Diglons are heterodimeric proteins composed of IgLON subunits, and Diglon-CO inhibits neurite outgrowth from cerebellar granule cells

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Summary

IgLONs are a family of four cell adhesion molecules belonging to the Ig superfamily that are thought to play a role in cell-cell recognition and growth-cone migration. One member of the family, opioid-binding cell-adhesion molecule (OBCAM), might act as a tumour suppressor. Previous work has shown that limbic-system-associated protein (LAMP), CEPU-1/Neurotrimin and OBCAM interact homophilically and heterophilically within the family. Here, we show that, based on their relative affinities, CEPU-1 might be both a homo- and a heterophilic cell adhesion molecule, whereas LAMP and OBCAM act only as heterophilic cell adhesion molecules. A binding assay using recombinant IgLONs fused to human Fc showed that IgLONs are organized in the plane of the membrane as heterodimers, and we propose that IgLONs function predominantly as subunits of heterodimeric proteins (Diglons). Thus, the four IgLONs can form six Diglons. Furthermore, although singly transfected cell lines have little effect on neurite outgrowth, CHO cell lines expressing both CEPU-1 and OBCAM (Diglon-CO) inhibit neurite outgrowth from cerebellar granule cells.

Key words: Cell adhesion, Ig superfamily, Neurite outgrowth, Tumour suppressor gene, GPI anchor, Membrane protein complex

Introduction

Cell adhesion molecules (CAMs) of the Ig superfamily are involved in many cellular processes during development, including regulation of cell proliferation, differentiation and migration (Brummendorf and Lemmon, 2001). Within the nervous system, various members of the Ig superfamily play defined roles in axon growth and guidance, and cell-cell recognition during synapse formation. Many CAMs associate laterally and function in cell surface molecular complexes. For example, L1 can associate with neuropilin-1 to form the Sema3A receptor (Castellani et al., 2000; Castellani et al., 2002), as well as interacting with, and signalling via, the fibroblast growth factor receptor (Walsh and Doherty, 1997). Glycosylphosphatidyl inositol (GPI)-anchored glycoproteins cluster in lipid rafts (Friedrichson and Kurzchalia, 1998; Varma and Mayor, 1998), encouraging their lateral association (Harris and Siu, 2002), and many or all will interact in cis with a transmembrane glycoprotein (Malhotra et al., 1998; Tansey et al., 2000).

IgLONs are a family of four CAMs belonging to the Ig superfamily. Limbic-system-associated protein (LAMP) was the first member to be identified, followed by opioid-binding CAM (OBCAM), Neurotrimin (NTM)/CEPU-1 and Kilon/Neurotractin (Funatsu et al., 1999; Levitt, 1984; Marg et al., 1999; Schofield et al., 1989; Spaltmann and Brummendorf, 1996; Struyk et al., 1995). Each member has been variously characterized in terms of molecular structure, molecular binding interactions, expression and ability to modify neurite outgrowth and cell adhesion. Typically, all four IgLONs consist of three Ig domains and are anchored in the plasma membrane via a GPI anchor. The GPI anchor sequesters them to the detergent-insoluble, cholesterol-rich lipid rafts, and the ability of IgLONs to signal to the interior of the cell will be influenced by this location and their requirement for a transmembrane receptor (Simons and Toomre, 2000). LAMP and CEPU-1 also exist as α and β isoforms, where the β isoform has an additional 11 or 12 amino acids between the third Ig domain and the GPI anchor; the function of this extra peptide is unknown (Brummendorf et al., 1997; Spaltmann and Brummendorf, 1996). The chick orthologue of Kilon can also be found as a two-Ig-domain form (Marg et al., 1999) and, furthermore, there is a secreted isoform of CEPU-1, CEPU-Se, that is missing the GPI anchor entirely (Lodge et al., 2001).

Early experiments indicated that LAMP and NTM/CEPU-1 were homophilic CAMs and, more recently, it has been shown that all IgLONs interact heterophilically within the family (Gil et al., 2002; Lodge et al., 2000; Marg et al., 1999; Zhukareva and Levitt, 1995). Expression of the different members has been examined in the developing nervous system of chick embryos and embryonic and postnatal rats. In general, two or more IgLONs are often expressed within a specific tissue at any particular time, and this is clearly observed in the chick retina, where expression of LAMP, OBCAM and NTM/CEPU-1 has been examined. LAMP and CEPU-1 are found in the outer plexiform layer, whereas LAMP and OBCAM are found in the inner plexiform layer (Lodge et al., 2000). Recently, it has been shown that IgLONs are expressed outside the nervous system,
and one member of the family, OBCAM, might act as a tumour suppressor in ovarian epithelial cancer (Sellai et al., 2003).

The ability of individual members of the IgLON family to modulate neurite outgrowth has been investigated by several groups. In some cases, little or no response has been observed and, in others, moderate enhancement or inhibition has been seen (Gil et al., 1998; Hancox et al., 1997; Lodge et al., 2001; Mann et al., 1998; Marg et al., 1999; McNamee et al., 2002). Interestingly, the most striking results were obtained with GP55, a glycoprotein isolated from adult brain that contains a mixture of all members of the IgLON family (Clarke and Moss, 1994). In this case, complete inhibition of neurite outgrowth from dorsal root ganglion (DRG) and forebrain neurons was seen, and this inhibition was reversed by pertussis toxin, suggesting the involvement of a G-protein-coupled receptor (Clarke and Moss, 1997; Wilson et al., 1996). Nevertheless, experiments with isolated members of the family failed to exert any similar activity (McNamee et al., 2002). One puzzling result obtained from this study was the inability of sympathetic nerves to adhere to LAMP and OBCAM, despite expressing LAMP, CEPU-1 and OBCAM (McNamee et al., 2002). This suggested an incomplete understanding of the molecular interactions of the IgLON family.

