

Activation of the MAPK Signal Cascade by the Neural Cell Adhesion Molecule L1 Requires L1 Internalization*

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L1-mediated axon growth involves intracellular signaling, but the precise mechanisms involved are not yet clear. We report a role for the mitogen-activated protein kinase (MAPK) cascade in L1 signaling. L1 physically associates with the MAPK cascade components Raf-1, ERK2, and the previously identified p90^{rsk} in brain. *In vitro*, ERK2 can phosphorylate L1 at Ser¹²⁰⁴ and Ser¹²⁴⁸ of the L1 cytoplasmic domain. These two serines are conserved in the L1 family of cell adhesion molecules, also being found in neurofascin and NrCAM. The ability of ERK2 to phosphorylate L1 suggests that L1 signaling could directly regulate L1 function by phosphorylation of the L1 cytoplasmic domain. In L1-expressing 3T3 cells, L1 cross-linking can activate ERK2. Remarkably, the activated ERK localizes with endocytosed vesicular L1 rather than cell surface L1, indicating that L1 internalization and signaling are coupled. Inhibition of L1 internalization with dominant-negative dynamin prevents activation of ERK. These results show that L1-generated signals activate the MAPK cascade in a manner most likely to be important in regulating L1 intracellular trafficking.

Immunoglobulin superfamily cell adhesion molecules (IGSF CAMs)¹ provide permissive and instructive cues for neuronal migration and neurite outgrowth during the formation of precise connections between neurons and their targets. The functional state and surface expression of IGSF CAMs on extending axons can be altered in response to the complex and changing environments that the axons traverse. For example, axonal expression of L1 is dramatically up-regulated on the contralateral side of commissural axons once they cross the floorplate in the developing mouse spinal cord (1). Precise regulation of CAM expression has also been implicated in synapse formation

and modification. Adhesion mediated by the invertebrate *Aplysia* CAM (apCAM) is regulated in culture by internalization during long term facilitation, a cellular model of learning involving structural alteration of synapses (2, 3). The internalization of apCAM is mediated through activation of the mitogen-activated protein kinase (MAPK) signaling cascade, which results in the phosphorylation of the apCAM cytoplasmic domain by MAPK (4). Genetic studies in *Drosophila* also support the idea that synaptic plasticity is modulated by CAM cell surface expression (5).

L1 is an IGSF CAM that has been implicated in a number of developmentally important processes including neuronal cell migration (6), axon outgrowth (7), and axon fasciculation (8, 9). Mutations in the human L1 gene cause abnormal brain development, characterized by mental retardation and defects in central nervous system axon tracts such as the corpus callosum and corticospinal tract (Ref. 10 and for review see Ref. 11). L1 is also expressed in adult mammals in regions such as the hippocampus and cerebellum, which undergo continual remodeling of synaptic connections, suggesting a possible role for L1 in these processes (12). This idea is supported by studies linking L1 to hippocampal long term potentiation (13) and spatial learning (14).

Two mechanisms have been proposed for regulating adhesion by L1 family members, both of which can be modulated by phosphorylation events. First, the L1 cytoplasmic domain (L1CD) contains an ankyrin-binding domain that shares homology with other CAMs including vertebrate NrCAM and neurofascin, as well as *Drosophila* neuroglian (15). The binding of ankyrin to the L1 subfamily has been shown to stabilize L1-mediated homophilic adhesion (15) and changes in the phosphorylation state of a critical tyrosine in the ankyrin-binding domain of neurofascin can regulate neurofascin-mediated adhesion (16). Second, L1 adhesion may be regulated at the level of cell surface expression (17). The neuronal form of L1 contains an alternatively spliced exon encoding four amino acids (RSLE) within the L1CD (18), which contributes to a tyrosine based sorting/endocytosis motif (YRSL) (19). This sequence enables the L1CD to directly bind the μ 2 subunit of the adaptin complex AP-2, linking L1 to the clathrin-mediated endocytotic pathway (20). Adaptin proteins are dynamically regulated by phosphorylation (21–23), and examples from G-protein-coupled receptors have demonstrated the importance of phosphorylation of both receptors and intracellular machinery in regulating endocytosis (24, 25). Consequently, it is likely that phosphorylation may also regulate L1 internalization.

L1 contains multiple potential phosphorylation sites and is phosphorylated *in vivo* (26). To understand the role of phosphorylation in L1 function, we have focused on kinases that inter-

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¹ The abbreviations used are: IGSF, immunoglobulin superfamily; CAM, cell adhesion molecule; apCAM, *Aplysia* CAM; NCAM, neural cell adhesion molecule; NgCAM, neural-glia CAM; NrCAM, neural-glia-related CAM; L1CD, L1 cytoplasmic domain; FGF, fibroblast growth factor; FGFR, FGF receptor; MBP, myelin basic protein; RSP, Raf synthetic peptide; MAPK, mitogen-activated protein kinase; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; HPLC, high pressure liquid chromatography.

act with L1. Previously, we identified two kinases, CKII and p90^{rsk}, that coimmunoprecipitate with L1 and phosphorylate L1 at Ser¹¹⁸¹ and Ser¹¹⁵², respectively (27, 28). p90^{rsk} is a distal component of the mitogen-activated kinase (MAPK or ERK) signal cascade, which raises the possibility that L1 may interact with additional components of this pathway.

The MAPK cascade is activated by a wide range of extracellular stimuli, and ERK kinases can phosphorylate many proteins, including transcription factors, membrane proteins, cytoskeletal proteins, and other kinases. The ERKs are activated by tyrosine kinase receptors, G-protein-linked receptors, and protein kinase C-dependent pathways, and the best resolved pathway involves the sequential activation of Ras, Raf, MEK, ERK, and p90^{rsk} (for review see Ref. 29). Activation of the upstream components occurs at the plasma membrane. However, recent evidence suggests that distal components including ERK and p90^{rsk} require internalization of the receptor tyrosine kinase or G-protein linked receptor to be fully activated (30–32). Finally, ERK activation has been implicated in the regulation of cell motility. For example, integrin-mediated activation of the MAPK cascade can influence cell motility through the phosphorylation of myosin light chain kinase by ERK (33).

We present evidence that two additional components of the MAPK cascade, ERK2 and Raf-1, associate with L1. ERK2 phosphorylates L1 and can be activated in L1-expressing 3T3 cells by L1 cross-linking antibodies. The activated ERK colocalizes with endocytosed L1. The activation of ERK by cross-linking cell surface L1 is prevented if endocytosis of L1 is blocked. This suggests that one function of the interaction between ERK and L1 may be in regulating L1 intracellular trafficking because only internalized L1 can be phosphorylated by activated ERK.

