Journal of Cell Science 109, 3129-3138 (1996)
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JCS3455

A family of glycoproteins (GP55), which inhibit neurite outgrowth, are members of the Ig superfamily and are related to OBCAM, neurotrimin, LAMP and CEPU-1

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SUMMARY

We have previously identified a glycosylphosphatidylinositol-linked glycoprotein of 55 kDa (GP55) which inhibits neurite outgrowth. We now provide evidence that GP55, isolated from adult chick brain, consists of at least two bands, both of which are active, i.e. block outgrowth of neurites from chick dorsal root ganglion neurons. An antiserum raised against the adult proteins reverses the inhibition and preliminary experiments suggest that GP55 is restricted to the nervous system, increases during development from very low levels at embryonic day 10 and is most abundant after hatching. Immunofluorescence reveals that GP55 is expressed on neurons cultured from an embryonic day 14 chick brain but is barely detectable on embryonic day 10 dorsal root ganglion neurons or embryonic day 8 forebrain neurons; the neurons which respond to substrate-bound GP55. Peptide sequencing revealed considerable homology with OBCAM, a protein previously identified on the basis of binding opiates. Nested polymerase chain reaction using primers to the OBCAM sequence and internal primers to GP55 peptides produced two different polymerase chain reaction fragments with homology to OBCAM. A full length clone (E19S) corresponding to one polymerase chain reaction product and a partial length clone (E14S) corresponding to the second have been isolated from an embryonic chick brain library. Both are members of the immunoglobulin superfamily and have (or are expected to have) three C2 domains. E19S has 90% homology with LAMP at the amino acid level. This sequence only partially matches the peptides from the adult protein and hence is probably not a major component of the adult proteins. E14S (GP55-A) has 83% homology to OBCAM at the amino acid level over the region sequenced. The sequence matches several of the peptides from the adult protein and is hence likely to correspond to a major component of the adult proteins. Thus members of the GP55 family are related to OBCAM, neurotrimin, LAMP and a recently discovered chick protein CEPU-1. Our results suggest molecules within this family are capable of acting as cell adhesion molecules and inhibitors of neurite outgrowth.

Key words: GP55, Inhibitory molecule, IgLON, Ig superfamily, LAMP, OBCAM, Neurotrimin, CEPU-1

INTRODUCTION

A variety of cell surface molecules have been shown to be vital for guiding a growth cone towards its eventual target including molecules which stimulate neurite outgrowth and molecules which inhibit or block axonal growth (Keynes and Cook, 1995). Inhibitory proteins are thought to play a role in channelling growing axons along the appropriate permissive tracts towards their eventual target (Wilson et al., 1993). In some cases they may act as a signal to demonstrate that a particular region of the nervous system is fully developed and further growth of axons in this region is not required. The best characterised inhibitory protein is collapsin (Luo et al., 1993) which is a member of the semaphorin family. One member of the semaphorin family, sema 1 which has been shown to play a very precise role in guiding axons in the early stages of limb bud formation provides a good example of how this group of molecules is likely to contribute to axonal guidance (Kolodkin et al., 1993). A number of other inhibitory proteins have been identified based on their ability to collapse growth cones, for example, a peanut lectin-binding glycoprotein from posterior somites (Davies et al., 1990), a 33 kDa glycosylphosphatidylinositol (GPI)-linked glycoprotein and a 25 kDa protein (RAGS) from tectum (Stahl et al., 1990; Drescher et al., 1995). Some inhibitory proteins are expressed in the adult central nervous system (CNS) and may be an important factor in preventing the regeneration of CNS neurons after damage (Schnell and Schwab, 1990). NI-35 has been shown to block regeneration of CNS axons and may be the major inhibitor in CNS myelin. However, it has recently been suggested that myelin associated glycoprotein (MAG) may be partially responsible for the inhibitory activity in CNS myelin (McKerracher et al., 1994; Mukhopadhyay et al., 1994) although the relative importance of MAG remains controversial (Bartsch et al., 1995). MAG, a relatively abundant protein, is a member of the Ig superfamily of cell adhesion molecules. It was initially shown to stimulate neurite outgrowth from embryonic DRG neurons, however, it has

now been shown to inhibit neurite outgrowth from adult DRG neurons and CNS neurons. This suggests that neurons must possess more than one receptor for MAG and that its activity may depend crucially on the receptors expressed by the neurons in question and the resultant second messenger systems which are activated. Proteins capable of a dual role i.e. inhibiting one group of neurons whilst being permissive for others may be of vital importance at specific decision points in the developing nervous sytem. An excellent example of this is in the emerging story of netrin-1 which promotes neurite outgrowth from commissural axons (Serafini et al., 1994) but acts as a chemorepellant for trochlear motor axons (Colamarino and Tessier-Lavigne, 1995). These effects are mediated by distinct receptors (Hedgecock et al., 1990). It is therefore becoming clear that there is no definite division between molecules which stimulate neurite outgrowth and molecules which inhibit neurite outgrowth.

We have recently identified a GPI-linked glycoprotein (GP55) from adult chick brain which inhibits the growth of neurons from both the CNS and the PNS (Clarke and Moss, 1994). GP55 will also inhibit the adhesion of neurons to a favourable substrate although it does not affect the adhesion of Schwann cells or chick heart fibroblasts. Both activities of GP55 can be reversed by the action of pertussis toxin, although not by the β-oligomer alone, suggesting both phenomena are mediated by a GTP-binding protein (G protein) probably G_o (Clarke and Moss, 1997). GP55 is equally active against embryonic DRG neurons and forebrain neurons.

In this paper we have begun to characterise the expression and the molecular structure of GP55. There are at least three members of the family based on peptide sequences and cDNA sequences. Here we describe one complete sequence and one partial sequence. Both of these demonstrate that the GP55 glycoproteins are members of the IgLON family which is composed of LAMP (Pimenta et al., 1995b), OBCAM (Schofield et al., 1989) and neurotrimin (Struyk et al., 1995) and a recently discovered chick protein CEPU-1 (Spaltmann and Brummendorf, 1996).

MATERIALS AND METHODS

Protein purification

Membrane skeleton fractions were prepared as described previously (Allsopp and Moss, 1989). The detergent-insoluble membrane skeleton, which is enriched in GPI-linked glycoproteins (Moss and White, 1992), was isolated from adult chick brain and the GPI-linked glycoproteins were released by treatment with 50 mU/ml phosphatidylinositol specific phospholipase C (PiPLC). The concanavalin A (ConA) binding glycoproteins from the resulting supernatant were isolated on a ConA-affinity column. The ConA eluate was separated on a hydroxylapatite column on a Beckman system gold HPLC and the T-cadherin from the T-cadherin/GP55 peak was removed using an anti-T-cadherin affinity column. GP55 was finally isolated from a size exclusion column (Clarke and Moss, 1994). On some occasions the eluant from the ConA-column was separated by preparative SDS-PAGE and the GP55 band(s) electroeluted. GP55 was deglycosylated by treatment with 5 mU N glycopeptidase F and 5 mU endo-*N*-acetylgalactosaminidase in 25 mM sodium phosphate, pH 7, 0.05 mM EDTA, 0.05% Triton X-100, 8 mM CHAPS, and protease inhibitor cocktail for 18 hours at 37°C. The isolated protein was digested with V8 protease and the peptides separated by reverse phase high performance liquid chromatography (HPLC). Individual peptides were sequenced using an ABI automated sequencer.

