The EMBO Journal Vol.18 No.17 pp.4744–4753, 1999

Pathological missense mutations of neural cell adhesion molecule L1 affect homophilic and heterophilic binding activities

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Mutations in the gene for neural cell adhesion molecule L1 (L1CAM) result in a debilitating X-linked congenital disorder of brain development. At the neuronal cell surface L1 may interact with a variety of different molecules including itself and two other CAMs of the immunoglobulin superfamily, axonin-1 and F11. However, whether all of these interactions are relevant to normal or abnormal development has not been determined. Over one-third of patient mutations are single amino acid changes distributed across 10 extracellular L1 domains. We have studied the effects of 12 missense mutations on binding to L1, axonin-1 and F11 and shown for the first time that whereas many mutations affect all three interactions, others affect homophilic or heterophilic binding alone. Patient pathology is therefore due to different types of L1 malfunction. The nature and functional consequence of mutation is also reflected in the severity of the resultant phenotype with structural mutations likely to affect more than one binding activity and result in early mortality. Moreover, the data indicate that several extracellular domains of L1 are required for homophilic and heterophilic interactions.

Keywords: axonin-1/cell adhesion molecules/F11/L1CAM/X-linked hydrocephalus

Introduction

Neural cell recognition molecule L1 is a member of the immunoglobulin (Ig) superfamily implicated in a variety of processes in neurohistogenesis, including neurite elongation, axon fasciculation and migration of neuronal precursors. It is the founder member of a subgroup of cell adhesion molecules (CAMs), which are related by structure and sequence; each consisting of six Ig-like domains, five fibronectin type III (FNIII)-like domains and a highly conserved cytoplasmic tail (reviewed in Kamiguchi and Lemmon, 1997; Brümmendorf *et al*., 1998). The importance of L1 in development is confirmed by its association with a neurological disease in man and by the results of gene disruption experiments in mice. In man, mutations in the *L1* gene have been found to be responsible for a clinically variable X-linked recessive disorder described as either X-linked hydrocephalus, MASA syndrome or spastic paraplegia type I (SPG1) (Rosenthal *et al*., 1992; Jouet *et al*., 1994; Vits *et al*., 1994). The cardinal features of these disorders are varying degrees of mental retardation and spasticity, frequently accompanied by congenital hydrocephalus and flexion deformities of the thumbs (reviewed in Kenwrick *et al*., 1996; Fransen *et al*., 1997). A related phenotype that is partially dependent on genetic background is seen in the mouse *L1* knockout lines (Cohen *et al*., 1997; Dahme *et al*., 1997; Fransen *et al*., 1998a). These observations not only confirm the importance of L1 for neural development but also highlight areas where L1 may have a pivotal role. For example, malformation of the corticospinal tract in both mouse and man may explain the spasticity seen in both species (Cohen *et al*., 1997). Furthermore, abnormal guidance of these axons across the midline confirms a role for L1 not only in growth, but also in correct guidance of developing axons of subsets of neurons. Moreover, malformations such as underdevelopment of the anterior vermis of the cerebellum and fused thalami may be the result of abnormal migration of a subset of cells (Yamasaki *et al*., 1995; Fransen *et al*., 1998a).

L1 mediates its effects on the host neuron through interaction with extracellular ligands and transduction of a variety of signalling events through associated proteins (reviewed in Brümmendorf and Rathjen, 1996; Kamiguchi and Lemmon, 1997; Kenwrick and Doherty, 1998). Biochemically, the extracellular domains of L1 are capable of binding to a variety of ligands, which include L1, itself, other neural members of the Ig superfamily, integrins and extracellular matrix components. The biological significance of many of these interactions is unknown, but for some, a role in neurite outgrowth has been demonstrated. Promotion of neurite outgrowth from a variety of explanted neuronal subtypes by substrate L1 is dependent on homophilic interaction at the neuronal surface (Lemmon *et al*., 1989; Doherty *et al*., 1995).

L1 shares many of its ligands with related molecules of overlapping function. Thus, the emerging view is that L1 is part of a network of dynamically interacting molecules which may form complexes at the cell surface that influence cell morphology and behaviour. Although several L1 interactions are possible, and probably relevant to L1 function, very little is known about how L1 is able to interact with so many different ligands and the precise nature of complexes that may form at neuronal surfaces. In this regard it is of primary importance to understand how each pair of interacting molecules bind. Only then can the interdependency of binding and potential complex formation be properly investigated.

Human mutations of L1 may provide valuable insight into the role of individual domains in L1 function. In

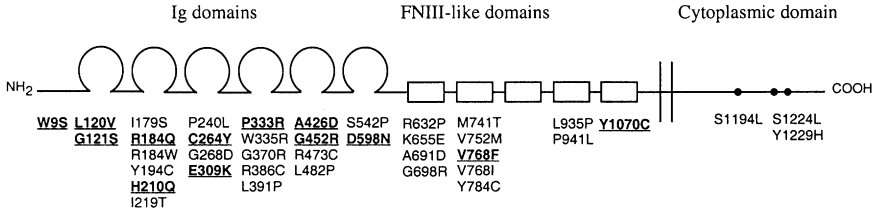


Fig. 1. Missense mutations in relation to the domain structure of L1. Mutations in bold and underlined are studied here.

particular, missense mutations highlight individual residues/regions of functional significance and provide a reservoir of variation that can be correlated with functional effect through *in vitro* assays. Thirty-nine different missense mutations have been described, the majority of which affect the extracellular, and therefore ligand-binding, region of L1 (Figure 1, compiled from references in the L1 mutation database http://dnalab-www.uia.ac.be/dnalab/11/, Du *et al*., 1998b and Saugier-Veber *et al*., 1998). They are distributed across 10 out of 11 ectodomains, which suggests that the integrity of most domains is required for correct L1 ligand interaction. In this report we examine the consequences of L1 pathological mutations for L1 ligand binding for the first time using expression systems that allow the production of glycosylated mammalian L1 extracellular domains. This allows the effects of individual changes to be studied in the context of correctly folded, intact L1 and enables us to discuss models for L1 homophilic and heterophilic interaction at the cell surface. We have concentrated on three ligands; L1 itself and the related GPI-anchored CAMs axonin-1 (also known as TAG-1 and TAX-1) and F11 (F3 or contactin) for the following reasons. First, homophilic binding is known to be important for neurite outgrowth in response to external L1 in *in vitro* assays using several different types of neuron (reviewed by Kamiguchi and Lemmon, 1997). Secondly, a characteristic feature of all vertebrate L1 subgroup members is their binding to F11 or to axonin-1 (Brümmendorf *et al*., 1993; Morales *et al*., 1993; Suter *et al*., 1995; Volkmer *et al*., 1996, 1998; Sakurai *et al*., 1997; Kunz *et al*., 1998). Thirdly, axonin-1 on the neuronal surface is required for L1-promoted neurite outgrowth and is assumed to be complexed with L1 in the membrane of a responsive neuron (Buchstaller *et al*., 1996; Rader *et al*., 1996; Stoeckli *et al*., 1996). The results presented here demonstrate for the first time that the human pathology may be due to different types of L1 malfunction and by inference, therefore, both homophilic and heterophilic binding are important for correct nervous system development. We show that many domains are involved in both homophilic and heterophilic binding and the data allow us to propose novel models for L1 interactions.

