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Early cortical plate specific glycoprotein in a marsupial species belongs to the same family as fetuin and α_2HS glycoprotein

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Two related glycoproteins, fetuin in species of the order Artiodactyla (cattle, sheep, pig) and α_2HS glycoprotein in the human [(1987) Cell Tissue Res. 248, 33-41] have a very specific distribution in the developing brain. We have isolated and determined the first 15 N-terminal residues of a similarly distributed glycoprotein in the developing brain of the tammar wallaby (*Macropus eugenii*). The degree of homology is the same between wallaby glycoprotein and α_2HS glycoprotein as between fetuin and α_2HS glycoprotein (46%). Antibodies made to synthetic peptides of fetuin were used to identify the wallaby glycoprotein. A polyclonal antibody to the purified glycoprotein was used for immunocytochemical identification of brain cells positive for this protein.

α_2HS glycoprotein; Fetuin; Brain development; (Marsupial)

1. INTRODUCTION

The presence of two related glycoproteins, fetuin and α_2HS glycoprotein [1], in the developing brain of animals such as sheep and cattle as well as in the human brain, expressed at a similar stage of cortical plate development suggests that such a glycoprotein may play a developmental role [2]. The tammar wallaby offers an excellent animal model for developmental studies because it is born at an extremely early stage of brain development; morphological work on its brain growth [3] showed that the development of the cortical plate is similar in marsupial and eutherian mammals. We have therefore investigated the possibility that a glycoprotein similar to that found in the fetal brain of eutherian mammals may be present in the developing brain of the tammar. We have separated the corresponding glycoprotein from tammar plasma, determined its N-terminal sequence for comparative purposes and used antibodies to map its distribution in the developing cortical plate.

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2. MATERIALS AND METHODS

Tammar plasma and brains were obtained from The Division of Wildlife and Rangelands Research (CSIRO, Canberra, Australia) by courtesy of Dr C.H. Tyndale-Biscoe and Dr L.A. Hinds.

2.1. *Immunocytochemistry*

Brains from 10-15-day pouch young were fixed in Bouin's fixative and embedded in paraffin wax as described in [3]. Serial sections of 8 μm were cut in a coronal plane and stained with antibodies using the peroxidase-antiperoxidase method (see [2]).

2.2. *Separation of wallaby glycoprotein*

The method used was in principle that of Marti et al. [4] for bovine fetuin.

Homogeneity of the final fraction was checked on SDS-polyacrylamide gels [5] and used for raising anti-tammar glycoprotein antibodies.

2.3. *Synthetic peptides derived from bovine fetuin*

The sequences to be synthesized were chosen from

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hydrophilicity plots [6] of the known amino acid sequence data for fetuin [1].

[Aba^{14}]-fetuin (4–19) (**1**), [Lys^{83},Aba^{96}]-fetuin (84–97) (**2**), [Aba^{190},Aba^{201}]-fetuin (189–203) (**3**) and fetuin (188–203) (**4**) were synthesised by standard solid-phase methods [7] and removed from the solid support with HF in the presence of anisole. Fetuin (188–203) was oxidised at high dilution to form the disulphide bridge between Cys^{190} and Cys^{201}. All four peptides were purified to homogeneity by reverse-phase medium-pressure liquid chromatography. Amino acid analyses of their hydrolysates gave the expected ratios.

Peptides **1**–**4** were coupled via the side-chain amino groups of their Lys residues to succinylated hen egg ovalbumin (Sigma) which was activated by conversion into the *p*-nitrophenyl ester [8]. Low-molecular-mass materials were removed by dialysis and the conjugates were isolated by lyophilisation. Hapten content was determined by amino acid analysis and found to be 32.5% (w/w) for **1**, 23.5% for **2**, 24.2% for **3** and 18.9% for **4**. Aba = 2-aminobutanoic acid, which was used to avoid difficulties with cysteine.

2.4. *Automated N-terminal sequence analysis*

Prior to sequence analysis the wallaby glycoprotein was further purified on a 12% acrylamide gel (10.2 × 8.2 cm) run in SDS [5]. The 62 kDa glycoprotein was detected by staining with Coomassie blue, recovered by electroelution [9] and precipitated with 9 vols ethanol (15 h, −20°C). Sequence analysis was performed using an Applied Biosystems gas-phase sequencer (model 470A) equipped with a PTH analyser (model 120A).

