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The purification and characterization of a Thy-1-like glycoprotein from chicken brain

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We have purified from chicken forebrain a membrane glycoprotein that is enriched in purified synaptic membranes and has an apparent mol.wt. of 22800 in 15% sodium dodecyl sulphate/polyacrylamide gels. This molecule was compared with rat and human brain Thy-1 glycoproteins purified by the same procedure in order to determine whether it could be a homologue of Thy-1. Although polyvalent heterologous antisera raised against the rat and chicken molecules showed no immunological cross-reactivity with the other glycoprotein, a great deal of physical and chemical similarity was demonstrated between the chicken glycoprotein and rat Thy-1. Their apparent molecular weights, subcellular localization and amino acid and amino sugar compositions are very similar. C.d. spectra show that both molecules contain predominantly a β-sheet and structure with no detectable α-helix. Electrophoretic analysis of the CNBr-cleaved molecules under reducing and non-reducing conditions shows that both molecules contain intramolecular disulphide bridges. Taken together these results suggest that the chicken brain glycoprotein is an immunologically distinct homologue of the mammalian Thy-1 glycoproteins.

Reif & Allen (1964) discovered a shared antigenic determinant between mouse thymus and brain, which they called Θ and which is now generally referred to as Thy-1. The molecular identity of the Thy-1 antigen was first determined from rat tissues. It was shown that all of the antigenic activity in rat brain and thymus could be recovered in a low-molecular-weight glycoprotein that contains approx. 30% (by weight) carbohydrate (Letarte-Muirhead *et al.*, 1975; Barclay *et al.*, 1975, 1976). The function of Thy-1 is not known, but it has been postulated to be involved in specific cell–cell interactions (Dulbecco *et al.*, 1979; Cohen *et al.*, 1981). The Thy-1 glycoproteins from rat thymus and brain appear to contain a common polypeptide chain of 111 amino acids, which has been sequenced (Campbell *et al.*, 1981), but different carbohydrate chains (Barclay *et al.*, 1976). Homologous glycoproteins have also been purified from mouse and human tissues (McClain *et al.*, 1978; Cotmore *et al.*, 1981).

Abbreviation used: SDS, sodium dodecyl sulphate.

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Although homologous glycoproteins exist in other mammalian species the tissue distribution of the Thy-1 glycoproteins in different species varies widely and only one tissue, the brain, consistently expresses high levels of Thy-1 in all species so far examined (for review, see Campbell *et al.*, 1981). The interspecies variation is best illustrated by lymphoid tissue. In mice, Thy-1 is present on thymocytes and T lymphocytes but absent from stem cells (Raff, 1971), whereas in rats, Thy-1 is present on thymocytes and a small subset of bone marrow cells (Williams, 1976) but is absent from T lymphocytes (Acton *et al.*, 1974). By contrast, dog thymocytes express only reduced amounts of Thy-1 and Thy-1 is not detectable at all on human thymocytes (Dalchau & Fabre, 1979). The presence of Thy-1 in non-mammalian vertebrate species has not yet been reported.

Wick & Shauenstein (1974) claimed that chickens did not possess a Θ antigen because chicken brain and thymus had no cross-reactive antigenic determinants, a finding confirmed by Rostas & Jeffrey (1977). In view of the subsequent data on the lack of expression of Thy-1 on the thymocytes of some species the conclusions of Wick & Shauenstein (1974) were clearly not valid. Acton *et al.* (1978)

reported the presence of Thy-1 in synaptic plasma membranes and synaptic junctions prepared from mouse brain. The subsequent demonstration by Rostas *et al.* (1979) that the protein and glycoprotein composition of synaptic junctions prepared from different species was highly conserved during evolution, and that chicken synaptic plasma membrane and synaptic junction fractions contain a concanavalin A-binding glycoprotein of similar molecular weight to Thy-1, suggested that chickens may in fact express Thy-1 in brain but not in thymus. The aim of the present work was to purify the concanavalin A-binding glycoprotein from chicken synaptic junctions and determine whether this molecule is an avian homologue of Thy-1.

Materials and methods

Chemicals

Sodium deoxycholate and bovine serum albumin were purchased from Sigma Chemical Co., St Louis, MO, U.S.A. Lentil lectin–Sepharose 4B and Sephacryl S-200 (superfine) were from Pharmacia Fine Chemicals, Uppsala, Sweden. Concanavalin A was from Boehringer–Mannheim, Munich, Germany. SDS was from BDH Chemicals, Poole, Dorset, U.K. CNBr was from Ajax Chemicals, Sydney, N.S.W., Australia. [^{125}I]Iodine was from New England Nuclear (Searle Nucleonics, Sydney, N.S.W., Australia).

Animals

Cockerel chickens (4 days old) of the 'Hyline' commercial egg-laying breed, whose predominant genetic stock is New Hampshire Red × White Leghorn, were obtained from K & L Thomas' Hatcheries, Cranbourne, Victoria, Australia. Adult Sprague–Dawley rats of either sex (approx. 250g) were obtained from the Monash University animal house. Human post-mortem frontal cortex was obtained from an elderly male, courtesy of Prince Henry's Hospital, Melbourne, Victoria, Australia.

Purification of Thy-1 glycoproteins

Our procedure for the purification of Thy-1 glycoproteins from chicken, rat and human brain was adapted from the methods of Barclay *et al.* (1975) and McClain *et al.* (1978). Unless otherwise stated all procedures were carried out at 4°C.