Results

To investigate whether extracellular missense mutations in L1 affect L1 ligand interactions, 13 substitutions distributed across the protein were chosen (shown bold and underlined in Figure 1). Through comparison with models of L1 domain structure, six of these mutations are predicted to affect the conformational integrity of individual domains as they involve key structural residues (Figure 1 and Table I; Bateman *et al*., 1996). Six are predicted to only affect surface properties of individual domains and one affects the signal peptide. Mutations were engineered into mammalian expression vectors pcDNA3 and pIG 5.2 in the context of both full-length L1 and the extracellular domains, respectively. Transient transfection of COS cells with pcDNA3 constructs was used to determine whether any of the mutations prevented translocation of L1 to the cell surface. Immunofluorescence staining of non-permeabilized cells showed that all mutant proteins are expressed on the cell surface, with the exception of W9S, a mutation in the putative signal peptide (unpublished results). Constructs representing the remaining 12 mutations were used in homophilic and heterophilic binding assays.

Several adjacent domains of L1 are required for homophilic binding

The ability of the mutant proteins to bind homophilically was assessed using a fluorescent microsphere bead aggregation assay (Kuhn *et al*., 1991). Purified Fc–L1 chimeric protein was captured onto microsphere beads coated with anti-Fc antibody. Inclusion of the Fc tag at the C-terminus of the chimera ensures that the captured protein is preferentially in an orientation that mimics presentation at the cell surface, thus facilitating *trans* binding between L1 molecules. To demonstrate that equal amounts of protein were captured each time, the Fc–L1 proteins were captured on the beads then removed by boiling in SDS sample buffer and run on SDS–PAGE. Figure 2 shows that for different chimeras equal amounts of protein were captured and, therefore, any differences in bead aggregation will not be due to differential protein loading.

To assess the effects of individual mutations on homophilic binding, Fc–L1-coated microspheres were disaggregated to a single bead suspension and clustering was measured over time. Aggregation time courses for the 12 missense mutations and a histogram of bead aggregation at 30 min are shown in Figure 3. Only three mutations (L120V, E309K and Y1070C) had no significant effect on homophilic binding compared with wild type. All other mutations had variable, but highly reproducible, effects on binding. The Ig6 mutation, D598N, showed a small but reproducible 18% reduction. Eight mutations affecting residues in Ig1–5 and FNIII-like domain 2 had a dramatic effect on aggregation. The degree of disruption varied from 44% reduction for V768F to 90% for G121S at 30 min. The mutations that affect homophilic binding are distributed throughout the protein, located in Ig domains 1–6 and FNIII domain 2, suggesting that all of these domains are required for homophilic binding. Six of these

Table I. Clinical summary for mutations studied in relation to ligand binding

Mutation	Enlarged ventricles or hydrocephalus (cases)	Mental deficit	Spastic paraplegia	Thumb deformity	Deaths <1 year (cases)	% binding versus wild type		
						L1	F11	Ax-1
Structural								
G121S	2/2	+	+	–	0/2	10	40	35
R184Q	15/15	+	+	+	10/15	17	7	15
C264Y	5/5	ND	ND	+	5/5	42	6	19
P333R	1/1	ND	ND	+	0/1	14	13	11
G452R	3/3	+	+	+	2/3	20	26	10
V768F	2/2	+	+	+	0/2	56	31	56
	28/28				**17/28**			
Surface								
L120V	1/1	+	+	+	0/1	100	90	88
H210Q	1/5	+	+	+	0/5	29	161	238
E309K	2/2	+	+	+	0/2	96	26	30
A426D	0/1	+	?	+	0/1	18	19	7
D598N	0/3	+	+	+	0/3	82	19	26
Y1070C	4/4	+	+	_	1/4	99	177	162
	8/16				**1/16**			
Other								
W9S	2/2	+	+	+	1/2			

+, presence or absence of abnormality in at least one affected case. –, not apparent. ND, not assessed due to early mortality. ?, no information. Mutations are categorized into those that do and those that do not affect key structural residues. The W9S mutation is classified separately as this affects the signal peptide. Levels of ligand binding are expressed relative to 100% wild-type binding.

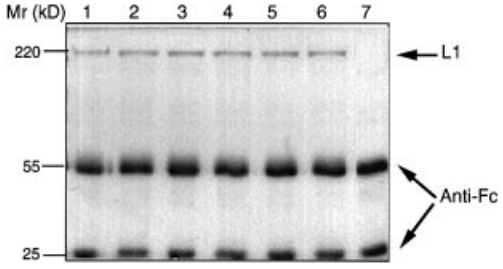


Fig. 2. L1–Fc chimeric proteins bind microspheres equally. Fc chimeric proteins were coupled to fluorescent beads coated with anti-Fc antibody. Protein was eluted in sample buffer, run on SDS–PAGE and visualized using Coomassie Brilliant Blue staining. Typical results for five proteins are shown. Lane 1, wild-type L1; lanes 2–6, mutant proteins C264Y, L120V, G121S, H210Q and R184Q, respectively; lane 7, anti-Fc coated beads only.

mutations (G121S, R184Q, C264Y, P333R, G452R and V768F) were predicted by Bateman *et al.* (1996) to affect the structure of individual L1 domains as they affect key residues required for correct folding; these mutations therefore probably affect binding by distorting domain conformation (Table I). H210Q, A426D and D598N affect residues with surface side chains and would not be predicted to affect individual domain structure. These mutations must therefore affect homophilic binding either through disrupting tertiary structure or through altering homophilic contact sites directly.