In this paper, we have analysed the homo- and heterophilic interactions of three members of the IgLON family. Heterophilic interactions for LAMP and OBCAM have higher affinity than homophilic interactions in trans, and all three IgLONs are shown to interact in cis, suggesting that they act predominantly as subunits of heterodimeric proteins. Furthermore, one dimeric IgLON (Diglon-CO) inhibits neurite outgrowth, reminiscent of the activity of GP55, whereas the IgLON subunits alone have no effect.

Materials and Methods

Materials

Antisera recognizing chicken LAMP, OBCAM and CEPU-1 were described previously (Lodge et al., 2000). α1LAMP-Fc, α2OB-CAM-Fc and α2CEPU-1-Fc (GenBank accession numbers for the respective IgLON sequences are Q98919, Q98892 and Q90773) were prepared either from stably transfected J558L mouse myeloma cells as described previously (Howard et al., 2002) or from calcium-phosphate-mediated transient transfection of HEK 293 human embryonic kidney cells, using methods described elsewhere (Chen and Okayama, 1988). CHO cell lines stably expressing cell surface α1LAMP, α2OB-CAM, α2CEPU-1 and α2CEPU-1 were prepared and cultured as described previously (Lodge et al., 2001).

Immunofluorescence microscopy

CHO cells were live stained with IgLON-Fc or IgLON-specific antisera, essentially as described previously (Lodge et al., 2000). Cells grown on coverslips were incubated at room temperature for 20 minutes in blocking buffer [0.12 M sodium phosphate buffer (pH 7.4), 1% bovine serum albumin (BSA)] containing antisera (1:100) or IgLON-Fc (25 μg ml⁻¹) and binding was detected by incubation with Texas-Red-conjugated anti-human IgG or goat anti-rat IgG (1:100; both from Jackson ImmunoResearch). Average fluorescence intensity of staining was measured using Metamorph (Universal Imaging Corporation), and values were normalized to background and statistics compiled using Excel (Microsoft).

Construction of GFP-tagged IgLON constructs

The plasmid vectors pSlax and pIRES EGFP were gifts from J. Gilthorpe (King’s College, London, UK). pOIG and pLIG, plasmid vectors expressing either chicken α1OB-CAM (GenBank accession number AF292934) or α1LAMP under the control of a β-actin promoter and enhanced green fluorescent protein (EGFP) downstream of an internal ribosome entry site (IRES) sequence were based on the modified pRES GFP vector. Polymerase chain reaction (PCR) using the primer pairs: 5'-TGGCCTGTGCACCATGGGGTCC-3' and 5'-ATCCAAAGTCGAGGAGCAGC-3' generated full-length α1OB-CAM immediately preceded by an NcoI site. Following cloning of the 1046 bp product into pCR®2.1-TOPO (Invitrogen), the α1OB-CAM open reading frame was ligated into the shuttle vector pSlax at BamHI and NcoI, then subcloned into pIRES GFP at Clal and NorI to produce pOG. Full-length α1LAMP was amplified by PCR using the primer pairs: 5'-AAGCTTCCATGTAQCGAGGC-3' and 5'-TCTAGATTAACACTTGTGAGTGAGGC-3', and the 1032 bp product was cloned into pCR®2-TOPO (Invitrogen). The α1LAMP open reading frame was subcloned directly into pIRES GFP at EcoRI and analysed for the correct orientation by restriction enzyme mapping to produce pLIG.

Transient transfection of singly transfected CHO cells

Wild-type or stably transfected IgLON-CHO cells were seeded at 1.5×10⁴ cells per well in a 24-well plate, grown overnight and transfected with pLIG or pOIG using FuGENE 6 (Roche) at a 1:3 (weight/volume) ratio according to the manufacturer’s instructions. Cells were incubated in the complex for 48 hours and analysed by immunofluorescence microscopy.

Preparation of CEPU-1/OBCAM-expressing CHO cells

α2CEPU-1 was cloned into the plasmid vector pBudCE4.1 (Invitrogen), downstream of the EF-1α promoter at the XhoI and KpnI sites to generate pBud(α2C). α2OB-CAM was cloned into pBud(α2C), downstream of the CMV promoter at the HindIII and XhoI sites, to produce pBud(α2C-α2O). Wild-type CHO cells were transfected with pBud(α2C-α2O) using FuGENE 6 at a 1:3 (weight/volume) ratio and selected in 400 μg ml⁻¹ Zeocin™ (Invitrogen). Colonies were assayed by immunofluorescence microscopy with anti-CEPU-1 and anti-OBCAM antibodies and isolated by two rounds of dilution subcloning. Expression of CEPU-1 and OBCAM was confirmed by dot blots and western blots. Cells were extracted in 20 mM Tris-HCl pH 7.6, 2 mM EGTA, 1% NP-40 and Complete™ (Roche) at 37°C for 15 minutes and centrifuged at 50,000 g at room temperature for 15 minutes. For dot blots, 2 μl of supernatant was spotted on nitrocellulose and then processed as for a western blot (Lodge et al., 2000).

