Functional binding interaction identified between the axonal CAM L1 and members of the ERM family

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A yeast two-hybrid library was screened using the cytoplasmic domain of the axonal cell adhesion molecule L1 to identify binding partners that may be involved in the regulation of L1 function. The intracellular domain of L1 bound to ezrin, a member of the ezrin, radixin, and moesin (ERM) family of membrane–cytoskeleton linking proteins, at a site overlapping that for AP2, a clathrin adaptor. Binding of bacterial fusion proteins confirmed this interaction. To determine whether ERM proteins interact with L1 in vivo, extracellular antibodies to L1 were used to force cluster the protein on cultured hippocampal neurons and PC12 cells, which were then immunolabeled for ERM proteins. Confocal analysis revealed a precise pattern of codistribution between ERM and L1 clusters in axons and PC12 neurites, whereas ERMs in dendrites and spectrin labeling remained evenly distributed. Transfection of hippocampal neurons grown on an L1 substrate with a dominant negative ERM construct resulted in extensive and abnormal elaboration of membrane protrusions and an increase in axon branching, highlighting the importance of the ERM–actin interaction in axon development. Together, our data indicate that L1 binds directly to members of the ERM family and suggest this association may coordinate aspects of axonal morphogenesis.

Introduction

L1 is an axon-specific member of the Ig family of cell adhesion molecules (CAMs)* that functions in many aspects of neuronal development including cell migration, axon extension, fasciculation, guidance, and myelination (Hortsch, 1996). L1 is expressed predominantly in the developing nervous system (Salton et al., 1983) and is polarized in its distribution immediately after axon formation (van den Pol and Kim, 1993). The functional importance of this CAM is emphasized by the neurological syndromes (e.g., MASA and X-linked hydrocephalus) that result from mutations of the single gene encoding L1 in humans.

L1 family members exhibit a conserved structural organization consisting of extracellular Ig and fibronectin type III domains, a single transmembrane segment, and a cytoplasmic domain ranging in size from 85 to 148 residues. Binding interactions with the intracellular and extracellular regions have the ability to transduce signals in both directions across the plasma membrane (Hortsch et al., 1998). The extracellular domain of L1 binds a variety of ligands, which include L1 itself, other members of the Ig superfamily, integrins, and extracellular matrix components (Brümmendorf and Rathjen, 1994; Montgomery et al., 1996). Cytoplasmic binding partners include cytoskeletal-associated proteins, protein kinases, and complexes associated with endocytosis and protein trafficking.

In particular, the cytoplasmic domain contains an ankyrin binding site, which couples L1 to the underlying actin cytoskeleton in a manner that can be negatively regulated by phosphorylation (Davis and Bennett, 1994; Hortsch et al., 1998). This interaction is suspected to stabilize L1 within the axonal membrane, an idea supported by the finding that axons eventually degenerate in mutant mice lacking ankyrin. However, initial axonal outgrowth and differentiation and the targeting of L1 to axons are relatively normal during early development in these mice, indicating ankyrinB is not required for these events (Scotland et al., 1998). Alternative interactions with the cytoskeleton are likely to mediate some L1 functions during the earliest stages of axonal development. The cytoplasmic region also contains a neuronal-specific sequence, RSLE (Arg-Ser-Leu-Glu), that arises from alternative...
splicing and appears to be essential for sorting L1 to the axonal growth cone (Kamiguchi and Lemmon, 1998). This sequence also participates in the AP2 adaptor-mediated endocytosis of L1 (Kamiguchi et al., 1998), which is required for activation of the mitogen-activated protein kinase signaling pathway that governs L1-mediated neurite outgrowth (Schafer et al., 1999).

Here, we identify a novel direct interaction between the cytoplasmic domain of L1 and the cytoskeletal-associated protein, ezrin. We further report that disruption of the ezrin–actin interaction dramatically alters neuronal morphology, suggesting that the L1–ezrin–actin interaction may be functionally important in the initial dynamic process of axonal outgrowth and neuronal differentiation.