F11 and axonin-1 may interact with L1 in a similar manner

L1 and its chick counterpart NgCAM interact heterophilically with the Ig superfamily members F11 and axonin-1 (Kuhn *et al*., 1991; Brümmendorf *et al*., 1993), two structurally related neural Ig superfamily members which have been implicated in axon fasciculation and neurite outgrowth (Brümmendorf and Rathjen, 1995; Rader and Sonderegger, 1998). Because histopathogenesis in patients includes malformation of axon tracts, we investigated whether interactions with these ligands are impaired by disease-causing mutations. For this analysis we assessed binding of F11- and axonin-1-coated microspheres to wild-type and mutant L1 protein expressed on the surface of eukaryotic cells. A mixed-bead aggregation assay would have been inappropriate in this case as it would have been complicated by the ability of L1-coated beads to self aggregate. For each assay COS cells were transfected with engineered plasmids to generate a mixed confluent layer of L1-expressing and non-expressing cells and then F11- or axonin-1-coated microspheres were bound. L1-expressing cells and bead binding were detected by two-colour immunofluorescence. A pilot experiment was conducted to show that F11 and axonin-1 coated beads bind to L1-expressing cells and not to untransfected cells (Figure 4).

For the analysis of mutant protein an automated image analysis system was developed to quantify microsphere binding to large numbers of L1-expressing cells (>200 cells for at least five experiments). A comparison of mutants requires that equal amounts of L1 protein are expressed on the cell surface. Therefore cells were chosen for analysis which expressed similar amounts of protein on the surface relative to a wild-type control performed in parallel (Figure 5D). These analyses showed that different disease-associated mutations have distinct effects on heterophilic ligand binding. For axonin-1 (Figure 5A) and F11 (Figure 5B), only the L120V mutation, which involves conservative exchange of a surface residue in the first Ig domain, had no effect on binding. All other mutations result in either decreased (G121S, R184Q, C264Y, E309K, P333R, A426D, G452R, D598N, V768F) or increased (H210Q and Y1070C) binding. Interestingly, the profile of binding activities of L1 mutants with respect to L1–axonin-1 interaction was similar to that observed for L1–F11 binding with one exception. Mutation H210Q which increased ligand binding, showed a more pronounced affect on axonin-1 than on F11 binding ($P = 0.06$). The heterophilic binding profile for mutations does

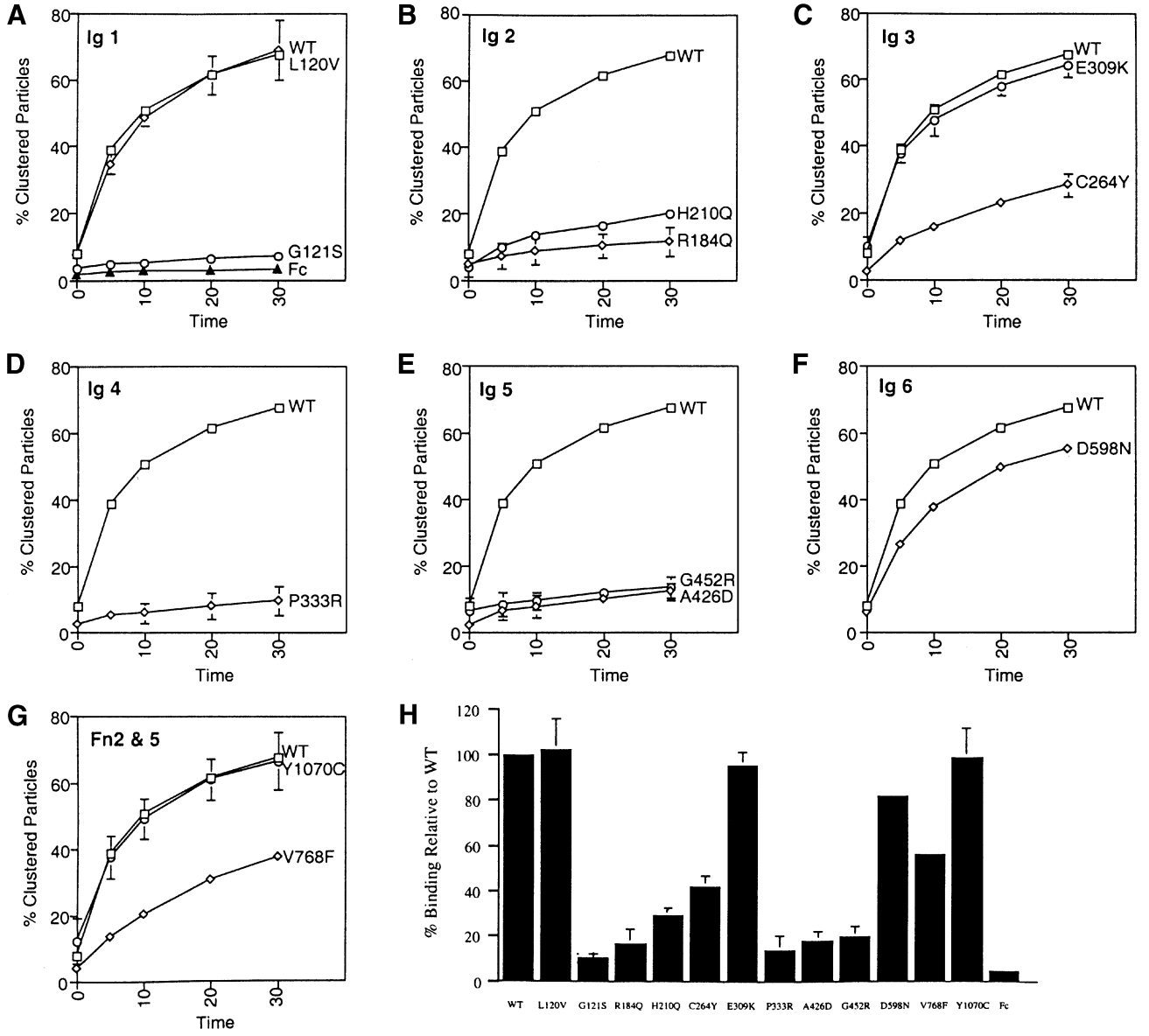


Fig. 3. Analysis of the homophilic binding of L1 wild-type and mutant Fc chimeric proteins. The homophilic binding capacity of wild-type and mutant L1–Fc chimeric proteins was assessed by FACS analysis as described in Materials and methods. Error bars represent the SEM of at least three independent analyses. (**A**–**G**) Homophilic binding of the mutant proteins and an anti-Fc only control, standardized to wild type for mutations in Ig domains 1–6 and FNIII domain 2, respectively. (**H**) A histogram comparing the per cent wild-type aggregation at 30 min for mutant proteins and anti-Fc coated beads.

not mirror that of homophilic binding. As outlined above, six of these mutations possibly disrupt the integrity of Ig domains 1–5 and FNIII-like domain 2 suggesting the involvement of extensive regions of L1 in interactions with axonin-1 and F11. The deleterious effects of surface mutations E309K, A426D and D598N in the third, fifth and sixth Ig domains support this proposal.