Neuronal cell culture and adhesion and outgrowth assays

Cerebellar granule cells (CGCs) were prepared from embryonic-day 15 (E15) chick cerebellum, essentially as described (Cambray-Deakin, 1995). Briefly, cerebella were shredded with forceps, incubated with 0.25% trypsin (GIBCO) and the cerebellar pieces were triturated in Hank’s balanced salt solution (HBSS) containing 3 mg ml⁻¹ BSA, 0.25% glucose, 1.4 mg ml⁻¹ MgSO₄, 330 ng ml⁻¹ DNase I (Sigma-Aldrich) and 4 mg ml⁻¹ soybean trypsin inhibitor (Sigma-Aldrich). Cells were overlaid on a gradient of 4% BSA in HBSS, centrifuged at 180 g for 5 minutes and plated in CGC culture medium [Dulbecco’s modified Eagle’s medium containing 5% foetal calf serum (FCS) (HyClone), 2 mM GlutaMAX™-I (GIBCO), 25 mM KCl, 0.9% glucose, 0.1 mg ml⁻¹ penicillin, 100 U ml⁻¹ streptomycin and 2.5% whole chick embryo extract, prepared as described (Howard and Bronner-Fraser, 1985)]. Neuronal adhesion assays were performed as described previously (McNamee et al., 2002), using 9×10⁵ CGC per well. Experiments were carried out in duplicate (4 hours) or triplicate (18 hours), counting blind five randomly chosen
microscope fields (1 mm²) per coverslip. For neuronal outgrowth assays, wild-type or stably transfected IgLON-CHO cell lines were seeded onto coverslips in a 24-well plate at 2.5x10⁵ cells per well and grown to confluence. CGCs at a cell density of 1x10⁵ cells per well were plated onto CHO cell monolayers and, after 28-31 hours at 37°C in a 5% CO₂ atmosphere, cells were stained with anti-chicken GAP-43 at 1:500 (Allsopp and Moss, 1989), followed by Texas-Red-conjugated anti-rabbit IgG (1:25; Jackson ImmunoResearch). Triplicate experiments, comprising three coverslips per CHO cell type, were carried out, and the proportion of neurite outgrowth was determined, counting blind at least 100 neurons per coverslip. Neurite lengths from 50 fields per experiment were measured using Metamorph, excluding any neurites less than 10 µm in length, and statistics were compiled and analysed with Excel and SPSS using one-way analysis of variance (ANOVA).

Reverse-transcription PCR
Total RNA was isolated from E16 chick embryo cerebellum using TRIzol® (GIBCO) and cDNA was made using SuperScript™ II (Invitrogen) according to the manufacturer’s instructions. IgLON transcripts were amplified using the following primer pairs: LAMP, 5'-GGTACAGGGATGACACCAGGAT-3' and 5'-TGGCCAATTGTGACTCCAGCTTG-3', 153 bp product; OBCAM, 5'-GTCGGAGAAGGACTATGGCAACT-3' and 5'-CACCTCCTATCTAAAGTCGAGGA-3', 190 bp product; CEPU-1, 5'-GACGACAAGCGCTTGCTTAACT-3' and 5'-CCACTGACGCTTTGGACAGTG-3', 242 bp product; and CEPU-Se, 5'-GACGACAAGCGCTTGCTTAACT-3' and 5'-TTGTTGGCCAGCACCTCTGTCACTC-3', 532 bp product.

Preparation and flow cytometry of IgLON-coated polystyrene beads
Carboxylated fluorescent microparticles (Polysciences) were covalently coupled to 400 µg ml⁻¹ protein G (Calbiochem) using the carbodiimide method (Polysciences data sheet 238C) and blocked with 5% ovalbumin at room temperature for 30 minutes with end-to-end mixing. 2.5 µg ml⁻¹ IgLON-Fc or Ox40-HuIg Fc control protein (gift from S. Marshall-Clarke, Liverpool University, Liverpool, UK) were added to a 20% suspension of microparticles in borate buffer and gently agitated at room temperature for 15 minutes. After sonication to obtain single particles, microparticles were allowed to aggregate for 15 minutes, diluted in FACSFlow® (Becton Dickinson) and analysed on a FACSVantage™ SE (Becton Dickinson) equipped with a laser configured to 488 nm ultraviolet light (Coherent Enterprise) and a model 127 He/Ne laser tuned to 633 nm (Spectra-Physics). Forward scatter, side scatter, FL1 (Yellow Green beads) and FL4 (Brilliant Blue beads) parameter data were collected. 10,000 events per sample were analysed using CellQuest™ Pro 4.0 (Becton Dickinson). Statistics were analysed with SPSS using one-way ANOVA.

Cell-based ELISA
Wild-type or stably transfected IgLON CHO cell lines were seeded into a 96-well plate at 3x10⁴ cells per well and grown for 18 hours at 37°C. Cells were incubated at room temperature for 1 hour in fresh culture medium containing 0 µg ml⁻¹, 0.1 µg ml⁻¹, 1 µg ml⁻¹ or 10 µg ml⁻¹ chimaeric IgLON-Fc, washed three times in PBS and fixed in 4% paraformaldehyde, 120 mM sucrose for 10 minutes. IgLON-Fc binding was detected by incubation with horseradish-peroxidase-coupled anti-human Fc (1:5000; DAKO) at room temperature for 1 hour. After 30 minutes in the presence of 3,3',5,5'-tetramethylbenzidine substrate (Sigma-Aldrich), colour development was stopped with 1 M H₂SO₄ and the absorbance read at 450 nm.