SDS-PAGE, electroelution and western blotting

Proteins were separated by SDS-PAGE using a discontinuous buffer system and 0.5 mm gels (Matsudaira and Burgess, 1978); proteins were detected by Coomassie brilliant blue or by silver stain (Morrisey, 1981). For electroelution of GP55 fractions containing GP55 were run under non-reducing conditions on a 0.75 mm 10% SDS-PAGE gel. The gel was stained with 0.05% Coomassie for 1 hour, destained with 40% methanol and washed in water. The bands at 58 kDa and 53 kDa were excised and stored at 4°C until required. Electroelution of GP55, F11 or blank pieces of gel were carried out using a BT1000 Biotrap electroelution chamber (Schleicher and Schuell, Anderman UK) as per the manufacturer's instructions. To assay for GP55 expression tissues were homogenised in 10 mM Tris[hydroxymethyl] aminomethane (Tris), 10 mM ethylene glycol vis (b-aminoethyl) ether-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 5% Nonidet P40 (NP40), washed once in 10 mM Tris, 10 mM EGTA and the 50,000 ***g*** pellet resuspended in 20 mM 3[M-morpholino]propanesulphonic acid, 5 mM EGTA, 0.16% NP40. This suspension was digested with 50 mU/ml PiPLC at 37°C for 2 hour and centrifuged at 50,000 ***g*** for 1 hour. Protein concentrations were determined by the Bradford assay and the GP55 was stored at −20°C until required. Western blotting was carried out essentially as described before (Kyhse-Anderson, 1984), detection was carried out as described previously (Clarke et al., 1993).

Polyclonal antiserum production

PiPLC supernatant corresponding to 200 μg GP55 was subjected to preparative SDS-PAGE and the bands corresponding to GP55 were excised, fixed in 45% methanol, 10% acetic acid and thoroughly washed in phosphate buffered saline (PBS). The excised bands were then homogenised in PBS and 50 μg of GP55/per rat was injected sub-cutaneously into Wistar strain rats. The antisera were screened by enzyme linked immunosorbent assay (ELISA) and western blotting.

Preparation of frozen sections

Dissociated pieces of tissue from freshly killed chicks of relevant age were fixed for at least 2 hours in 4% (w/v) formaldehyde in 0.12 M phosphate buffer, pH 7.4, at room temperature. The tissue pieces were incubated in 6%, 12% and 18% (w/v) sucrose then frozen in Cryo-M-Bed (Bright Instruments, UK) and stored at −70°C until use. Sections (10 μm) were cut on a Bright cryostat (Bright Instruments, UK) and the sections were adhered to glass coverslips precoated with 0.2% gelatin, 0.02% chromic potassium sulphate. Tissue sections were then incubated in 0.1 M glycine, pH 7.4, for 15 minutes at room temperature followed by a 15 minute incubation in PBS containing 10% foetal calf serum (FCS). Sections were then incubated sequentially in immune or control serum (1:200) in 10% FCS in PBS then goat anti-rat fluorisothiocyanate (FITC) (1:200) in 10% FCS in PBS. Sections were mounted in Airvol™ on glass slides and examined under oil immersion using a Leitz photomicroscope.

Tissue culture

E9-E11 DRG neurons were grown as described previously (Allsopp and Moss, 1989) except that occasionally cells were grown on 13 mm glass coverslips coated with 10 μg/ml laminin for 2 hours. E7-E8 forebrain neurons were grown as described previously (Aizenman et al., 1986). Cells were grown at approximately 15,000 cells/cm^2 in 5% CO_2 at 37°C on 13 mm glass coverslips which had been coated with 10 μg/ml poly-L-lysine for 2 hours. E14 forebrains were treated in the same way as E7-E8 but were cultured in Dulbecco's modified eagle medium (DMEM), 10% FCS, 100 i.u./ml penicillin and 100 μg/ml streptomycin. E14 forebrain cells were grown on 13 mm glass coverslips for 4-5 days.

Immunofluorescence staining was carried out on cultured cells by incubating the cells sequentially with: (1) rat 1 serum (1:200) in PBS and 10% FCS; (2) goat anti-rat FITC (1:200) in PBS and 10% FCS; and (3) 4% formaldeyde in PBS.

Inhibition assay

Cells were grown on 13 mm glass coverslips coated with 15 μl of nitrocellulose dissolved in methanol (Lagenaur and Lemmon, 1987), air dried, and coated with 20 μl 10 μg/ml laminin for 30 minutes. Some coverslips were then coated with 10 μl of one of the following: 100 μg/ml electroeluted GP55, native GP55, electroeluted top or bottom band of GP55, electroeluted F11 or an equivalent volume of electroeluted control gel for 30 minutes. Coverslips were then blocked in PBS, 1% bovine serum albumin (BSA) for 30 minutes before being washed briefly in Hanks' balanced salt solution (HBSS). DRG neurons were grown on these coverslips at 600 cells/cm^2 in 24-well plates with 500 μl of culture medium. For experiments to test the activity of the antiserum, coverslips were either coated with 20 μl immune rat serum or control rat serum of appropriate dilution (typically 1:50) after GP55 and before the coverslips were blocked with BSA.

Quantitation of neurite outgrowth was performed as described previously (Clarke et al., 1993). Briefly, neuronal cell bodies were considered suitable for quantitation if they were single, phase-bright approximately spherical cells. Cells that had extended neurites were counted if: (1) neurites had emerged from a distinguishable cell body; (2) processes were at least twice the diameter of the cell body; and (3) the emerging neurites were not in contact with any non-neuronal cells. The number of cells extending neurites were compared to the total number of cell bodies in the field of view (~1 mm^2) and this was counted in triplicate for each coverslip.

Cloning and sequencing

Nested PCR was carried out using primers based on the OBCAM sequence and primers based on the GP55 peptides. The sense primer was based on amino acids 49-57 of the OBCAM sequence and was 5′CAAGGAGAGAGCGCCACACTCAGATC ^{T}/C3′; the antisense primer was based on amino acids 256-264 and was 5′TGGCCAGCCTGGTGTCTTCCTTGAACCA3′. PCR was carried out for 35 cycles at 94°C, 60°C and 72°C for one minute each using 2 mM $MgCl_2$ and cDNA from either an E14 or E19 chick brain. The PCR products of approximately 650 bp were used as template in a second PCR experiment. In this case the degenerate primers were based on the GP55 peptide sequences YAGNDF and FQWFKE. The sense primer was 5′TA ^{T}/C GCIGGIAA ^{T}/C TT ^{T}/C3′ and the antisense primer was 5′^{T}/C TC ^{T}/C TT ^{G}/A AACCA ^{T}/C TG ^{G}/A AA3′. PCR was carried out for 35 cycles at 94°C, 42°C and 72°C for one minute each using cDNA from the previous PCR experiment. The resulting PCR products were cloned into pCRII using the TA cloning kit (Invitrogen). The insert size in plasmids from both experiments were determined and gave a mixture in each case of 550 bp and 600 bp. In both cases the 550 bp inserts were similar but not identical to OBCAM whereas the 600 bp insert was unrelated. Two related sequences were obtained and were designated E14S^{PCR} and E19S^{PCR} depending on the age of the cDNA from which they were originally derived.