Crude membrane preparation and solubilization in deoxycholate. The forebrains of 50 chickens, 35 rats or 25g of human frontal cortex were homogenized at 1000rev./min with a motor-driven Teflon pestle in 10vol. of 0.32M-sucrose, pH7. Nuclei and cellular debris were removed by centrifugation for 16000*g*-min and the pellet obtained was washed once with the same sucrose solution. The pooled supernatants were centrifuged for 8×10^6 *g*-min and the membrane pellet obtained was delipidated with acetone precooled to −20°C using a Servall Omnimixer. The resultant protein powder was extracted with 150ml of 2% (w/v) sodium deoxycholate/0.02% (w/v) NaN_3/0.01M-Tris/HCl, pH8.0. The mixture was frozen and stored over night at −20°C. After thawing it was centrifuged at 8×10^6 *g*-min to remove the deoxycholate-insoluble material. Before column chromatography, the deoxycholate concentration of this solution was lowered to 0.5% by the addition of 0.26% (w/v) deoxycholate/0.02% (w/v) NaN_3/0.01M-Tris/HCl buffer, pH8.

Chromatography. The detergent extract was applied to a lentil lectin–Sepharose 4B column (8.0cm × 2.6cm) at a constant flow rate of 30ml/h. The column was washed with 0.5% (w/v) deoxycholate buffer until the absorbance of the effluent had returned to the baseline value. The bound material was then eluted with 0.2M-1-methyl α-D-glucopyranoside in 1.0% (w/v) sodium deoxycholate buffer at 40ml/h. The eluted material was concentrated to a volume of approximately 5ml using an Amicon model 52 ultrafiltration cell and a PM 10 membrane at a pressure of 190kPa. The concentrate was applied to a Sephacryl S-200 column (84cm × 2.6cm) at 33ml/h. Fractions (4.5ml) were collected, pooled and concentrated using the Amicon ultrafiltration system. The Thy-1 glycoprotein fraction obtained at this stage was approx. 60–70% pure as judged by polyacrylamide-gel electrophoresis and was used for the production of antisera, immunodiffusion experiments, CNBr-cleavage experiments and c.d. spectra. However, for amino acid analyses, pure Thy-1 was isolated free of the contaminants by preparative SDS/polyacrylamide-gel electrophoresis.

SDS/polyacrylamide-gel electrophoresis. Slab gels of the required concentration were cast from acrylamide stock solutions containing 29.2% (w/v) acrylamide and 0.8% (w/v) *NN*-methylenebisacrylamide using the apparatus described by Studier (1973) and the discontinuous SDS buffer system developed by Laemmli (1970). Where gradient gels were required these were poured using the apparatus described by Kelly & Luttges (1975). Slab gels were stained in 0.2% (w/v) Coomassie Brilliant Blue R containing 50% (w/v) trichloroacetic acid for 20min followed by destaining in repeated washes of 7% (v/v) acetic acid.

Purification of Thy-1 by preparative electrophoresis. After gel filtration the final concentrate containing Thy-1 was applied as a single sample across the entire width of a 2mm thick 15% polyacrylamide slab gel. After electrophoresis thin strips were cut from each side of the gel slab and stained in the usual way, while the bulk of the gel was frozen at −70°C. After destaining the strips,

they were aligned with the thawed bulk of the gel and the part of the gel containing the Thy-1 band was excised with a razor blade. The unstained gel containing the Thy-1 band was immediately cut into small pieces and polymerized into cylindrical gels containing the same polyacrylamide-gel concentration. Dialysis bags were placed over the bottom of each cylindrical gel and, using the same buffer system, the Thy-1 glycoprotein was electrophoretically eluted from the gel at a constant current of 30mA into the dialysis bags. The dialysis bags were removed from the bottom of the cylindrical gels and the sample was exhaustively dialysed against 0.1% SDS to remove the glycine present in the electrophoresis running buffer. The sample was freeze-dried, rehydrated in a small volume and again dialysed against 0.1% SDS to reduce the SDS concentration in the sample. Blank samples prepared from cylindrical gels containing no Thy-1 glycoprotein were used as controls.

Amino acid analysis

Samples (200μg) of purified dialysed samples were hydrolysed in 6M-HCl at 110°C for 20, 24, or 72h. The hydrolysed samples were dried under vacuum, and amino acid analysis was performed in an automatic Jeol JLX-6AH amino acid analyser equipped with an integrator.

Protein estimation

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (concentration determined by using A_{280} 0.6 for a 1mg/ml solution) as standard.

Autoradiography

Concanavalin A-binding glycoproteins present in samples run on SDS/polyacrylamide gels were detected by autoradiography of gels incubated with ^{125}I-concanavalin A (Rostas *et al.*, 1977). Concanavalin A was iodinated by the chloramine-T method of Greenwood *et al.* (1963) and affinity-purified by the batch procedure described by Rostas *et al.* (1979).

CNBr cleavage

To 200μg of freeze-dried samples was added 200μl of 70% (v/v) formic acid containing excess CNBr. Uncleaved controls contained formic acid without CNBr. The samples were incubated at 20°C for 24h and the digests were then diluted with 1ml of water, freeze-dried and rehydrated twice to ensure removal of formic acid. Samples were then rehydrated in sample buffer and analysed by SDS/polyacrylamide-gel electrophoresis on 12–22% linear gradient gel slabs. The gels were stained for protein with Coomassie Blue and incubated with ^{125}I-concanavalin A to detect concanavalin A-binding glycoproteins.

