**L1 endocytosis is controlled by a phosphorylation-dephosphorylation cycle stimulated by outside-in signaling by L1**

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**Introduction**

The immunoglobulin superfamily cell adhesion molecule (IgSF CAM) L1 participates in several processes that are essential for the normal development of the nervous system. Processes such as neurite extension and neuronal migration require dynamic regulation of the cell adhesion mediated by L1. This is controlled in part by internalization of L1 to regulate the availability of L1 on the cell surface. L1 internalization is controlled by interactions of its cytoplasmic domain with signaling, cytoskeletal, and internalization machinery (Long and Lemmon, 2000).

The L1 cytoplasmic domain (L1CD)* is not required for adhesion, but it is needed for regulation of adhesion by signal transduction and internalization. Interestingly, mutations in the cytoplasmic domain of the human L1 gene impair the formation of major axon tracts in neural development (for review see Kamiguchi et al., 1998a). One important function of the L1CD that has been analyzed in detail is the regulation of endocytosis, intracellular trafficking, and cell surface distribution of L1. Neuronal L1 differs from L1 found in nonneuronal cells such as Schwann cells and neuroblastoma cell lines in that it contains four amino acids (RSLE) in the cytoplasmic domain encoded by the alternatively spliced exon 28 (Miura et al., 1991). The RSLE stretch immediately regulates L1 endocytosis through dephosphorylation of Y1176 is a critical regulatory point of L1-mediated adhesion and signaling.

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*Abbreviations used in this paper: 74-SH7-IR, 74-SH7 immunoreactivity; DRG, dorsal root ganglion; IR, immunoreactivity; L1CD, L1 cytoplasmic domain; PVDF, polyvinylidifluoride; Y1176, tyrosine 1176.
follows tyrosine 1176. The composite sequence (YRSLE) forms a tyrosine-based sorting motif that is required for endocytosis of L1 via clathrin-coated pits (Kamiguchi et al., 1998b). Like tyrosine-based sorting signals characterized in other proteins (Ohno et al., 1996), the YRSLE sequence found in L1 serves as a binding site for the µ2 chain of the clathrin-associated AP-2 complex in vitro, and a number of endocytic machinery components can be coimmunoprecipitated with L1. Furthermore, mutating tyrosine 1176 (Y1176) or removing the RSLE exon also prevents L1 from interacting with AP-2, and consequently prevents clathrin-mediated endocytosis of L1 (Kamiguchi et al., 1998b). In dorsal root ganglion (DRG) neurons growing on an L1-Fc substrate, clathrin-mediated endocytosis of L1 occurs preferentially at the rear of growth cones, suggesting local regulation of L1 endocytosis and that its recycling may be important in growth cone motility (Kamiguchi and Lemmon, 2000). Indeed, blocking AP-2-mediated internalization of L1 inhibits L1-based neurite growth in part by disrupting a gradient of L1 adhesivity from the growth cone periphery to the central domain (Kamiguchi and Yoshihara, 2001). Finally, the neuronal form of L1 (with YRSLE) is significantly less adhesive than the nonneuronal form due to its more rapid internalization and shorter dwell time on the cell surface (Long et al., 2001).

Phosphorylation is likely to play a critical role in regulating L1-mediated processes such as neurite outgrowth (Atashi et al., 1992). L1 is phosphorylated on both serine and tyrosine residues in cultured neurons, and clustering of L1 on cultured cells with cross-linking antibodies can trigger changes in both kinase and phosphatase activity (Klinz et al., 1995; Schaefer et al., 1999), as well as in the phosphorylation state of L1 (Zisch et al., 1995; Kunz et al., 1996). Several kinase activities coimmunoprecipitate with L1. We have identified three L1-associated kinases, CKII, p90rsk, and ERK2, that can phosphorylate certain serine residues in the L1CD in vitro (Wong et al., 1996a,b; Schaefer et al., 1999). Inhibition of p90rsk phosphorylation of L1 impairs DRG neurite outgrowth on L1 but not on laminin substrates. Furthermore, activation of the MAP kinase cascade, which includes p90rsk and ERK2, requires L1 endocytosis. Tyrosine kinases, such as p60src and FGF receptor, have also been implicated in L1 signaling (Ignelzi et al., 1994; Doherty and Walsh, 1996), and the receptor tyrosine kinase, EphB2, is capable of phosphorylating L1 (Zisch et al., 1997). Tyrosine phosphorylation may regulate associations between L1 and the cytoskeleton. For example, both L1 and the L1 family members neurofascin and NrCAM associate with the membrane cytoskeleton protein ankyrin, and phosphorylation of a tyrosine within the ankyrin binding site of neurofascin, which is conserved in L1, disrupts this association (Tuvia et al., 1997).