2.5. *Preparation of antibodies*

Antibodies to the purified fraction containing the glycoprotein as well as antibodies to fetuin fragments were raised in mice by intraperitoneal injection at 14-day intervals with Freund's adjuvant [1]. Rabbit polyclonal antibodies to whole tammar plasma were prepared as in [10].

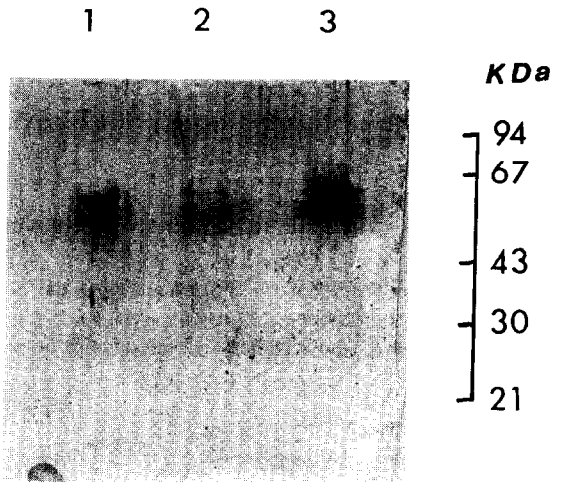

Fig.2. Comparison of bovine fetuin and wallaby glycoprotein by immunoblotting. Samples of separated glycoprotein (1–2 g, lane 1), calf fetuin (1 g, lane 2) and whole tammar wallaby plasma (0.5 l, lane 3) were run on 10% SDS reducing polyacrylamide gel and proteins were transferred to nitrocellulose [11]; additional protein-binding sites were blocked by incubation with 3% BSA in TBS (20 mM Tris-HCl, 0.5 M NaCl; pH 7.4) for 1 h. The paper was then incubated with a 1:100 dilution of the anti-peptide **1** (4–19) antiserum for 2 h followed by 1 h incubation with swine anti-mouse IgG (Dakopatts, Denmark) in 1:100 dilution. The paper was finally incubated with mouse PAP (Dakopatts) (diluted 1:500) and peroxidase activity visualized using 0.2 mg/ml diaminobenzidine/0.005% H_2O_2 in TBS [1]. The low-molecular-mass markers were obtained from Pharmacia.

3. RESULTS

3.1. *N-terminal sequence of wallaby glycoprotein*

Fourteen of the 15 amino-terminal residues of the wallaby glycoprotein were identified by automated sequence analysis of the electroeluted protein. This sequence is compared with the N-terminal sequences of human α_2HS glycoprotein and calf fetuin in fig.1. Displacement of the wallaby glycoprotein by one residue in relation to the N-terminal sequences of the two other proteins revealed a number of identities. Residues equivalent to Pro^4, Pro^{11}, Asp^{14} and Asp^{15} are found in both α_2HS glycoprotein and fetuin. Two additional residues, Arg^9 and Asn^{12}, are shared between the wallaby glycoprotein and α_2HS

	1				5					10					15
Wallaby glycoprotein	Arg	Ser	Pro	**Pro**	Leu	Pro	Pro	Val	**Arg**	Asp	**Pro**	**Asn**		**Asp**	**Asp**
α_2HS-glycoprotein	Ala	Pro	His	Gly	**Pro**	Gly	Leu	Ile	Tyr	**Arg**	Gln	**Pro**	**Asn**	Cys	**Asp**	**Asp**
Fetuin	Ile	Pro	Leu	Asp	**Pro**	Val	Ala	Gly	Tyr	Lys	Glu	**Pro**	Ala	Cys	**Asp**	**Asp**

Fig.1. Comparison of the N-terminal sequence of the 62 kDa wallaby glycoprotein with that of α_2HS glycoprotein and calf fetuin. Residues 1–15 of the wallaby glycoprotein were aligned with residues 2–16 of human α_2HS glycoprotein [13] and calf fetuin [1]. Identities are shown in bold type. No residue was identified at position 13 in the wallaby protein sequence which is consistent with the presence of a Cys residue in the α_2HS glycoprotein/fetuin sequences as the sample was not reduced and alkylated.