The λgt10 library made from E14 chick brain cDNA was screened using the two PCR products random prime labelled with [^{32}P]dCTP. One clone of 2.1 kb was identified using the E19S^{PCR} product and was hence designated E19S. A clone of 1.4 kb was identified using the E14S^{PCR} product and was hence designated E14S.

RESULTS

We have recently isolated a GPI-linked glycoprotein GP55 of approximately 55 kDa from the membrane skeleton from adult chicken brain (Clarke and Moss, 1994). This glycoprotein reduces the adhesion of neurons and inhibits neurite outgrowth of both DRG neurons or forebrain neurons when mixed with a favourable substrate. Neurite outgrowth from DRG explant is also inhibited by the addition of soluble GP55 (Clarke and Moss, 1997). GP55 sometimes appeared to consist of two

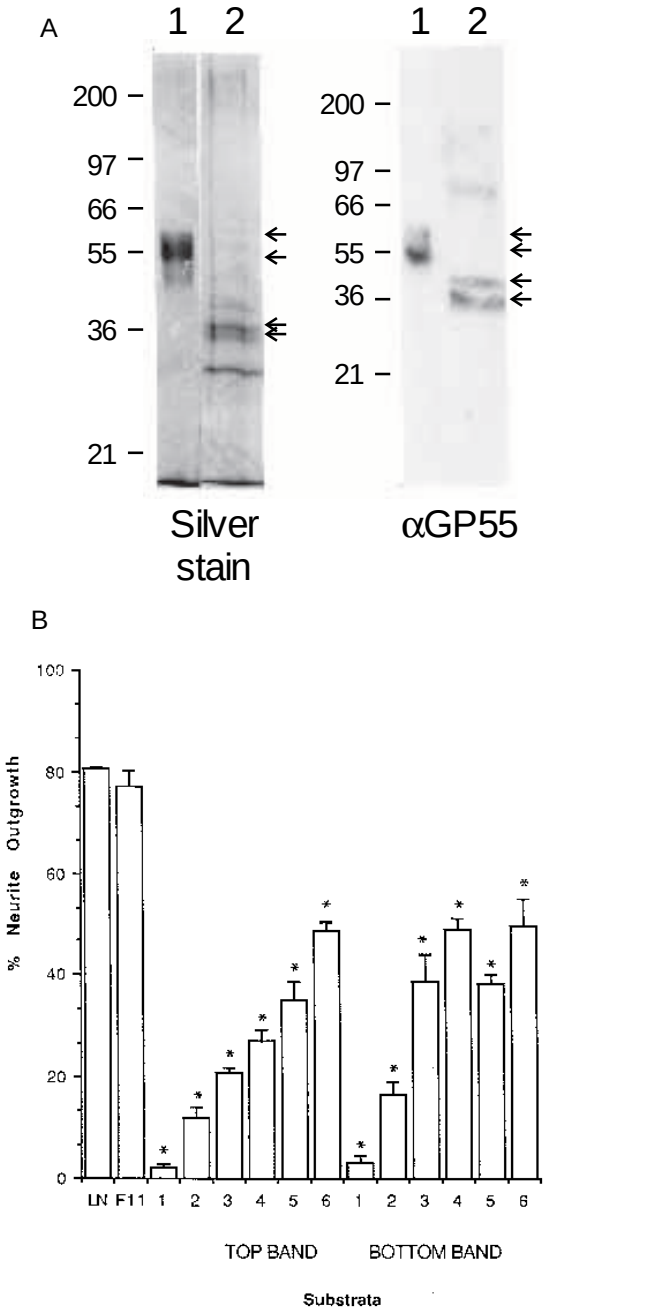
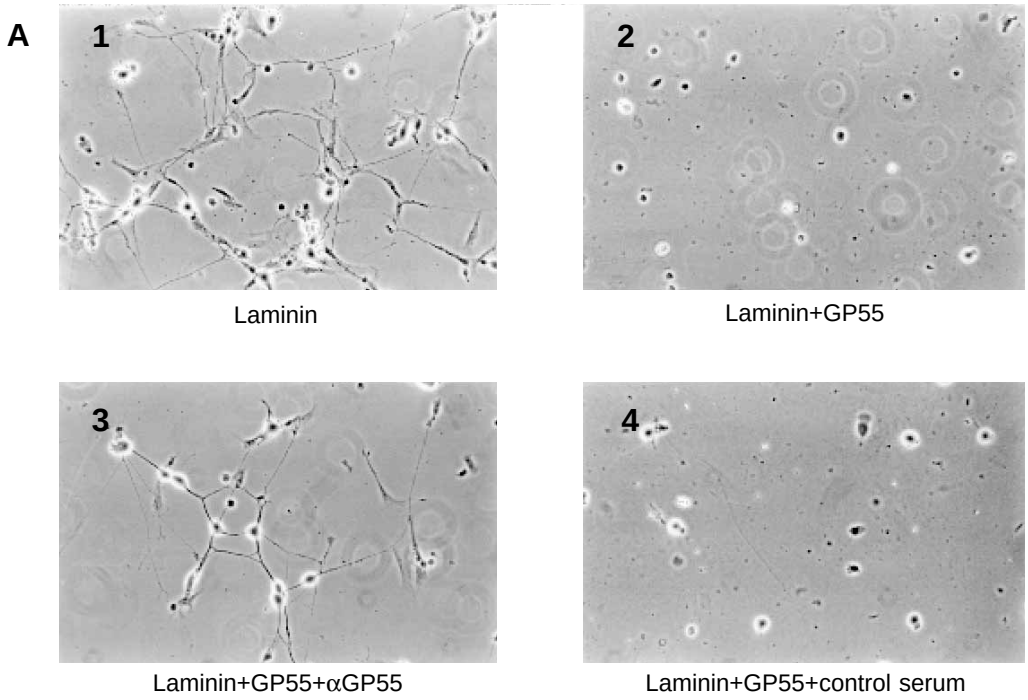


Fig. 1. (A) Deglycosylation of GP55 produces two bands of approximately 36-39 kDa on SDS-PAGE gels. In both cases lane 1 is pure GP55 and in both cases lane 2 is GP55 deglycosylated as described in Materials and Methods. There is an extra band on the silver stained SDS-PAGE which is not observed on the western blot probed with antiserum to GP55 which probably corresponds to the glycosidase enzyme. Bars indicate molecular mass in kDa. (B) The top and bottom bands of GP55 will inhibit DRG neurite outgrowth. DRG neurons were grown on laminin and mixed substrata containing various concentrations of electroeluted GP55 from either the top or bottom bands. Controls: (1) 10 μg/ml laminin; (2) 10 μg/ml laminin + electroeluted F11; (3-8) 10 μg/ml laminin + 100 μg/ml, 50 μg/ml, 25 μg/ml, 10 μg/ml, 5 μg/ml and 1 μg/ml top band GP55, respectively; (9-14) 10 μg/ml laminin + 100 μg/ml, 50 μg/ml, 25 μg/ml, 10 μg/ml, 5 μg/ml and 1 μg/ml bottom band GP55, respectively. Asterisks indicates significant difference compared to LN alone, P<0.001. Note the concentration dependency of inhibition.