Here, we present evidence that outside-in signaling dynamically regulates the functional state of the L1CD via dephosphorylation. We have characterized the mAb 74-5H7 that recognizes a subset of L1 at points of cell–cell contact and in vesicular structures. The 74-5H7 epitope is regulated by phosphorylation of L1 and maps to the region immediately preceding the tyrosine-based sorting motif. In vitro studies show that phosphorylation within the epitope prevents recognition by 74-5H7 and also binding of AP-2 to L1. We have identified the nonreceptor tyrosine kinase p60src as an L1-phosphorylating kinase that preferentially phosphorylates Y1176 of the tyrosine-based sorting motif in vitro. Cell–cell contact is able to induce changes in the phosphorylation state of L1 that is recognized by 74-5H7. These data demonstrate that L1–L1 binding induces a functional change in the L1CD mediated by a phosphorylation-dephosphorylation cycle.

**Results**

**mAb 74-5H7 recognizes a special fraction of L1 in neurons**

Chick DRG neurons growing in culture have L1 distributed over their entire surfaces (Fig. 1 B). When mAb 74-5H7 was used for immunocytochemistry, however, we were surprised to find that immunoreactivity (IR) was concentrated at...
binds to bacterially expressed L1CD (rL1CD), a 14-amino acid L1CD sequence, SEARPMKDETFGEY, immediately preceding the alternatively spliced (RSLE) region, but not to a peptide corresponding to the juxtamembrane region (KRSDK...VDS). (C) A series of peptides was synthesized containing alanine substituting for individual amino acids to determine which amino acid residues are critical for 74-5H7 binding to the SEARPMKDETFGEY sequence. Dot blot analysis shows that substituting alanine for lysine 1169, glutamine 1171, threonine 1172, phenylalanine 1173, or glycine 1174 inhibits 74-5H7 binding to the peptides. Additional peptides were synthesized containing phosphoserine 1163, phosphothreonine 1172, and phospho-Y1176. The dot blot shows that phosphorylation of threonine 1172 or Y1176 inhibits 74-5H7 recognition of the peptide, whereas phosphorylation of serine 1163 has no effect. (D) Western blot showing (lane 1) 74-5H7 binding to purified rat L1, (lane 2) that preincubation of the monoclonal antibody with the SEARPMKDETFGEY peptide competes for binding to purified rat L1. The 220- and 85-kD molecular mass markers are shown on the left.

Characterization of the 74-5H7 epitope

In principle, there are several possible explanations for the unique distribution of 74-5H7-IR, including a change in posttranslational modification such as phosphorylation, blocking of the epitope by protein–protein interactions, or conformational change. Two lines of evidence suggest that phosphorylation is the most likely explanation. First, 74-5H7 recognizes L1 by Western blot after fractionation by SDS-PAGE (Fig. 2 A). Under these conditions, posttranslational modifications to the L1CD would be preserved, whereas global conformational changes or protein associations would be lost. Second, bacterially produced, purified recombinant L1CD is recognized by 74-5H7 on Western blots (Fig. 2 B), indicating that the 74-5H7 epitope is part of the primary structure of the L1CD, suggesting that the epitope is masked by posttranslational modifications of L1 in vivo.

To determine which region of the cytoplasmic domain contained the 74-5H7 epitope, we prepared a series of plasmid constructs containing sequences coding for L1 with various cytoplasmic domain truncations, expressed them in myeloma cells, and examined which ones preserved the 74-5H7 epitope. Western blot analysis of extracts from myeloma cells expressing different L1 truncations shows that 74-5H7 recognizes full-length L1 and L1 truncated after Y1176, which leaves the first 33 amino acids of the L1CD intact (Fig. 2 A). The mAb also recognizes the nonneuronal form of L1 that lacks the alternatively spliced exon 28 (amino acids 1177–1181; unpublished data). In contrast, the 74-5H7 does not recognize L1 truncated after lysine 1147 that removes all but the first four amino acids of the cytoplasmic domain (Fig. 2 A). These results suggest that the 74-5H7 epitope resides in the first 33 amino acids of the L1 cytoplasmic domain.

A series of peptides was made to map the precise epitope recognized by 74-5H7. First, we synthesized two peptides spanning the first 33 amino acids of the cytoplasmic domain at points of cell–cell contact. Therefore, expression of the 74-5H7 epitope correlates with a functional state of L1 that occurs when L1 is engaged in adhesion at the cell surface and during trafficking.
and asked whether 74-5H7 recognized either on dot blots.
Our analysis indicates that 74-5H7 recognizes a 14 amino acid peptide, SEARPMKDETGEY (residues 1163–1176; Fig. 2 B), but not the peptide, KRSGKGGYSVKD-KEDTQVDS (residues 1144–1163), consisting of the first 20 amino acids of the L1CD. We then used synthetic peptides with alanine scanning substitutions through the 14 amino acid peptide to determine which residues are essential for recognition by 74-5H7. This analysis identified five amino acids, SEARPMKDETGEY (underlined) that when individually changed to alanine, abolish 74-5H7 binding (Fig. 2 C). Although only these five amino acids were identified as critical for 74-5H7 binding, we were unable to detect binding to peptides shorter than the SEARPMKDETGEY sequence, suggesting there may be structural contributions to the epitope from other residues (unpublished data). The SEARPMKDETGEY peptide also competed with purified L1 for 74-5H7 binding, confirming the importance of this sequence for mAb recognition of L1 (Fig. 2 D, lane 2). Thus the 74-5H7 epitope, which is present in L1 at points of cell–cell contact and in small vesicle-like structures, is positioned immediately NH2-terminal to the tyrosine-based sorting motif. This motif (YRSLE) is present in the neuronal form of L1 and is critical for regulation of L1 endocytosis (Kamiguchi et al., 1998b).

