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A MAP Kinase-Signaling Pathway Mediates Neurite Outgrowth on L1 and Requires Src-Dependent Endocytosis

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The neural cell adhesion molecule L1 mediates the axon outgrowth, adhesion, and fasciculation necessary for proper development of synaptic connections. Mutations of human L1 cause an X-linked mental retardation syndrome termed CRASH (corpus callosum hypoplasia, retardation, aphasia, spastic paraplegia, and hydrocephalus), and L1 knock-out mice display defects in neuronal process extension resembling the CRASH phenotype. Little is known about the biochemical or cellular mechanism by which L1 performs neuronal functions. Here it is demonstrated that clustering of L1 with antibodies or L1 protein in rodent B35 neuroblastoma and cerebellar neuron cultures induced the phosphorylation/activation of the mitogen-activated protein kinases (MAPKs) and extracellular signal-regulated kinases 1 and 2. MAPK activation was essential for L1-dependent neurite outgrowth, because chemical inhibitors [2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one and 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene] of the MAPK kinase MEK strongly suppressed neurite outgrowth by cerebellar neurons on L1. The nonreceptor tyrosine kinase pp60$^{c\text{-}src}$ was required for L1-triggered MAPK phosphorylation, as shown in *src*-minus cerebellar neurons and by expression of the kinase-inactive mutant Src(K295M) in B35 neuroblastoma cells. Phosphatidylinositol 3-kinase (PI3-kinase) and the small GTPase p21^{rac} were identified as signaling intermediates to MAPK by phosphoinositide and Rac–GTP assays and expression of inhibitory mutants. Antibody-induced endocytosis of L1, visualized by immunofluorescence staining and confocal microscopy of B35 cells, was blocked by expression of kinase-inactive Src(K295M) and dominant-negative dynamin(K44A) but not by inhibitors of MEK or PI3-kinase. Dynamin(K44A) also inhibited L1 antibody-triggered MAPK phosphorylation. This study supports a model in which pp60$^{c\text{-}src}$ regulates dynamin-mediated endocytosis of L1 as an essential step in MAPK-dependent neurite outgrowth on an L1 substrate.

Key words: neurite outgrowth; endocytosis; neural cell adhesion molecule; signal transduction; Src; MAP kinase; PI3-kinase

The cell adhesion molecule L1 functions broadly in regulating the growth of axons in developing neurons and in fostering learning in the adult brain (Persohn and Schachner, 1990; Scholey et al., 1993; Luthi et al., 1994). L1 is localized in the plasmalemma of growth cones and processes of developing neurons, on axons of mature nonmyelinated neurons, and in Schwann cells, astrocytes, and some hematopoietic cells (for review, see Schmid and Maness, 2000). L1 knock-out mice show axon guidance errors in the corticospinal tract (Cohen et al., 1997) and corpus callosum (Demyanenko et al., 1999), misoriented dendrites of cortical pyramidal cells (Demyanenko et al., 1999), degeneration of sensory axons (Haney et al., 1999), and enlarged ventricles (Dahme et al., 1997; Demyanenko et al., 1999). Many of these features are present in humans with the X-linked mental retardation syndrome termed CRASH (corpus callosum hypoplasia, retardation, aphasia, spastic paraplegia, and hydrocephalus), resulting from L1 gene mutations (Fransen et al., 1997).

Little is known about the mechanism by which L1 directs axonal growth or guidance, but the structural features of L1 suggest that it may differ from that used by other classes of receptors to stimulate neurite outgrowth. The L1 extracellular region mediates homophilic (L1–L1) and heterophilic binding via its six immunoglobulin-like and five fibronectin type III domains (Appel et al., 1993; Zhao et al., 1998). Its short cytoplasmic region of 110 amino acids lacks a tyrosine kinase domain but contains an actin-interaction domain (Dahlin-Huppe et al., 1997), an ankyrin-binding region (Davis and Bennett, 1994), and a neuronal-specific sequence, RSLE, which targets L1 to axons (Kamiguchi and Lemmon, 1998). This motif is also involved in binding adaptor protein 2, a clathrin adaptor, which enables L1 to be internalized in growth cones via receptor-mediated endocytosis (Kamiguchi et al., 1998). It is not known whether endocytosis of L1 plays any role in the biological functions of L1 or merely directs L1 to intracellular pathways for degradation.