rather than one band and as shown in Fig. 1A, deglycosylation of GP55 revealed at least two bands. Their molecular masses were in the range of 36-39 kDa compared to 53-58 kDa for the glycosylated proteins and the bands were much sharper as is typical for a deglycosylated protein. The two glycosylated bands could be partially separated by size exclusion chromatography on HPLC or by eluting from non-reducing preparative SDS-PAGE. The activity of both bands was assessed and both inhibit neurite outgrowth of DRG neurons (Fig. 1B) suggesting they may be two members of a family of proteins with similar activity or alternatively spliced variants of the same protein. Both bands display a concentration dependent activity with a similar EC50 both to each other and to that obtained for the whole band (Clarke and Moss, 1994). Thus the top band gave half maximal inhibition at 5 ng/ml compared to 5-25 ng/ml for the bottom band and 5-10 ng/ml for GP55 (Fig. 1B). The activity of the proteins was not affected by non-reducing SDS-PAGE and subsequent elution. We confirmed that F11 retained its ability to act as a suitable substrate for DRG neurons even when electroeluted (Fig. 1B) and that buffer obtained by electroeluting a blank piece of gel did not inhibit the growth of the neurons (data not shown).

To assist in the characterisation of this protein we have raised a rat antiserum which as shown in Fig. 1A, was specific for GP55. The antiserum detects both bands on a western blot and this was confirmed by running the electroeluted bands separately on SDS-PAGE (data not shown, see also Fig. 3). The antiserum also detects the deglycosylated proteins (Fig. 1A). When diluted 1:50, the antiserum largely reversed the inhibition due to GP55 as shown in Fig. 2A although the difference between this and



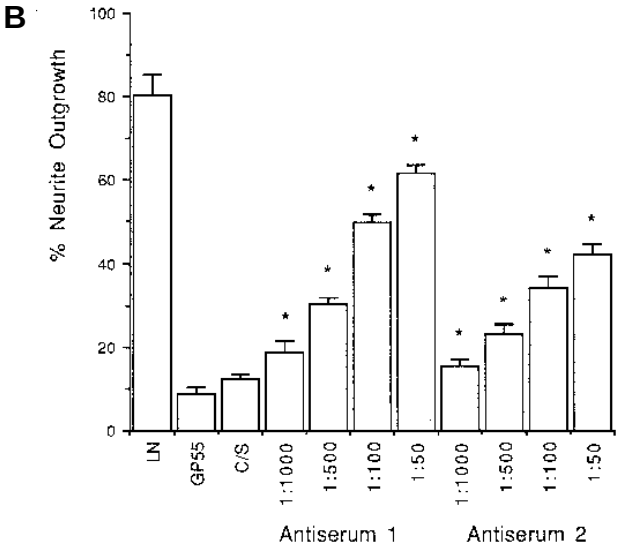


Fig. 2. Anti-GP55 reverses the outgrowth inhibition effect of GP55 in vitro. DRG neurons were cultured on 10 μg/ml laminin and mixed substrata containing 100 μg/ml GP55 and 1:50 dilution of sera. (A) Photomicrographs of E10 DRG neurons cultured on different substrata showing the effect of anti-GP55 on neurite outgrowth. (1) Laminin; (2) laminin + GP55; (3) laminin + GP55 + anti-GP55; (4) laminin + GP55 + control serum. ×160. Bar, 50 μm. (B) Quantitation of the effect of anti-GP55 antisera on the outgrowth of E10 DRG neurons. All coverslips were coated with 10 μg/ml laminin, and where indicated 100 μg/ml GP55. Some coverslips were also coated with various concentrations of anti-GP55 antisera. LN, laminin alone; GP55, laminin + GP55; C/S, LN + GP55 + control serum 1:50; remaining experimental conditions were LN + GP55 + immune serum at the dilution indicated. Asterisk indicates significant difference compared to LN + GP55 alone, $P<0.0001$. Note that the antisera could reverse GP55 mediated inhibtion in a concentration dependent manner, with antiserum 1 being more effective than antiserum 2.

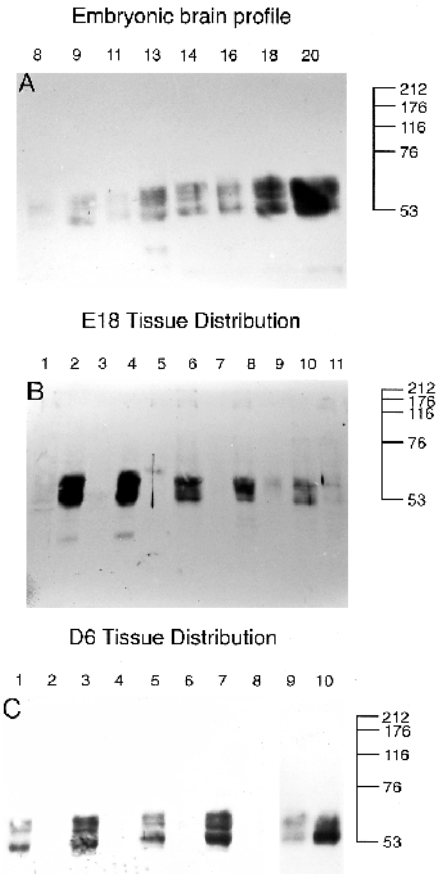


Fig. 3. (A) Developmental profile of GP55 expression in embryonic chick brain by western blot. PiPLC supernatants from different age chick embryo brains were probed with anti-GP55 antiserum. Embryonic age in days is indicated above each track. Expression appears to increase through development. Equal concentrations of protein were loaded per lane. (B) Tissue distribution of GP55 in embryonic day 18 chicks. PiPLC supernatants were isolated from different tissues and studied by western blot using anti-GP55 antiserum. (1) Lung, (2) optic tectum, (3) liver, (4) cerebrum, (5) liver, (6) cerebellum, (7) gizzard, (8) brain stem, (9) heart, (10) retina, (11) kidney. Equal concentrations of protein were loaded in each lane. GP55 is abundant in all areas of the CNS. (C) Tissue distribution of GP55 in post-hatched day 6 chicks. PiPLC supernatants were isolated from different tissues and studied by western blot using anti-GP55 antiserum. (1) Retina, (2) lung, (3) brain stem, (4) kidney, (5) cerebellum, (6) heart, (7) cerebrum, (8) liver, (9) spinal cord, (10) optic tectum. Equal concentrations of protein were loaded per lane. Higher expression levels were detected in optic tectum, cerebrum and brain stem. Retina and cerebellum show higher expression of the lower molecular mass band whilst cerebrum, spinal cord and brain stem show similar levels of all bands. However, the signal in some samples is beyond the linear range hence some caution in interpretation is required. Bars indicate molecular mass in kDa.

laminin alone was still significant. A 100 μg/ml concentration of GP55 will require at least an equivalent concentration of specific antibody in order to neutralise its activity so incomplete reversal at a 1:50 dilution of antiserum is not surprising. The sera from two rats were titrated out as shown in Fig. 2B. Serum 1 was more effective in reversing the inhibition than serum 2 i.e. a 1:50 dilution reversed the inhibition from 18% of control (no antibody) to 75% of control (serum 1) or 57% of control (serum 2). These results correlate with the results obtained by ELISA with the two sera, namely serum 1 could detect GP55 at 1:30,000 while serum 2 could only detect GP55 at 1:1,000.