Phosphorylation of L1 peptides inhibits 74-5H7 binding
The 14 amino acid peptide contains three potential phosphorylation sites, SEARPMKDETGEY (underlined). We synthesized peptides in which one of these residues was phosphorylated, and found that the peptide containing phosphorylated threonine 1172 was no longer recognized by 74-5H7. In addition, 74-5H7 recognition of phosphorylated Y1176 peptide was reduced over 80%, although the peptide containing phosphoserine 1163 was strongly reactive (Fig. 2 C). These results suggest that phosphorylation of one or both of these sites, threonine 1172 and Y1176, in L1 could be responsible for its inability to react with 74-5H7, and that the epitope may be transiently unmasked by dephosphorylation. However, phosphothreonine has never been reported in L1 (for review see Schaefer et al., 1999).

p60src phosphorylates the L1CD
Although phosphorylation of both threonine 1172 and Y1176 disrupt the 74-5H7 epitope, phosphorylation of threonine residues in L1 has not been reported either in vitro or in vivo (Salton et al., 1983; Faissner et al., 1985; Linnemann et al., 1988; Sadoul et al., 1989; Kunz et al., 1996; Wong et al., 1996a,b; Zisch et al., 1997). In contrast, at least two reports indicate that L1 is tyrosine phosphorylated in vivo (Heiland et al., 1996; Zisch et al., 1997). Therefore, we were interested in determining which kinase(s) can phosphorylate L1 on Y1176. The nonreceptor tyrosine kinase p60src has been implicated in L1 function (Ignelzi et al., 1994). Tyrosine phosphorylation of the CTLA-4 T-lymphocyte receptor endocytosis motif by src family kinases is implicated in regulation of its cell surface expression (Bradshaw et al., 1997). To determine if L1 could be a substrate for p60src, we tested the ability of purified p60src to phosphorylate recombinant L1CD in vitro, and found that L1 is phosphorylated by this kinase (Fig. 3 A). The phosphorylated L1CD was digested with endoproteinase Asp-N, and the resulting fragments were separated by reverse phase HPLC. One major peptide, DETGEYPYRSLESDN, was identified associated with radioactivity. The site of phosphorylation of this peptide was determined to be the seventh residue by assessing the elution of radioactivity using covalent sequencing supports to allow tracking of the radiolabeled residue(s); this corresponds to Y1176. Phosphorylated peptide fragments containing the other three tyrosines present within the L1CD did not coelute with radioactivity, indicating that p60src preferentially phosphorylates Y1176, an amino acid residue that plays a critical role in regulating L1 trafficking and endocytosis.

L1 Y1176 is phosphorylated in vivo
The finding that 74-5H7 recognizes dephosphorylated L1, and the restricted distribution of 74-5H7-IR relative to total L1 IR, predicts that some neuronal L1 is phosphorylated on Y1176 in vivo. To demonstrate that Y1176 was, indeed, phosphorylated in vivo, we produced a new Ra-pYRSLE antibody. This affinity-purified antibody showed strong binding to KDETGEpYRSLESDN and virtually no immunoreactivity for KDETGEYRSLESDN in ELISA assays (unpublished data). The Ra-pYRSLE also bound to rL1CD, phosphorylated using src, as did an anti-pY antibody (Fig. 3 A). Neither Ra-pYRSLE nor anti-pY antibody bound to unphosphorylated rL1CD. Finally, Ra-pYRSLE did bind to a band corresponding in molecular weight to L1 in human, rat, mouse, and chick brain homogenates prepared in the presence of tyrosine phosphatase inhibitors (Fig. 3 B and C), demonstrating that y1176 is, indeed, phosphorylated in vivo. L1, affinity purified from mouse brain using 74-5H7,
did not bind the Ra-pYRSLE as one would expect (unpublished data).

To support the idea that phosphorylation of the Y1176 was responsible for regulating the 74-5H7 epitope in vivo, fresh chick brains were homogenized in the presence and absence of a cocktail of tyrosine phosphatase inhibitors (0.1 mM ammonium molybdate, 0.2 mM phenyl arsine oxide, 5 nM microcystin, and 100 pM cypermethrin, 10 mM pervanadate; Tonks et al., 1988). The anti-pY antibody recognized two very prominent bands in the brain extract that were not L1 but served as indicators for the effectiveness of the phosphatase inhibitors. When the inhibitors were absent, a strong signal for the 74-5H7 epitope was seen, whereas the anti-pY antibody gave a relatively weak signal (Fig. 4, lane A). Alternatively, when the inhibitors were present, the 74-5H7-IR was relatively weak but the pY-IR was strong (Fig. 4, lane B). The simplest interpretation of this data is that the phosphatase inhibitors prevent dephosphorylation of Y1176 on L1 and thereby block 74-5H7 binding.