Results and discussion

The cytoplasmic domain of L1 binds to ezrin

The cytoplasmic domain of L1 fused to the DNA binding domain of LexA was used as “bait” to screen a yeast two-hybrid library constructed from PC12 cells that had been treated for 24 h with NGF. Interactions between the cytoplasmic domain of L1 and either rat AP2 (medium subunit μ.1) or the cytoskeletal protein ankyrinb were confirmed by this screen (Davis and Bennett, 1994; Kamiguchi et al., 1998). In addition, a strong and specific interaction was identified with ezrin (amino acids 1–406), a member of the ezrin, radixin, and moesin (ERM) family of proteins (Fig. 1).

The COOH terminus of all ERM proteins binds directly to F-actin, whereas the NH2 terminus (of ∼300 residues) shares homology with a domain common to members of the 4.1 protein superfamily and has, therefore, been termed the FERM (four-point-one, ERM) domain. FERM domains are thought to be general transmembrane protein-binding modules (Mangeat et al., 1999). The membrane–cytoskeleton linker function of ERM proteins is negatively regulated by an intramolecular association between the FERM and COOH-terminal domains that masks and inactivates their respective binding sites. Phosphorylation of the actin binding tail and exposure to polyphosphatidyl inositides are both believed to contribute to activation of these molecules, providing the means to dynamically regulate cytoskeletal–membrane interactions. Such tightly controlled interactions are suspected to underlie ERM involvement in the development of cell type–specific morphologies (for review see Bretscher, 1999).

Table 1. Summary of yeast two-hybrid interactions

| L1 + RSLE | L1–RSLE | L1ΔY | L1trunc | L1RSLEAS |
|-----------|---------|------|---------|---------|
| Ezrin     | +       | –    | –       | +       |
| Ankyrin   | +       | –    | –       | +       |
| AP2       | +       | +    | +       | +       |

The cytoplasmic domain of L1, with the RSLE alternatively spliced sequence, with a truncation at amino acid 1,182, or with serine1194 mutated to leucine, bound to ezrin. Baits: L1+RSLE (L1 COOH terminus with the RSLE alternatively spliced sequence), L1–RSLE (L1 COOH terminus without RSLE), L1ΔY (L1 COOH terminus with tyrosine adjacent to RSLE motif mutated to phenylalanine), L1trunc (L1 COOH terminus truncated at amino acid residue 1,182, E of the RSLE motif), L1RSLEAS (containing RSLE but with serine1194 of the rat sequence mutated to leucine). Trapped clones: ezrin, ankyrin, AP2.

Ezrin and AP2 interact with the same domain of L1

To further characterize the interaction between L1 and ezrin, several plasmids encoding the LexA DNA binding domain fused to the cytoplasmic domain of various L1 isoforms (full length, truncated, and mutant) were constructed. These plasmids were cotransformed in L40 with each of the rescued library plasmids and interactions assessed by β-galactosidase staining (Fig. 1). Our results indicate that the truncated L1 (L1 trunc), lacking the ankyrin binding domain (equivalent to MASA mutation truncation amino acid 1,180), binds to ezrin as effectively as the entire intracellular domain of L1. However, the L1 isoform lacking only the RSLE sequence fails to interact with ezrin. Mutating the tyrosine that precedes the RSLE sequence also prevents binding, whereas mutation of serine 1196 to leucine (equivalent to MASA mutation S1194L) does not affect binding (Table I). Identical data were obtained with AP2. Together these data suggest that ezrin binds to L1 at a site that is largely overlapping with that for AP2 and may even compete for the same domain in a way that could be regulated by phosphorylation, since enhanced ERM phosphorylation has been correlated with increased membrane–cytoskeleton association (Bretscher et al., 1997). Although the binding site has not been
mapped precisely, the YRSLE motif and select neighboring residues are conserved amongst L1 CAM family members including Ng-CAM, Nr-CAM, and neurofascin (Brophy, 2001). Only ezrin was isolated from the yeast two-hybrid library, but the NH2-terminal domains of moesin and radixin are very similar, and where it has been examined, the three ERM proteins have largely overlapping function so it is likely that all ERM members will show similar interactions with L1.

Ezrin and L1 bind in vitro
To confirm the interaction between L1 and ezrin in vitro, the L1 cytoplasmic domain was expressed as a glutathione S-transferase (GST) fusion protein, and the trapped ezrin cDNA was expressed as a His- and S-tagged bacterial fusion protein. We found that GST-L1 and GST-(L1 trunc) bind ezrin, whereas GST alone does not, thereby confirming the interaction identified using the yeast two-hybrid assay (Fig. 1).