C.d. spectra

C.d. spectra of the final concentrates from the Sephacryl column were obtained with a Jobin–Yvon dicrograph IIIS at 20°c over the wavelength range 190–250nm. Solutions of the proteins with 0.5% (w/v) deoxycholate/0.02% (w/v) NaN_3/0.01M-Tris/HCl, pH8.0, were used at a concentration of approx. 1mg/ml in cells of 0.1mm pathlengths. Spectra were corrected for baseline effects.

Immunodiffusion

Ouchterlony double immunodiffusion (Ouchterlony & Nillson, 1973) was carried out using agar gels containing 0.5% (w/v) sodium deoxycholate. All antigen samples were solubilized and diluted in 0.5% (w/v) sodium deoxycholate. Synaptic plasma membranes, synaptic junctions, post-synaptic densities and Triton X-100 soluble fractions were prepared from chicken brain by the methods of Cotman & Taylor (1972) and Cotman *et al.* (1974).

Production of antisera

Antisera against the chicken Thy-1-like glycoprotein were raised in 6-month-old female New Zealand White rabbits. The final concentrate from the gel-filtration column was freeze-dried and treated with ice-cold ethanol to remove the deoxycholate from the pellet. The pellet was resuspended in distilled water and emulsified with an equal volume of adjuvant. Each rabbit was injected with approx. 150μg of antigen in Freund's complete adjuvant given intramuscularly into one thigh. This was repeated 7 days later in the other thigh. After a further 3 weeks approx. 150μg of antigen in Freund's incomplete adjuvant was given subcutaneously and the animal was bled 1week later. Rabbit antiserum against purified Thy-1 glycoprotein from rat brain was a gift from Dr. R. J. Morris, National Institute from Medical Research, Mill Hill, London NW7 1AA, U.K.

Results

Purification of Thy-1-like glycoprotein

In order to maximize yields, a crude membrane fraction from whole brain instead of purified synaptic membranes was used as the starting material. The purification procedure was adapted from the methods of Barclay *et al.* (1975) and McClain *et al.* (1978) for purifying Thy-1 glycoproteins from rat and mouse brains respectively.

After delipidation approx. 80% of the protein in the acetone-powdered membranes was solubilized by the deoxycholate. Approx. 95% of the protein in the deoxycholate extract was not retained by the lentil lectin affinity column (Fig. 1*a*). Most of the protein that bound and was subsequently eluted with sugar

from the lentil lectin column was of high molecular weight, or aggregated, in deoxycholate because it emerged from the Sephacryl S-200 column in or shortly after the void volume (Fig. 1*b*). The pooled material indicated in Fig. 1(*b*) was taken as the final glycoprotein fraction. From chicken brain this fraction contained 6–8% of the protein applied to the Sephacryl column and consisted of predominantly one diffuse protein band on SDS/polyacrylamide gels (Fig. 2*b*). This band accounted for 60–70% of the total protein in the fraction, as judged by densitometry of Coomassie Brilliant Blue-stained gels. This glycoprotein had an apparent molecular weight that was very similar to that of rat brain Thy-1 (Table 1, Fig. 2*b*) and was purified from chicken brain within a yield of approx. 0.5mg of protein per 10g wet wt.

In order to validate the purification procedure and to provide Thy-1 glycoprotein to use as a reference standard in studies of the chicken glycoprotein, the same purification procedure was applied to rat and human brain. The final glycoprotein fraction ob-

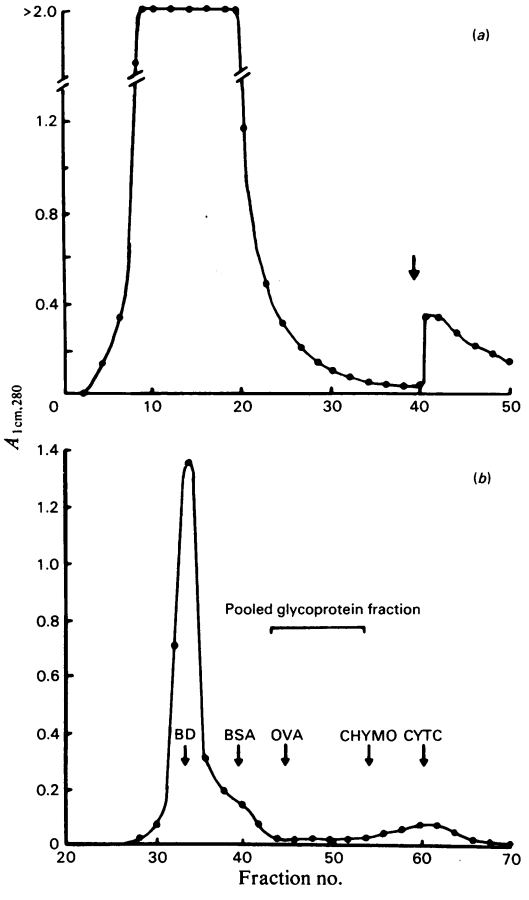

Fig. 1. *Purification of glycoprotein fraction*
Results shown are from a chicken brain preparation but are representative of preparations from rat and human. (*a*) Affinity chromatography of deoxycholate extract on lentil lectin–Sepharose 4B; 600ml of crude membrane extract was applied to a 50ml column (8.0cm × 2.6cm) at 30ml/h in 0.5% deoxycholate/0.02% NaN_3/0.01M-Tris/HCl, pH8.0. Elution of the bound material with 0.2M-1-methyl α-D-glucopyranoside/1.0% deoxycholate/0.02% NaN_3/0.01M-Tris/HCl, pH8.0, was begun at the point indicated by the arrow. (*b*) Chromatography of lentil lectin eluate on Sephacryl S-200; 5ml of concentrated eluate was applied to a column (84cm × 2.6cm) in 0.5% deoxycholate/0.02% NaN_3/0.01M-Tris/HCl, pH8.0, at 33ml/h. The fractions that were pooled to produce the final glycoprotein fraction are indicated, as are the elution positions of molecular-weight markers: BD, Blue Dextran; BSA, bovine serum albumin; OVA, ovalbumin; CHYMO, chymotrypsinogen; CYTC, cytochrome *c*.