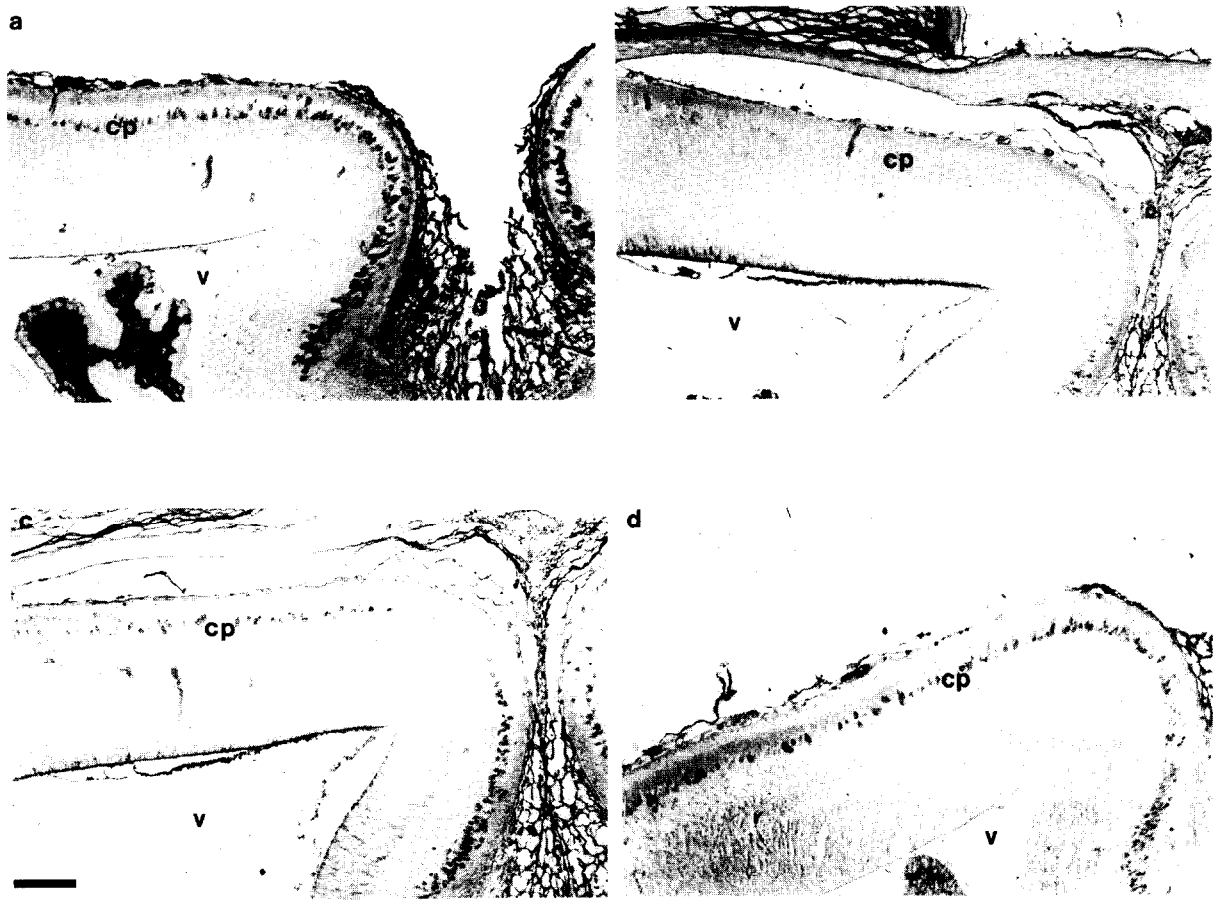

Fig.3. Immunoperoxidase staining in a 15-day postnatal tammar brain showing the characteristic pattern of cortical plate staining. (a,b) Stained with anti-serum to tammar plasma (1:200 dilution); in (b) the anti-serum has been preabsorbed with the separated glycoprotein prior to staining; (c) stained with anti-wallaby glycoprotein antiserum (1:100 dilution); (d) stained with anti-fetuin digest antibody D [1] (1:100 dilution). Magnification, × 19. CP, cortical plate; V, lateral ventricle; bar, 100 μm.

glycoprotein. The residue equivalent to Arg^9 is conservatively replaced by Lys in fetuin. No residue was identified at position 13 which is consistent with the occurrence of a Cys residue in the α_2HS glycoprotein/fetuin sequences as the wallaby glycoprotein was not reduced and alkylated.