Phosphorylation of L1 Y1176 inhibits L1 binding to AP-2

We have shown previously, using an in vitro peptide competition assay, that the peptide FGEYRSLESDEE blocks binding of a biotin-labeled TGN38 peptide to AP-2 purified from rat brain (Kamiguchi et al., 1998b). This, and other data, showed that the YRSLE sequence mediates L1 binding to AP-2 (Kamiguchi et al., 1998b). It is known that phosphorylation of a tyrosine in the tyrosine-based sorting motif of CTLA-4 can inhibit binding to AP-2 and, as a result, can inhibit clathrin-mediated endocytosis of the CTLA-4 (Shiratori et al., 1997). To test this for L1, we compared the ability of phosphorylated and unphosphorylated L1 peptides to compete with a peptide spanning the TGN38 tyrosine-based sorting motif for binding to AP-2 (Fig. 5). The FGEpYRSLESDEE peptide phosphorylated on Y1176 failed to block the interaction between TGN38 peptide and AP-2, indicating that L1 phosphorylated at Y1176 cannot bind to AP-2. In contrast, phosphorylation of S1181, which we have shown is the residue phosphorylated by CKII (Wong et al., 1996b), gave a peptide that retained its ability to block TGN38 binding to AP-2, demonstrating that CKII phosphorylation of L1 would not inhibit binding to AP-2.

74-5H7 binding to L1 is dynamically regulated by cell–cell contact, L1–L1 homophilic interactions, and L1 cross-linking

The results of immunocytochemical studies reported in Fig. 1 suggested that 74-5H7-IR might be induced by cell–cell contact, L1–L1 homophilic interactions, and L1 cross-linking. We have shown previously, using an in vitro peptide competition assay, that the peptide FGEYRSLESDEE blocks binding of a biotin-labeled TGN38 peptide to AP-2 purified from rat brain (Kamiguchi et al., 1998b). This, and other data, showed that the YRSLE sequence mediates L1 binding to AP-2 (Kamiguchi et al., 1998b). It is known that phosphorylation of a tyrosine in the tyrosine-based sorting motif of CTLA-4 can inhibit binding to AP-2 and, as a result, can inhibit clathrin-mediated endocytosis of the CTLA-4 (Shiratori et al., 1997). To test this for L1, we compared the ability of phosphorylated and unphosphorylated L1 peptides to compete with a peptide spanning the TGN38 tyrosine-based sorting motif for binding to AP-2 (Fig. 5). The FGEpYRSLESDEE peptide phosphorylated on Y1176 failed to block the interaction between TGN38 peptide and AP-2, indicating that L1 phosphorylated at Y1176 cannot bind to AP-2. In contrast, phosphorylation of S1181, which we have shown is the residue phosphorylated by CKII (Wong et al., 1996b), gave a peptide that retained its ability to block TGN38 binding to AP-2, demonstrating that CKII phosphorylation of L1 would not inhibit binding to AP-2.

Figure 5. L1 peptide phosphorylated at Y1176 cannot bind to AP-2. The μ2 chain of AP-2 was incubated with the YQRL peptide (see Materials and methods) in the absence (right-most band) or presence of competitor peptides (EpYRSLE, EYRSLEpS, and EYRSLE). The concentrations of each competitor peptide are indicated in the figures. The EYRSLEpS and EYRSLE peptides were strong competitors of the YQRL peptide, showing that they bind to the μ2 chain of AP-2, even at concentrations as low as 10 μM. In contrast, the EpYRSLE peptide did not act as a competitor, even at 1,000 μM.

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contact. To directly test this idea, we used a cell-pelleting paradigm to rapidly induce cell–cell contact and asked whether a change in 74-5H7-IR could be detected on Western blots. Mechanically dissociated chick brain cells were induced to reform cell–cell contacts by low speed centrifugation. When cells were lysed at various times after cell pelleting and the lysates were analyzed by Western blot, a clear increase in 74-5H7-IR was observed beginning as early as 5 min and continuing for up to 60 min. The increase was followed by a decrease toward basal levels by 3 h after the cells were pelleted (Fig. 6). The presence of intact L1 throughout the time course was confirmed by reprobing the blots with a polyclonal anti-L1 antibody (8D9). These results indicate the change in 74-5H7-IR is rapidly and transiently induced by cell–cell contact.

Although the cell pelleting experiments described above indicate that cell–cell contact dramatically increases 74-5H7-IR, the cell pelleting paradigm involving inducing contact among many types of cell surface receptors present on brain cells in addition to L1. Therefore, to ask whether the increase in 74-5H7-IR can be induced specifically by L1–L1 interactions, we used stable lines of myeloma cells transfected to express L1. These cells are normally nonadhesive, but form L1-dependent aggregates when they express L1 (Wong et al., 1995). Immunocytochemistry of myeloma cells expressing full-length L1 shows 74-5H7-IR localized to regions of cell–cell contact (Fig. 7 A), whereas labeling L1 with a polyclonal antibody raised against recombinant L1CD shows a more uniform cell surface distribution for L1 (Fig. 7 B). Colocalization of the two antibodies is shown in yellow at the cell–cell contact site (Fig. 7 C). This result is consistent with L1–L1 homophilic binding being sufficient to mediate changes in 74-5H7-IR at cell–cell contact sites.