EXPERIMENTAL PROCEDURES

Materials—Protease inhibitors, Pefabloc SC, leupeptin, and aprotinin, as well as horseradish peroxidase-conjugated goat anti-rabbit antibodies were purchased from Roche Molecular Biochemicals. Recombinant bacterially expressed ERK2 was obtained from Upstate Biochemicals, Inc. (Lake Placid, NY). Anti-ERK2 and anti-Ras monoclonal and polyclonal antibodies were purchased from Transduction Laboratories (Lexington, KY). Anti-phospho-specific ERK antibodies were purchased from New England Biolabs (Beverly, MA). Anti-Raf-1, B-Raf, and MEK1 were purchased from Santa Cruz Biotechnology. [³²P]H₃PO₄ was purchased from ICN Biochemicals (Irvine, CA). The anti-NCAM antibody was the gift of Dr. Urs Rutishauser (Sloan-Kettering, New York, NY). The 5G3 anti-human L1 monoclonal antibody was a gift from Dr. R. A. Reisfeld (Scripps Research Institute, La Jolla, CA). The 74–5H7 anti-L1 monoclonal antibody is described in Ref. 34. The rabbit anti-human L1 antibody has been described previously (35). The L1 cytoplasmic domain with a His₆ tag was expressed in *Escherichia coli* (28) and used to make a rabbit antibody. Monoclonal and rabbit anti-phosphorylated ERK antibodies were obtained from New England Biolabs. Raf synthetic peptide (RSP) was purchased from Promega (Madison, WI). The epidermal growth factor ERK site peptide, T669, as well as the tyrosine kinase inhibitors, erbstatin analog, and PPI were purchased from Calbiochem (La Jolla, CA). Immobilon-P polyvinylidene difluoride membrane was from Millipore (Marlborough, MA). RenaissanceTM enhanced chemiluminescent detection reagents were purchased from NEN Life Science Products. Bacterial expression vector pQE13 and Ni-NTA agarose beads were from Qiagen (Valencia, CA). All other chemicals were purchased through Sigma.

Cell Culture—NIH-3T3 cells (American Type Tissue Culture Collection, Manassas, VA) and dorsal root ganglia from embryonic day 10 chickens were cultured as described previously (20). Briefly, the L1-expressing NIH-3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc.) supplemented with 10% fetal calf serum and 600 µg/ml G418 (Life Technologies, Inc.) prior to serum starvation.

L1 Immunoprecipitation—Brains from P7 Harlan Sprague-Dawley rat pups or embryonic day 14 chick embryos were homogenized in 20 mM Tris, pH 7.4, 1 mM EGTA, 1 mM sodium orthovanadate, and 10 mM

p-nitrophenyl phosphate (TEV-PNP) containing 0.32 M sucrose, 200 mM Pefabloc SC, and 100 µg/ml aprotinin. The homogenates were separated by ultracentrifugation on a sucrose gradient for 45 min at 58,400 × *g* at 4 °C. The plasma membrane layer was washed in TEV-PNP and then centrifuged 30 min at 150,000 × *g* at 4 °C to pellet the membranes. The plasma membrane pellet was solubilized in TEV-PNP containing 1% Triton X-100 and centrifuged for 45 min at 150,000 × *g* at 4 °C to remove insoluble material. The solubilized membrane fraction was then incubated for >4 h at 4 °C with Sepharose beads conjugated to a monoclonal anti-L1 antibody, mAb 74–5H7 (34). The beads were washed with TEV-PNP containing 1% Triton X-100 twice followed by four washes with TEV-PNP before use in kinase assays or Western blot analysis.

L1CD Preparation—The cytoplasmic domain of human L1, comprising residues 1144–1257, was cloned into the pQE13 bacterial expression vector to produce a recombinant L1CD containing a hexahistidine epitope at the N terminus. This protein was expressed in *E. coli*, and L1CD was purified from the bacteria by Ni²⁺ affinity chromatography using nickel-nitrilotriacetic acid agarose beads, using the manufacturer's protocols.

In Vitro Kinase Assay—Kinase phosphorylation reactions were carried out with L1 immunoprecipitates in TEV-PNP buffer containing 10 mM MgCl₂, 2 mM MnCl₂, 5 mM [γ-³²P]ATP, and myelin basic protein (MBP), ERK substrate peptide (T669), or RSP for 30 min at room temperature. The reactions were stopped by the addition of sample buffer and boiling for 5 min. MBP was separated from other proteins in the reaction by SDS-PAGE, and the radiolabeled MBP was visualized by autoradiography. T669 is a synthetic peptide derived from a potential ERK site on the epidermal growth factor receptor and has the sequence ERELVEPLTPSGEAPNQALLR (36). RSP is a synthetic peptide with the sequence IVQQFGFQRRSNNNGKLTN, which corresponds to a potential autophosphorylation site in the Raf-1 kinase. A tyrosine has been replaced at position seven by a phenylalanine to prevent tyrosine phosphorylation of the substrate (37). The peptides were separated from other proteins in the reaction on a Tris-Tricine SDS-PAGE system (38) modified with a 19–33% linear gradient resolving gel and visualized by autoradiography.

Western Blot Analysis—L1 immunoprecipitates were mixed with sample buffer and boiled for 5 min. The samples were then separated by SDS-PAGE. The proteins were transferred to Immobilon-P membrane, and the membrane was then blocked with 5% nonfat dry milk in Tris-buffered saline. The commercial primary antibodies were used as recommended by the manufacturer. The membrane was incubated with primary antibodies for 1 h at room temperature with shaking and washed with 0.1% Tween-20 in Tris-buffered saline. The membrane was then probed with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:1000 in 5% milk/0.05% Tween-20/phosphate-buffered saline) for 1 h, washed, and then visualized by chemiluminescence. The Western blots were scanned onto a Macintosh power PC using a AGFA duoscanner, and images were analyzed with NIH Image.

In the experiments designed to detect ERK activation, NIH-3T3 cells stably transfected with full-length human L1 (20) were plated at a density of 2 × 10⁵ cells/60-mm dish. Prior to stimulation, the cells were maintained in low serum, 0.5% fetal calf serum in DMEM for 48 h followed by 2 h in serum-free DMEM. At all times the tissue culture medium was maintained at 37 °C and equilibrated with CO₂. The cells were then treated with rabbit polyclonal anti-L1 antibody for various periods. After the treatments, cells were directly extracted from the tissue culture dishes with 300 µl of sample buffer supplemented with 1 mM sodium orthovanadate. The sample was boiled for 5 min followed by sonication with a vibrating probe sonicator to shear DNA. The samples were separated by SDS-PAGE and analyzed by Western blot as above. Blots were first probed with the anti-phosphorylation-specific ERK antibodies and then stripped and re-probed with other antibodies recognizing both phosphorylated and unphosphorylated forms of ERK (total ERK) to compare loading between lanes and relative ERK activation levels.