A requirement for the nonreceptor tyrosine kinase pp60$^{c\text{-}src}$ in L1-mediated neurite outgrowth has been identified in *src*-minus neurons (Ignelzi et al., 1994), but how pp60$^{c\text{-}src}$ regulates neurite growth on L1 is not known. pp60$^{c\text{-}src}$ is tethered by N-terminal myristylation to the cytoplasmic face of the plasma membrane and interacts functionally with a variety of receptor types (Maness et al., 1996). On the basis of the recent finding that pp60$^{c\text{-}src}$ is required for endocytosis and signaling of the β-adrenergic receptor (Ahn et al., 1999), we have investigated the hypothesis that pp60$^{c\text{-}src}$ regulates the internalization of L1 as a critical determinant of L1-mediated neurite outgrowth. Like the β-adrenergic receptor, L1 clustering activates mitogen-activated protein kinases (MAPKs) in neuronal cells (Schmid et al., 1997; Schaefer et al., 1999), but it is not known whether MAPK has a

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role in neurite outgrowth or other physiological functions of L1. L1 triggering also modulates intracellular levels of phosphoinositides, pH, and Ca^{2+} (Schuch et al., 1989; von Bohlen und Halbach et al., 1992) and activates tyrosine phosphatases in growth cones (Atashi et al., 1992; Klinz et al., 1995), suggesting that an array of signaling intermediates may coordinate the complex intracellular program that allows growth cones to navigate through the developing brain and form synaptic connections.

Here we demonstrate that MAPK activation triggered by L1 in neuronal cells is required for neurite outgrowth on L1 and is mediated via $pp60^{c\text{-}src}$ and the small GTPase $p21^{rac}$ and phosphatidylinositol 3-kinase (PI3-kinase). Furthermore, it is shown that $pp60^{c\text{-}src}$ functions by controlling dynamin-mediated endocytosis of L1 as an essential step in MAPK activation. This L1 pathway has the potential to regulate both actin cytoskeletal dynamics in growth cones and gene expression in the nucleus, events that may coordinate to regulate the growth and navigation of neuronal processes.

MATERIALS AND METHODS

Antibodies and plasmids. Antibodies used in this research were mouse monoclonal antibody Neuro4 against the L1 extracellular region (the gift of John Hemperly, Becton Dickinson); monoclonal antibody HA.11 directed against the hemagglutinin (HA)-epitope tag (Babco, Richmond, CA); anti-active MAPK polyclonal antibody (Promega, Madison, WI) specific for dually phosphorylated, activated MAPK; extracellular signal-regulated kinase 1 (ERK1) polyclonal antibody K-23 and Raf-1 and B-Raf antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); and anti-active p38 antibody (New England Biolabs, Beverly, MA). The following cDNAs were subcloned into the vector pcDNA3 for expression in B35 neuroblastoma cells: Fak-related nonkinase (FRNK) and Fak(397F) (Michael Schaller, University of North Carolina, Chapel Hill, NC); Ras(15A), RhoA(19N), Rac1(17N), and Cdc42(17N) (Channing Der, University of North Carolina, Chapel Hill, NC); Src(K295M) (Sara Courtneidge, Sugen); PI3-kinase Δp85 (A. Baldwin and M. Mayo, University of North Carolina, Chapel Hill, NC); dynamin(K44A) (Marc Caron, Duke University, Durham, NC); c-Raf-1(621A and R89L) and B-Raf (621A and R89L) (D. Morrison, National Cancer Institute, Frederick, MD); and HA-tagged ERK2 (Melanie Cobb, University of Texas Southwestern Medical School, Dallas, TX). Other plasmids used encoded enhanced cyan fluorescence protein (Invitrogen, San Diego, CA) and glutathione *S*-transferase (GST) plasmids encoding the c-Jun (1–79)–GST fusion protein (Shelton Earp, University of North Carolina, Chapel Hill, NC) and the Rac-binding domain (RBD)–GST fusion protein (Richard Cerione, Cornell University, Ithaca, NY).