Results
LAMP-Fc does not bind to CGCs that express LAMP, OBCAM and CEPU-1 on their surface. We have shown previously that sympathetic neurons, which express LAMP, OBCAM and CEPU-1, bind only to CEPU-1-Fc in cell adhesion assays (Lodge et al., 2000; McNamee et al.,...
Fig. 2. Recombinant IgLON-Fc proteins show differential interactions with CGCs. CGCs adhere preferentially to CEPU-1-Fc and fail to adhere to LAMP-Fc. Dissociated CGCs were incubated on coverslips sequentially coated with protein A and recombinant IgLON-Fc for (A) 4 hours (n=2) or (B) 18 hours (n=3), and adhering cells were counted. After 4 hours, the number of cells adhering to CEPU-1-Fc was approximately 60% of that adhering to poly-L-lysine and was significantly above background (P<0.0001), whereas adhesion to LAMP and OBCAM was similar to background. After 18 hours, adhesion to CEPU-1-Fc had increased to 80% of the poly-L-lysine control and adhesion to OBCAM-Fc was now 50% of the poly-L-lysine control and significantly above background (P<0.0001). Adhesion to LAMP-Fc remained similar to background. (C) Live staining of CGC with IgLON-Fc revealed a punctate stain with both CEPU-1-Fc and OBCAM-Fc, but LAMP-Fc staining was similar to the secondary antibody alone. In keeping with the adhesion results shown above, the CEPU-1-Fc staining was more intense than that observed with OBCAM-Fc. Bar, 25 μm.

Fig. 3. IgLON homodimers have different binding affinities. Fluorescent beads coupled to protein G coated with recombinant LAMP-Fc, OBCAM-Fc or CEPU-1-Fc were allowed to aggregate for 15 minutes and analysed by flow cytometry. (A) CEPU-1-Fc-coated beads showed the best aggregation, with only 22% of beads remaining single, compared with 30% for OBCAM-Fc and 45% for LAMP-Fc. An unrelated lymphocyte protein Ox40-HuIg Fc gave 68%, and protein-G-coupled beads gave 80% (n=4). CEPU-1 and OBCAM were significantly different from LAMP (P<0.004 and P<0.04, respectively). (B) CEPU-1-Fc-coated beads formed larger aggregates than LAMP-Fc-coated beads. The number of single CEPU-1-Fc beads was small compared with single LAMP beads, and the number of CEPU-1 aggregates with three or more beads was much larger.
In order to investigate whether the relative affinity improved with time and approached the activity of CEPU-1-Fc, we tested whether CGC also show a similar discrepancy in their interactions with members of the IgLON family. CGCs express LAMP, OBCAM and CEPU-1 as shown by reverse-transcription PCR (RT-PCR), and immunofluorescence staining revealed that all three are located on their surface (Fig. 1). Nevertheless, cell adhesion assays showed that CGCs bound well to CEPU-1-Fc and only weakly to LAMP-Fc at both 4 hours and 18 hours (Fig. 2A,B). The initial adhesion to OBCAM-Fc at 4 hours was relatively low, but this improved with time and approached the activity of CEPU-1-Fc after 18 hours (Fig. 2B). To investigate whether the relative affinity of protein-protein interactions between the different IgLON molecules varied, the ability of the chimaeric proteins to bind to the surface of CGCs was tested. Although weaker than binding with antibody, both CEPU-1-Fc and OBCAM-Fc binding was observed, but LAMP-Fc binding to the surface of CGC was not detectable (Fig. 2C). This latter experiment strongly suggested that the difference in cell adhesion is primarily due to variations in protein binding rather than indirect effects.

**Differential affinity of homophilic interactions**

The unexpected interactions between the recombinant chimaeric proteins and neurons suggested an incomplete understanding of the molecular complexes formed by this family, and prompted a comparison of the relative affinity of the homophilic interactions of LAMP, OBCAM and CEPU-1. Recombinant IgLONs attach to protein-G-coupled fluorescent polystyrene beads by their Fc tail, leaving both IgLON heads available for binding. Aggregation experiments showed that CEPU-1-Fc interacted with higher affinity, because fewer single beads were observed (Fig. 3). LAMP-Fc had the lowest affinity for itself as judged by the larger number of single beads (Fig. 3A) and smaller size of the aggregated beads (Fig. 3B), whereas the OBCAM-Fc had the highest affinity for itself as judged by the larger number of single beads (Fig. 3A) and smaller size of the aggregated beads (Fig. 3B).

**Fig. 4.** LAMP binds with highest affinity to OBCAM and CEPU-1. CHO cell lines were grown in 96-well plates and incubated with varying concentrations of chimaeric proteins. (A) For LAMP-CHO, strong binding was observed with OBCAM-Fc and CEPU-1-Fc, both of which displayed almost identical absorbance for each concentration tested. However, with LAMP-Fc, the absorbance remained at background levels, even at 10 μg ml⁻¹ (−0.75), as predicted by the previous result. The next highest was CEPU-1-Fc at 10 μg ml⁻¹ (−0.35) and the lowest was OBCAM-Fc at 10 μg ml⁻¹ (−0.2). (C) For CEPU-CHO, highest absorbance was observed with LAMP-Fc at 10 μg ml⁻¹ (−0.8), followed by CEPU-1-Fc at 10 μg ml⁻¹ (−0.7) and the lowest was OBCAM-Fc at 10 μg ml⁻¹ (−0.35), as expected. Error bars lie within the symbol for some points. (D) Taking together the relative affinities of the homophilic interactions from Fig. 3 and the comparative affinity of the homo- and heterophilic interactions, we propose this hierarchy for the relative affinity of IgLON interactions in trans.