Peptide Sequencing—Recombinant L1CD (10 µg) was phosphorylated with recombinant ERK2 in TEV-PNP containing 10 mM MgCl₂, 2 mM MnCl₂, 5 mM ATP, and 5 µCi of [γ-³²P]ATP. The samples were then digested with endoproteinase Asp-N for 18 h at 37 °C, and the resulting peptides were separated by HPLC on a C-18 reverse phase column. Fractions were collected and analyzed for protein concentration and radioactivity. The fractions containing significant protein and radioactivity were then sequenced on an ABI protein sequencer.

Indirect Immunofluorescence—L1-expressing NIH-3T3 cells (20) cultured on two-chamber plastic slides (Lab-Tek, Naperville, IL)

coated with fibronectin ($5 \mu\text{g}/\text{cm}^2$; Roche Molecular Biochemicals) were maintained in 0.5% serum in DMEM for 48 h followed by 2 h in serum-free DMEM. Then the cells were treated with either rabbit polyclonal anti-L1 antisera or preimmune sera for 20 min and processed for immunocytochemistry to examine the subcellular distribution of phosphorylated ERK. Following fixation with 4% formaldehyde and permeabilization with 0.02% Triton X-100, the cells were incubated with mouse monoclonal anti-phospho ERK (1:500; New England Biolabs) at 4°C for 16 h. Phosphorylated ERK was then visualized with Texas Red-conjugated anti-mouse IgG (1:100; Molecular Probes, Eugene, OR).

In some experiments, the cells were double-labeled for L1 and phosphorylated ERK to analyze colocalization. Differential labeling of cell surface and internalized L1 was performed as described previously (20). In the experiment designed to double-label cell surface L1 and phosphorylated ERK, live cells were incubated with rabbit polyclonal anti-L1 antibody for 1 h at 37°C , followed by incubation with Oregon Green-conjugated anti-rabbit IgG (1:200; Molecular Probes) for 1 h at 4°C . Subsequently, the cells were fixed with 4% formaldehyde for 30 min, permeabilized, and blocked with a mixture of 10% horse serum and 0.02% Triton X-100 in phosphate-buffered saline. The cells were then incubated with mouse monoclonal anti-phospho ERK overnight at 4°C followed by Texas Red-conjugated anti-mouse IgG (1:100).

In the experiment designed to double-label internalized L1 and phosphorylated ERK, live cells were incubated with rabbit polyclonal anti-L1 antibody for 1 h at 37°C , followed by incubation with unconjugated anti-rabbit IgG ($200 \mu\text{g}/\text{ml}$; Molecular Probes) for 1 h at 4°C . The cells were fixed, permeabilized, and incubated with mouse monoclonal anti-phospho ERK overnight at 4°C . Then the cells were incubated with a mixture of Texas Red-conjugated anti-mouse IgG (1:100) and Oregon Green-conjugated anti-rabbit IgG (1:200). The labeled cells were mounted with SlowFade (Molecular Probes), and images taken with a Zeiss LSM 410 confocal laser microscope (Zeiss, Göttingen, Germany) using an argon/krypton laser (excitation lines, 488 and 568 nm) and a $100\times$ Plan-Neofluor, numerical aperture 1.3, oil objective.

Transfection of L1-3T3 Cells with Dominant-negative Dynamin—Transfection of cDNA encoding for K44A dynamin or β -galactosidase was done using recombinant adenovirus vectors (kind gift of Dr. Jeffrey E. Pessin, The University of Iowa, Iowa City, IA). Production of concentrated adenovirus and infection of NIH-3T3 cells were done as described previously (32). Briefly, 85–90% confluent 293 cells (American Type Culture Collection) were infected with adenovirus and incubated for 36–48 h. The cells were collected and lysed by repeated freezing and thawing, and concentrated adenovirus (1 ml of cell lysate/10-cm culture dish of 293 cells) was prepared. Then, L1-expressing 3T3 cells (50–60% confluent) plated on fibronectin-coated 35-mm dishes were infected with $50 \mu\text{l}/\text{dish}$ of concentrated adenovirus medium. After 48 h incubation, the cells were serum-starved, treated with anti-L1 antibody, and processed for Western blot analysis to detect phosphorylated ERK. Immunocytochemistry of infected 3T3 cells showed that approximately 95% of the cells expressed the transgene products (data not shown).

RESULTS

Raf-1 and ERK Activity Associates with L1—At least two distinct kinase activities have previously been shown to coimmunoprecipitate with L1 (27, 39, 40). These have been identified as CKII (27) and $\text{p}90^{\text{rsk}}$ (28), which phosphorylate L1 at Ser¹¹⁸¹ and Ser¹¹⁵², respectively. $\text{p}90^{\text{rsk}}$ is a distal component of the MAPK signaling cascade and has been found to associate with ERK in PC12 cells, in *Xenopus* oocytes, and in COS cells transfected with $\text{p}90^{\text{rsk}}$ isoforms (41–43). These findings raise the possibility that L1 may associate with other kinases involved in the activation of $\text{p}90^{\text{rsk}}$, such as ERK. To determine whether any other kinases in the MAPK pathway associate with L1, Western blots of L1 immunoprecipitates from rat brain membrane preparations were probed for Ras, Raf-1, B-Raf, MEK-1, ERK, and $\text{p}90^{\text{rsk}}$ (Fig. 1A). The results demonstrate that Raf-1 and ERK2, in addition to the previously identified $\text{p}90^{\text{rsk}}$, are associated with L1. As a control for the stringency of the wash conditions, the abundant IGSF CAM, NCAM, was shown not to coimmunoprecipitate with L1. The MAPK cascade components, Ras, B-Raf, and MEK-1, were not detected in the L1 immunoprecipitates (data not shown). The predominant bands in silver-stained L1 immunoprecipitates