Cell cultures and methods of L1 clustering. To produce a cell line stably expressing human L1 (with RSLE), we transfected rat B35 neuroblastoma cells (Schubert et al., 1974) with human neuronal L1 cDNA in the pcDNA3 vector (gift of John Hemperly, Becton Dickinson). After selection in 0.5 mg/ml G418, 17 clones were assayed for L1 expression by Western blotting, and L1 localization on the cell surface was verified by indirect immunofluorescence staining with Neuro4 antibodies. One clone (designated B35-L1) exhibiting intermediate levels of L1 expression was chosen for the studies reported here. B35-L1 cells were maintained in DMEM containing 10% fetal bovine serum (FBS; HyClone, Logan, UT) and G418 (0.25 mg/ml). Primary cultures of mouse cerebellar neurons were prepared from postnatal day 8 Sv129/C6B57 hybrid mice by the method of Schnitzer and Schachner (1981) and grown in DMEM with 10% FBS (HyClone), 25 mM KCl, and penicillin/streptomycin. The medium was replaced with OptiMEM (Life Technologies, Gaithersburg, MD), and cells were incubated for 8 hr before initiating signaling. For clustering of L1 on B35-L1 cells and cerebellar neurons, L1 monoclonal antibody Neuro4 (30 μg) or L1–Fc fusion proteins (50 μg) were preincubated with $F(ab')_2$ fragments of secondary antibodies raised against Fc fragments of mouse or human IgG for 1 hr at 4°C in 50 μl of OptiMEM. The complexes were added directly to cells in a 60 mm dish containing 1 ml of medium at 37°C. Clustering of L1 into patches on the cell surface was observed by indirect immunofluorescence staining (data not shown).

MAPK phosphorylation assays. Cells were stimulated with the indicated L1 antibodies or fusion proteins, rinsed once with cold HBSS, and extracted in Nonidet P-40 (NP-40) lysis buffer containing 1% NP-40, 0.25% (w/v) Na-deoxycholate, 50 mM HEPES, pH 7.4, 137 mM NaCl, 1 mM Na-EDTA, 10 mM NaF, 1 mM Na-orthovanadate, 10 mM *p*-nitrophenylphosphate, 1 mM phenylmethylsulfonyl fluoride, 10 mM β-glycerophosphate, 10 μg/ml leupeptin, 0.1 TIU/ml aprotinin, 1 μg/ml pepstatin, 2 nM calyculin A, and 10% (v/v) glycerol. Lysates were clarified by centrifugation at 14,000 × *g* for 20 min at 4°C. Protein concentration was determined using the bicinchoninic acid assay (Pierce, Rockford, IL). Proteins in cell extracts were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked for 2 hr in 1% bovine serum albumin (BSA) in Tris-buffered saline containing 0.05% Tween 20 (TTBS) and then incubated overnight at 4°C either with anti-active MAPK antibodies or p38 MAPK antibodies in 1% BSA in TTBS. After washing in TTBS, the membrane was incubated with goat anti-rabbit IgG horseradish peroxidase conjugate in TTBS for 1 hr. The membrane was again washed in TTBS, and immune complexes were detected using enhanced chemiluminescence (ECL; NEN) with Sterling x-ray film (BioWorld). Membranes were stripped and reprobed in the same manner with ERK antibodies to detect total ERK protein.

For assaying phosphorylation of HA-tagged ERK2, B35-L1 cells were transfected for transient expression with an HA-tagged ERK2 plasmid with or without plasmids expressing dominant-negative mutants. Subconfluent cultures were transfected in 60 mm dishes containing OptiMEM using Lipofectamine according to the manufacturer's instructions (Life Technologies). Signaling experiments were initiated 36–40 hr after transfection. Cells were lysed in NP-40 lysis buffer, and HA-tagged ERK2 was immunoprecipitated from cell extracts (500 μg) with anti-HA antibody (Babco) for 1.5 hr, followed by Protein G-Sepharose for 0.5 hr at 4°C. Immune complexes were washed in lysis buffer and subjected to SDS-PAGE, followed by immunoblotting with anti-active MAPK antibody. For all assays, the exposed bands on x-ray film were quantitated by densitometric scanning and analysis with Image-Quant NT software. MEK inhibitors [2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one (PD98059; 50 μM; New England Biolabs) and 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126; 10 μM; Promega)] or a PI3-kinase inhibitor [Ly294002 (10–20 μg; Promega)] dissolved in dimethylsulfoxide (DMSO; <0.01%) was added to cells for 1 hr before signal initiation. The Rho inhibitor C3 botulinum endotoxin (20 μg) was added with Lipofectamine (5 μg) to cells for 4 hr before stimulation. Control cultures received the same concentration of DMSO or Lipofectamine.

For assaying the activity of c-Jun N-terminal kinase (JNK), cell extracts (100 μg of protein) in NP-40 lysis buffer were incubated with c-Jun (1–79)–GST fusion protein for 4 hr at 4°C. Immune complexes were washed twice with lysis buffer and twice with kinase buffer (25 mM HEPES, pH 7.4, 25 mM $MgCl_2$, 25 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na-orthovanadate, and 2 mM *p*-nitrophenylphosphate) and then incubated in kinase buffer with 50 μM ATP and 0.5 μCi of [γ^{32}P]ATP (ICN) at 30°C for 30 min. Proteins were separated on SDS-PAGE. The dried gel was exposed to x-ray film, and exposed bands were quantitated by densitometric scanning and analysis with Image-Quant NT software.