The cell pelleting data indicate that the 74-5H7 epitope is rapidly and transiently induced. Previous work from our lab has shown that L1 can be phosphorylated at multiple sites (Wong et al., 1996a,b; Schaefer et al., 1999). Regulation of the epitope was preserved in myeloma cells expressing L1 with only the first 33 amino acids of the cytoplasmic domain, indicating that the potential posttranslational modification was restricted to this region of the L1CD (Fig. 8). Similar results were obtained with myeloma cells expressing full-length L1 but not L1 expressing 3T3 cells or Cos-7 cells.

To determine whether the change in 74-5H7-IR can be induced by L1 binding in neurons, we used L1 cross-linking polyclonal antibodies to mimic L1 binding. Cross-linking L1 with antibodies has been shown to induce changes in a number of intracellular second messengers including Ca\(^{2+}\), IP\(_3\), and IP\(_3\) (von Bohlen und Halbach et al., 1992), as well as to activate the MAPK signaling cascade (Schaefer et al., 1999) and phosphatase(s) (Klinz et al., 1995). In addition, cross-linking L1 or the L1 heterophilic binding partners, axonin-1 and F3/F11, can also regulate the phosphorylation state of L1 (Zisch et al., 1995; Kunz et al., 1996). 74-5H7-IR was observed at points of cell–cell contact in cultured neurons without L1 antibody cross-linking (Fig. 9 B). 74-5H7-IR was dramatically up-regulated by cross-linking L1 on DRG neurons in culture with polyclonal anti-L1 antibodies (8D9) (Fig. 9 C) as compared with isolated neurons without L1 antibody cross-linking (Fig. 9 A). In a variation of the L1 cross-linking experiment, we took mechanically dissociated chick brain cells, preincubated them at 4°C with cross-linking polyclonal L1 antibodies (8D9), and then brought the cells to 37°C to induce cross-linking of L1 on the cell surface. The cells were lysed after various times and the L1 was immunoprecipitated. The immunoprecipitates were analyzed by Western blot and a clear time course for 74-5H7-IR was observed. 74-5H7-IR for L1 peaked at 5 min and returned to baseline levels by 30 min (Fig. 9 D). Together, these results indicate that the 74-5H7 epitope is dynamically regulated on cell surface L1, and that it can be induced by L1–L1 homophilic binding and L1 cross-linking.
Discussion

We have presented evidence that L1 homophilic binding and L1 cross-linking at the cell surface induce a transient change in the phosphorylation state of the L1CD that can regulate its association with the endocytic machinery. This regulation of L1 internalization provides a means to control adhesion and facilitate growth cone movement. Our present studies depend in large measure on mAb 74-5H7. Several lines of evidence showed that this antibody binds to a subset of L1 and binding to L1 is negatively regulated by phosphorylation. First, several epitope mapping experiments demonstrate that 74-5H7 recognizes the sequence SEARPMKDETGFGEY, and that phosphorylation of Y1176 inhibits recognition. Second, L1 purified from brain extract in the presence of phosphatase inhibitors has weak 74-5H7-IR, whereas L1 purified in the absence of inhibitors shows stronger 74-5H7-IR. We showed that the L1CD could be phosphorylated by src on Y1176 and that an antibody specific for pYRSLE showed that the corresponding epitope on L1 was present in brain. Immunohistochemical studies of growth cones with 74-5H7 revealed that this form of L1 is restricted, being absent from filopodia and concentrated in vesicle-like structures. Finally, experiments using neurons and L1 expressing myelomas showed that cell contact or antibody cross-linking of L1 caused significant but transient changes in the phosphorylation state of L1 on Y1176. Together, these data show that L1 mediated contact causes an outside-in signal that alters the phosphorylation state of L1 and, in turn, alters L1 internalization and adhesion, a form of inside-out signaling.

The SEARPMKDETGFGEY sequence, where 74-5H7 binds, is perfectly conserved in human, mouse, rat, chicken, and fugu fish L1 homologues. Although the complete SEARPMKDETGFGEY sequence is not conserved in other vertebrate L1 homologues, acidic residues NH2-terminal to Y1176 and a hydrophobic residue at the Y-3 position are conserved not just in direct L1 homologues, but also in the L1 family members Nr-CAM and neurofascin (Hortsch, 1996). Previous data implicate p60src in L1 function, because src-/- neurons extend poorly on L1 substrates, suggesting their response to L1 is impaired (Ignelzi et al., 1994). Our finding that p60src can phosphorylate Y1176 provides potential mechanistic insight into this phenotype. We suggest that L1 in src-/- neurons grown on L1 may be relatively hypophosphorylated at Y1176. This could alter the regulation of L1 endocytosis; disruption of L1 trafficking could in turn impair neurite outgrowth on L1. Suter and Forscher (2001) recently showed that p60src activity could be involved in a positive feedback loop strengthening CAM interactions with their cytoskeletal linkages. Our data support a similar role for p60src in which it could phosphorylate L1 under certain conditions, thereby inhibiting internalization and increasing adhesion.