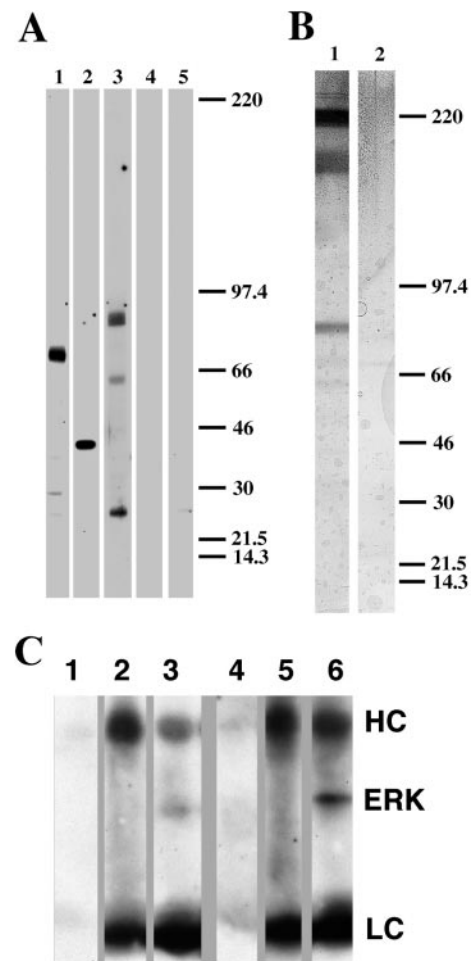


FIG. 1. Western blots of L1-associated kinases from rat brain. A, rat brain membrane proteins adsorbed to anti-L1 (74–5H7 mAb) conjugated Sepharose beads were separated by SDS-PAGE, blotted onto Immobilon-P, and probed with polyclonal antibodies against Raf-1 (lane 1), ERK2 (lane 2), $\text{p}90^{\text{rsk}}$ (lane 3), NCAM (lane 4), and goat anti-rabbit horseradish peroxidase secondary antibody only (lane 5). Locations of standard markers in kDa are indicated at right. B, representative silver stains of anti-L1 (74–5H7 mAb) bead immunoprecipitates (lane 1) and Sepharose bead control (lane 2). C, embryonic day 14 chick brain membrane extracts (lanes 1–3) or P7 rat brain membrane extracts (lanes 4–6) were incubated with anti-L1 (74–5H7 mAb) conjugated Sepharose beads (lanes 3 and 6), anti-NCAM conjugated Sepharose beads (lanes 2 and 5), or unconjugated Sepharose beads (lanes 1 and 4). ERK was only found in association with the anti-L1 beads in both chick and rat brain. HC, Ig heavy chain; LC, Ig light chain.

correspond to L1 products of 220, 135, and 80 kDa, indicating that associated kinases are present well below stoichiometric levels and may associate with a specific subset of L1 (Fig. 1B). To demonstrate that the ERK association with L1 in brain was specific, anti-L1-coated beads, anti-NCAM-coated beads, and uncoated beads were incubated with detergent extracts from P7 rat brains and embryonic day 14 chick brains. ERK was found in the L1 immunoprecipitations but not in the NCAM immunoprecipitations or bead controls (Fig. 1C). Similar results were found for Raf-1 (data not shown).

To determine the activity of the kinases identified by Western blot analysis, L1 immunoprecipitates from rat brain were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and either Raf substrate (RSP) or the ERK substrates (MBP or T669, an ERK substrate peptide derived from the epidermal growth factor receptor). The L1 immunoprecipitates were able to phosphorylate all three substrates consistent with the Western blot results (Fig. 2).

ERK2 Phosphorylates LICD—Previous work has demon-

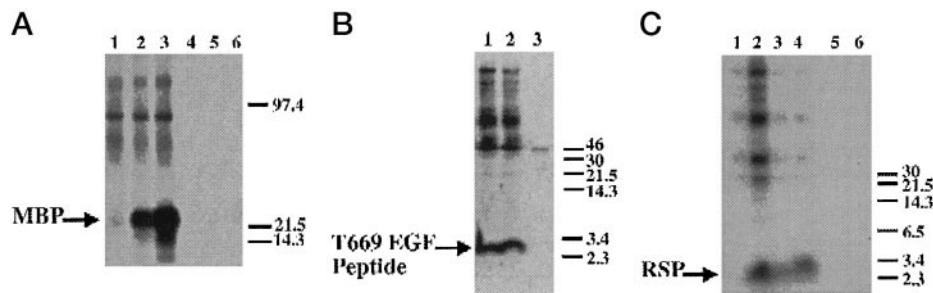


FIG. 2. L1-associated kinase activities from rat brain. *In vitro* phosphorylation of two ERK substrates, MBP and T669, and a Raf-1 substrate, RSP, by kinase activities coimmunoprecipitating with L1 on 74–5H7 mAb-coated Sepharose beads is shown. **A**, autoradiograph of MBP phosphorylation by L1 immunoprecipitates resolved by SDS-PAGE showing no MBP (lane 1), 1 μ g of MBP (lane 2), and 4 μ g of MBP (lane 3). Lanes 4, 5, and 6 are kinase reactions using rat brain membrane extracts adsorbed to unconjugated Sepharose beads with 0, 1, and 4 μ g of MBP, respectively. **B**, autoradiograph of ERK substrate peptide T669 phosphorylation by L1 immunoprecipitates resolved by Tris-Tricine SDS-PAGE showing 1 μ g of T669 (lane 1), 0.5 μ g of T669 (lane 2), and rat brain membrane Sepharose bead kinase reactions with 1 μ g of T669 (lane 3). **C**, autoradiograph of Raf-1 substrate peptide RSP phosphorylation by L1 immunoprecipitates resolved by Tris-Tricine SDS-PAGE showing no RSP (lane 1), 10 μ g of RSP (lane 2), 2.5 μ g of RSP (lane 3), and 5 μ g of RSP (lane 4). Lanes 5 and 6 are kinase reactions using rat brain membrane extracts adsorbed to uncoated Sepharose beads with 0 and 10 μ g RSP, respectively. Molecular mass markers are indicated to the right.

strated that L1 is phosphorylated on at least two serines (27, 28). Our earlier studies showed that endoproteinase Asp-N digested L1 from L1 immunoprecipitation kinase reactions and from *in vivo* metabolically labeled L1 both contained at least three radiolabeled peptide fragments. The major peaks of radioactivity ran at 30–32, 48–54, and 58–64 min on reverse phase HPLC. The 30–32-min peak contains a peptide fragment containing Ser¹¹⁵² that can be phosphorylated by p90^{rsk} (28), and the 58–64-min peptide fragment peak contains peptides with Ser¹¹⁸¹ that can be phosphorylated by CKII (27). We reasoned that the newly characterized Raf-1 and/or ERK2 kinase activities associated with L1 may account for the phosphorylated peptide peak of 48–54 min. ERK2 was the most likely candidate because the L1CD contains a potential proline directed phosphorylation site at Ser¹²⁴⁸. In addition, Sonderegger and colleagues (40) found that the synthetic substrate peptide, syntide-2, which can act as a competitive substrate for Raf-1, did not affect the phosphorylation of chicken L1 (Ng-CAM) by L1-associated kinase activities.