Assay of phosphoinositides. PI3-kinase activity was assayed by immunoprecipitating phosphotyrosine-modified PI3-kinase from cell extracts with phosphotyrosine antibodies and measuring the production of phosphoinositides from phosphatidylinositol in an immune complex kinase assay (Myers et al., 1993). Cell extracts (800 μg) were incubated with phosphotyrosine antibody 4G10 for 8 hr at 4°C and then for 1 hr with Protein G-Sepharose. Immunoprecipitates were collected and washed twice with buffers I, II, and III (buffer I, 20 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, and 100 μM Na-orthovanadate; buffer II, 100 mM Tris, pH 7.4, 500 mM LiCl, and 100 μM Na-orthovanadate; and buffer III, 10 mM Tris, pH 7.4, 100 mM NaCl, and 100 μM Na-orthovanadate). Phosphatidylinositol (0.5 mg/ml; Avanti Polarlipids) was sonicated in 20 mM Tris, pH 7.4, and 1 mM Na-EGTA and incubated with the immune complexes for 10 min at room temperature in kinase reaction buffer (total volume of 50 μl) containing 20 μM ATP, 1 μCi of [γ^{32}P]ATP (3000 Ci/mmol), 10 mM $MgCl_2$, 20 mM Tris, pH 7.4, and 100 mM NaCl. The reaction was terminating by adding 6N HCl (15 μl) and 1:1 (v/v) chloroform:methanol (120 μl). Samples were vortexed for 30 sec and centrifuged at 10,000 × *g* for 1 min. Aliquots (30 μl) of the chloroform phase were spotted on SilicaG60 plates (Whatman, Maidstone, UK) and subjected to thin-layer chromatography in chloroform:methanol:25% (w/w) NH_4OH:water (60:47:2:11.3, v/v/v/v). Commercially available phosphatidylinositol 4-phosphate (PIP; Sigma, St. Louis, MO) was used to indicate the location of the product. The plate was dried and exposed to

x-ray film. Phospholipids were visualized by incubating the thin-layer plate with iodine vapors, and radioactive spots were scraped from the plate and quantitated for ^{32}P incorporation in a scintillation counter.

Assays for GTP-bound p21^{rac} and Raf kinase activity. For measurement of activated, GTP-bound Rac (Bagrodia et al., 1998), B35-L1 cells were stimulated with L1 antibodies or normal IgG as described; then lysates (500 μg) were incubated for 30 min at 4°C with 20 μg of a purified GST fusion protein (RBD–GST) consisting of the Rac-binding domain of PAK1 together with glutathione Sepharose. RBD–GST binds only to the activated form of Rac (Rac–GTP) but not to inactive Rac–GDP. RBD–GST/Rac–GTP complexes were collected by centrifugation and analyzed by SDS-PAGE and Western blotting with anti-Rac antibodies (Transduction Laboratories, Lexington, KY).

To assay Raf activation, we subjected lysates in NP-40 lysis buffer (800 μg) to immunoprecipitation using polyclonal antibodies specific for c-Raf or B-Raf (Santa Cruz Biotechnology) and Protein G-Sepharose. In a coupled assay measuring myelin basic protein (MBP) phosphorylation (Upstate Biotechnology, Lake Placid, NY), Raf immune complexes were preincubated with inactive MEK and MAPK for 30 min at 37°C and then with MBP and [$\gamma^{32}P$]ATP for 10 min at 37°C. MBP was adsorbed to Whatman P81 paper and quantitated for incorporation of radioactivity by scintillation counting. An activated form of Raf provided by the manufacturer was used as a positive control. The amounts of Raf immunoprecipitated were determined in parallel by immunoblotting with Raf antibodies.

Measurement of neurite outgrowth. Cultures of mouse cerebellar neurons consisting chiefly of granule cells were prepared from postnatal day 7 or 8 pups by the method of Schnitzer and Schachner (1981). Immunoaffinity-purified L1 protein from adult mice brain was applied in 10 μl spots onto nitrocellulose-coated plastic coverslips (22 × 22 mm^2) in 35 mm Petri dishes as described (Ignelzi et al., 1994). After blocking with 1% bovine serum albumin, 2 × 10^6 cerebellar cells were added per coverslip in 2 ml of basal medium with Eagle's salts (Life Technologies) with 10% heat-inactivated horse serum, 2.5 gm/l glucose, and penicillin/streptomycin. No neurons attached to uncoated areas of the nitrocellulose. The MEK inhibitors PD98059 and U0126 were dissolved in DMSO and added at the time of plating at 50 or 10 μM final concentration, respectively, resulting in a final concentration of 0.1% DMSO. Control cultures received the same concentration of DMSO. Cells were fixed after 24 hr with 4% *p*-formaldehyde and then mounted on microscope slides. The length of the longest neurite per cell was measured using a microscope-mounted image processor with cursor overlay. Only neurites longer than ~10 μm and not in contact with other cells were measured. Neuron attachment to L1 was assayed by counting at least 100 cells from 30 or more randomly selected fields. Data were obtained from three independent experiments.