Western blot analysis of GP55 expression was carried out using the polyclonal antiserum. A developmental profile of GP55 in embryonic chick brain (Fig. 3A) shows that GP55 is first detected around E9/10 but only reaches significant levels late in development. Expression reaches highest levels around post-hatched day 5 after which it plateaus or may decrease a little (data not shown). The tissue distribution of GP55 at both E18 and post-hatched day 6 in chick was examined and at E18, GP55 is expressed in all major areas of the CNS including the cerebrum, cerebellum, brain stem, optic tectum and spinal cord (Fig. 3B). In addition we could detect GP55 expression at low levels in kidney, gut, heart and skeletal muscle. At D6 GP55 is expressed in much higher levels in the cerebrum, cerebellum, brain stem, tectum, spinal cord and retina but we could not detect any expression of GP55 in non-neural tissues using this technique (Fig. 3C). At D6 an interesting feature of GP55 expression is that we can detect at least 3 immunoreactive bands which are expressed to different degrees in different tissues.

From these results we went on to look at the expression of GP55 in more detail in the retina and cerebellum using frozen sections. In day 6 post-hatched retina, GP55 is predominately localised in the retinal ganglion cell (RGC) fibre layer, the inner plexiform layer and the outer plexiform layer (Fig. 4A). The bipolar cells of the inner nuclear layer also stain as do the RGC cell bodies. The staining pattern at E17 was similar but much less intense while at E9 no staining could be observed. In day 6 post-hatched cerebellum, GP55 is particularly abundant in the molecular layer. Granule cells also stain although Purkinje cells appear to be negative (Fig. 4B). In keeping with this observation the white matter is also largely unstained. This pattern is repeated at E18 although the immunoreactivity is much less intense. The staining pattern of both the retina and the cerebellum is consistant with GP55 being expressed on neurons. In cultures from E14 forebrain which contain a mixture of cells including neurons and astrocytes GP55 was expressed on cells with a typical neuronal morphology although maybe not all neurons (Fig. 4C,D). In contrast E10 DRG neurons and E7/8 neurons which are inhibited by GP55 express barely detectable levels of GP55 (data not shown).

In order to begin to analyse the structure of GP55 we isolated the protein and obtained a number of peptide sequences most of which had homology to OBCAM. OBCAM is a GPI-linked molecule identified on the basis of binding opiates. It has a molecular mass of 58 kDa, is a member of the Ig superfamily of cell adhesion molecules based on sequence homology and has three C2 domains (Schofield et al., 1989). Thus, it seemed possible that GP55 was either the chicken equivalent of OBCAM or a related molecule. Interestingly, we obtained two peptides which appeared to correspond to the same region of the OBCAM molecule again suggesting there may be more than one gene product (Fig. 5).

Nested PCR was carrried out using primers based on the OBCAM sequence and primers based on the GP55 peptide sequences (see Materials and Methods) using cDNA obtained from E14 or E19 chick brain as a template. Two PCR products of slightly different size were obtained in each case and the shorter PCR products ($E14S^{PCR}$ or $E19S^{PCR}$) showed considerable homology to OBCAM but were not identical to OBCAM or each other. $E14S^{PCR}$ was used to screen an E14 chick brain λgt10

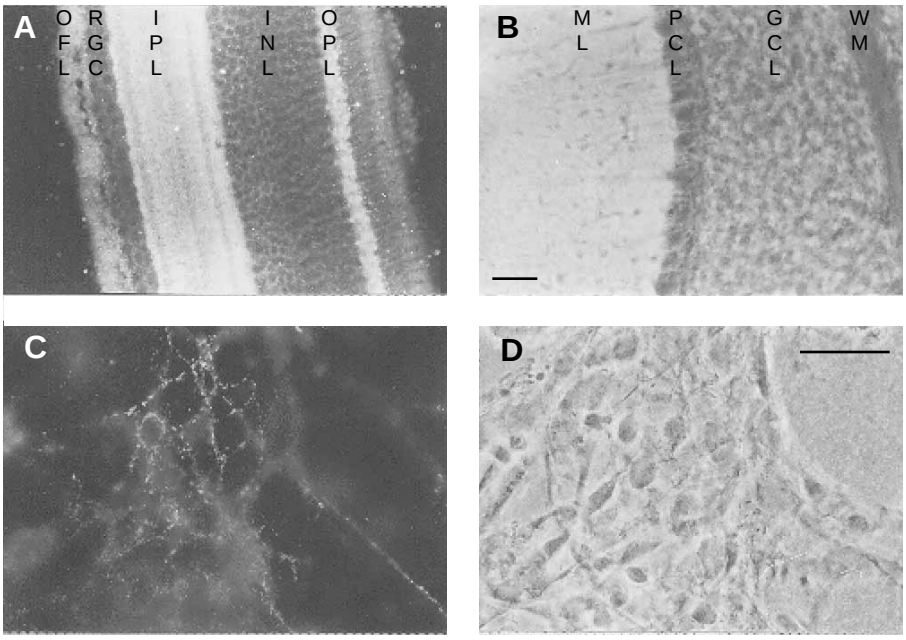


Fig. 4. GP55 is expressed on neurons. Immunofluorescence was carried out as described in Materials and Methods. A 1:200 dilution of the rat anti-GP55 antiserum was used to detect the GP55 followed by a 1:200 dilution of goat-anti-rat FITC. (A) Post-hatched day 6 chick retina. Staining can be observed in the inner and outer plexiform layers (IPL, OPL) and the optic fibre layer (OFL). There is also some staining of the cells of the inner nuclear layer (INL) and the retinal ganglion cell bodies (RGC). ×160. (B) Post-hatched day 6 chick cerebellum. Staining can be observed in the molecular layer (ML) and granule cell layer (GCL) but the Purkinje cell layer (PCL) and white matter (WM) appear predominantly negative. ×160. Bar, 50 μm. (C) Embryonic day 14 chick forebrain neurons stained with anti-GP55 antiserum shows staining on cells with neuronal morphology. ×320. (D) The same field as C under phase contrast microscopy. ×320. Bar, 50 μm.