In our quantification of heterophilic L1 interactions, we have used F11 and axonin-1 isolated from chick brain as it can be isolated in sufficient quantities for comprehensive investigations. To examine the possibility that avian and mammalian proteins differ in their binding profiles with respect to mutant human L1, binding of chick F11 was compared with that of F3 (the mouse orthologue of F11). Microspheres coated with F3–Fc fusion protein were incubated with COS cell transfectants as described above. Mutants H210Q and Y1070C showed increased binding of F3–Fc, L120V showed wild-type binding and all other mutants, in particular E309K, showed strongly reduced binding of the mouse protein (Figure 5C). Thus, mutations in L1 affected the binding of chick and mouse F11/F3 in a similar manner suggesting that L1 binding sites on these proteins have been conserved during evolution.

Discussion

We have surveyed the ligand-binding effects of a series of pathological mutations in L1 and found that the majority of missense mutations in extracellular domains affect homophilic interactions, heterophilic binding to other cell adhesion molecules of the immunoglobulin superfamily or both. The effects of these mutations allow us to draw conclusions regarding the nature of different L1 ligand interactions and these will be discussed in turn.

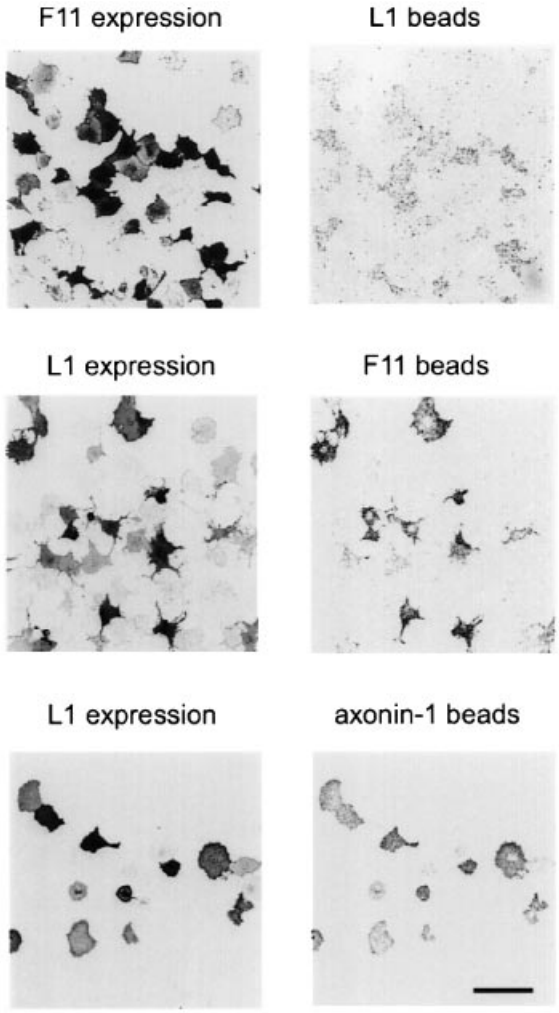


Fig. 4. Interactions of L1 with the neural Ig superfamily members F11 and axonin-1. COS cells were transfected with F11-expressing plasmids to generate confluent mixtures of F11 expressing and non-expressing cells which were incubated with microspheres coated with L1–Fc fusion protein (top). The left-hand side shows expression of F11 (red fluorochrome) whereas the right-hand side shows distribution of L1–Fc beads (yellow fluorescence). Inverted images are shown. Similarly, L1-expressing COS cells but not untransfected cells were found to bind beads coated with F11 (second row) and L1-expressing cells bound axonin-1 (third row). Scale bar, 200 μM.

Most missense mutations interfere with homophilic binding

The nine mutations that disrupt homophilic binding reside in several domains (Ig1, Ig2, Ig3, Ig4, Ig5, Ig6 and FNIII-like domain 2), indicating that the integrity of all of these domains is required for wild-type binding, although the contribution of each may vary. For those mutations that effect key structural residues, i.e. G121S, R184Q, C264Y, P333R, G452R and V768F, their effect may not be local but may spread to conformationally linked domains. However, structural mutations are possibly limited in their ability to transmit conformational change beyond a single domain and its boundaries and, therefore, these results still implicate a large portion of the molecule in homophilic interaction. In contrast, two mutations, H210Q in Ig2 and A426D in Ig5, severely reduce homophilic binding although they are highly unlikely to disrupt domain structure. These two mutations may highlight important contact sites in L1–L1 interactions or in the formation of interdomain tertiary structure. The D598N mutation in Ig6, that has a modest but significant effect on homophilic binding also affects a surface site.

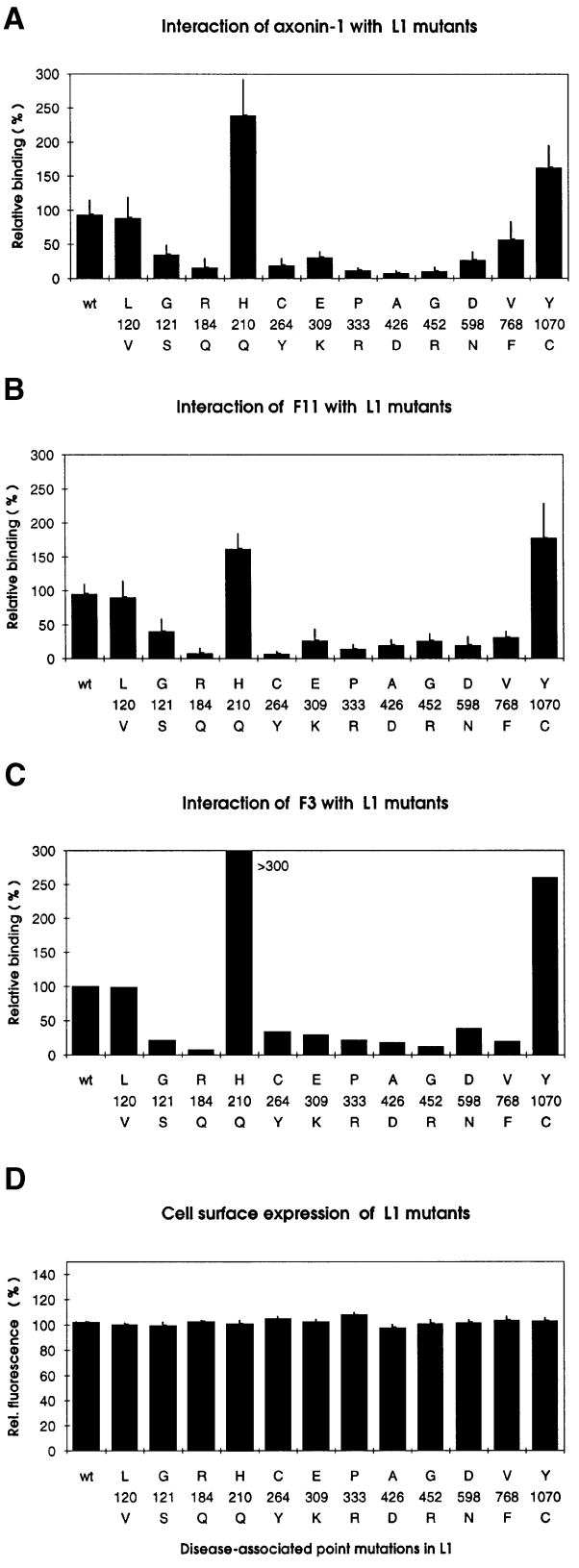
Additional evidence for the involvement of several domains in homophilic binding comes from consideration of the insect Ig superfamily adhesion molecule hemolin and domain deletion studies on chick NgCAM (Kunz *et al*., 1998). X-ray crystallography of the four Ig-domain hemolin protein indicates that a horseshoe structure can be adopted which is stabilized by interactions of the first with the fourth and of the second with the third domain (Su *et al*., 1998). This structure is possible due to the small seven-residue spacer region between Ig domains 2 and 3, which is also found in mammalian L1 and chick NgCAM. Moreover, many of the key residues required for hemolin folding are conserved in L1 subgroup members. There is a high degree of conservation at the putative intramolecular contact sites for L1 and hemolin and therefore it seems possible that the first four domains of L1 can also adopt a horseshoe structure. Interestingly, 50% of the known pathological human missense mutations in the first four domains of L1 lie within the regions defined as intramolecular binding sites for hemolin even though these binding faces comprise only 13% of the residues. Those mutations that lie within these regions include the G121S, R184Q and C264Y, changes which drastically affect homophilic binding. We therefore propose a model in which the first four domains of L1 adopt an intramolecular hemolin-like fold and the extracellular domains overlap in an antiparallel fashion (Figure 6). The exact degree of overlap cannot be determined on the basis of these data and will require further analysis of mutated constructs. The effect of the V768F mutation suggests that antiparallel overlap may involve this domain although it is also possible that this mutation has an indirect effect through affecting the presentation of Ig domains.