ERM family expression peaks during neural differentiation
We examined the developmental time course of ezrin and moesin expression using immunoblots of both cultured hippocampal neurons and NGF-treated PC12 cells (Fig. 2). Ezrin and moesin levels are highest in the youngest (3–5 d in culture) hippocampal neurons, a time period notable for axonal polarization, outgrowth, and differentiation, and decrease dramatically after the first week in culture. In PC12 cells, ERM expression is highest in cultures treated with NGF for 3–5 d and decreases after 10 d of NGF treatment. Interestingly, the peak expression of the ERM family in both cell types occurs during neuronal process outgrowth and precedes that reported for ankyrin, which peaks at postnatal day 10 in cerebellar cell cultures (Kunimoto, 1995). These results suggest that the interaction between L1 and ezrin may play a role in the earliest events of neuronal morphogenesis.

L1 and the ERM family codistribute in vivo
In sections from P0 rat brain, L1 immunolabeling is most intense in axon tracts and in cell somata, residing in relatively undifferentiated regions like the cerebral cortex. This distribution is consistent with previous reports (Persohn and Schachner, 1990; Demyanenko et al., 1999). ERM labeling is more extensive, reflecting expression in several cell types.
Figure 3. **L1 strongly interacts with ERM proteins in vivo.** (A and C) After forced L1 clustering, labeling exists as distinct patches along the axons (arrows). (Note that an axon from a neighboring cell can be seen wrapped around the cell soma and dendrites, a common phenomena in hippocampal cultures). (B and D) Clustering is also evident within axons labeled for the ERM proteins. ERM labeling within dendrites remained smooth. Fluorescence intensity profiles taken along axons in which L1 had been forced to cluster (E) confirmed these observations. Red, green, and blue lines represent L1, ERM, and DIC labeling profiles, respectively. Similarly, forced L1 clustering (G and I) in differentiated PC12 cells resulted in clustering of ERM labeling (H and J). Quantification of labeling along neurites confirmed these observation (F). Bar: (A and B) 16 μm; (C and D) 11 μm; (G and H) 12 μm; (I and J) 8 μm.
Double labeling shows the proteins are codistributed in several regions (Fig. 2, C–E).

**ERM family concentrates in structures undergoing rapid morphogenesis**

In hippocampal neurons, immunolabeled ERMs are distributed in axons and dendrites but are most prominent in actin-rich structures like axonal and dendritic growth cones (Goslin et al., 1989; Paglini et al., 1998). Their distribution in cultured fibroblasts and epithelial cells is analogous; ERM proteins are expressed and concentrated at cell surface structures, such as microvilli, ruffling membranes, and cell–cell/cell–matrix adhesion sites (Bretscher, 1999). In neurons, occasional foci of ERM labeling are also noted along processes that are sometimes associated with branch points (unpublished data), a pattern consistent with the notion that ERM interactions are critical for the genesis of cell specific morphologies. Unlike L1, the ERM family is not polarized to axons, indicating there is likely to be an alternative binding partner(s) for the ERM family in dendrites.

**L1 and the ERM family interact in vivo**

To examine interactions between L1 and the ERM family in vivo, antibodies were used to force cluster L1 on the surface of hippocampal neuron axons and on PC12 neurites, at 37°C, in the presence of phenylarsine oxide, a general inhibitor of endocytosis (Doré et al., 1997), or at 12°C (Harder et al., 1998), and the effect on ERM distribution was assessed. All three treatments yield similar results. However, to rule out the contribution of endocytosis quantification data are restricted to 12°C and phenylarsine oxide experiments. In both cell types, antibody-induced capping dramatically changes the labeling pattern for L1 from a smooth continuous distribution to distinct focal clusters (Fig. 3). Double labeling for L1 and the ERM family reveals a precisely coincident pattern of clusters that in the hippocampal neurons is restricted to axons. Digital fluorescence profiles of L1 and ERM-labeled clusters within axons and neurites confirm the distinct colocalization pattern as exhibited by shared peaks in fluorescence intensity (hippocampal neurons correlation coefficient = 0.736, p = <0.0001; PC12 cells correlation coefficient = 0.744, p = <0.0001). PCR analysis was used to confirm that all rat PC12 cells used express only full-length (+RSLE) L1 (Miura et al., 1991; Takeda et al., 1996). Labeling for spectrin, another cortical actin-associated protein, was not altered by the forced clustering of L1 and remained smooth throughout the axon (Fig. 4, A–D). L1-forced clustering investigations were also performed in Schwann cells, which express exclusively an L1 variant lacking the cytoplasmic RSLE sequence (Martini et al., 1994; Takeda et al., 1996). In these cells, L1 clustering did not alter ERM protein labeling (Fig. 4, E and F). Together these data provide strong evidence that L1 and ERM proteins interact in vivo, that this interaction is specific for the ERM family of actin binding proteins, and that it requires the RSLE miniexon.