We tested the ability of recombinant ERK2 to phosphorylate recombinant L1CD *in vitro* to determine whether L1 could be a substrate for ERK2 phosphorylation. The phosphorylated L1CD was digested with endoproteinase Asp-N, and the resulting fragments were separated by reverse phase HPLC. Two peaks of radioactivity were detected, a minor peak associated with peptides that eluted at 48–51 min, and a major peak associated with peptides that eluted at 56–59 min (Fig. 3A). The minor peak (48–51 min) was composed primarily of a peptide with the sequence DIKPLGSDDSLA along with a small amount of a peptide with the sequence DETFGEYRSLESDN. The major peak (56–59 min) was comprised of two peptides with the sequences DETFGEYRSLESDNEEKAFGSSQPSLNG and DGSFIGQYSGKKEKEAAGGNDSSGATSPINPAVAL. These peptides correspond to phosphorylated peptides eluting at 58–64 min in previous experiments where L1 was purified from brains of rat pups injected with [³²P]orthophosphate (27). The site of phosphorylation of the 48–51-min fragments was determined to be the seventh residue (underlined) of the DIKPLGSDDSLA peptide by assessing the elution of radioactivity using covalent sequencing supports to allow tracking of the radiolabeled residue(s). The minor ERK2 phosphorylation site thus corresponds to Ser¹²⁰⁴ in L1. The site of phosphorylation in the major peak was determined to be the 27th residue (underlined) of the DGSFIGQYSGKKEKEAAGGNDSSGATSPINPAVAL peptide corresponding to Ser¹²⁴⁸ in the L1 (Fig. 3B). Thus, ERK2 can phosphorylate two serines in the L1CD. Our earlier studies indicated that L1 is phosphorylated on at

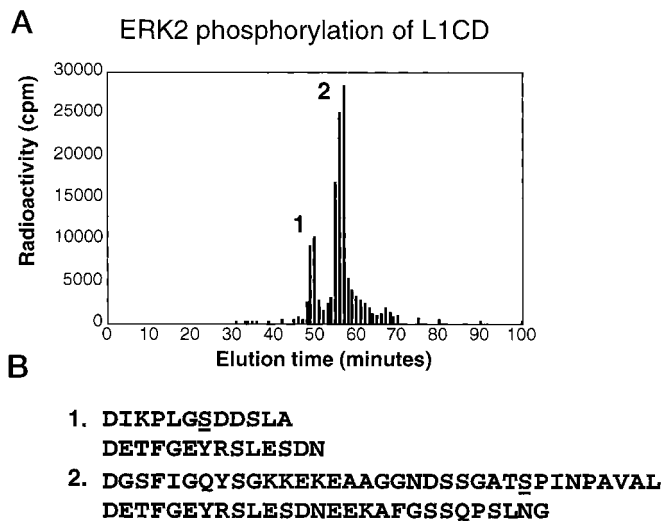


FIG. 3. L1 is a substrate for ERK2 phosphorylation. Recombinant L1CD was phosphorylated *in vitro* by recombinant ERK2 with radiolabeled ATP. Proteolytic fragments of L1CD were obtained by digestion with endoproteinase Asp-N. **A**, the resulting fragments were separated by reverse phase HPLC, and the eluted fractions were assayed for radioactivity. **B**, the ERK2 phosphorylated L1CD peptide peak from 48–51 min and 56–59 min were sequenced. The radioactivity from peak 1 was associated with the seventh residue and that from peak 2 with the 27th residue corresponding to Ser¹²⁰⁴ and Ser¹²⁴⁸, respectively. These serines are underlined in the sequences shown.

least four sites *in vivo* (27). We have shown previously that two sites correspond to sites phosphorylated by p90^{rsk} (Ser¹¹⁵²) and CKII (Ser¹¹⁸¹). In this current study we find that the two sites phosphorylated by ERK, Ser¹²⁴⁸ and Ser¹²⁰⁴, correspond to two additional sites phosphorylated in post-natal rat brain (27).

L1 Cross-linking Can Activate ERK—To determine whether L1 can activate the MAPK cascade, we used L1-expressing NIH-3T3 cells, which provide a simplified system for biochemical analysis. This system is particularly useful because basal activity of the MAPK pathway can be acutely down-regulated in the NIH-3T3 cells by serum starvation. Antibodies raised against the extracellular domain of CAMs have been extensively used to mimic CAM binding events and to stimulate CAM-mediated signaling (for examples see Refs. 44–49). Polyclonal anti-L1 antiserum was used to cross-link L1 in NIH-3T3 cells that were stably transfected to express L1. Western blot analysis of lysates from these cells, using antibodies that specifically recognize the phosphorylated and activated form of ERK, demonstrates that polyclonal rabbit anti-L1 antiserum

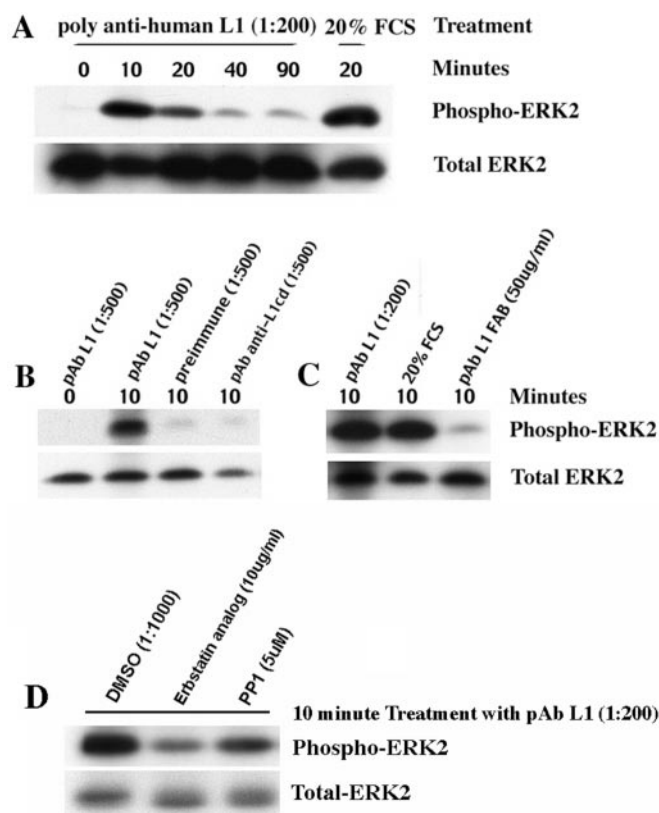


FIG. 4. L1 cross-linking activates ERK in L1-expressing NIH-3T3 cells. Serum-starved cells were treated with various reagents and ERK activation assayed by Western blot analysis. *A*, time course of ERK activation following addition of rabbit polyclonal anti-human L1 antiserum. *B*, control showing that incubation with preimmune rabbit antiserum and another rabbit antiserum raised against the L1 cytoplasmic domain (pAb anti-L1CD) did not result in ERK activation. *C*, rabbit polyclonal anti-human L1 Fab does not activate ERK to the same extent as the intact polyclonal. *D*, tyrosine kinase inhibitors reduce the activation of ERK by L1 cross-linking polyclonal antibodies. Serum-starved cells were preincubated with either carrier Me_2SO (*DMSO*), erbstatin analog (receptor tyrosine kinase inhibitor), or PP1 (a Src family tyrosine kinase inhibitor). *FCS*, fetal calf serum.

activates ERK, whereas monovalent Fab fragments derived from the polyclonal serum did not greatly activate ERK (Fig. 4, *A* and *C*). This result indicates that cross-linking of L1 on the cell surface is necessary for activation of the signaling cascade. Two negative controls, polyclonal rabbit preimmune sera and polyclonal rabbit antiserum raised against the cytoplasmic domain of L1, did not activate ERK (Fig. 4*B*). 20% fetal calf serum was used as a positive control for stimulating the MAPK pathway. The ERK antibodies used in these studies recognize both ERK1 and ERK2. However, ERK2 was the predominate kinase activated by L1 cross-linking as determined by molecular mass. In a limited number of cases, we did observe some ERK1 activation in addition to ERK2 (data not shown). These results are consistent with the observation that L1 isolated from rat brain membranes coimmunoprecipitates with ERK2 (Fig. 1*A*). Maximum activation was observed within 10 min of stimulation with a low level of continued activation until 90 min.