**Fig. 5.** Do IgLONs form heterodimers in cis? We propose that IgLONs preferentially form heterodimers on the surface of neurons. The homodimersic IgLON-Fc proteins might not bind to the heterodimers because dimerization induces a conformational change that interferes with binding, because of steric hindrance or because the affinity of one head to one of the heterodimeric subunits is too low to stabilize the complex.
Diglon-CO inhibits neurite outgrowth whereas OBCAM-Fc gave intermediate values. These results mirrored the ability of the chimaeric proteins to bind to the surface of CGCs and sympathetic neurons. Nevertheless, they do not provide an adequate explanation for why LAMP-Fc does not bind to the surface of neurons, because both CEPU-1 and OBCAM are expressed and should be available to bind LAMP-Fc. This prompted us to examine the heterophilic interactions of members of the IgLON family.

Some heterophilic interactions predominate over homophilic interactions (in trans)

To quantify the relative affinity of the homo- and heterophilic interactions, we used a cell-based ELISA assay (Fig. 4). These experiments demonstrated that LAMP bound with highest affinity to OBCAM and CEPU-1 (Fig. 4B,C). By contrast, the OBCAM/CEPU-1 heterophilic complexes were less stable than the heterophilic complexes involving LAMP (Fig. 4B,C). The homophilic interactions of both OBCAM and LAMP were weak (Fig. 4A,B), whereas CEPU-1/CEPU-1 homophilic interactions had relatively high affinity (Fig. 4C), confirming the results obtained from the bead aggregation experiments. It is unclear why CEPU-1/CEPU-1 interactions should have higher affinity than other homophilic interactions, but the recombinant CEPU-1 used in these experiments includes the β exon (whereas the LAMP used was the α isoform), so βCEPU-1 might have a role as a homophilic CAM as well as a heterophilic CAM. It remains to be seen whether this is due to...

Fig. 6. Heterodimeric proteins form on the surface of CHO cells expressing both LAMP and OBCAM. CHO cells stably expressing LAMP or CEPU-1 were transiently transfected with pOIG. Cells were stained with LAMP-Fc, OBCAM-Fc or CEPU-1-Fc. (A) LAMP-CHO cells bound OBCAM-Fc with high affinity but, when OBCAM was expressed, staining was specifically reduced (arrow). Non-transiently transfected cells have either LAMP monomers or dimers on their cell surface but, after expression of OBCAM, LAMP is sequestered by OBCAM in heterodimers. OBCAM:LAMP heterodimers no longer stabilize OBCAM-Fc binding. If OBCAM expression exceeds LAMP expression then OBCAM monomers or dimers will also be available, but these will similarly fail to stabilize OBCAM-Fc binding. (B) LAMP-CHO cells bound CEPU-1-Fc with high affinity but, when OBCAM was expressed, staining was specifically reduced (arrow). CEPU-1-Fc binds well to cells expressing only LAMP, but binding is destabilized by the introduction of OBCAM and the subsequent incorporation of LAMP into LAMP:OBCAM dimers. (C) LAMP-CHO cells bound LAMP-Fc very weakly, but expression of OBCAM increased LAMP-Fc binding owing to greater retention of LAMP-Fc by the LAMP-OBCAM dimers (compared with LAMP homodimers) and perhaps the presence of free OBCAM, which would bind LAMP-Fc with relatively high affinity. Introduction of OBCAM into CEPU-1-CHO cells resulted in little change in fluorescence intensity. (D) LAMP-Fc bound with similar (high) affinity to CEPU-1-CHO and to the OBCAM:OBCAM dimers that might form. However, it is unclear from this experiment whether CEPU-1:OBCAM dimers also form and whether LAMP-Fc can interact with them. Intense fluorescence staining of all cells was observed. (E) OBCAM-Fc and (F) CEPU-1-Fc stained CEPU-1-CHO cells less intensely than LAMP-Fc but, again, OBCAM expression did not change the intensity of fluorescence staining. It is important that, in these last three experiments, transient transfection of OBCAM cDNA was insufficient on its own to change the ability of the chimaeric proteins to bind. For all cases, expression of EGFP alone did not alter IgLON binding. Bar, 25 μm. The fluorescence intensity of untransfected and pOIG-transfected LAMP-CHO cells was quantified. Transfection of OBCAM significantly reduced the binding of (G) OBCAM-Fc or (H) CEPU-1-Fc as judged by the reduction in fluorescence intensity. (I) The fluorescence intensity of untransfected and pOIG-transfected CEPU-1-CHO cells was quantified. Transfection of OBCAM did not significantly alter the binding of LAMP-Fc as judged by fluorescence intensity. (J) The expression of OBCAM on pLIG-transfected CHO cells was unchanged as judged by immunofluorescence staining with antisera to OBCAM.
the β exon or whether CEPU-1 is different in this respect from LAMP and OBCAM. Taken together, these experiments revealed a hierarchy for the homo- and heterophilic interactions as shown in Fig. 4D.