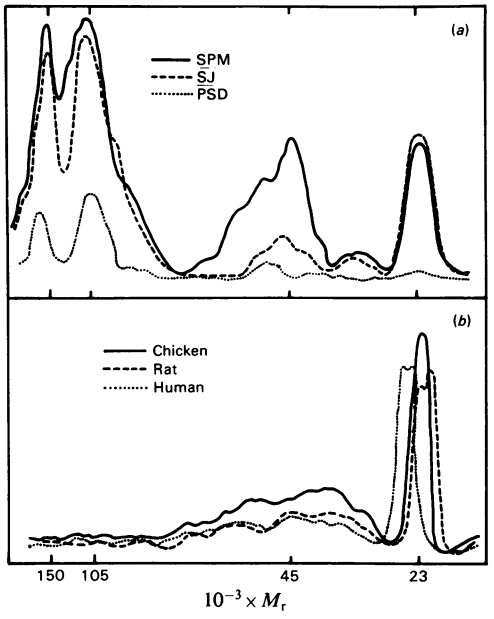

Fig. 2. *Electrophoresis of glycoproteins of chicken brain synaptic membrane fractions and final glycoprotein fractions from chicken, rat and human brain*
(*a*) Autoradiogram scan of ^{125}I-concanavalin A bound to 55μg each of synaptic plasma membranes (SPM), synaptic junctions (SJ) and post-synaptic densities (PSD) from chicken brain electrophoresed in an 8–17.5% linear/exponential polyacrylamide gel slab. The autoradiogram was scanned at 520nm. (*b*) Electrophoretogram of the final glycoprotein fractions from chicken, rat and human brain separated in an 8–17.5% linear/exponential polyacrylamide gel slab. The gel was stained with Coomassie Brilliant Blue and the photograph of the gel was scanned at 520nm.

tained from rat brain contained at least 70% Thy-1, as identified by immunoreactivity with an antiserum raised against rat brain Thy-1 glycoprotein prepared by the method of Barclay *et al.* (1975), and by its electrophoretic mobility on SDS/polyacrylamide gels. The yield of rat brain Thy-1 was the same as that for the chicken brain glycoprotein. The procedure also produced the human Thy-1 homologue in similar yield and purity from post-mortem brain. In this case the molecule was identified as the Thy-1 homologue by its cross-reactivity with the anti-(rat Thy-1) serum and by its electrophoretic mobility on SDS/polyacrylamide gels (Cotmore *et al.*, 1981).

Immunological cross-reactivity

The simplest test of homology between the chicken brain glycoprotein and rat brain Thy-1 is whether antisera raised against one of the glycoproteins would react with the other glycoprotein. An antiserum was raised in rabbits against the final glycoprotein fraction from chicken brain, and a specific antiserum against rat brain Thy-1 was kindly provided by Dr. R. J. Morris. Both antisera produced a single precipitin line by double immunodiffusion (Fig. 3*a*) and a single precipitin arc by immunoelectrophoresis (results not shown) against their respective antigens. The cross-reactivities of these sera with the glycoprotein fractions are shown in Fig. 3(*a*). The anti-(chicken glycoprotein) serum showed no cross-reactivity with either rat or human brain glycoprotein fractions. The anti-(rat Thy-1) serum cross-reacted with the human but not with the chicken glycoprotein fraction. The lack of immunological cross-reactivity between the rat and chicken glycoproteins was confirmed by immunofluorescence and a solid-phase enzyme-linked immunoassay (P. L. Jeffrey, D. I. Greig & J. A. P. Rostas, unpublished work).

Identity of the purified glycoprotein with the concanavalin A-binding glycoprotein of synaptic membranes

The purified chicken brain glycoprotein bound ^{125}I-concanavalin A (Fig. 5) and the bands on the autoradiogram were coincident with the protein band stained with Coomassie Brilliant Blue. The chicken brain glycoprotein had the same electrophoretic mobility in SDS/polyacrylamide gels as the low-molecular-weight concanavalin A-binding glycoprotein of synaptic membranes (Fig. 2). The relative amounts of the concanavalin A-binding glycoprotein in the three synaptic membrane fractions (synaptic plasma membranes, synaptic junctions, post-synaptic densities) are indicated by the densitometric scans of the autoradiograms produced after the binding of ^{125}I-concanavalin A to equal amounts of each of the fractions after electrophoresis in SDS/polyacrylamide gels (Fig. 2*a*). The molecule is a major glycoprotein of both synaptic plasma membrane and synaptic junction fractions but is barely detectable in post-synaptic density fractions. It must be remembered that although these concanavalin A-binding glycoproteins are major