How do these data compare with previous studies on homophilic interaction? Several models have been proposed, the most prominent of which is one involving Ig2 self-binding in an anti-parallel fashion where Ig1 and Ig3 may have, at most, a stabilizing effect (Zhao and Siu, 1995; Zhao *et al*., 1998). Although our data endorse the involvement of Ig2 this model seems highly improbable as it cannot explain the effect of mutations in other domains. Our results have something in common with those produced by Holm *et al.* (1994), which also implicated several domains in homophilic binding although the model they suggest would not explain all of the results described here. Differences in these data sets may reflect the experimental design. Earlier studies mainly used *Escherichia coli*-produced protein and only single, or few isolated L1 domains. None of these soluble proteins was checked for correct folding and it is conceivable that they do not adopt a native structure. Our data have been derived using mammalian protein with each missense mutation analysed in the context of the entire 11-domain extracellular region.

The profile of mutations affecting heterophilic binding of L1 to F11 and axonin-1 is overlapping but distinct from that affecting L1–L1 interaction

A difference in heterophilic versus homophilic binding properties is not surprising in view of models which

suggest *cis* interaction of F11 and axonin-1 with L1 subgroup members (Rader *et al*., 1996; Sakurai *et al*., 1997). Again, the data presented here implicate several domains. For axonin-1 and F11 the integrity of all Ig domains, as well as the second FNIII-like domain, is clearly required. This result is compatible with a model for involvement of Ig domains 2 and 3 as well as the third FNIII-like domain in binding of chick NgCAM to axonin-1 (Kunz *et al*., 1998) as structural alteration of the second FNIII fold may have a reverberating effect on the third FNIII-like domain. Most interesting are the results obtained for two mutations E309K and H210Q that have almost reciprocal effects on homophilic versus heterophilic binding. E309K protein while having wild-type L1 binding, significantly reduces interaction with both F11 and axonin-1. As E309K affects a surface site on L1 this mutation may highlight a contact site for the heterophilic ligands. In contrast, H210Q increases the ability of L1 to bind F11 and axonin-1, although this surface mutation almost destroys homophilic binding. Mapping of these two surface residues onto the hemolin structure indicates that they would have side chains emanating from opposite sides of the horseshoe, supporting the involvement of different sides of the L1 molecule in homophilic versus heterophilic interaction. Interestingly, recent studies suggest that NgCAM may simultaneously interact both homo-



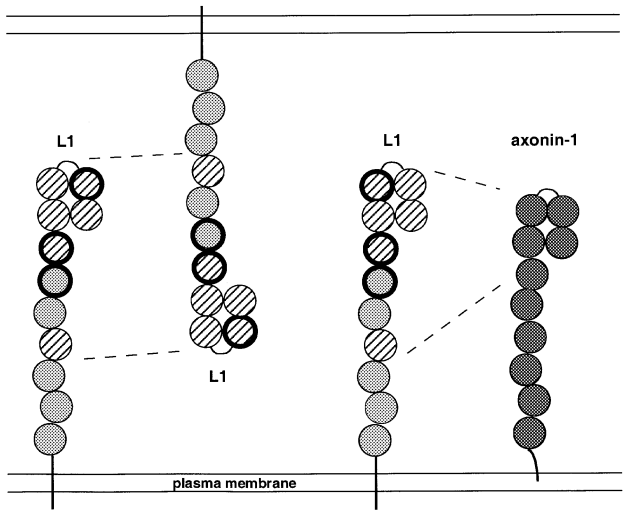


Fig. 6. A summary of mutation effects in relation to L1 homophilic and heterophilic binding. Models of homophilic and axonin-1 interactions are shown with the positions of mutations that reduce binding depicted by hatched domains (structural changes) or bold outlines (surface changes). Axonin-1 is known to interact with L1 *in cis* through the first four domains of the GPI-linked protein. The first four domains of both L1 and axonin-1 may adopt a horseshoe structure similar to that observed for hemolin as many of the key structural residues are conserved between these proteins. Both interactions possibly involve several domains of the L1 protein. The exact extent of L1 involvement cannot yet be defined but possibly involves several Ig domains and may extend as far as the second FNIII-like domain where a structural mutation affects both interactions. For simplicity, mutations that increase heterophilic binding are not depicted here.