library and one partial length clone of 1.4 kb was obtained. The sequence matched three of the peptides (GP55-2, 3b and 4 in Fig. 5) obtained from the adult protein (Fig. 6) with the exception of one amino acid which was a lysine instead of a valine. This difference is most likely to be due to an error in the original peptide sequence. GP55-1, however, only matches 11 out of the 14 amino acids suggesting that this peptide like GP55-3a originates from the second related protein which co-purifies with the E14S gene product. Confirmation that E14S corresponds to one of the adult GP55 proteins is based primarily on asparagine 157 in GP55-3a which is unique to E14S among the members of the IgLON family. E14S is therefore referred to as GP55-A. A 2.1 kb clone, E19S, which encoded the whole of the open reading frame (ORF) was obtained by screening the library with E19S^{PCR} product and the sequence is shown in Fig. 7. It has an ORF of 1,014 nucleotides coding for 338 amino acids which will give an expected molecular mass of 37 kDa. The peptides obtained from the adult protein only partially matched the E19S deduced amino acid sequence and none were identical suggesting E19S is neither of the two proteins isolated from adult brain. Both clones have considerable homology with OBCAM, neurotrimin and LAMP from rat and CEPU-1 from chick as shown in Fig. 8 and Table 1. E19S may indeed be the chicken equivalent of LAMP. A comparison of the first, second and third C2 domains between members of the IgLON family sequenced so far indicates that the first and second domains are well conserved and it is the third domain which is most distinct. Interestingly, this is the region where two alternatively spliced variants have been identifed in LAMP and CEPU-1 (Spaltmann and Brummendorf, 1996) (Pimenta et al., 1995a). Essential structural features are conserved including six cysteines which form the three C2 domains and five putative glycosylation sites although E19S has a total of 8 potential glycosylation sites. The sequence around the signal peptide cleavage site is well conserved although there is some variation in predicting the cleavage site (Fig. 8). We have based our prediction for the cleavage site on that identified for LAMP by sequencing the N-terminal of the protein. Both sequences are compatible with the GPI-linkage for which we already have evidence. One unusual feature of GP55-A is the possession of a consensus sequence for a glycosaminoglycan chain. It remains to be seen whether this sequence is used for attachment of a gag chain although the presence of a basic amino acid may argue against this. Both GP55-A and E19S(LAMP) also have low but significant homology (22-28%) with a variety of cell adhesion molecules including N-CAM, F11 and MAG.

DISCUSSION

We have shown previously that GP55 will inhibit neurite outgrowth when bound to the substrate in the presence of laminin, poly-L-lysine and G4 (Clarke and Moss, 1994). In this paper we have demonstrated that the adult protein consists of

BOTH BANDS

GP55-1	**Y**	T	?	L	Y	A	G	N	D	**F**	**Y**	?	I	**D**
OBCAM	S	T	I	L	Y	A	G	N	D	K	W	S	I	L
GP55-2	**I**	**T**	**G**	**I**	**T**	R	**E**	**E/Q**	?	G	E	Y		
OBCAM	I	S	D	I	K	R	D	Q	S	G	E	Y		
GP55-3a	**V**	T	V	N	**F**	**A**	P	**T**	I	**Q**	**E**	**L**	K	
GP55-3b	**V**	T	V	N	Y	P	P	Y	I	?	**N**	A	**V**	
OBCAM	I	T	V	N	Y	P	P	Y	I	S	K	A	K	
GP55-4	F	Q	W	F	K	E								
OBCAM	F	Q	W	F	K	E								

TOP BAND

GP55	A	M	D	**F**	V	T	V	R
OBCAM	A	M	D	N	V	T	V	R

Fig. 5. Comparison of peptides obtained from GP55 total band and GP55 top band with OBCAM. Amino acid differences are shown in bold. The regions used to design GP55 specific primers are underlined. Note the two peptides 3a and 3b, which correspond to the same region of OBCAM.

Table 1. Amino acid sequence homology between members of the IgLON family

	GP55-A	E19S	OBCAM	Neurotrimin	LAMP	CEPU-1
GP55-A	–	58%	83%	73%	57%	72%
E19S	58%	–	55%	56%	90%	54%

Amino acid sequence homology between OBCAM, neurotrimin, LAMP, CEPU-1, GP55-A and E19S. Data show that GP55-A is most closely related to OBCAM, and E19S is likely to be the chicken equivalent of LAMP. The GP55-A sequence is incomplete and the cDNA clone lacks approximately 200 nucleotides in the N-terminal ORF region.

at least two bands both of which are active in blocking neurite outgrowth. The two bands could be due to heterogeneity of glycosylation, alternative splicing as has been observed for CEPU-1 (Spaltmann and Brummendorf, 1996) and LAMP (Pimenta et al., 1995a) or they could be due to two different gene products. The latter is more likely because we obtained two different peptide sequences which clearly corresponded to the same region of the protein and because deglycosylation of GP55 again resulted in two bands. However it is possible there may be alternatively spliced forms. Peptide sequences were initially obtained from the mixture of proteins and attempts to obtain unique sequences from either the top or the bottom band were unsuccessful with the exception of one peptide AMDFVTVR which was obtained from the top band. GP55-A is most similar to OBCAM (83%) and may be the chicken equivalent of this protein. In general the N-terminal of of the IgLONs is more