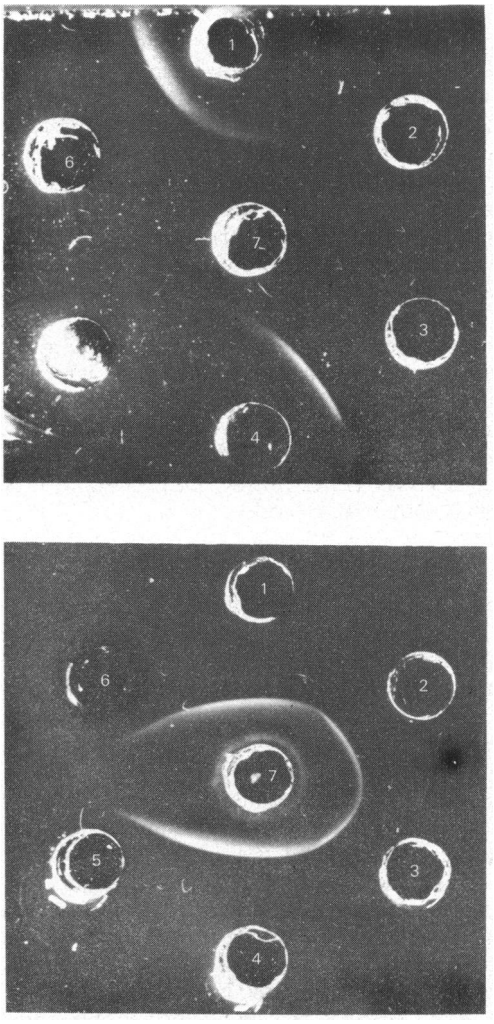

Fig. 3. *Species cross-reactivity of anti-(chicken glycoprotein) and anti-(rat Thy-1) sera (a) and reactivity of anti-(chicken glycoprotein) with chicken brain synaptic membrane fractions (b) analysed by immunodiffusion*
(*a*) The wells contained: 1, anti-(rat Thy-1); 2 and 3, chicken glycoprotein fraction (1.1 mg/ml); 4, anti-(chicken brain glycoprotein); 5 and 6, rat glycoprotein fraction (1.0 mg/ml); 7, human glycoprotein fraction (1.8 mg/ml). (*b*) The wells contained: 1 and 4, chicken glycoprotein fraction (1.1 mg/ml); 2, synaptic plasma membranes (3.9 mg/ml); 3, synaptic junctions (5.5 mg/ml); 5, post-synaptic densities (4.0 mg/ml); 6, Triton-soluble fraction from synaptic plasma membranes (1.3 mg/ml); 7, anti-(chicken glycoprotein).

glycoproteins in these membrane fractions they are very minor proteins (Kelly & Cotman, 1977; Rostas *et al.*, 1979). The reactivity of the antiserum raised against the chicken brain glycoprotein fraction with the synaptic membrane fractions was consistent with the distribution of the low-molecular-weight concanavalin A-binding glycoprotein (Fig. 3*b*): a strong single precipitin line was obtained with synaptic plasma membranes and synaptic junctions but post-synaptic densities showed little, if any, reactivity. The lines were continuous with each other and with the line produced against the final glycoprotein fractions. The weak precipitin line formation with the Triton X-100-soluble fraction from synaptic plasma membranes is probably due to the low protein concentration of this sample. In view of the small size of the glycoprotein, the presence of good precipitin lines probably indicates that the molecule is in an aggregated form in the agarose gel; low protein concentrations and the presence of a second detergent type would be expected to inhibit such interactions. The relative immunoreactivities of this antiserum with the synaptic membrane fractions has been confirmed using a solid-phase enzyme-linked immunoassay (P. L. Jeffrey, D. I. Greig & J. A. P. Rostas, unpublished work).

Physical and chemical similarities between the chicken glycoprotein and rat Thy-1

Apparent molecular weight. The glycoproteins isolated from the brains of the three species all had similar apparent molecular weights on SDS/polyacrylamide gels (Fig. 2*b*, Table 1). The molecules from rat and human brain usually migrated as a doublet, especially on gradient gels. This is in agreement with the results of Kuchel *et al.* (1978) and Cotmore *et al.* (1981). By contrast, the chicken glycoprotein appeared as a single diffuse band. Table 1 shows the apparent molecular weights of the chicken and rat glycoproteins on polyacrylamide gels of different acrylamide concentrations as determined by calibration against standard proteins run in the same gels. As reported by Williams *et al.* (1977), rat Thy-1 shows a progressive decrease in apparent molecular weight with increasing acrylamide gel concentration. The chicken brain glycoprotein shows the same behaviour and to the same extent.

Table 1. *Apparent molecular weight of chicken glycoprotein and rat Thy-1*

	Apparent molecular weight		
Acrylamide (%) ...	15	12.5	10
Chicken glycoprotein	22800	23700	24100
Rat brain Thy-1	23300	23700	24100
	21900	22800	23500

C.d. spectra

The secondary structure of Thy-1 is unusual for a membrane glycoprotein in that it contains no detectable α-helix and consists mainly of a β-pleated sheet. This results in a highly characteristic c.d. spectrum (Campbell *et al.*, 1979). The spectra of the chicken, rat and human glycoprotein fractions are shown in Fig. 4. All exhibited a negative peak at approx. 213nm and had the same form, which indicated primarily β-structure with no detectable α-helix (Greenfield & Fasman, 1969). The spectrum for the rat brain glycoprotein fraction agrees well with the data of Campbell *et al.* (1979) in form and amplitude, but appears to be slightly blue-shifted since these authors found the negative maximum to occur at 217nm. The reason for this difference is probably the presence of small quantities of contaminating proteins in the glycoprotein fraction and/or the presence of the detergent buffer solution. The important observation, however, is that the chicken glycoprotein showed a similar c.d. spectrum to that of rat Thy-1. The magnitude of the negative peak of the human glycoprotein's c.d. spectrum was considerably less than that of the other two, but was still within the range exhibited by homologous proteins (Campbell *et al.*, 1979).