Fig. 5. The effect of disease-associated mutations on binding of L1 to F11 and axonin-1. COS cells were transiently transfected with expression plasmids encoding wild-type or mutant L1. Binding of chick axonin-1 (**A**), chick F11 (**B**) and mouse F3–Fc-coated beads (**C**) to the transfectants was quantified with an image analysis system. Values are expressed as per cent wild-type binding with mean and error bars (SEM) calculated from at least five independent analyses (A and B) and with mean values from two independent experiments (C). Differences in F11 and axonin-1 binding are not due to differences in amounts of surface L1 as cell-surface expression levels were equivalent (**D**).

philically and heterophilically utilizing different regions of protein for each interaction (Kunz *et al*., 1998). It is conceivable that H210Q may destabilize a conformation of L1 that contains hidden F11 and axonin-1 binding sites, thus increasing heterophilic binding. Such masked sites have been proposed for axonin-1 itself (Rader *et al*., 1996).

The second mutation that leads to increased binding is Y1070C, which, interestingly, is one of several pathological L1 mutations that introduce a free cysteine at the protein surface. *In vivo*, this type of mutation may promote inappropriate disulfide bond formation inside or outside the cell, thereby disturbing L1 mobility, availability or downstream function. Inappropriate disulfide bond formation plays a role in hereditary diseases linked to the fibroblast growth factor receptors (FGFRs; Galvin *et al*., 1996; Neilson and Friesel, 1996) and potentially in the neurological phenotype resulting from mutations in Po (reviewed in Chothia and Jones, 1997); both FGFRs and Po are Ig superfamily members. The availability of a free cysteine may also contribute to the enhanced binding seen in *in vitro* assays through enhanced presentation of natural or masked binding sites as, in these assays, L1 is mobile within the COS cell membrane. Finally, H210Q and Y1070C may qualitatively change a local binding site for heterophilic ligands.

Our observation that mammalian F11 (F3, contactin) shows essentially the same binding profile with respect to human L1 mutants as avian F11 is not surprising as Ig superfamily CAMs from different vertebrate species have been found to interact functionally (reviewed in Brümmendorf and Rathjen, 1995). For example, human axonin-1 (TAX-1) promotes neurite outgrowth of chick dorsal root ganglion neurons (Hasler *et al*., 1993).

Perhaps the most striking observation is that L1 mutations affect binding to axonin-1 and F11 in a similar fashion and thus these two proteins may interact with L1 in a very similar, although not identical, manner. The implication from this observation is that axonin-1 and F11 are unlikely to interact with L1 at the same time.

The L120V mutation

One conservative mutation, L120V, had no effect on homo- or heterophilic binding. At the nucleotide sequence level the L120V mutation creates a potential cryptic donor splice site within exon 4 of the coding region of L1 (ag**c**tgggc to ag**g**tgggc). Moreover, the new site has a higher consensus value (CV = 0.885) for a mammalian donor splice sequence (consensus = aggtaagt) than does the natural intron 4 site gggtgcga (CV = 0.799; Krawczak *et al*., 1992). Thus it is possible that this nucleotide change exerts its affect on L1 function by affecting mRNA splicing rather than by disruption of ligand interactions. Approximately 15% of disease-causing mutations in L1 are due to effects on mRNA splicing and a silent (G308G) coding region L1 mutation affecting splicing has been reported recently (Du *et al*., 1998a).

The W9S mutation interferes with surface expression

In theory, missense mutations could be affecting protein stability or trafficking in addition to ligand interaction. W9S, a substitution within the hydrophobic region of the putative signal peptide, was predictably found to affect surface expression (data not shown). Most mutations, however, did not eliminate cell-surface expression on COS cells, although subtle effects on protein trafficking or metabolism could be contributing to patient phenotype.

Structural, functional and phenotypic correlation

Phenotype/genotype correlation for patients with L1 mutations has been confounded by a degree of intrafamilial variation in clinical presentation. Nevertheless, some correlation of early mortality with type of mutation has been observed. In particular, mutations that eliminate L1 from the cell surface are more likely to result in severe hydrocephalus and early mortality (Yamasaki *et al*., 1997; Fransen *et al*., 1998b). Fransen *et al.* also conducted an analysis on 13 cases that suggested that key residue mutations might be more devastating than those affecting surface sites. This conclusion is supported by the clinical details summarized in Table I. All 28 patients with structural mutations presented with hydrocephalus or enlarged ventricles and 17 of these did not survive past 1 year. In contrast, only 8/16 patients with surface mutations developed enlarged ventricles and all survived. We now have the opportunity to compare the structural nature of each mutation with the ability of L1 to bind to itself, axonin-1 or F11. Clearly, mutations that affect the structural integrity of individual domains result in the diminution of both homophilic and heterophilic binding. Those that affect surface residues have less predictable consequences. A426D inhibits binding by all ligands in a similar manner to a structural mutation. H210Q, E309K and D598N, however, primarily affect either heterophilic or homophilic interaction. As the surface residue mutations tend to be associated with a less morbid phenotype, this may be the result of interference with some rather than all of L1′s many functional interactions. The Y1070C mutation, which does not eliminate binding for any of the ligands tested, is found in a family where three out of four affected cases survived to adulthood.

In conclusion, we have demonstrated that the majority of missense mutations in the extracellular domains of L1 affect ligand interactions and some of them have distinct effects on the binding of different ligands. The fact that pathological mutations can affect either homophilic or heterophilic interactions alone suggests that both forms of L1 binding activity are important *in vivo* and that some aspects of patient pathology are due to disturbances in cell-surface interaction. Domains of human L1 involved in heterophilic binding have not been explored previously and our data indicate that interactions of F11 and axonin-1 with L1 may be similar. The observation that several domains are involved in homophilic binding is in stark contrast to previous results and the data indicate that new models for L1–L1 and L1–axonin-1/F11 interactions will need to be explored. Distinct surface residues that affect binding may highlight contact sites between proteins and serve as a foundation for directed mutagenesis and peptide inhibition experiments aimed at investigating L1 interactions.