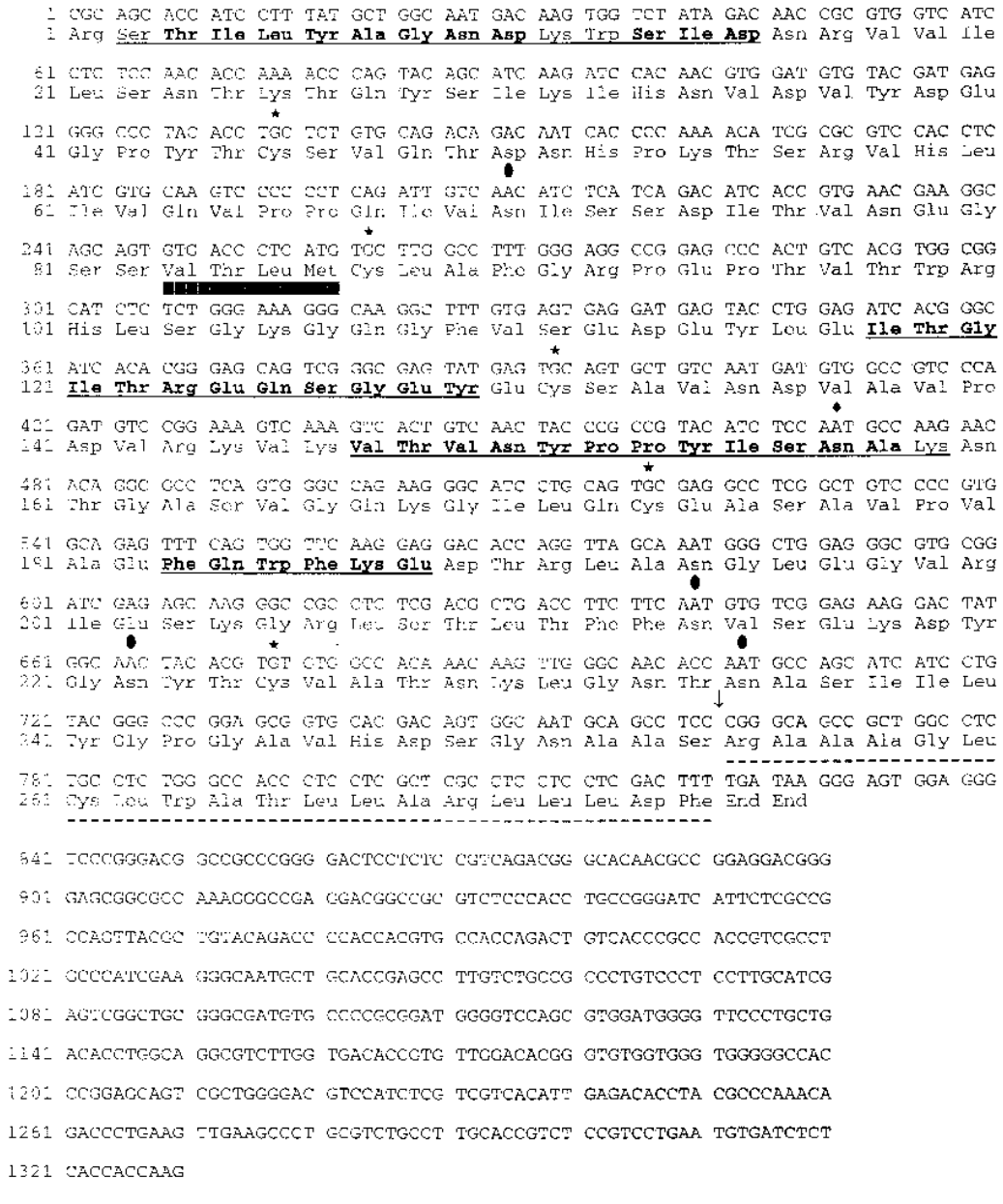


Fig. 6. The cDNA sequence of E14S (GP55-A) and deduced amino acid sequence. E14S lacks approximately 200 nucleotides of the ORF including those which code for the putative signal peptide. The potential N-linked glycosylation sites are depicted by filled ovals. The C-terminal hydrophobic tail is indicated by a dashed line and an arrow indicates the cleavage site for the GPI-anchor. The consensus sequence for a GAG chain is indicated by the filled squares and the cysteines which form the three C2 domains are marked with asterisks. Peptide sequences obtained from GP55 are underlined and the amino acid matches are shown in bold. Where an amino acid residue was uncertain in the peptide sequence we have indicated a match if the amino acid matched the consensus sequence.

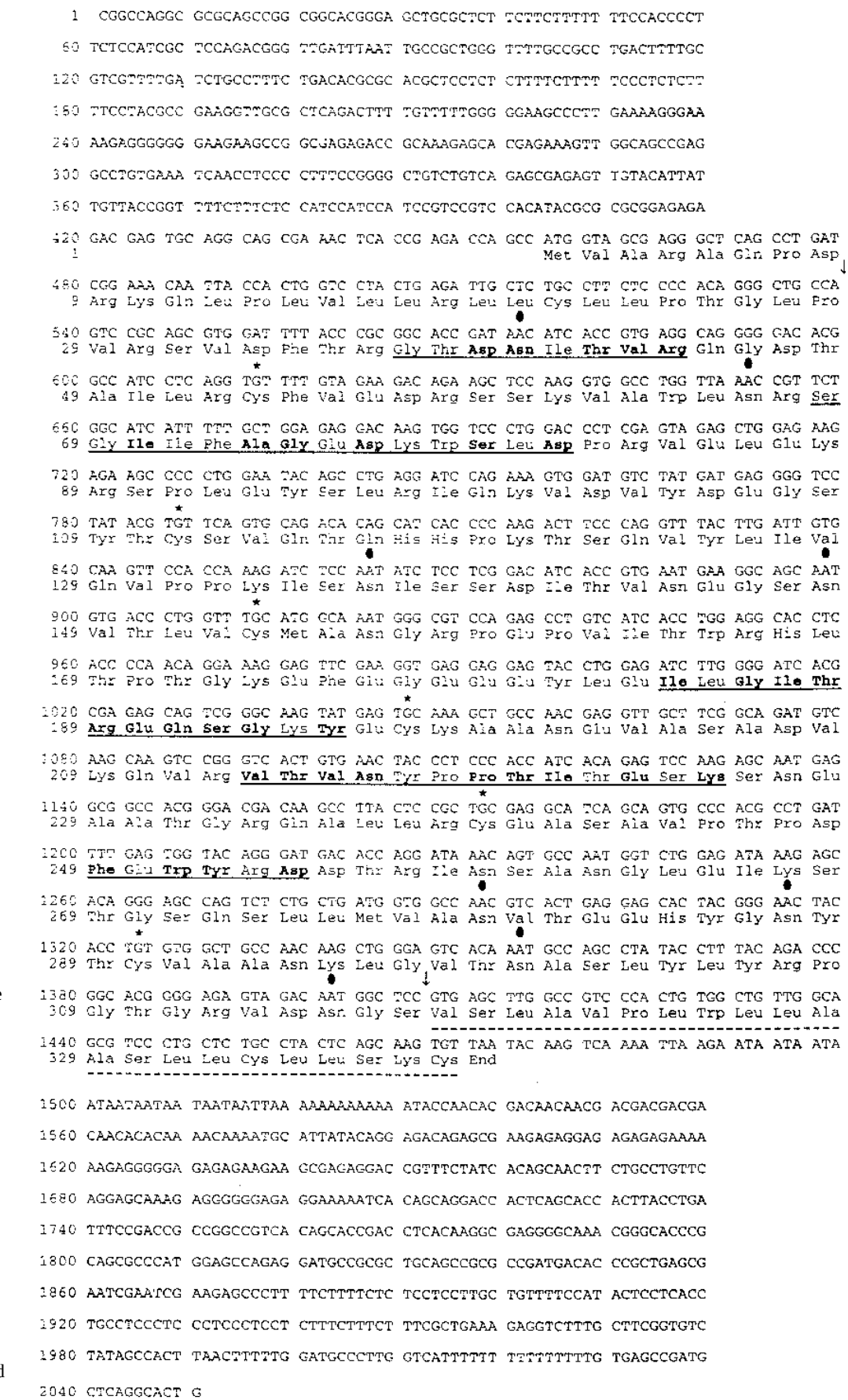


Fig. 7. The cDNA sequence of E19S and deduced amino acid sequence. The putative cleavage site for the signal peptide and the cleavage site for the GPI-anchor are marked by arrows. The potential N-linked glycosylation sites are depicted by filled ovals. The C-terminal hydrophobic tail is indicated by a dashed line and the cysteines which form the three C2 domains are marked with asterisks. Peptide sequences obtained from GP55 are underlined and the amino acid matches are shown in bold. Where an amino acid residue was uncertain in the peptide sequence we have indicated a match if the amino acid matched the consensus sequence.