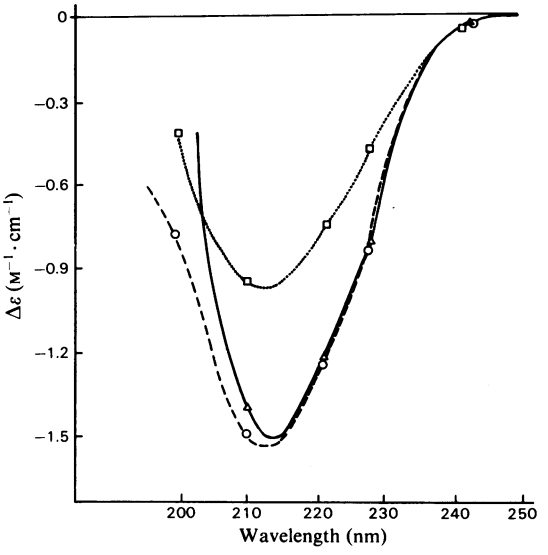

Fig. 4. *C.d. spectra of glycoprotein fractions from chicken* (△), *rat* (○) *and human* (□) *brain*
Spectra were run at 20°C using a cell of 0.1mm pathlength in the presence of 0.5% deoxycholate/0.02% NaN_3/0.01M-Tri/HCl, pH8.0, at a protein concentration of 1mg/ml.

Amino acid composition

The rat Thy-1 and chicken Thy-1-like glycoproteins were electrophoretically purified from the final glycoprotein fractions and analysed for amino acid content as described in the Materials and methods section. The results are shown in Table 2, together with a previously published amino acid analysis of rat Thy-1 (Barclay *et al.*, 1976) and the amino acid composition determined from the complete sequence of the rat molecule (Campbell *et al.*, 1981). The serine, threonine and methionine values were extrapolated back to zero time of hydrolysis to account for the degradation of these residues under prolonged acidic conditions. The valine, isoleucine and phenylalanine values were taken as found in the 72-h hydrolysate, and the values for the other amino acids were averaged over the three hydrolysis times. The hexosamine values were taken from the 20-h hydrolysate. It is clear that the chicken glycoprotein has a very similar amino acid and amino sugar composition to that of the rat Thy-1 glycoprotein (Table 2). Cysteine residues are very important in the structure of Thy-1, since they form two intrachain disulphide links. Cysteine was not measured directly but the chicken molecule clearly contains cysteine residues as indicated by the CNBr-cleavage experiments.

CNBr cleavage

A characteristic feature of the rat Thy-1 molecule is that it can be cleaved by CNBr at the single methionine residue 84 into two peptides, which are held together by internal disulphide bonds so that they can only be resolved by electrophoresis if the sample is first reduced (Kuchel *et al.*, 1978). Fig. 5 indicates that the chicken brain glycoprotein has very similar properties. When electrophoresis was carried out without reduction the apparent molecular weight of the cleaved sample was unchanged. However, in the presence of 2-mercaptoethanol lower-molecular-weight peptides appeared. ^{125}I-Concanavalin A binding to the cleaved rat Thy-1 sample revealed a carbohydrate distribution similar to that obtained by Kuchel *et al.* (1978) using periodic acid/Schiff staining. The peptide pattern obtained after cleavage was the same if Coomassie Blue or ^{125}I-Concanavalin A was used to visually detect it; Fig. 5 shows the picture obtained with the

Table 2. *Amino acid composition of chicken and rat preparations*

Abbreviations used: ND, not determined; Cys(O_3H), cysteic acid.

	Amino acid content (residues/100 residues)			
	Chicken glycoprotein	Rat glycoprotein	Rat Thy-1*	Rat Thy-1†
Asx	12.4	12.9	12.7	13.5
Glx	9.2	11.3	9.1	9.0
His	2.6	3.3	4.1	3.6
Lys	7.6	7.3	6.9	7.2
Arg	5.7	6.8	7.5	8.1
Thr	8.8	9.0	7.6	9.0
Ser	10.4	9.0	7.4	7.2
Pro	2.9	4.3	3.6	2.7
Ala	5.0	3.5	3.3	1.8
Cys(O_3H)	ND	ND	3.1	3.6
Gly	5.5	7.6	6.0	3.6
Tyr	4.7	2.2	2.0	1.8
Val	6.1	7.5	7.3	8.1
Ile	5.8	5.6	3.9	4.5
Leu	11.0	11.4	10.4	11.7
Phe	2.8	3.6	4.0	3.6
Met	2.0	0.9	1.1	0.9
Glucosamine	7.1	11.7	8.3	
Galactosamine	1.9	1.4	1.0	

* From Barclay *et al.* (1976).

† Calculated from the amino acid sequence of rat brain Thy-1 in Campbell *et al.* (1981).