IgLONs form dimers in the plane of the membrane

Nevertheless, this hierarchy of binding does not explain the original observation that LAMP-Fc binds weakly or not at all to CGCs and sympathetic and DRG neurons (McNamee et al., 2002). Even though LAMP-Fc does not bind well to itself, it is clear that LAMP-Fc should bind well to CEPU-1 and OBCAM, which are both present on the surface of CGC. To explain our observations, we need to consider the possible interactions between IgLONs in the plane of the membrane. It has been shown previously that NTM/CEPU-1 will form homodimers in the plane of the membrane (Struyk et al., 1995). We have also observed the formation of homodimers and homotetramers for LAMP and OBCAM, as well as CEPU-1, when cells are confluent (data not shown). We suggest that the tetramers are formed from two pairs of homodimers that have formed in cis, and that these dimers then interact in trans. Thus, in response to external stimuli, all three IgLONs form homodimers and tetramers. When two or more IgLONs are expressed, is it possible that heterophilic dimers might form in cis? And, if so, does this explain why LAMP-Fc does not bind to the neuronal cell surface (Fig. 5)?

Do IgLONs form heterodimers in cis?

This question was investigated by transiently transfecting OBCAM or LAMP into CHO cell lines already expressing one IgLON. The dimeric recombinant proteins were used to detect the presence of monomers that are available to form homodimers (Fig. 6). In the first series of experiments, OBCAM was transiently transfected into LAMP-CHO cells. LAMP monomers and dimers bind OBCAM-Fc with high affinity, whereas OBCAM monomers and dimers bind OBCAM-Fc with low affinity. Crucially, we propose that OBCAM:LAMP heterodimers will also fail to bind OBCAM-Fc owing to their lower affinity for one head, steric hindrance or conformational changes. Thus, OBCAM-Fc can be used to determine whether LAMP monomers or dimers remain available after expression of OBCAM, or whether they are irreversibly sequestered into LAMP:OBCAM dimers. When OBCAM was transiently transfected into LAMP-CHO cells, OBCAM-Fc binding was substantially reduced, a result that can only be accounted for by the irreversible sequestration of LAMP into LAMP:OBCAM dimers (Fig. 6A,G). LAMP expression on the surface of the cells was unchanged by the expression of OBCAM, as judged by immunofluorescence staining with LAMP antisera (data not shown). Likewise, the binding of CEPU-1-Fc was reduced by the introduction of OBCAM (Fig. 6B,H). Again, the formation of LAMP:OBCAM dimers in cis results in the destabilization of the tetrameric complex formed with CEPU-1-Fc, owing to failure of the homodimeric protein to bind to the heterodimer. By contrast, LAMP-Fc binding increased when OBCAM was co-expressed (Fig. 6C), probably owing to overexpression of OBCAM compared with the LAMP already present on the cell surface.

When OBCAM was expressed in CEPU-1-CHO cells, no substantial change in the binding of LAMP-Fc, CEPU-1-Fc or OBCAM-Fc was observed. Because the affinity of all three chimaeric proteins is similar for both CEPU-1 and OBCAM, this experiment does not demonstrate clearly whether CEPU-1:OBCAM dimers form. However, the increase in overall IgLON concentration owing to the transient expression of OBCAM does not cause a detectable change (Fig. 6D-FI), supporting our hypothesis that the differences in fluorescence intensity seen with other combinations are caused by the formation of heterodimers and not by changes in the overall level of IgLON expression. The expression of a second IgLON and GFP does not appreciably affect the expression of the first, as shown by staining OBCAM-CHO cells, transiently expressing LAMP and GFP, with OBCAM antisera (Fig. 6J).

To confirm the formation of LAMP:OBCAM dimers and test for the formation of LAMP:CEPU-1 dimers, we repeated these experiments but transiently transfected LAMP into OBCAM-CHO and CEPU-1-CHO cells. Similar results to those observed in Fig. 6 were obtained (Fig. 7). The expression of LAMP in OBCAM-CHO cells destabilized LAMP-Fc binding, again suggesting that LAMP dimerizes with OBCAM in cis (Fig. 7A). Similarly, expression of LAMP in CEPU-1-CHO cells destabilized LAMP-Fc binding, providing evidence for the formation of LAMP:CEPU-1 dimers in cis (Fig. 7B). In both cases, LAMP expression enhanced binding of OBCAM-Fc and CEPU-1-Fc probably because of overexpression of LAMP compared with CEPU-1 or OBCAM (Fig. 7C,D). In conclusion, these experiments provide evidence for the formation of LAMP:OBCAM and LAMP:CEPU-1 heterodimers in cis, suggesting that each IgLON protein is a subunit of a heterodimeric glycoprotein formed from two GPI-anchored polypeptide chains.

It cannot be determined from these experiments whether CEPU-1:OBCAM dimers in cis are also formed. Therefore, we prepared CHO cell lines stably expressing both αCEPU-1 and OBCAM (CO-CHO). CO-CHO cells expressed both OBCAM and CEPU-1, as shown by immunofluorescence and dot blots (data not shown). We tested the ability of these cells to modify neurite outgrowth from CGCs compared with the CHO cells expressing αCEPU-1, βCEPU-1 or OBCAM alone. Outgrowth on wild-type or LAMP-, OBCAM- and αCEPU-1- or βCEPU-1-expressing CHO cells varied between 28.6% and 34.6%, and the differences were not statistically significant. Outgrowth on the CO-CHO cells was 17.4%, a reduction of 45% from the average of the αCEPU-1-CHO and OBCAM-CHO values, respectively (Fig. 8A). Thus, co-expression of CEPU-1 and OBCAM on CHO cells resulted in activity not seen with either alone. We propose that CEPU-1:OBCAM dimers are the functional molecule, and their ability to inhibit neurite outgrowth is reminiscent of the activity originally observed with the mixture of IgLONs (GP55) (Clarke and Moss, 1994; Wilson et al., 1996).