The 74-5H7 epitope is adjacent to the tyrosine-based motif, Yxx0 (0 is a bulky hydrophobic amino acid) in the L1CD that mediates L1 endocytosis (Kamiguchi et al., 1998b). Studies of tyrosine-based sorting motifs in other proteins indicate that the tyrosine has to be in a nonphosphorylated state for the signals to be active in sorting and endocytosis; i.e., phosphorylation of the tyrosine prevents binding of adaptin proteins to the motif (Boll et al., 1996; Ohno et al., 1996). Indeed, our in vitro peptide competition experiments using peptides derived from L1 show that a Y1176-phosphorylated peptide corresponding to the potential p60src phosphorylation site, FGEPYRSLEpSDNEE, is no longer able to bind the µ2 chain of AP-2, whereas a serine 1181-phosphorylated peptide corresponding to the CKII phosphorylation site, FGEPYRSLEpSDNEE, retains its capacity to associate with µ2. It is highly unlikely that T1172 influences L1–AP-2 interactions because there is no evidence suggesting it is phosphorylated, and extensive studies show that the fourth residue in the amino-terminal direction does not influence µ2 binding to tyrosine-based sorting motifs (Boll et al., 1996). Our results show that the 74-5H7-IR is inhibited by phosphorylation of Y1176 and that src family kinases may mediate this. Similarly, clathrin-mediated endocytosis of the T lymphocyte receptor, CTLA-4, has been shown to be negatively regulated by phosphorylation of the critical tyrosine in the endocytosis motif (Bradshaw et al., 1997; Shiratori et al., 1997) and this is likely by src family kinases (Miyatake et al., 1998). Overall, our results imply that only L1 that can bind 74-5H7 is capable of interacting with the clathrin-mediated endocytic machinery. This is consistent with the finding that in growth cones growing on L1 substrates, 74-5H7-IR is at the rear of growth cones, where L1 is internalized and colocalizes with Eps15, a marker for clathrin-coated pits (Kamiguchi et al., 1998b).

We have shown previously that inhibition of L1 endocytosis dramatically increases L1-mediated adhesion (Long et al., 2001). L1 at the rear of growth cones growing on L1 is less adhesive than L1 at the front of the growth cones, but this differential is not observed on growth cones growing on N-cadherin (Kamiguchi and Yoshihara, 2001). Therefore, phosphorylation of Y1176 provides a rapid but reversible means of increasing L1-mediated adhesion.

The hypothesis that the 74-5H7 epitope is regulated by phosphorylation/dephosphorylation involves the implication of L1 phosphatases in its regulation and is consistent with early results that show that cross-linking L1 activates tyrosine and serine phosphatases (Klinz et al., 1995). A number of potential candidate phosphatases exist. These include phosphotyrosine phosphatases (PTPases) that prefer substrates containing acidic residues NH2-terminal to the phosphotyrosine such as PTP1B, and rat brain PTP1 (for review see Zhou and Cantley, 1995). Another candidate is the receptor protein tyrosine phosphatase β because of its ability to bind heterophilically with L1 family CAMs. The phosphatase may also be coexpressed in some of the same cell types as L1 (Peles et al., 1998). It has been suggested that acidic residues NH2-terminal to the phosphotyrosine residue are a hallmark for targeted dephosphorylation (Pinna and Ruzzene, 1996) and that the number, nature, and position of the acidic residues may create consensus sequences that are recognized selectively by individual PTPases (Pinna and Donella-Deana, 1994).

The simplest interpretation of our biochemical analysis of the 74-5H7 epitope is that unengaged L1 is phosphorylated on Y1176, and that Y1176 becomes dephosphorylated subsequent to L1 ligand binding. Dephosphorylation allows interaction with AP-2 and clathrin-mediated endocytosis. In
this paradigm, the time course of 74-5H7-IR observed in the cell pelleting experiments could reflect internalization and subsequent recycling (and rephosphorylation) of L1. Our immunohistochemical observations of the distribution of 74-5H7-IR suggest the situation is more complex, however, and that L1–L1 binding does not always induce L1 endocytosis. First, L1 can mediate stable axon fasciculation, suggesting it is not invariably endocytosed at points of L1–L1 binding. Second, although growing DRG neurons on L1 increases L1 internalization and subsequent recycling (Kamiguchi and Lemmon, 2000; Kamiguchi and Yoshihara, 2001), endocytosis of L1 occurs preferentially at the rear of the growth cone. 74-5H7-IR is colocalized with vesicle-like structures in these neurons, rather than just at points of contact between the growth cone and the L1-Fc substrate (Kamiguchi et al., 1998b). These observations suggest that dephosphorylation of Y1176 and L1 endocytosis in growth cones are not regulated solely by L1–L1 binding, but also by restricted localization of the dephosphorylation machinery. We cannot rule out the possibility that, in vivo, the 74-5H7 epitope in some L1 is masked by interactions with other proteins, such as cytoskeletal components (Brophy, 2001). Thus, although 74-5H7 clearly identifies a subset of L1 in cells that are dephosphorylated at Y1176, there may be other populations of L1 that are dephosphorylated at Y1176, but not accessible to the antibody and thus not visualized in immunocytochemical experiments.