**Disruption of ezrin–actin interaction alters morphogenesis**

Functional studies of the ERM family indicate they play an essential role in the establishment of characteristic cell type–specific morphology. Previous work has shown that overexpression of the NH₂ terminus of ezrin alone fused to GFP (N-Ez–GFp) acts as a potent dominant negative (Crepaldi et al., 1997), presumably by disabling the ERM–actin link. In the current investigation, N-Ez–GFp–transfected neurons, grown on L1 substrates for 72 h, exhibit significantly more axonal branching compared with GFp-transfected neurons (mean axonal branches N-Ez–GFp = 3.0, GFp = 1.0; p = 0.0051) and compared with transfected neurons cultured on either laminin or poly-l-lysine. Independent of substrate, cells transfected with N-Ez–GFp elaborate numerous fine protrusions that resembled extended filopodia more than genuine branches (mean number fine protrusions on L1 substrate, N-Ez–GFp = 99.2, GFp = 0.3; p < 0.0001) (Fig. 5). These protrusions range in length from 2 to 25 μm and are immunopositive for L1 and endogenous ERM proteins. The phenomena becomes more prevalent in axons as the neurons mature (unpublished data). The protrusions we observe resemble the long fragile processes seen when similar N-ERM constructs are overexpressed in insect cells, NIH3T3 cells, or MDCK cells (Martin et al., 1997; Amieva et al., 1999; Woodward and Crouch, 2001) and together suggest the L1–ERM–actin interaction may be playing an important functional role in membrane and cytoskeleton stabilization required for appropriate axonal arborization, branching, and early neuronal morphogenesis.

The self-associating activity exhibited by the full-length ERM family members has inhibited the search for their binding partners using standard coimmunoprecipitation methods (Bretscher et al., 1997; Vaheri et al., 1997). We too were unable to coimmunoprecipitate these proteins despite using a variety of different approaches. However, collectively our results indicate that the ezrin–L1 interaction is
Figure 5. Expression of ezrin NH\textsubscript{2} terminus dominant negative causes abnormal morphogenesis. (A) Transfection of neurons grown on an L1 substrate with a construct encoding the NH\textsubscript{2} terminus of ezrin fused to GFP resulted in increased axonal branching and the growth of abnormal numbers of long branchlets and filopodia-like protrusions. High power insets of boxed region show localization of L1 throughout N-Ez–GFP protrusions. (B) Transfection of neurons cultured on laminin had no significant effect on branching; however, similar filopodia-like protrusions were noted. High power images of boxed regions show localization of endogenous ERM proteins concentrated at the tips of N-Ez–GFP protrusions. (C) Transfection of hippocampal neurons cultured on an L1 substrate with the pEGFP-F’ vector (no insert) indicates normal growth at DIV 3. Inset shows high power image of normal axonal growth. Bar: (A) 30 μm; (A, insets) 11 μm; (B) 24 μm; (B, insets) 17 μm; (C) 24 μm; (C, inset) 10 μm.
Materials and methods

Yeast two-hybrid
Libraries were screened as described previously (Bartel et al., 1993). The DNA binding domain of LexA was coupled to the cytoplasmic domain of rat L1, NILE (amino acids 1,146–1,259) in the plasmid pBTM116-KN-Ad2. The activation domain was fused to two PC12 cDNA libraries, either randomly or oligo dt primed, in plasmid pGADGH constructed using RNA isolated from rat PC12 cells treated for 24 h with NGF (cDNA libraries provided by Drs. S. Tsui and S. Hagleoua, State University of New York, Stony Brook, NY). The sites and specificity of in vivo binding were characterized by cotransforming the yeast strain L40 with each of the rescued library plasmids and one of a series of plasmids encoding LexA fusion proteins.