Although initiation of neurite outgrowth is blocked in some CGCs, approximately 17% do still extend neurites. To test whether neurite extension is affected by CEPU-1:OBCAM dimers in these neurons, we compared the lengths of the neurites grown on wild-type, CEPU-1-CHO, OBCAM-CHO and CO-CHO cells. CEPU-1-CHO and CO-CHO cells showed a small reduction in neurite length compared with the wild type, and neurites on OBCAM-CHO cells were slightly longer (Fig. 8B). Nevertheless, these differences are small and the
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Based on affinity, LAMP dimers should interact with CEPU-1:OBCAM dimers. Surprisingly, LAMP-Fc did not bind to the CO-CHO cells in the cell-based ELISA any better than to the LAMP-CHO cells, and binding was significantly worse than to the CEPU-1-CHO or OBCAM-CHO cells (Fig. 8C). This suggests further evidence that CEPU-1 and OBCAM exist principally as heterodimers on the surface of the CHO cells and suggests that, upon dimerization, one or both subunits either have their binding site for LAMP obscured or adopt a different conformation. In conclusion, CEPU-1:OBCAM dimers might also exist on the surface of CGC, but LAMP-Fc binding is not stabilized.

Discussion

We started with the observation that recombinant LAMP-Fc
Fig. 8. CEPU-1/OBCAM-expressing CHO cells inhibit neurite outgrowth from CGCs. CGCs were seeded at 1×10^5 cells per well onto confluent CHO cell monolayers growing on coverslips and incubated at 37°C in a 5% CO2 atmosphere for 28-31 hours. Cells were fixed, permeabilized and stained with chicken-GAP-43 antibody to identify the neurons. (A) At least 100 individual neurons were counted per coverslip, comparing the number with and without neurites. CO-CHO cells inhibited the number of neurons that had extended neurites by 40% (with respect to the average of the CEPU-1- and OBCAM-expressing CHO cells) and 45% (with respect to the average of the CEPU-1- and OBCAM-expressing cell lines) (*P<0.001 compared with CEPU-1- or OBCAM-expressing CHO cells and P=0.02 compared with wild-type CHO cells). (B) At least 220 random neurites greater than 10 µm long were measured per CHO cell line using Metamorph. Neurites from CGCs seeded on OBCAM-CHO were slightly longer and those on CEPU-1- and CO-CHO slightly shorter, respectively, than those from CGCs seeded on wild-type CHO cells (*n=5). (C) CHO cell lines were grown in a 96-well plate and incubated with 1 µg ml–1 LAMP-Fc. LAMP-Fc bound well to CEPU-1- and OBCAM-expressing CHO cells, but its affinity for CO-CHO cells was comparable to that of the LAMP-CHO cells.

does not bind to the surface of neurons. This was contrary to predictions based on previous studies that showed homo- and heterophilic binding of almost all members of the family. This prompted careful analysis of the relative affinities of both homo- and heterophilic IgLON interactions in transfected CHO monolayers growing on coverslips and incubated at 37°C in a 5% CO2 atmosphere for 28-31 hours. Cells were fixed, permeabilized and stained with chicken-GAP-43 antibody to identify the neurons. CO-CHO cells inhibited the number of neurons that had extended neurites by 40% (with respect to wild-type CHO cells) and 45% (with respect to the average of the CEPU-1- and OBCAM-expressing cell lines) (*P<0.001 compared with CEPU-1- or OBCAM-expressing CHO cells and P=0.02 compared with wild-type CHO cells). (B) At least 220 random neurites greater than 10 µm long were measured per CHO cell line using Metamorph. Neurites from CGCs seeded on OBCAM-CHO were slightly longer and those on CEPU-1- and CO-CHO slightly shorter, respectively, than those from CGCs seeded on wild-type CHO cells (*n=5). (C) CHO cell lines were grown in a 96-well plate and incubated with 1 µg ml–1 LAMP-Fc. LAMP-Fc bound well to CEPU-1- and OBCAM-expressing CHO cells, but its affinity for CO-CHO cells was comparable to that of the LAMP-CHO cells.

The key conclusion of this paper is that IgLONs form and function as heterophilic dimers in the plane of the membrane. We propose that each IgLON protein is predominantly a homophilic CAM. The key conclusion of this paper is that IgLONs form and function as heterophilic dimers in the plane of the membrane. We propose that each IgLON protein is predominantly a homophilic CAM. The key conclusion of this paper is that IgLONs form and function as heterophilic dimers in the plane of the membrane. We propose that each IgLON protein is predominantly a homophilic CAM. The key conclusion of this paper is that IgLONs form and function as heterophilic dimers in the plane of the membrane. We propose that each IgLON protein is predominantly a homophilic CAM.
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A

B

C

Fig. 9. Proposed IgLON and Diglon CAMs. We have shown that LAMP associates in cis with either OBCAM or CEPU-1, and that CEPU-1 and OBCAM also associate in cis; we also propose that the same combinations might be possible for Kilon. (A) The proposed dimeric IgLONs (Diglons). (B) Diglon-LO might, in principle, bind to one or more of the other Diglons. (C) It is unclear whether IgLONs will also function as independent CAMs; this will be the case if cells express only one member of the family or unequal amounts of two or more IgLONs. Based on the results presented here, the most likely combinations are shown. LAMP and OBCAM are unlikely to function as homophilic CAMs, but a cell expressing only LAMP will adhere effectively to an adjacent cell expressing only OBCAM or CEPU-1. It remains to be seen whether CEPU-1 is unique in acting as a homophilic CAM or whether this is an activity restricted to the minor isoforms of CEPU-1 (and LAMP) that have a β exon.