Materials and methods

Clinical details of patients with L1 mutations

The clinical details for families with mutations W9S, G121S, R184Q, H210Q, C264Y, E309K, G452R, D598N, V768F and Y1070C have been

Table II. Mutagenesis strategy

Mutation	1st round PCR primers	2nd round PCR primers	R.E. digest
W9S	V1 (cactaaagggaacaaaagctggag) W9Smp (gaggagaggc**g**acacgtaccg)	L1 (atggctctggggcttgtgcag)	*Eco*RI–*Sph*I
L120V	V1 L120Vmp (gcggtgccca**c**cttattgctg)	L1	*Eco*RI–*Sph*I
G121S	G121Smp (caataagctg**a**gcaccgccat) L2 (ccgttctggcccatcgtcac)	V2 (ccgaattccggcgccgggaaagat) *Eco*RI–*Bgl*II	
R184Q	R184Qmp (caggacgagc**a**ggtgacgatg) L1	L3 (gtaccagtcgccccactctggc)	*Bgl*II–*Sph*I
H210Q	H210Qmp (acatctgcca**g**gcccacttcc) L1	L3	*Bgl*II–*Sph*I
C264Y*	L4 (atggtcgtggcgctgcggtacg) L5 (tgctgtgcttcctctgactg)	L6 (cagagcctctccggatctac) L1	*Bgl*II–*Sph*I
E309K	L7 (gcatgattgacaggaagccgc) E309Kmp (cagcggtact**t**gccatcatcc)	L8 (acgtagatgtaggcattgcg)	*Sph*I–*Sex*AI
P333R	L9 (cagaaccacaacaag) P333Rmp	L10 (tggccacgcagctgtagttgccctg)	*Sph*I–*Bsm*I
A426D	L11 (ccgagaactcactgggca) A426Dmp (aggatcttg**g**ctggcagct)	L10	*Sph*I–*Bsm*I
G452R	L11 G452Rmp (gcacaggcgctc**c**gaaggc)	L10	*Sph*I–*Bsm*I
D598N	L12 (gatgcaactcagatcactcaggg) D598Nmp (ccaccacat**t**cagttcggta)	L4	*Bbr*PI–*Bbr*PI
V768F*	L13 (gactgccaagtccagggcaggcc) L14 (gtactcgccgaaggtctcatc)	L15 (ctggagtcctgcagaagacc) L5	*Sma*I–*Eag*I
Y1070C*	L13 L14	L16 (ctacgtgctctcctaccacc) 17 (aagccagcaggagggagcctca)	*Kpn*I–*Sac*I

Mutant clones for each mutation were generated either using three-primer mutagenesis or by nested PCR from patient cDNA (marked with asterisks). Primers are sense (top line) or antisense (bottom line) with respect to L1 coding sequence. Primers containing a mutation are suffixed with mp and the changed base is bold and underlined. Mutated cassettes of gene were cloned into wild-type by replacement of sections flanked by restriction enzymes (R.Es) that cut only once in L1 cDNA.

summarized previously (Krawczak *et al*., 1992; Jouet *et al*., 1993, 1994; Vits *et al*., 1994; Jouet and Kenwrick, 1995). Details from these references together with unpublished information on families with L120V, A426D and P333R (present authors and E.Fransen and S.Forrest, personal communication) have been used to compile Table I. The L120V and P333R mutations are both unpublished and result from nucleotide changes C358G and C998G, respectively, of the L1 coding sequence (numbered according to Hlavin and Lemmon, 1991).

Clone construction

A 3.9 kb *L1* cDNA clone containing from 14 bp upstream of the first methionine codon to 206 bp downstream of the stop codon was obtained from J.Hemperly at Becton Dickinson. Derivatives of this clone have been used previously for the production of functional L1 (Doherty *et al*., 1995). For expression of full-length *L1* in mammalian cells the 3.9 kb *Eco*RI insert was cloned from pBluescript (pBS-L1) into the *Eco*RI site of pcDNA3 (5.4 kb, Invitrogen). For the production of the Fc–L1 chimeric protein, a clone containing only the extracellular domains of L1 up to the first residue of the transmembrane domain was cloned into a modified version of the pIG1 (Simmons, 1993). The vector had been modified to include a splice donor site downstream of the inserted gene and delete *Eco*RI and *Pst*I sites using adapter-mediated mutagenesis (pIG 5.2, a gift from Pat Doherty). Full-length *L1* cDNA was first modified in a pBluescript derivative in order to retain the extracellular domains on an *Eco*RI–*Eco*RV fragment that could be inserted into pIG 5.2 in-frame with the portion of the vector encoding the Fc portion of human IgG1. Briefly, an artificial *Eco*RV site was inserted after the region encoding the fifth fibronectin domain using the following PCR-directed mutagenesis strategy. A 222 bp PCR product was generated from *L1* cDNA using primers NL6V (agtggcgaagatatcagggagcct, a modified antisense *L1* primer containing an *Eco*RV site) and L3 (caggttccatatcttgttca). This was then cut with *Eco*RV and *Sac*I and used to replace the 3′ end of *L1* from a natural *Sac*I site located at position 3218 in the fifth FNIII domain. The resultant clone encoded for 1114 amino acids of the mature protein with six natural residues and one introduced aspartic acid following the final FNIII domain. Modified versions of pBluescript (Stratagene) containing a single *Eco*RI site or *Eco*RI, *Eco*RV and *Hin*dIII sites in place of the multiple cloning site were also engineered for easy manipulation of the *Eco*RI or *Eco*RI–*Eco*RV fragments of *L1* in *in vitro* mutagenesis experiments. Deletion of the multiple cloning site allowed the use of additional restriction enzyme sites when cassetting mutated sections of *L1* into the wild-type clone.

Plasmid clones containing mutated *L1* were engineered using one of two different methods. Where cDNA from lymphoblastoid cell lines of patients was available, nested PCR reactions were conducted as described previously (Jouet *et al*., 1994) to amplify a section of *L1* containing the mutant base. This was cloned into wild-type L1 using appropriate flanking restriction enzyme sites (Table II). Otherwise, PCR-directed *in vitro* mutagenesis was performed on a small section of the gene. The mutated cassette was then inserted into wild-type *L1* using appropriate natural restriction enzyme cleavage sites. *In vitro* mutagenesis was conducted using a three primer method modified from that described by Ke and Madison (1997). Initially a megaprimer was produced using 30 cycles of PCR on wild-type *L1* cDNA template using *pfu* (Stratagene) or BIO-X-ACT™ (Bioline) proof-reading DNA polymerases with a mutagenic primer and flanking *L1* primer. An aliquot of megaprimer was then used in conjunction with a third flanking primer in a second round PCR. In this way an *L1* fragment containing the mutation was produced. This was then cut with appropriate restriction enzymes for replacement of the same region in the wild-type clone. Mutated cassettes were used to replace wild-type sections of *L1* (Table II). Clones were sequenced in two directions across the mutated cassettes using the Prism™ dye terminator kit (Perkin-Elmer) and *L1* gene primers and analysed on an ABI 373 semi-automated DNA sequencer.

Cell transfection and immunofluorescent staining

COS-7 cells were cultured until confluent in Dulbecco's modified Eagle's medium (DMEM; Sigma D-5671) containing, 200 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum (FCS). The cells were harvested and plated to sub-confluence on 4-well chamber slides (Nunc), transfected with 0.5 μg of DNA (purified using QIAprep spin miniprep kit) using lipofectin reagent (Gibco-BRL) and incubated overnight. The medium was replaced and the cells were further incubated for 24 h to allow expression of the protein. For examination of cell-surface expression, cells were fixed with 3% paraformaldehyde prior to antibody staining. The cells were incubated with 1:500 dilution of rabbit anti-L1 polyclonal antibody (a gift from Vance Lemmon, Cleveland) followed by anti-rabbit FITC-conjugated IgG (1:30, Sigma).