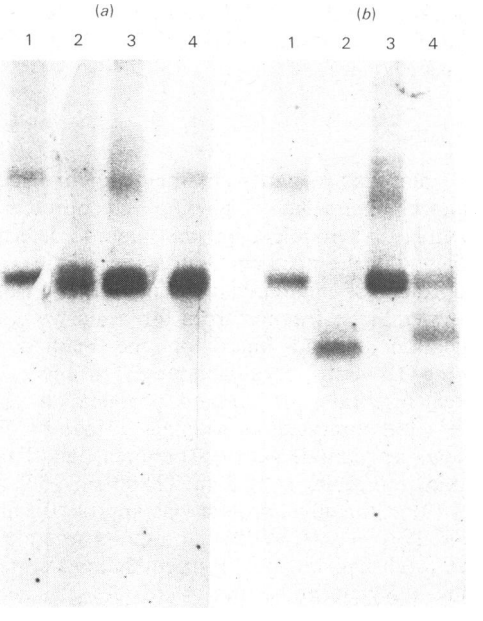

Fig. 5. *CNBr cleavage of chicken and rat glycoprotein fractions*

Autoradiograms of ^{125}I-concanavalin A bound to chicken and rat glycoproteins electrophoresed in 12–22% linear SDS/polyacrylamide slab gels run under non-reducing (*a*) or reducing (*b*) conditions. The gels contained: 1, control chicken glycoprotein; 2, cleaved chicken glycoprotein; 3, control rat glycoprotein; 4, cleaved rat glycoprotein.

latter, more sensitive, method. The major carbohydrate-containing fragment obtained from the chicken glycoprotein had a slightly lower apparent molecular weight than the major glycopeptide from rat Thy-1, but whether this indicates a different location of the methionine residue or merely a difference in the bound carbohydrate is not clear. As the amino acid analysis indicated that the chicken molecule may have two methionine residues it was possible that the CNBr cleavage would produce three peptides. However, these would only be detectable if (*a*) the combined effects of the peptide chains and their attached carbohydrate residues resulted in distinctly different electrophoretic mobilities for each peptide and (*b*) one of the methionine residues was not so close to one end of the molecule that the resulting small peptide was lost from the gel during the fixation and staining. Thus the lack of three clearly visible peptides after CNBr cleavage is not evidence against the existence of two methionine residues. Similarly, the fact that identical cleavage conditions resulted in almost complete cleavage of the chicken molecule, whereas a substantial proportion of the rat Thy-1 remained uncleaved (Fig. 5; Kuchel *et al.*, 1978), is consistent with the existence of two methionine residues in the chicken molecule, but does not prove it, since a single methionine residue could simply be in a less chemically protected position in the chicken molecule.

Discussion

Our aim was to purify the major low-molecular-weight concanavalin A-binding glycoprotein of chicken brain synaptic membranes and to determine whether it is the chicken homologue of the Thy-1 glycoprotein of mammalian brain. Starting from a crude membrane fraction from forebrain we used a purification method based on the methods for isolating Thy-1 from rat and mouse brains. From chicken forebrain the method produced a glycoprotein that migrated as a single diffuse band on SDS/polyacrylamide gel electrophoresis and had an apparent molecular weight of 22800 on 15% gels. The yield of this glycoprotein was approx. 0.5 mg of protein/10 g wet wt. When the method was applied to rat forebrain and post-mortem human cortex it produced glycoproteins that were identified as rat and human Thy-1 respectively in yields similar to that of chicken and comparable with that reported by others for rat and human Thy-1 (Barclay *et al.*, 1975; Cotmore *et al.*, 1981).

The first task in our investigation was to establish that the glycoprotein purified from the crude membrane fraction was the same as the major low-molecular-weight concanavalin A-binding glycoprotein from synaptic membranes. The purified glycoprotein had the same electrophoretic mobility as the synaptic membrane glycoprotein (Fig. 2) and it also bound concanavalin A (Fig. 5). An antiserum raised against the isolated glycoprotein fraction showed immunological reactivity against synaptic membrane fractions that contain the concanavalin A-binding glycoprotein but not against those that did not. Fig. 2(*a*) shows that purified synaptic plasma membranes and synaptic junctions contain considerable quantities of the glycoprotein but isolated post-synaptic densities do not. Fig. 3(*b*) shows that by immunodiffusion the antiserum against the purified glycoprotein fraction reacted strongly with synaptic plasma membranes and synaptic junctions but not with post-synaptic densities. We have now confirmed and extended this result by using immunofluorescence histochemistry and quantitative enzyme-linked immunosorbent assay ('ELISA') based on the inhibition of indirect binding assays of Morris & Williams (1975). The glycoprotein is 100% membrane bound and present in brain with the highest specific activity in purified synaptic plasma membranes and present in high concentration in areas of tissue known to contain large numbers of synapses (P. L. Jeffrey, D. I. Greig & J. A. P. Rostas, unpublished work). Considering all the-above data, there seems little doubt that the purified glycoprotein is in fact the major low-molecular-weight concanavalin A-binding glycoprotein of synaptic membranes. The second task was to look for evidence of homology between the chicken brain glycoprotein and the Thy-1 isolated from rat brain using the same procedure.

With the availability of specific polyvalent antisera the simplest proof of homology would have been the demonstration that the two glycoproteins were immunologically cross-reactive. Fig. 3 shows that neither the anti-(chicken glycoprotein) nor the anti-(rat Thy-1) serum recognized the other glycoprotein. Nevertheless the anti-(rat Thy-1) serum showed good reactivity with the human Thy-1 homologue, even though only a minority of the antibodies in such sera are directed against cross-reactive antigens (Dalchau & Fabre, 1979; Cotmore *et al.*, 1981; Kemshead *et al.*, 1982). Although immunological cross-reactivity would have been strong evidence in favour of homology, this is not a necessary condition for homology. Homologous molecules from quite closely related species can sometimes be immunologically distinct. The major high-molecular-weight glycoprotein of lymphoid cells in the mouse and rat, the T200 glycoprotein and the leucocyte common antigen, are not immunologically cross-reactive (Trowbridge & Mazauskas, 1976; Sunderland *et al.*, 1979). The Thy-1 glycoprotein itself has several antigenic determinants, some of which vary even between strains of mice (Williams *et al.*, 1977). Only 20–30% of the

antibodies in a polyvalent antiserum against rat thymocyte Thy-1 recognize human brain Thy-1 (Cotmore *et al.*, 1981) and monoclonal antibodies that are specific for each of the rat and human glycoproteins have been produced (McKenzie & Fabre, 1981). Thus we compared the chemical and physical properties of the chicken glycoprotein and rat brain Thy-1 in order to find some evidence for homology.