The proposal that GPI-anchored proteins form a heterodimeric membrane protein is novel. Previous work and current theories suggest that GPI-anchored glycoproteins are introduced into the plasma membrane as monomers, possibly in lipid shells, which then cluster in lipid rafts (Anderson and Jacobson, 2002; Harris and Stu, 2002). In some cases, this clustering can result in lateral associations, leading to dimer formation and the subsequent creation of higher-order oligomeric complexes. The Dictyostelium adhesion molecule gp80 oligomerizes in lipid rafts (Harris et al., 2001), and axonin-1 is thought to form an adhesion lattice by homophilic cis and trans interactions (Freigang et al., 2000; Kunz et al., 2002). The typical size and stability of lipid rafts is contentious, but recent proposals that rafts are less than 5 nm in size and contain three to four GPI-anchored proteins of more than one molecular species are particularly interesting and fit well with our data (Sharma et al., 2004). Axonin-1/TAG-1 also forms interactions in cis with the transmembrane glycoproteins NgCAM (Kunz et al., 1998) and L1 (Malhotra et al., 1998), and this is a common theme, because it provides a mechanism for GPI-anchored glycoproteins to signal intracellularly (Castellani et al., 2002; Malhotra et al., 1998; Olive et al., 1995; Tansey et al., 2000). In these cases, it is not clear whether the binding partner is selected on the cell surface depending on the partners expressed and/or in response to external stimuli. Our evidence raises the possibility that IgLONs might form into heterodimeric proteins in vesicles before arrival at the cell surface, and the option to form homodimers is unavailable.

In early experiments, GP55 inhibited the initiation of neurite outgrowth from 100% of DRG neurons. GP55 consisted of a mixture of all the IgLONs in adult chick brain, so it is likely that all the Diglons were present. Here, we have tested the activity of Diglon-CO and shown that it blocks initiation of neurite outgrowth from a subpopulation of CGCs. Other Diglons might act on different subpopulations, and it will be interesting to assay the relative expression of each IgLON subunit in individual CGCs. The neurites that do grow on Diglon-CO-expressing cells are slightly shorter than neurites experiments revealed the formation of LAMP:CEPU-1 dimers. It was not possible to determine whether CEPU-1:OBCAM dimers form using this approach, but a cell line expressing both IgLONs inhibited neurite outgrowth from CGC, an activity not seen with either molecule alone. Previous experiments in which DRG neurons were grown on a mixture of LAMP-, OBCAM- and CEPU-1-Fc proteins combined with laminin also showed no inhibitory activity (McNamee et al., 2002). This suggests that simply mixing CEPU-1 and OBCAM together, without the option of forming dimers, is insufficient to generate activity. Furthermore, LAMP-Fc failed to bind to the CO-CHO cells, a result best explained by the formation of a heterodimer. We have not tested the ability of Kilon to form heterodimers in cis but, based on the information about its trans interactions, we suggest that Kilon will also form heterodimers with CEPU-1, LAMP and OBCAM. We can now explain why LAMP and OBCAM-Fc do not bind detectably to the surface of neurons. LAMP-Fc will not bind to Diglon-LO, -LC or -CO, presumably because of steric hindrance or conformational changes. We propose that LAMP-Fc will not bind to Diglon-KO, -KC and -LK for similar reasons. Thus, the only homodimeric protein that does bind to neurons is CEPU-1-Fc, suggesting that CEPU-1 might be the only IgLON to form homodimers.
on wild-type and OBCAM-CHO cells, but the lengths are similar to those on CEPU-1-CHO cells alone. Thus, the blocking of neurite outgrowth initiation is the most prominent activity of Diglon-CO. However, it remains to be seen whether established growth cones from neurons that do respond to Diglon-CO will also be inhibited. The molecular identity of the receptor on CGG is not known, although a Diglon rather than an IgLON is the most likely candidate. The results described in this paper will substantially change the way in which the activities of the IgLON family are investigated. Cell lines expressing two or more members of the family and heterodimeric recombinant proteins will be required to characterize the involvement of IgLONs in initiation and guidance of axons, cell-cell recognition and control of cell division.

It is now crucial to investigate the complement of IgLONs expressed on the surface of individual cells in order to determine which Diglons are present and, where more than two IgLON subunits are expressed, which combinations are preferred. Is the expression of the different gene products coordinated or can LAMP-OBCAM heterodimers exist alongside excess OBCAM? In Fig. 9, we suggest that the four IgLONs might form six Diglons, each of which might interact with itself or one or more of the other five Diglons. In addition, if some cells do express a single IgLON, each member of the family has a choice of two (or, in the case of CEPU-1, three) other IgLONs that it might interact with. The putative transmembrane receptor that Diglons or IgLONs interact with in cis has not yet been identified but, depending on whether there is a single receptor or several related receptors, the IgLONs and Diglons might mediate a considerable range of cellular responses.

The experiments described here are particularly timely, because recent reports that IgLONs are expressed beyond the nervous system and that OBCAM might act as a tumour suppressor gene (Sellar et al., 2003) will increase the potential because recent reports that IgLONs are expressed beyond the development and growth of neurons, but not non-neuronal cells via a G-protein-coupled receptor. Eur. J. Neurosci. 9, 334-341.

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