COS cell protein production

COS-7 cells plated onto 4 × 150 mm culture dishes at a density of ~3 × 10^6 cells per dish and incubated overnight in DMEM plus 10% FCS. The cells were transfected with 10 μg DNA (either L1-Fc derivatives or F3-Fc) per dish using 400 μg/ml DEAE dextran and 100 μM chloroquine. After 3 h the medium was aspirated and the cells incubated with 10% dimethylsulfoxide (DMSO) in phosphate-buffered saline (PBS) for 2 min, the DMSO/PBS was aspirated and replaced with 20 ml DMEM containing 10% IgG free FCS. After 24 h, the medium was replaced with 25 ml DMEM containing 1% IgG free FCS and the chimeric proteins were allowed to accumulate in the medium for 6 days. An expression plasmid encoding a fusion protein of F3 (mouse orthologue of F11; Gennarini *et al*., 1989) with human IgG Fc domains (Buttiglione *et al*., 1998) was kindly provided by Catherine Faivre-Sarrailh and Geneviève Rougon (CNRS, Marseille, France).

Purification of recombinant proteins

Medium containing Fc-chimeric proteins was subjected to protein A–Sepharose affinity chromatography (1 ml HiTrap columns, Pharmacia Biotech). Proteins were recovered by elution with 100 mM glycine pH 3.0 in 0.5 ml fractions and analysed by SDS–PAGE. The protein-containing fractions were combined and dialysed against PBS. The protein concentration was estimated using Bio-Rad DC protein assay reagents.

Homophilic binding assay

Bioclean fluorescent microspheres (Red, 0.6 μm, Duke Scientific Corps) were coated with anti-human IgG antibody (Fc specific; sigma, I-2136). Briefly, 250 μl of fluorescent microspheres was incubated for 1 h at 37°C with 50 μl of antibody and 700 μl of PBS. The beads were subsequently washed twice with PBS/5% FCS, incubated for a further 30 min at room temperature then washed twice before being stored in 250 μl PBS/0.02% azide. These prepared antibody-coated beads were used throughout the study. For each L1–Fc protein sample, 10 μl of antibody-conjugated beads were washed twice with PBS/5% FCS and sonicated for 2 min in iced water to ensure bead dissagregation. The microspheres were incubated with 2.5 μg of wild-type or mutant L1–Fc protein and made up to 50 μl final volume with PBS; these were then incubated for 2 h at 37°C to allow capture of the protein. Excess unbound protein was removed by washing three times with PBS/FCS. As a control to ensure that equivalent concentrations of the L1–Fc mutant and wild-type chimeric proteins were captured onto the anti-Fc coated beads, 30 μl of beads were coated and the protein was then removed by resuspending in 10 μl SDS sample buffer and subjected to SDS–PAGE. For the homophilic binding assay, L1–Fc-coated beads were dissaggregated to a single bead suspension by trituration with a Hamilton syringe followed by sonication in iced water for 30 min. To allow homophilic aggregation to occur, the dissaggregated L1–Fc beads were incubated at 37°C. Samples were taken in triplicate from 0 to 30 min and diluted 1:500, then 1:10, in PBS. These samples were then analysed using a Becton Dickinson FACSort. Ten thousand particles were sampled for each time point and the percentage of particles with fluorescence equal to two or more single beads (clusters) was measured. Each mutant protein was assayed at least three times in parallel with wild-type to control for variation between experiments. The results were then standardized to a wild-type binding curve generated using 15 independent experiments.

Quantitation of bead binding to transfected COS cells

F11 and axonin-1 were released from embryonic chick brain plasma membranes by phosphatidylinositol-specific phospholipase C and were isolated by immunoaffinity chromatography essentially as described previously (Volkmer *et al*., 1996). Coupling of chick F11, mouse F3–Fc and chick axonin-1 to fluorescent beads (0.5 μm), transfection of COS cells and bead binding assays were performed as outlined by Brümmendorf *et al*. (1993), with the exception that cells were maintained in DMEM/1% FCS before bead incubation and were fixed with 3% formaldehyde for 15 min at room temperature before staining with polyclonal antibodies. Interactions between L1 and F11 or axonin-1 may occur *in cis* on the same cell membrane, or *in trans*. For this reason proteins were coupled randomly to microspheres rather than being presented via an adapter antibody. Because COS cells are heterogeneous with respect to size, form, surface properties and expression levels of heterologously expressed proteins, large numbers of cells have to be evaluated in order to quantify bead binding. This was achieved with image analysis procedures derived from a system described in detail previously (Treubert and Brümmendorf, 1998). In all experiments designated for automated image processing great care was taken to ensure that cells were forming uninterrupted confluent monolayers. After bead incubation, washing of the cell monolayer and fixation, L1-expressing cells (which represented 10–50% of cells in the confluent monolayer) were identified by immunofluorescence analysis with polyclonal antibodies directed to human L1 (Wolff *et al*., 1988). Images were captured separately for L1-expressing cells (Cy3-fluorochrome, red fluorescence) and beads (yellow fluorescence) avoiding spectral overlap. L1-expressing cells were identified automatically and distinguished from untransfected cells by a fluorescence intensity threshold. Fluorescent beads were identified using size exclusion criteria and an intensity threshold. Automated comparison of each cell image with its corresponding bead image allowed the calculation of bead density on L1-expressing cells and background density on untransfected cells. Background binding, which was <5% of wild-type L1 binding both for axonin-1 and F11 beads, was subtracted. For chick F11 and axonin-1 at least five independent analyses were performed for each combination of ligand and L1 mutant and in each analysis at least 200 transfected cells were evaluated. Mouse F3–Fc was analysed in two independent experiments. Data were compared using the Mann–Whitney U-test implemented in the Statview program (Abacus Concepts, Inc., Berkeley, CA). To check cell surface expression levels of different L1 mutants, fluorescence intensity of the L1-expressing cells was quantified in parallel to bead binding measurements.

Acknowledgements

The authors thank Cyrus Chothia for invaluable advice on the structure of Ig superfamily molecules, Patrick Doherty and Natalia Drocopoulos for helpful discussion and provision of protocols. We are very grateful to Catherine Faivre-Sarrailh and Geneviève Rougon for kindly providing the F3–Fc expression plasmid, and to Dieter Jobsky and Mechthild Henning for excellent technical assistance. This work was generously supported by the European Union (BIO4-CT96-0450) and the Wellcome Trust.

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Received March 1, 1999; revised June 30, 1999; accepted July 6, 1999