Immunofluorescence staining for endocytosis of L1. For experiments in which cell surface L1 and endocytosed L1 were differentially labeled in living cells, a modification of the method of Kamiguchi et al. (1998) was used. Rat B35-L1 neuroblastoma cells (20,000 cells/well) were plated in LabTek II chamber slides (Nunc, Naperville, IL) coated with poly-D-lysine. After 24 hr, cells were transfected using Lipofectamine with a plasmid encoding enhanced cyan fluorescence protein (0.15 μg of cDNA) together with a plasmid expressing one of the following (0.25 μg of cDNA): wild-type c-Src, kinase-inactive Src(K295M), dominant-negative dynamin(K44A), or dominant-negative PI3-kinase (Δp85). At 24 hr after the start of transfection, cells were incubated for 45 min at 37°C with 25 μg/ml Neuro4 IgG or normal mouse IgG to induce endocytosis. Cells were washed with cold DMEM and then incubated with goat anti-mouse IgG conjugated to rhodamine for 30 min at 4°C to label L1 on the cell surface. The cells were rinsed with HBSS and fixed in 4% *p*-formaldehyde for 30 min. After washing cells once with 0.1 mM glycine in PBS and twice with PBS, cells were blocked with goat anti-mouse IgG for 1 hr at 4°C. After rinsing once in cold PBS, the cells were again fixed in 4% *p*-formaldehyde for 10 min at room temperature. Cells were washed once with 0.1 mM glycine in PBS and twice with PBS, and the membrane was permeabilized with 0.1% Triton X-100 in PBS containing 10% goat serum for 1 hr at room temperature. Cells were washed once with PBS and incubated with anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) for 1 hr to label cytoplasmic L1. After additional washes, slides were mounted with Vectashield. Cells were viewed in a Zeiss LSM10 confocal laser microscope equipped with an argon laser (excitation lines of 488 and 514 nm) resulting in an optical thickness of 0.5 μm at the University of North Carolina Microscopy Services Facility, Department of Pathology (Chapel Hill, NC; Dr. Bob Bagnell, Director).