Previously, others have shown that L1 may signal through the fibroblast growth factor receptor (FGFR) (50), and p60^{src} is implicated in L1-mediated neurite outgrowth (51). These two tyrosine kinases are also capable of activating the MAPK cascade. We used tyrosine kinase inhibitors directed against the FGFR (erbstatin analog) (52, 53) or Src family kinases (PP1) (54) to characterize additional components of the L1-initiated signal transduction cascade. A 15-min pretreatment with either of the tyrosine kinase inhibitors reduced the activation of

ERK stimulated by the L1 antibodies compared with cells pretreated with the carrier alone (dimethyl sulfoxide) (Fig. 4*D*). These results indicate that a receptor tyrosine kinase (perhaps FGFR) and a nonreceptor tyrosine kinase of the Src family are both likely to be involved in L1-stimulated MAPK activation.

Immunocytochemical studies also demonstrated that L1-cross-linking antibodies activate ERK in L1-expressing NIH-3T3 cells (Fig. 5, *A–C*). Confocal sections through these cells showed that activated ERK was present in a punctate intracellular pattern suggestive of intracellular vesicles (Fig. 5*C*). ERK activated by 20% fetal calf serum was present in a similar punctate staining pattern in the cytosol (not shown). No accumulation of activated ERK was observed at the plasma membrane, although the system used may not have the sensitivity to detect a diffuse plasma membrane distribution of activated ERK (Fig. 5, *D–F*).

Endocytosed L1 Colocalizes with Activated ERK—Because some activated ERK coimmunoprecipitated with L1 (Fig. 2), but ERK activated by L1 cross-linking was detected in vesicular structures rather than at the plasma membrane (Fig. 5*C*), we wondered whether L1 and activated ERK colocalize in intracellular compartments. To examine whether endocytosed L1 colocalizes with phosphorylated ERK in NIH-3T3 cells, live cells were incubated with anti-L1 antibodies at 37 °C to allow endocytosis of antibody-bound L1. We have previously shown that, under these conditions, anti-L1 antibodies specifically label endocytosed L1 (20). Subsequently, the cells were permeabilized and processed for immunocytochemistry of activated ERK (Fig. 5*H*) and the endocytosed anti-L1 antibodies (Fig. 5*G*). A subset of the vesicles containing endocytosed L1 were also immunoreactive for activated ERK (Fig. 5*I*). Analysis of many cells indicated that about 52% of the vesicles containing endocytosed L1 colocalized with activated ERK (26 cells with an average of 10 endocytosed L1 vesicles/cell were counted). These vesicles most likely represent endosomes because endocytosed L1 colocalizes with endosomal markers (20). In contrast, no colocalization of activated ERK and cell surface L1 was observed (Fig. 5*F*) when cell surface L1 was immunolabeled by live cell staining prior to permeabilization.

Inhibition of L1 Internalization Prevents ERK Activation—The fact that activated ERK is only colocalized with internalized L1 raised the possibility that L1 internalization is required for L1-mediated ERK activation. It has been shown that a dominant-negative form of dynamin (K44A dynamin) specifically blocks clathrin-mediated endocytosis (55, 56) and that transfection of K44A dynamin into L1-expressing 3T3 cells inhibits L1 endocytosis by over 80% (20). Therefore, we tested whether K44A dynamin transfection could inhibit L1-mediated ERK phosphorylation induced by anti-L1 cross-linking and observed almost complete inhibition of ERK activation as assessed using the anti-phospho-ERK antibody (Fig. 6). This result strongly suggests that L1 endocytosis is required for L1-mediated ERK activation.

DISCUSSION

Cell adhesion molecules are multifunctional in the sense that they promote dynamic processes such as growth cone pathfinding and cell motility under some conditions, whereas under different conditions they maintain stable cell-cell associations and axon fasciculation. These multiple functions may involve differences in the adhesive state of CAMs. The ability of CAMs to either promote cell motility or stabilize cell morphology may be regulated by CAM-independent signals or signal transduction pathways initiated by the CAMs themselves. We have presented several pieces of evidence in support of the conclusion that the kinase ERK2 may be involved both in the regulation of L1 function by phosphorylation of L1 and as an