In summary, we have used 74-5H7 to show that L1 is dephosphorylated at Y1176 where L1 is internalized and trafficking in growth cones. We have also shown that the phosphorylation state of Y1176 determines whether L1 can interact with the clathrin internalization machinery. A variety of experiments showed that the phosphorylation/dephosphorylation state of L1 is regulated by cell contact and restricted to specific sites in neurons. The phosphorylation of the L1CD on Y1176 by p60src that is important for L1-mediated neurite outgrowth raises the possibility that the regulation of L1 internalization by phosphorylation and dephosphorylation represents a critical regulatory point for L1. The ability of L1–L1 adhesion to influence its endocytosis is reminiscent of how other surfaces can influence integrin expression on neurites (Condic and Letourneau, 1997). In *Aplysia*, the distribution of apCAM, an IgSF CAM member, is acutely regulated by endocytosis during learning-induced synaptic remodeling (Bailey et al., 1992), whereas in *Drosophila*, changing the distribution of another IgSF member, fascicillin II, alters synapse size (Schuster et al., 1996). It appears that regulated endocytosis may be a general mechanism underlying CAM functions in dynamic processes such as cell migration, neurite outgrowth, and synaptic plasticity.

Materials and methods

Materials

Protease inhibitors, Pefabloc SC, leupeptin, and aprotinin, as well as HRP-conjugated goat anti-rabbit antibodies were from Boehringer. Texas red- and Oregon green-conjugated anti-rabbit IgGs were from Molecular Probes, Inc. Anti–phosphotyrosine antibody (4G10) and recombinant human p60src were from Upstate Biotechnology. The anti-L1 antibodies were produced in the laboratory and include polyclonal anti-chicken L1 antibody (8D9) (Lemmon and McLoon, 1986), polyclonal anti-human L1 antibody (Hlavin and Lemmon, 1991), polyclonal anti-recombinant L1 cytoplasmic domain, and 74-5H7 mouse monoclonal anti-L1 (Lemmon et al., 1989). The new rabbit anti-74-5H7 antibody was made in conjunction with Eurogentec [32P]HPO42 was purchased from ICN Biomedicals. Gensys synthesized the L1 cytoplasmic domain synthetic peptides used to characterize mAb 74-5H7 and the custom alanyl substitution peptides as well as the phosphopeptides directly onto a solid cellulose-based support using SPOT® technology. The Immo- bilon-P polyvinylidenefluoride (PVDF) membrane was from Millipore. Renaissance enhanced chemiluminescent detection reagents were from NEN Life Science Products. Tissue culture reagents were from Gibco BRL. The following tyrosine phosphate inhibitors were used: microcystin and cyster-methrin (Calbiochem), molybdate, phenylarsine oxide, and orthovanadate (Sigma-Aldrich). All other chemicals were purchased from Sigma-Aldrich.

DRG cell culture

DRG were dissected from embryonic day 10 chicks and dissociated with 0.1% collagenase, 0.1% DNase in CaCl2/MgCl2 free HBSS and grown on glass coverslips sequentially coated with poly-l-lysine, 10 μg/ml goat anti-human IgG (Fc specific), and L1-Fc chimera (Kamiguchi et al., 1998b). Some DRG neurons were grown on glass coverslips coated with poly-l-lysine and 10 μg/ml laminin.

L1-expressing myeloma cell lines

L1-expressing myeloma cells were described previously (Wong et al., 1995). Cell surface expression of L1 by transfected myeloma was evaluated by live cell staining immunofluorescence, and stable cell lines were cloned using FACS® and subsequently sorted by FACS® to produce lines with identical expression levels.

Chick brain cell suspension

Whole brains were dissociated from E10 chick embryos and the meninges were carefully removed. The brains were then broken up into small fragments by passing them through a 300-μm nylon mesh. The fragments were incubated on ice in DME supplemented with 1 mM EDTA for 5 min, and then run through 50-μm nylon mesh. The fragments were then pelleted by centrifugation, washed with DME/10% FCS, and pelleted again to remove the EDTA. A single cell suspension was obtained by trituration with flame-polished Pasteur pipettes. The cells were centrifuged through a DME/50% FCS cushion to remove cell fragments and vesicles. Finally, the cells were resuspended in DME/10% FCS, counted, and plated in 15-cm tissue culture dishes in a tissue culture incubator (37°C, 5% CO2) for 1 h.