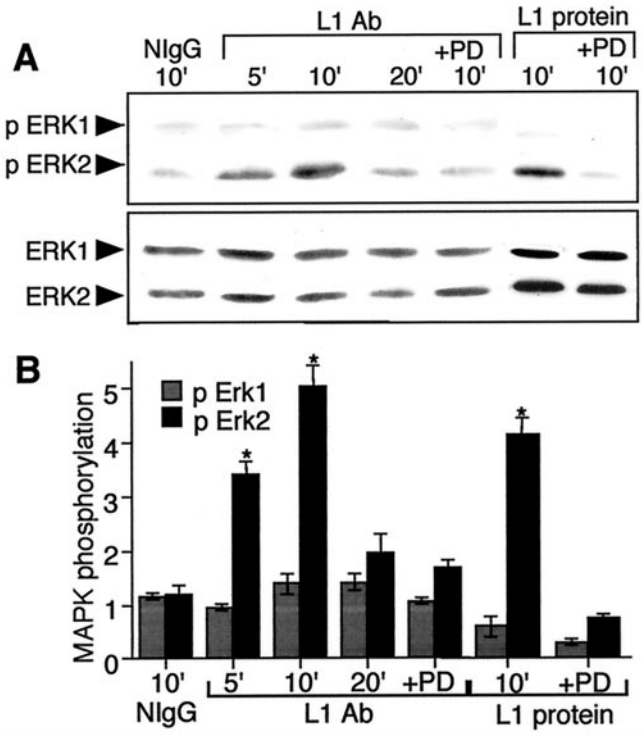

Figure 1. Clustering of L1 in cerebellar neurons activates ERK2. *A, Top,* Cerebellar cultures were incubated with nonimmune mouse IgG (*NIgG*), monoclonal antibodies against L1 (Neuro4) complexed with F(ab′)$_2$ fragments of anti-mouse IgG (*L1 Ab*), or L1–Fc fusion protein complexed with F(ab′)$_2$ fragments of anti-human IgG *(L1 protein*). Where indicated (*+PD*), cells were treated with the MEK inhibitor PD98059 (*PD*; 25 μM). Cell extracts were subjected to SDS-PAGE and immunoblotting with anti-active MAPK antibodies specific for phosphorylated ERKs (*pERK1* and *pERK2*). *Bottom*, The same nitrocellulose filter was stripped and reblotted with antibodies recognizing phosphorylated and nonphosphorylated ERK proteins (ERK1 and ERK2). This experiment was repeated twice with similar results. *B*, Densitometric quantification of the ERK phosphorylation in *A* is shown in arbitrary units of phosphorylation relative to that of the nonimmune IgG control. Error bars indicate SEs based on three experiments; an *asterisk* denotes statistical significance ($p < 0.05$).

RESULTS

L1 clustering activates the MAPK pathway in cerebellar neurons

L1 signal transduction was investigated in primary cultures of mouse cerebellar neurons, which express endogenous L1 and are comprised of >90% granule cells (Schnitzer and Schachner, 1981). Signaling by cell adhesion molecules of the Ig superfamily, such as neural cell adhesion molecule (NCAM), integrins, and certain receptor tyrosine kinases, is initiated by the clustering of receptors with multivalent ligands or antibodies (Heldin, 1995). L1 molecules were clustered on the surface of cerebellar neurons with antibodies against the L1 extracellular domain; then cell lysates were analyzed by immunoblotting with phospho-specific MAPK antibodies recognizing a dually phosphorylated/activated form of the extracellular-regulated kinases ERK1 and ERK2. These MAP kinases are activated by dual phosphorylation of Thr 202 and Tyr 204 (Crews et al., 1992; Marshall, 1994). L1 clustering in cerebellar neurons stimulated ERK2 dual phosphorylation fivefold, increasing rapidly within 5–10 min and then declining to nearly basal levels by 20 min (Fig. 1). The decline in phosphory-

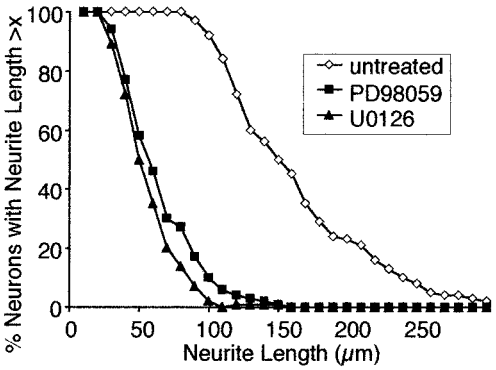

Figure 2. Effect of MEK inhibitors on L1-dependent neurite outgrowth by cerebellar neurons. Mouse cerebellar neurons were plated on purified L1 protein adsorbed to nitrocellulose-coated coverslips and allowed to extend neurites for 24 hr without or with MEK inhibitors (50 μM PD98059 or 10 μM U0126). Cells were fixed, neurite lengths were measured, and results were plotted in a neurite length distribution curve. Two hundred or more cells were analyzed for each condition (see Table 1 for additional data). This experiment was repeated twice with similar results.

lation could be caused by activation of a dual specificity tyrosine/threonine phosphatase such as MAPK phosphatase (MKP-1) (Fuller et al., 1997) or L1-triggered phosphatase activities present in a subcellular fraction enriched in growth cone membranes (Klinz et al., 1995). L1 clustering did not induce phosphorylation of ERK1 in the cerebellar neuron cultures even though similar levels of ERK1 and ERK2 were indicated by immunoblotting with antibodies against ERK1 and ERK2 protein (Fig. 1*A*). Nonimmune IgG complexes had no effect on ERK phosphorylation. Multivalent L1 protein, consisting of the L1 extracellular region fused to the Fc portion of human IgG, also stimulated the phosphorylation of ERK2 approximately four- to fivefold in cerebellar neurons with kinetics similar to that induced by L1 antibody complexes (Fig. 1). ERK2 phosphorylation in cerebellar neurons induced by cross-linked L1 antibodies or L1–Fc protein was effectively inhibited by the inhibitor PD98059, which is specific for MEK (MAP kinase kinase) (Fig. 1). PD98059 binds the inactive form of MEK1 and to a lesser extent MEK2, preventing the MEK activation required for MAPK phosphorylation (Alessi et al., 1995).