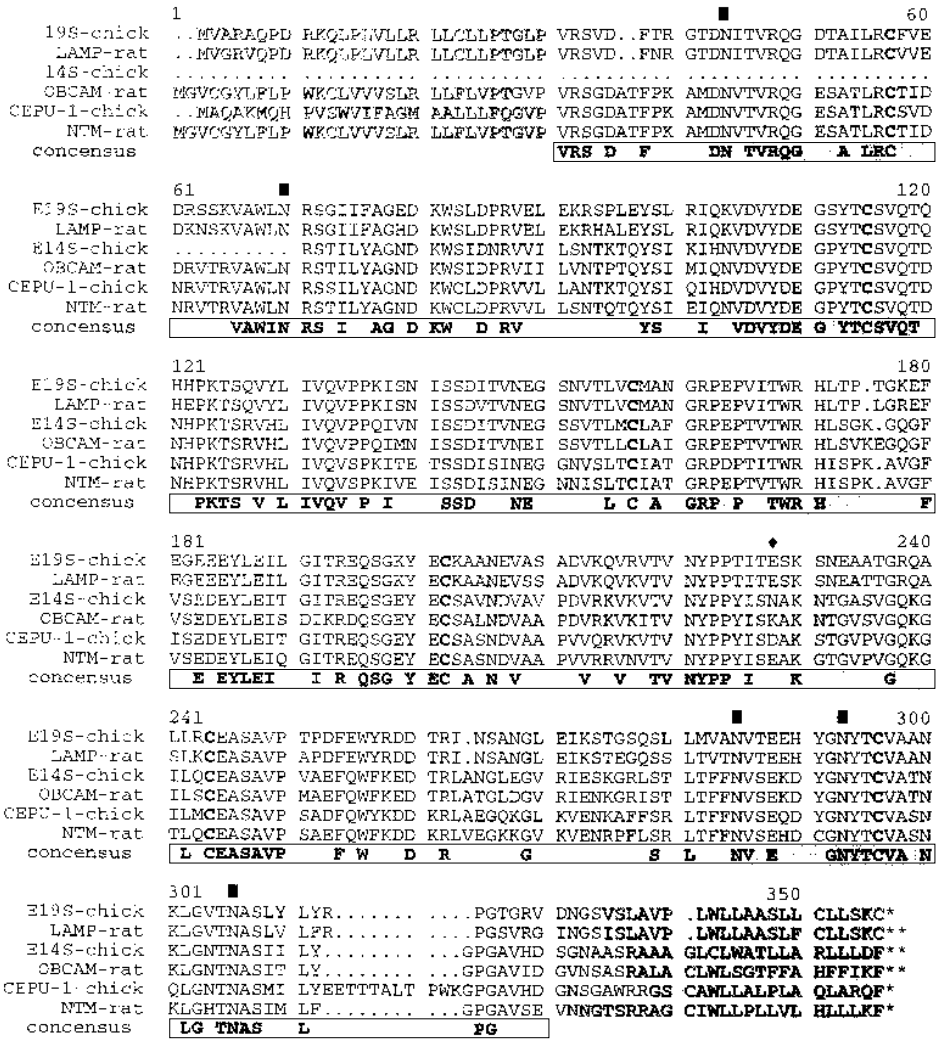


Fig. 8. Amino acid sequence comparison between the coding regions of E19S (LAMP), rat-LAMP, E14S (GP55-A), rat-OBCAM, chick-CEPU-1 and rat neurotrimin. The boxed regions indicate the signal peptide and the hydrophobic tail removed upon attachment of the GPI-anchor. Putative N-linked glycosylation sites are shown by the filled squares, cysteines which form the three C2 domains are shown in bold and the diamond marks the position of the asparagine which is so far unique to the peptide obtained from GP55 and the E14S (GP55-A) sequence. Asterisks indicate the stop codons.

highly conserved thus the overall homology may be higher. GP55-A also corresponds to one of the adult proteins because it matches several of the peptides precisely. Unfortunately the GP55-A sequence does not extend to the N terminus of the protein hence we cannot compare the one peptide known to originate from the top band with this sequence. It therefore remains to be seen which band this sequence corresponds to. We have rescreened the λgt10 library in order to find a clone to complete the sequence, however, although this resulted in obtaining a full length 3.2 kb clone it has proved to be different from GP55-A and is likely to be a third member of the family. One point of interest with respect to the GP55-A sequence is the potential glycosaminoglycan (gag) chain attachment site. It remains to be seen whether this site is used, however, it may be unlikely since removal of the O- and N-linked sugars reduces the molecular mass to that expected for the polypeptide chain alone. E19S is most similar to LAMP and is probably the chicken equivalent of LAMP. LAMP acts as a molecular recognition cue for functionally related neurons. It will be interesting to observe whether E19S has a similar localisation to that observed for LAMP in the rat (Zacco et al., 1990). The E19S sequence does not match exactly any of the adult peptides and is probably not a major component of the adult protein.

Our evidence suggests that GP55 is expressed on neurons and may be restricted to neurons. It is abundant in the CNS and there may be small amounts in the PNS. It is unclear whether any non-neural tissues express GP55 or whether the trace amounts observed are due to innervation. GP55 appears relatively late in development although we have not yet looked for localised expression early in development. This may be an interesting avenue to explore because neurons earlier in development appear to respond to GP55. Molecules which inhibit neurite outgrowth are suspected to play an important role during development in guiding axons to their appropriate targets. Whilst this may be one possible function of GP55, its abundance at the time neurons are making and stabilising their connections suggests additional roles perhaps in synaptogenesis. Furthermore it is interesting to note that GP55 will inhibit the adhesion of neurons to a favourable substrate (Clarke and Moss, 1997) and at a lower concentration than we have so far observed will inhibit neurite outgrowth. Thus between 0.5 μg/ml and 25 μg/ml GP55 the adhesion of neurons is reduced by 90%. However, at 0.5 μg/ml essentially all neurons which adhere extend neurites whilst at 25 μg/ml less than 50% of neurons extend neurites. Even at 100 μg/ml GP55 the number of neuronal cell bodies adhering is 10-15% of control (Clarke and Moss, 1994).

Of the three members of the IgLON family identified in rat, LAMP is the only one so far whose function has been analysed.

Levitt and colleagues have shown that LAMP is a homophilic cell adhesion molecule which will stimulate outgrowth of neurons which express LAMP but appears to neither stimulate nor inhibit the growth of LAMP negative neurons (Zhukareva and Levitt, 1995). We have shown that GP55 will inhibit outgrowth of GP55 negative neurons and it remains to be seen whether GP55 positive neurons respond in the same way. Unlike LAMP, GP55 must be able to interact with a heterophilic receptor on the neuronal cell surface. Evidence from earlier work (Clarke and Moss, 1994, 1997) indicates that GP55 must interact either directly or indirectly with a G protein coupled receptor. One interesting possibility is that GP55 may bind to another member of the IgLON family and that this complex may activate a G protein coupled receptor. Superficial observation of the E14 forebrain cultures suggests that GP55 positive neurons can interact with each other. It has been suggested that this family of glycoproteins may act as specific molecular labels for different classes of neurons (Struyk et al., 1995; Zhukareva and Levitt, 1995; Spaltmann and Brummendorf, 1996). Our results are compatible with this concept because these molecules could act as CAMs to enhance correct synaptic connections whilst inhibiting or repelling interactions between incorrect neuron pairs. It will be interesting to determine whether members of the GP55 family will bind to themselves, to other members of the family and to unrelated receptors. It is interesting to note that the only chick member of this family identified so far, CEPU-1, labels cerebellar Purkinje cells which are conspicuously unlabelled by our antisera to GP55 (Spaltmann and Brummendorf, 1996). The mechanism by which interactions between members of this family may initiate second messenger pathways is unclear, however, experiments are underway to provide further evidence for a heterophilic G protein-coupled receptor.

We thank Dr M. Birch for help with sequencing the PCR fragments, Dr M. Wilkinson for the peptide sequences, Mr K. Lobotesis for assistance in screening the cDNA libraries and Mr D. Bassi and Mr R. Reid for assistance with the figures. This work was supported by an MRC grant G9308076N, a Royal Society Fellowship to D.J.M. and a British Council grant to D.S.K. The EMBL accession number for E14S (GP55) is Y08170 and for E19S (LAMP) is Y08171.

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(Received 7 August 1996 – Accepted 9 September 1996)