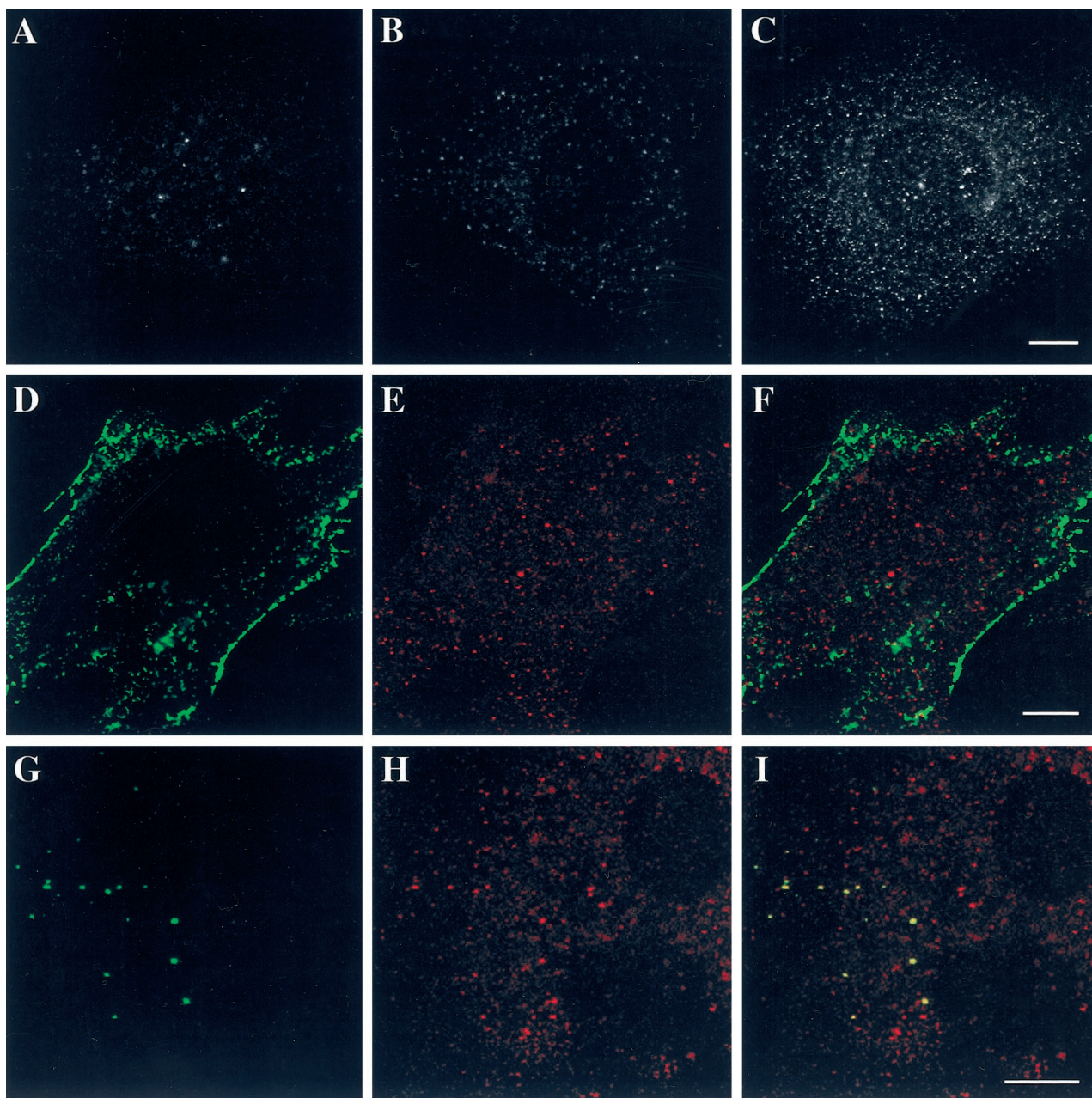


FIG. 5. Confocal sections (0.83 μm in thickness) of L1-transfected NIH-3T3 cells. A–C, the cells were immunolabeled for phosphorylated ERK before (A) or after 20 min of treatment with preimmune sera (B) or anti-L1 antiserum (C). D–F, the cells were double-labeled for cell surface L1 (D) and phosphorylated ERK (E). A superimposed image (F) shows no significant colocalization of the two. G–I, the cells were double-labeled for endocytosed L1 (G) and phosphorylated ERK (H). A superimposed image (I) shows colocalization of the two as evidenced by yellow. Bar, 10 μm .

intermediary in L1-mediated signaling. First, ERK2 coimmunoprecipitates with L1 from brain lysates. Second, *in vitro*, ERK2 phosphorylates L1 at Ser¹²⁰⁴ and Ser¹²⁴⁸, which are conserved residues in the L1 family of cell adhesion molecules and are likely phosphorylated *in vivo* (28). Third, L1 cross-linking leads to ERK2 activation. Fourth, activated ERK2 colocalizes with endocytosed L1. Finally, if L1 internalization is blocked with dominant-negative dynamin, ERK2 is not activated.

ERK2 Phosphorylation of L1—Our analysis of metabolically labeled L1 isolated from rat brain indicates that the ERK2 sites are phosphorylated *in vivo* (28); however, the effects of phosphorylation at these sites on L1 function are unknown. One possible function of serine phosphorylation of L1 by MAPK cascade kinases is in the regulation of its association with the

cytoskeleton. Ser¹²⁰⁴ is in a highly conserved region of the L1CD responsible for binding to the cytoskeletal protein ankyrin (57). A 25-residue internal deletion in the cytoplasmic domain of the L1 family member neurofascin, which encompasses the analogous region in L1 containing Ser¹²⁰⁴, completely eliminates ankyrin binding activity (58). Bennett *et al.* (58) also found that phosphorylation of a tyrosine in this region inhibits neurofascin binding to ankyrin. These findings raise the possibility that phosphorylation of Ser¹²⁰⁴ by ERK2 could also regulate L1 association with the cytoskeleton. In a similar vein, Ser¹¹⁵², which is phosphorylated by p90^{rsk}, is immediately adjacent to the membrane-proximal region of the L1CD critical for interactions with actin stress fibers in L1-transfected B28 glioma cells (59). One hypothesis that encompasses these observations is that the MAPK cascade components,

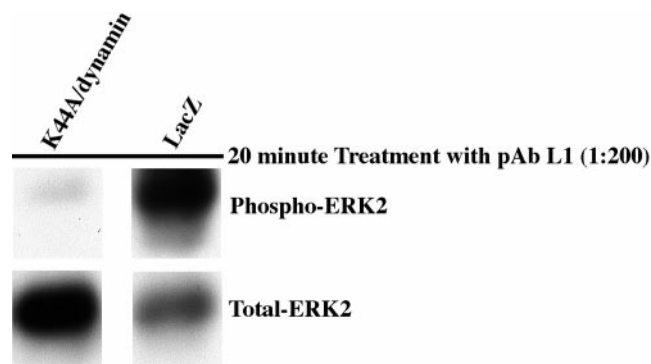


FIG. 6. Internalization of L1 is required to activate ERK. L1-expressing NIH-3T3 cells were transfected with K44A dominant-negative dynamin or a LacZ control and then treated with anti-L1 antibodies for 20 min to cross-link the cell surface L1. The cells expressing K44A-dynamin showed almost no phosphorylated ERK, whereas the LacZ expressing cells have a robust response.

ERK2 and $p90^{rsk}$, mediate a coordinated and transient change in the phosphorylation state of L1 that regulates its interactions with the cytoskeleton.

It is also interesting to note that Ser¹²⁴⁸ is located N-terminal to a proline which forms part of a minimal consensus motif, S(PXXP), for a SH3 domain-binding site (60). Although it is not known whether L1 can use this region to interact with proteins containing SH3 domains, ERK2 phosphorylation could modulate the association of L1 with potential binding partners (61).

ERK2 is a proline-directed kinase that is best able to phosphorylate substrates containing proline in the C-terminal residue adjacent to the phosphorylation site (62, 63). Ser¹²⁴⁸ is located N-terminal to a proline, making it a good ERK2 phosphorylation consensus site. Ser¹²⁰⁴ phosphorylation by ERK2, on the other hand, is an example of ERK2 phosphorylation of a noncanonical phosphorylation site. Two other examples of phosphorylation of noncanonical sites by ERK have been reported (61, 64). A possible explanation for this comes from structural analysis of ERK2, which reveals that it contains two regions for interacting with substrates: a proline specificity region as well as another region in the C-terminal domain of the kinase that forms a substrate-binding groove (65). Furthermore, MAPKs bind short proline-containing peptides relatively poorly, suggesting that a longer sequence with additional structural determinants is necessary for optimal binding and phosphorylation by the kinase (66, 67). It may be that ERK2 is capable of using other regions of the L1CD to stabilize its interaction with L1 and phosphorylate Ser¹²⁰⁴.