An intact MAPK pathway is required for neurite outgrowth on L1

To determine whether the MAPK pathway participated in L1-directed neurite outgrowth, primary cultures of mouse cerebellar neurons (postnatal day 7–8) were analyzed for neurite outgrowth on purified mouse L1 protein adsorbed to nitrocellulose-coated dishes as described previously (Ignelzi et al., 1994). L1-stimulated neurite outgrowth was inhibited by the MEK inhibitors PD98059 and U0126 (Fig. 2, Table 1). Mean neurite length decreased 56% in the presence of PD98059 and 66% with U0126 (Fig. 2). U0126 was slightly more effective than PD98059, probably because U0126 inhibits active and inactive forms of MEK (Favata et al., 1998). The residual neurite outgrowth observed in the presence of MEK inhibitors suggested that there may be both MAPK-dependent and -independent pathways for neurite growth on L1. Neither PD98059 nor U0126 affected cerebellar neuron attachment to purified L1 protein (Table 1; Cells/mm^2), the ability of cerebellar neurons to initiate neurite growth (Table 1; Cells with neurites/mm^2; % cells with neurites), or the morphology of the neurons. Cells did not adhere to or extend neurites on areas of the nitrocellulose-coated dishes without L1 protein. Effects on fasciculation could not be evaluated in these sparsely plated cultures. These results reveal a functional role for the MEK–MAPK pathway in L1-dependent neurite outgrowth by cerebellar neurons.

L1 activates ERK1 and ERK2 in B35-L1 neuroblastoma cells

The rat neuroblastoma cell line B35 (Schubert et al., 1974) is a useful CNS model for investigating signaling pathways of neural cell adhesion molecules (Schmid et al., 1999). The B35 cell line displays differentiated neuronal properties such as membrane excitability and expression of enzymes for neurotransmitter metabolism (Schubert et al., 1974) and is more easily transfected than primary neurons. B35 cells express little or no L1 as detected by immunoblotting and immunofluorescence staining with L1 antibodies. To define the L1-signaling pathway for MAPK activation further, a B35 cell line was generated that stably expressed the RSLE-containing neuronal isoform of L1 (B35-L1 cells).

L1 molecules were clustered on the surface of B35-L1 cells with L1 antibody complexes; then cell lysates were analyzed for dual phosphorylation of endogenous MAPKs by immunoblotting with phospho-specific MAPK antibodies. Phosphorylation of both ERK1 and ERK2 increased four- to fivefold within 10 min of L1 clustering, whereas nonimmune IgG had no effect (Fig. 3*A,B*). Phosphorylation was rapid and transient with similar kinetics for ERK1 and ERK2. Clustering of L1 on the cell surface was necessary for MAPK activation, because monovalent Fab fragments of L1 antibodies did not induce ERK phosphorylation (data not shown). The ability of L1 to stimulate phosphorylation of ERK1 and ERK2 in B35-L1 cells, in contrast to the phosphorylation of only ERK1 in cerebellar neurons, suggested that these pathways were differentially regulated. Similar results were obtained during NCAM stimulation in cerebellar neurons and B35 cells expressing NCAM140 (Schmid et al., 1999). Selective activation of ERK2 and not ERK1 in neuronal cells also occurs during stimulation of protein kinase C or NMDA receptors (English and Sweatt, 1996). Differential MAPK regulation was characterized previously in yeast in which mating and pseudohyphal differentiation are independently controlled by the MAPKs Fus3 and Kss1 (Madhani et al., 1997).

To address the specificity of L1 activation of the MAPK-signaling pathway, B35-L1 cells were assayed for L1-dependent phosphorylation of the MAP kinases, JNK, and p38, which are activated in response to multiple cellular stressors (Waskiewicz and Cooper, 1995). No increase of JNK phosphorylation was observed within 5–20 min of L1 clustering, whereas sorbitol-treated cells as a positive control showed prominent JNK activity (Fig. 3*C*). Similarly, the phosphorylation of p38 MAPK was not increased over basal levels compared with anisomycin-treated cells as positive controls (Fig. 3*D*).

L1 activates the MAPK pathway via PI3-kinase and Rac

To investigate whether L1 clustering activated PI3-kinase in L1 antibody-triggered B35-L1 cells, the production of phosphoinositides by PI3-kinase was quantitatively determined. During activation, PI3-kinase becomes phosphorylated on tyrosine and can be immunoprecipitated with phosphotyrosine antibodies. Incubation of immune complexes from L1 antibody-stimulated B35-L1 cells with phosphatidylinositol and [γ^{32}P]ATP produced radiolabeled phosphatidylinositol 3-phosphate, which was sepa-

Table 1. Inhibition of neurite outgrowth on L1 by MEK inhibitors

	Mean neurite length (μm ± SEM)	*n*	Cells/mm^2	Cells with neurites/mm^2	Cells with neurites (%)
Untreated	152 ± 12*	253	38	34	90
PD98059 (50 μM)	67 ± 4*	264	36	33	91
U0126 (10 μM)	52 ± 1*	200	35	32	90

n represents the number of cells measured for mean neurite length. Mean neurite lengths were obtained in three independent experiments. More than 200 cells were scored for neurite length in each condition.

