-1 it was indirectly shown that asparagine at positions 23, 75 and 99 was linked to carbohydrate (Williams & Gagnon, 1982), and for those positions the proposed sequence requirement for carbohydrate attachment, i.e. -Asn-Xaa-Thr/Ser- (Struck & Lennartz, 1980), was fulfilled. No other positions showed this sequence. It is therefore likely, especially since thymocyte Thy-1 seems to contain more carbohydrate than the brain counterpart, that the

same three positions are also occupied by carbohydrate in the thymocyte molecule. It should be stressed, however, that the values for carbohydrate content, weight and composition are means, since a considerable heterogeneity has been shown to exist (Carlsson & Stigbrand, 1982). This could mean that one or two chains are missing at any position in some of the molecules or that the structures of the three oligosaccharide chains differ between molecules.

Further studies on the carbohydrate structure of mouse lymphocyte Thy-1 are required to elucidate changes in the glycosylation pattern during differentiation of T-lymphocytes and furthermore to establish the basis for the heterogeneity of the Thy-1 molecule.

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