L1 Activation of ERK—The MAPK signaling cascade can be activated by a wide range of extracellular stimuli including those transmitted through receptor tyrosine kinases (for review see Ref. 29). The ability of L1 cross-linking to activate ERK2 combined with the ability of ERK2 to phosphorylate L1 raises the possibility that the phosphorylation state and function of L1 is regulated by heterologous extracellular signals. This is an especially appealing idea because L1 expression can be regulated by cell contact, electrical activity, and growth factors (17, 68). Therefore, signals that activate the MAPK cascade independent of L1-mediated binding, which activate the MAPK cascade, could regulate the L1 phosphorylation state. Neurite outgrowth-promoting growth factors such as nerve growth factor and FGF activate the MAPK cascade. However, the MAPK cascade targets involved in neurite outgrowth are only partially defined. Our results with both ERK2 and $p90^{rsk}$ (28) suggest that L1 could be one of these targets. An important issue is whether activation of ERK2 through an L1-initiated mechanism is equivalent to ERK2 activation by

other stimuli, such as growth factor receptors. If L1-activated ERK is equivalent to that activated by other signaling pathways, L1 binding could synergize with growth factor effects. Perhaps not, because our data show that L1 can regulate the localization and activity of MAPK signaling cascade components through their association with the endocytosed and trafficking L1.

Characterized signaling pathways that activate ERK2 involve activation of tyrosine kinases. L1 lacks intrinsic tyrosine kinase activity, so for it to activate ERK2, it is likely that L1 is at least transiently coupled either directly or indirectly to a tyrosine kinase. Two tyrosine kinases that can trigger MAPK cascade activation, the FGFR and $p60^{src}$, have both been implicated in L1-mediated neurite outgrowth. An extensive series of studies have established that the FGFR can be activated by L1-homophilic binding and L1-mediated FGFR activation stimulates neurite outgrowth (53, 69, 70). A similar signaling pathway is also utilized by other growth factor receptors including the epidermal growth factor receptor, platelet-derived growth factor receptor, and insulin-like growth factor-1 receptor to induce cell motility (71–73). The nonreceptor tyrosine kinase $p60^{src}$ also appears to be involved in some aspects of L1-mediated signaling because neurons from $p60^{src}$ knockout mice are impaired in their ability to extend neurites on L1 *in vitro* (51). Work in other systems has shown that FGFR and $p60^{src}$ signaling can be coupled. $p60^{src}$ can associate with the FGFR and is activated following treatment with FGF in 3T3 cells (74, 75). Furthermore, microinjection of a function blocking antibody against $p60^{src}$ inhibits FGF-induced neurite outgrowth in PC12 cells (76). Our initial characterization of the L1-initiated signal transduction cascade with the tyrosine kinase inhibitors erbstatin analog and PP1 also indicates that both receptor tyrosine kinases and nonreceptor tyrosine kinases contribute to L1 signaling and ERK activation.

Interestingly, ERK activation has been correlated with the down-regulation of cell adhesion. For example, integrin-mediated adhesion is down-regulated by activation of the MAPK cascade through a mechanism that does not involve gene regulation (77). In the case of epidermal growth factor-stimulated, integrin-mediated cell motility, activation of ERK is correlated with integrin deadhesion and disassociation of focal adhesion plaques, which is required for efficient cell migration (78). Furthermore, down-regulation of apCAM cell surface expression is mediated by ERK phosphorylation of apCAM, which targets the CAM for endocytosis and degradation (4). Both of these events involve reducing cell adhesion and are correlated with cell movement or terminal sprouting. In the case of L1, activation of ERK2 may regulate the intracellular trafficking of L1, suggested by the colocalization of activated ERK2 and L1 in vesicles, facilitating L1-mediated migration and/or neurite outgrowth.

ERK Activation Is Linked to Endocytosis—Recent studies suggest that some of the downstream MAPK cascade components, specifically ERK and $p90^{rsk}$, require endocytic trafficking of the receptor to be fully activated. For instance, the receptor tyrosine kinases epidermal growth factor receptor and insulin-like growth factor I receptor, as well as some G-protein-coupled receptors, require clathrin-mediated internalization to fully activate ERK (79, 80). In all of these cases, blocking internalization appeared to inhibit the signaling pathways downstream of Raf. We have previously shown that L1 associates with clathrin-mediated endocytosis machinery both *in vitro* and *in vivo* and that endocytosis of L1 occurs via a clathrin-dependent pathway in NIH-3T3 cells (20). Here we report that activated ERK is only colocalized with internalized L1 and that when internalization of L1 is blocked with a dominant-negative

form of dynamin, activation of ERK is blocked. Thus, L1 activation of ERK involves clathrin-mediated internalization, similar to the epidermal growth factor receptor and insulin-like growth factor I.

Clathrin-mediated endocytosis machinery may recruit signaling molecules to endocytosing receptors. p60^{src} can associate with cell trafficking machinery including dynamin, synapsin-1, and α -adaptin (81). Furthermore, p60^{src} is primarily localized to endosomes (82) from which it can be recruited to plasma membrane sites including focal adhesions (83). In growth cones, p60^{src} is primarily localized in vesicular structures (84, 85). It may be that L1 recruits signal transduction components such as the tyrosine kinase p60^{src} through its interaction with the endocytosis machinery.

Conclusions—The findings reported in this paper demonstrate that ERK is associated with L1 *in vivo* and phosphorylates L1 *in vitro* at residues that are phosphorylated *in vivo* and that L1 cross-linking activates ERK but not if L1 internalization is inhibited. These data provide a framework for understanding several disparate reports concerning L1-mediated signaling and L1-stimulated neurite growth. 1) Both the FGFR and p60^{src} have been found to influence L1-mediated neurite growth and both can activate ERK. 2) The two ERK phosphorylation sites in the L1 cytoplasmic domain are in or near the ankyrin-binding site. 3) ERK can activate p90^{rsk1}, which phosphorylates L1 in a region important for neurite growth and association with the actin cytoskeleton. 4) Activated ERK is associated with internalized L1 but not L1 at the cell surface. A key function of L1-mediated activation of ERK and p90^{rsk1} may be to phosphorylate trafficking L1, preventing it from interacting with the actin and ankyrin cytoskeleton. This regulation of L1 trafficking may be important for L1 based axon extension.

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