The apparent molecular weights of rat Thy-1 and the chicken glycoprotein, as determined by electrophoretic mobility in SDS/polyacrylamide gels, were very similar (Table 1), more similar in fact than the apparent molecular weights of the rat and human homologues of Thy-1 (Fig. 2*b*). Both the rat and human Thy-1 glycoproteins migrate as a doublet under standard electrophoresis conditions but the chicken molecule migrates as a single diffuse band. These differences are well within the range variability exhibited by known homologues of Thy-1. Thy-1 from AKR mouse brain migrates as a doublet in SDS/polyacrylamide gels, whereas Thy-1 from CBA mouse brain migrates as a single band (Cotmore *et al.*, 1981). This variation is presumably due to heterogeneity in the bound carbohydrate, since Barclay *et al.* (1976) have shown that Thy-1 from rat brain and thymus, which have significantly different apparent and real molecular weights (Kuchel *et al.*, 1978), have an identical protein chain with different bound carbohydrate. The chicken brain glycoprotein probably also exhibits heterogeneity in its carbohydrate, since in an SDS/urea/sodium phosphate/acrylamide-gel system the single band resolves into a clear doublet (K. A. Bailey & P. L. Jeffrey, unpublished work).

The molecular structure of Thy-1 is unusual for a membrane glycoprotein. Thy-1 has considerable sequence homology with immunoglobulin domains and is thought to be anchored in the membrane by covalently linked lipid at its *C*-terminal rather than by a hydrophobic portion of its polypeptide chain (Campbell *et al.*, 1981). The protein chain contains a great deal of β-structure and no detectable α-helix (Campbell *et al.*, 1979). The β-strands are held in place by two intrachain disulphide bridges and most of the polypeptide's surface is probably covered by the large *N*-linked carbohydrate chains (Williams & Gagnon, 1982). The striking similarity between the c.d. spectra of the rat and chicken molecules (Fig. 4) provides strong evidence in favour of homology. Indeed, as was the case with the molecular-weight comparisons, the spectra of the chicken and rat molecules correspond more closely than do the spectra of the human and rat homologues of Thy-1.

A comparison of the chemical properties of rat Thy-1 and the chicken glycoprotein also supported the idea of homology between the two molecules. Rat Thy-1 contains three *N*-linked and no *O*-linked carbohydrate structures and this is reflected in the fact that thymocyte Thy-1 contains no *N*-acetylgalactosamine, whereas approximately one-third of its bound carbohydrate is *N*-acetylglucosamine (Barclay *et al.*, 1976). However, brain Thy-1 does contain a small amount of *N*-acetylgalactosamine, which is thought to be associated with the covalently linked lipid because it is found only in the *C*-terminal peptide, which contains no serine or threonine residues. Our analysis of the amino sugar content of rat Thy-1 agreed well with that reported by Barclay *et al.* (1975) and was very similar to that for the chicken glycoprotein. The fact that both glycoproteins bind to concanavalin A and lentil lectin indicates that both have *N*-linked carbohydrate structures that contain a fucose residue in an $\alpha 1 \rightarrow 6$ linkage to the asparagine-linked *N*-acetylglucosamine (Kornfeld *et al.*, 1981). Amino acid analysis of the chicken glycoprotein and rat Thy-1 after acid hydrolysis also indicated similarity between the two glycoproteins. A crucial feature of Thy-1 structure is its intrachain disulphide bridges so that the presence of such bridges is an important requirement for homology between the two glycoproteins. Although we did not measure cysteine content directly, our experiments on CNBr cleavage clearly demonstrate that the chicken molecule possesses internal disulphide bridges. When rat brain Thy-1 is cleaved by CNBr at its single methionine residue, the two peptides do not separate unless the cleaved molecule is reduced (Fig. 5; Kuchel *et al.*, 1978). The same behaviour is observed in the chicken molecule, indicating that the cleaved peptides are held together by internal disulphide bonds (Fig. 5). Moreover the amino acid composition indicates two methionine residues per molecule in the chicken rather than the single residue present in the rat Thy-1, so that the ability of the resulting peptides to be held together in the absence of reduction suggests the presence of more than one disulphide bridge.

Taken together these results strongly suggest that despite the lack of immunological cross-reactivity the chicken brain glycoprotein is a homologue of the mammalian Thy-1 glycoproteins. Quantitative analyses of the development of the expression of the chicken glycoprotein as well as its subcellular and regional distribution in the central nervous system (P. L. Jeffrey, D. I. Greig & J. A. P. Rostas, unpublished work) further supports this contention. However, definitive statements on homology will have to await the determination of the amino acid sequence of the chicken glycoprotein. Based on an analysis of sequence homologies between rat Thy-1 and immunoglobulin domains (Cohen *et al.*, 1981) it has been suggested that these molecules all evolved from a common ancestor, which has given rise to a number of different molecules whose functions are

associated with recognition. The presence of a Thy-1 homologue in birds may provide a test of this proposal and a valuable comparison for current theories of protein evolution in general.

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