3.2. *Immunological comparison of wallaby glycoprotein and calf fetuin*

Fig.2 shows an immunoblot of whole tammar plasma, separated glycoprotein and calf fetuin against antibody raised to peptide **1** {[Aba^{14}]-fetuin (4–19)}. It has previously been shown using crossed immunoelectrophoresis and immunoblotting [1,2] that antibodies to fetuin and α_2HS do not cross-react easily. Antisera to neither of these proteins cross-reacted with tammar plasma. On the other hand antibodies to the three synthetic peptides **1**, **2** and **4** reacted very strongly with both wallaby plasma and separated glycoprotein. This is illustrated for peptide **1** in fig.2. The two bands seen in fig.2 may be due to storage degradation but are clearly visible both in plasma and in the separated fraction and may represent two forms of the same protein. We also observed some cross-reactivity between tammar material (fig.3) and antibodies raised against trypsin-digested fetuin [1] but, as mentioned above, there was no cross-reactivity using antisera to native fetuin or α_2HS glycoprotein.

3.3. *Immunocytochemical localization of wallaby glycoprotein in the developing brain*

Fetuin and α_2HS glycoprotein have been previously suggested to be markers for the early cortical plate cells in several species [2]. At a similar stage of brain development in the tammar, it is possible to demonstrate a strong positive cellular staining using antibodies to whole wallaby plasma or to the separated glycoprotein (fig.3a,c). This staining can be abolished if the antiserum to the whole tammar plasma is absorbed with the separated glycoprotein prior to immunocytochemical staining of the section (fig.3b). Similar staining can also be obtained using antibodies prepared against trypsin-digested fetuin [1] (fig.3d). Cells stained with those antibodies appear around day 10 postnatal and are the first cortical plate cells. These cells eventually contribute to layer V and particularly layer VI in the mature cortex (see [12]).

4. DISCUSSION

The molecular mechanisms by which cells in the brain differentiate and form functional connections are largely unknown. The finding of a specific family of glycoproteins (fetuin-α_2HS glycoprotein) with a characteristic distribution in the cells of the early cortical plate of the mammalian neocortex suggests that these glycoproteins may have a role to play in the early stages of formation of the neocortex. Our finding of a related glycoprotein with a similar distribution in the developing brain of a marsupial opens up the possibility of some functionally orientated studies, because of the accessibility of the immature neonates of this species.

We have shown from earlier work [1,2] that the antigenic relationship between these highly homologous proteins is difficult to investigate as the cross-reactivity is weak. We have therefore raised antibodies to peptide fragments chosen for their predicted antigenicity [6]. Using these antibodies it was possible to show that a glycoprotein from tammar plasma shares antigenic domains with fetuin and α_2HS glycoprotein (fig.2). The N-terminal sequence of the wallaby glycoprotein, aligned as in fig.1, shows that 6 (or probably 7) out of 15 amino acids are identical with those in the corresponding α_2HS glycoprotein sequence. This is the same level of homology as that seen between α_2HS glycoprotein and fetuin.

In a previous report describing the partial sequence of calf fetuin we showed that 109/161 residues were identical between fetuin and human α_2HS glycoprotein [1]. The calf fetuin sequence is now 90% complete (Christie, D.L. et al., unpublished) and a similar degree of homology exists throughout the sequence. There seems to be little doubt that these two proteins are derived from a common ancestral gene. In the present report, immunological criteria and partial sequence data suggest that a protein from wallaby serum should be grouped in the same family as fetuin and α_2HS glycoprotein. While the likely function of these proteins remains unclear it is of interest that the three proteins share a very specific distribution in the developing brain.

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