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 p90RSK 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)† 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 μ2 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-
act with L1. Previously, we identified two kinases, CKII and p90rsk, that communoprecipitate with L1 and phosphorylate L1 at Ser1181 and Ser1152, respectively (27, 28). p90rsk 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 p90rsk (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 p90rsk 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 crosslinking 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 L1 was obtained from Upstate Biotechnologies, 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]Pi-Pi, PO4, was purchased from ICN Biomedicals (Irvine, CA). The anti-NCAM antibody was the gift of Dr. Urs Rutishauser (Stanford, New York, NY). The 5G3 anti-human L1 monoclonal antibody was a gift from Dr. R. A. Reisfeld (Scirppis 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 (GSK) was phosphorylated with recombinant ERK2 in TEV-PNP 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 reaction was 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 IVQQFGFQRRSNNGKLTN, which corresponds to a potential autophosphorylation site in the Raf-1 kinase. A tyrosine kinase was then 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 at 150 rpm. The membrane was then washed with TEV-PNP containing 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 with chemiluminescence. The Western blots were scanned onto a Macintosh power Mac 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 × 104 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 recombinant anti-L1 antibody for various periods. After the treatments, the 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 reprobed with other antibodies recognizing both phosphorylated and unphosphorylated forms of ERK (total ERK) to compare loading between lanes and relative ERK activity 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 Glu-4.13-restricted and then digested with trypsin. Phosphorylated 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.
coated with fibronectin (5 μg/cm²; 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 μg/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, Gottingen, Germany) using an argon/krypton laser (excitation lines, 488 and 568 nm) and a 100 × Plan-Neofluor, numerical aperture 1.3, oil objective.

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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 p80 rsk (28), which phosphorylate L1 at Ser1181 and Ser1152, respectively. p80 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 p80 rsk isoforms (41–43). These findings raise the possibility that L1 may associate with other kinases involved in the activation of p90 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 homogenate preparations were probed for Raf, RafB, Raf, MEK-1, ERK, and p90 rsk (Fig. 1A). The results demonstrate that Raf-1, and ERK2, in addition to the previously identified p90 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 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. Immunoprecipitates were blotted unto Immobilon-P, and probed with polyclonal antibodies against Raf-1 (lane 1), ERK2 (lane 2), p90 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 (74–57H 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 (74–57H mAb) conjugated Sepharose beads (lanes 3 and 6), anti-NCAM conjugated Sepharose beads (lanes 2 and 5), or un conjugated 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.

ERK2 Phosphorylates L1CD—Previous work has demonstrated that L1 associates with Ras and Raf, and that phosphorylation of L1 results in ERK activation. The results presented below support the hypothesis that L1CD associates with Ras and Raf, and that phosphorylation of L1CD results in ERK activation.
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 corresponding to phosphorylated peptides eluting at 30–32, 48–54, and 58–64 min on reverse phase HPLC. Two peaks of radioactivity were detected, a minor peak associated with the sequences DETFGEYRSLESDNEEKAFSSQPSLNG 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 RSP phosphorylation by L1 immunoprecipitates resolved by Tris-Tricine SDS-PAGE showing no RSP (lane 1), 10 μg of RSP (lane 2), 2.5 μg of RSP (lane 3), and 5 μg 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 μg RSP, respectively. Molecular mass markers are indicated to the right.

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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 μg of MBP (lane 2), and 4 μg of MBP (lane 3). Lanes 4, 5, and 6 are kinase reactions using rat brain membrane extracts adsorbed to uncoated Sepharose beads with 0, 1, and 4 μg of MBP, respectively. B, autoradiograph of ERK substrate peptide T669 phosphorylation by L1 immunoprecipitates resolved by Tris-Tricine SDS-PAGE showing 1 μg of T669 (lane 1), 0.5 μg of T669 (lane 2), and rat brain membrane Sepharose bead kinase reactions with 1 μg of T669 (lane 3). C, autoradiograph of Raf-1 substrate RSP phosphorylation by L1 immunoprecipitates resolved by Tris-Tricine SDS-PAGE showing no RSP (lane 1), 10 μg of RSP (lane 2), 2.5 μg of RSP (lane 3), and 5 μg 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 μg RSP, respectively. Molecular mass markers are indicated to the right.

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 p90k (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 postnatal 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
ERK activation following addition of rabbit polyclonal anti-human L1

Serum-starved cells were treated with various reagents and as the intact polyclonal. polyclonal anti-human L1 Fab does not activate ERK to the same extent cells were preincubated with either carrier Me2SO (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 the fibroblast growth factor receptor (FGFR) (50), and p60 src is is activated by L1 cross-linking as determined by molecular mass. 20% fetal calf serum (FCS), control showing that incubation with preimmune rabbit antiserum raised against the cytoplasmic domain (pAb anti-L1CDD) 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 Me2SO (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. 4B). 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 predominant 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 communoprecipitates with ERK2 (Fig. 1A).

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 p60src 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. 4D). 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. 5C). 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. 5C), 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. 5H) and the endocytosed anti-L1 antibodies (Fig. 5G). A subset of the vesicles containing endocytosed L1 were also immunoreactive for activated ERK (Fig. 5F). 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. 5F) 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 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. 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-L1CDD) 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 Me2SO (DMSO), erbstatin analog (receptor tyrosine kinase inhibitor), or PP1 (a Src family tyrosine kinase inhibitor). FCS, fetal calf serum.
intermediary in L1-mediated signaling. First, ERK2 coimmunoprecipitates with L1 from brain lysates. Second, in vitro, ERK2 phosphorylates L1 at Ser\textsuperscript{1204} and Ser\textsuperscript{1248}, 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 ERK 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. Ser\textsuperscript{1204} 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 Ser\textsuperscript{1204}, completely eliminates ankyrin binding activity (58). Bennett \textit{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 Ser\textsuperscript{1204} by ERK2 could also regulate L1 association with the cytoskeleton. In a similar vein, Ser\textsuperscript{1152}, which is phosphorylated by p90\textsuperscript{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,
ERK2 and p90\textsuperscript{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 Ser\textsuperscript{1248} 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). Ser\textsuperscript{1248} is located N-terminal to a proline, making it a good ERK2 phosphorylation consensus site. Ser\textsuperscript{1207} 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 Ser\textsuperscript{1207}.

**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\textsuperscript{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\textsuperscript{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\textsuperscript{src} also appears to be involved in some aspects of L1-mediated signaling because neurons from p60\textsuperscript{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\textsuperscript{src} signaling can be coupled. p60\textsuperscript{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\textsuperscript{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\textsuperscript{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
Activation of MAPK by the Neural Cell Adhesion Molecule L1

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. p60src can associate with cell trafficking machinery including dynamin, synapsin-I, and α-adaptin (81). Furthermore, p60src is primarily localized to endosomes (82) from which it can be recruited to plasma membrane sites including focal adhesions (83). In growth cones, p60src is primarily localized in vesicular structures (84, 85). It may be that L1 recruits signal transduction components such as the tyrosine kinase p60src 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 p60src 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 p90rsk1, 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 p90rsk1 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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