In vitro binding
“Bait” inserts were cloned into the plasmid pGEX-5 x 2 and all “trapped” cDNAs cloned into the plasmid pET-30b. Protein expression was induced with IPTG, and bacterial proteins were isolated by sonication in PBS (GST cDNAs cloned into the plasmid pET-30b. Protein expression was induced to the extracellular region of L1 (neat supernatant, mouse monoclonal, ASC45, Developmental Studies Hybridoma Bank) was applied to live cultures for 15 min. Protein clustering was amplified by the application of a biotinylated anti-mouse secondary antibody (1:200; Vector Laboratories) for 2 h. Cells were then fixed and double labeled for ERMs proteins (13H9; 1:20; or spectrin (rabbit polyclonal [240/235], 1:20; Chemicon). (Note that Ezrin specific antibodies used for Western blots could not be used for immunohistochemistry.) L1 labeling was visualized with a streptavidin-Cy3-conjugated antibody (IgG). Immunolabeling and transfections (Leica TCS-SP UVII). Correlation coefficients were calculated from fluorescence intensity profiles taken along 20–30-μm lengths of labeled axons (hippocampal neurons) or neurites (PC12s) from three different experiments.

Cell cultures
Dissociated hippocampal neurons were prepared from hippocampi of embryonic day 18 Sprague Dawley rats as described previously (Benson et al., 1994). Cells were plated at a density of 3,600 cells/cm² on poly-L-lysine and laminin (25 μg/ml) or L1-Fc substrates (2 μg/ml). L1 substrates were prepared according to Angellis et al. (1999). The plg expression plasmid containing an insert of L1-Fc was provided by Dr. J.L. Salzer (New York University School of Medicine). Neurons were maintained in Neurobasal medium containing B27 supplements. PC12 cells were cultured on poly-L-lysine and collagen-coated coverslips in RPMI 1640 containing 10% horse serum and 5% FCS and differentiated using NGF as described previously (Salton et al., 1983). Schwann cells were prepared as described (A.F. Svenningsen and L. Pedraza, personal communication), and their identification was confirmed using immunoblotting for S100 (rabbit polyclonal, 1:250; Dako).

Immunoblots
Cultured hippocampal neurons (3–25 d growth) were homogenized as described previously (Benson and Tanaka, 1998). PC12 cells were NGE treated and harvested in 2x SDS sample buffer (0–20 d differentiation). Equal protein amounts were fractionated on 7.5% SDS-polyacrylamide gels then transferred to PVDF paper. Blots were probed with either goat anti–IgM-FITC (Jackson ImmunoResearch Laboratories) or in the presence of 20 μm phenylalanine oxide, both without permeabilization. Confocal scanning laser microscopy was used to investigate all immunolabeling and transfections (Leica TCS-SP UVII). Correlation coefficients were calculated from fluorescence intensity profiles taken along 20–30-μm lengths of labeled axons (hippocampal neurons) or neurites (PC12s) from three different experiments.

Construction of mutant cDNA encoding the NH2-terminal domain of ezrin
The NH2-terminal domain of ezrin was obtained by digesting of the complete coding sequence of human ezrin (a gift from Dr. M. Arpin, Institut Curie, Paris, France) (Algain et al., 1993) with HindIII and Xmal. This fragment (1,042 bp) was inserted, in frame, into the corresponding sites in the vector pEGFP-N1 (CLONTECH Laboratories, Inc.) and sequenced. Neurons on different substrates (see Results) were transfected at plating using Effectene (QIAGEN) according to the manufacturer’s instructions and fixed after 24–72 h. Control transfections using pEGFP-F’ (CLONTECH Laboratories, Inc.), a farnesylated form of EGFP that is targeted to the plasma membrane, or plasmid without insert were performed concurrently. All processes of pEGFP-F’ and six N-Ez–GFP–transfected neurons grown on L1 or laminin or poly-L-lysine substrates were traced and analyzed using the Neurolucida 2000 system (MicroBrightField, Inc.). Endogenous Ezrin and L1 distribution was labeled in transfected cells using the antibodies described above. Experiments were repeated in three different hippocampal cultures.

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