Cell pelleting and L1 cross-linking time course experiments

The chick brain cells in suspension were transferred to 15 ml-conical tubes (5 × 105 cells in 5 ml), centrifuged in a clinical centrifuge for 30 s, and then replaced into the tissue culture incubator. At each time point (0, 5, 15, 30, 45, 60, 120, and 180 min), pelleted cells were removed from the incubator. The cells were extracted in 20 mM Tris, pH 7.4, 1 mM EDTA, 2 mM sodium orthovanadate, and 10 mM p-nitrophenyl phosphate (TEV-PNP) containing 0.25% SDS, 1% NP-40, 40 mM sodium fluoride, and protease inhibitors (200 μM Pefabloc SC, 1 μg/ml leupeptin, 100 μg/ml aprotinin, and 5 μg/ml turkey trypsin inhibitor). The cell extracts were incubated for 30 min on ice and then centrifuged in a microfuge at maximum speed for 10 min at 4°C. SDS-PAGE sample buffer was then added to the supernatants, and the samples were boiled for 5 min. The samples were separated by SDS-PAGE (5–15% gradient gel), transferred to a PVDF membrane, and the membrane was blocked with 5% evaporated nonfat milk, 0.1% Tween 20, in TBS. The membrane was incubated with 74-5H7, washed, probed with HRP-goat anti–mouse antibodies, and reactive proteins were visualized by chemiluminescence. Blots were stripped and reprobed with polyclonal anti-L1 antibodies to assess protein loading. The intensity of the bands was quantified using densitometry and NIH Image analysis software. In some experiments, samples were divided in half and immunostained with 74-5H7 and polyclonal anti-L1 antibodies separately. The same 74-5H7-IR time course was observed as divided with the stripping and reprobing procedure.

In L1 cross-linking experiments, chick brain cells in suspension (105/ml) were preincubated on ice with polyclonal anti-L1 (8D9) cross-linking antibodies for 30 min. The cells were brought to 37°C for various periods of time to induce L1 clustering by the antibodies, spun down in a microfuge for 30 s, extracted, and the L1 was immunoprecipitated with protein A beads for 1 h.

Immunocytochemistry of DRG neurons and L1-expressing myeloma cells

Cells were fixed with a modified Bouin’s solution containing 75 ml saturated picric acid, 5 ml formalin, 5 ml glacial acetic acid, and 20 ml distilled water for 5 min. The cells were washed with PBS and permeabilized...
and blocked in a solution of 10% horse serum/0.02% Triton X-100 in PBS for 1 h. Cells were incubated in primary antibodies overnight at 4°C. Polyclonal anti-L1 antibodies were added to undiluted 74-5H7 hybridoma culture supernatant. In some experiments, live cells were incubated for 1 h with polyclonal anti-L1 antibodies (1:250) to cross-link L1 on the cell surface. The cells were then rinsed with prewarmed tissue culture medium, fixed, and stained with 74-5H7, followed by fluorescent secondary antibodies (Schafer et al., 1999).

**Confoal microscopy**

Images of DRG neurons and L1-expressing myeloma cells were taken with a confocal laser microscope (model LSM 410; ZEISS), using an argon/krypton laser (excitation lines, 488 and 568 nm) and a 100x Plan-Neofluor, NA 1.3, oil objective.

**Peptide sequencing**

20 μg recombinant L1CD, prepared as previously described (Wong et al., 1996b), was phosphorylated with recombinant human p60src in TEV-PNP containing 10 mM MgCl₂, 2 mM MnCl₂, 5 mM ATP, and 20 μCi γ-[³²P]ATP. The L1CD was isolated by running the sample on SDS-PAGE (5–15% gradient gel), transferring the proteins to PVDF membrane and cutting out the radiolabeled L1CD band. The sample was then digested with endoprotease Asp-N for 18 h at 37°C, and the resulting peptides were separated by HPLC on a C-18 reverse phase column. The fractions containing significant protein and radioactivity were then sequenced on an ABI protein sequencer (University of Kentucky, Macromolecular Structural Analysis Facility).

**Rabbit anti-phosphoYRSLE (pYRSLE) production and characterization**

A new affinity-purified rabbit antibody to pYRSLE was made by immunizing a rabbit with the peptide KDETFGEpYRSLESDN and then purifying the antibody over a column with the same peptide. The bound antibodies were eluted and run over a peptide column with KDETFGEYRSLESDN to remove antibodies that recognize unphosphorylated L1. To demonstrate that the Ra-pYRSLE antibody recognized the appropriate epitope, recombinant L1CD was phosphorylated with src and ATP, as above, run on an SDS gel, transferred to PVDF membrane, and Western blots were done.

**Studies on the effect of phosphorylation of the L1CD on interactions with AP-2**

The photoactivation cross-linking reaction was performed as described previously (Rapport et al., 1999; Kamiguchi et al., 1999b). In brain AP-2 complexes were purified from calf brain and then incubated with a peptide including the tyrosine-based sorting motif from TGN38, biotin, and a photo-activatable cross-linking reagent. Competitor peptides (Receptor Genetics) were simultaneously added at varying concentrations. The peptides included FGEYRSLESDNNE, which corresponds to amino acids 1173–1185 of the L1CD, and two phosphopeptides, FGEYRSLESDNNED and FGEYRS-LEpSDNN. After allowing the mixtures to incubate in the dark, they were cross-linked with UV. The samples were then run on SDS-PAGE, transferred to nitrocellulose, and incubated with streptavidin-conjugated HRP (Boehringer). The labeled bands were detected via chemiluminescence.

**ECL films for densitometry were scanned with an imaging**

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