*Statistically significant differences in means of treated and untreated samples using a *t* test ($p < 0.05$).

rated from reactants by thin-layer chromatography (Fig. 4*A*). The amount of activated PI3-kinase increased two- to threefold within 3–8 min after antibody triggering. A similar extent of increase in PI3-kinase activity occurs in cells treated with epidermal growth factor (EGF), platelet-derived growth factor, or phorbol esters and is effective in mediating their physiological functions (Conricode, 1995; Nave et al., 1996; Cross et al., 1997). To determine whether PI3-kinase was involved in regulating L1-triggered ERK phosphorylation, a dominant-negative PI3-kinase mutant (Δp85) was cotransfected with HA–ERK2 for transient expression in B35-L1 cells. This dominant inhibitory mutant contains a deletion in the PI3-kinase regulatory subunit (p85) that abolishes its binding to the catalytic subunit (p110) (Hara et al., 1994). Stimulation of B35-L1 cells expressing this mutant with L1 antibodies resulted in complete inhibition of ERK2 phosphorylation (Fig. 4*B*). Treatment of B35-L1 cells with 10 μM Ly294002, a selective chemical inhibitor of PI3-kinase (Vlahos et al., 1994), prevented L1 antibody-induced phosphorylation of endogenous ERK1 and ERK2 (Fig. 4*D*).

Rac and Cdc42 are members of the Rho family of small GTPases that regulate actin cytoskeletal dynamics (Ridley et al., 1992), signal to JNK (Vojtek and Cooper, 1995), and cause cross-cascade activation of the ERK pathway (Frost et al., 1997). PI3-kinase contributes to the activation of Rac and Cdc42 by providing phosphoinositides that bind the plextrin homology domain of specific guanine nucleotide exchange factors (GEFs) (Quilliam et al., 1995). To investigate the role of small GTPases in L1-stimulated MAPK activation, B35-L1 cells were cotransfected with HA-tagged ERK2 and dominant-negative Rac1, Ccd42, and RhoA plasmids, which act by sequestering respective GEFs. ERK2 phosphorylation in antibody-triggered B35-L1 cells was effectively blocked by the expression of dominant-negative Rac1(17N) but not RhoA(19N) or Cdc42(17N) mutants (Fig. 4*B*). C3 botulinum toxin, which inhibits RhoA, B, and C but not Rac or Cdc42 (Braun et al., 1989; Chrzanowska-Wodnicka and Burridge, 1996), also had no effect on L1-dependent ERK phosphorylation (Fig. 4*D*). The participation of Rac in L1 signaling was confirmed in an assay that directly measured the production of activated, GDP-bound Rac (Bagrodia et al., 1998). Cell lysates from L1 antibody-treated B35-L1 cells were incubated with purified RBD–GST fusion protein, which selectively binds Rac–GTP and not Rac–GDP. The amount of Rac–GTP pulled down by the RBD–GST complexes was significantly increased after L1 stimulation of B35-L1 cells (Fig. 4*C*). These results supported a role for Rac in L1 signaling to MAPK.

L1-triggered MAPK activation in B35-L1 neuroblastoma cells differed from NCAM140 signaling to MAPK in its independence from Ras and the focal adhesion kinase p125^{fak}. Cotransfection of B35-L1 cells with HA–ERK2 and a dominant-negative Ras(15A) plasmid, which sequesters Ras–GEFs (Quilliam et al., 1994), had no effect on L1 antibody-stimulated ERK phosphorylation, whereas it strongly inhibited MAPK activation by EGF (Fig. 4*B*). Expression of a Ras dominant-negative mutant (Ras17N) or a competitive N-terminal Raf peptide that binds Ras (Brtva et al., 1995) also had no effect (data not shown). Under the same conditions, Ras(15A) and Ras(17N) interfered strongly with NCAM140-dependent MAPK activation in B35 cells (Schmid et al., 1999). In a coupled immune complex kinase assay for Raf-induced phosphorylation of MBP (Fucini et al., 1999), neither c-Raf nor B-Raf became activated to a significant degree (>10% over the normal IgG control) during L1 antibody stimulation of B35-L1 cells. Dominant-negative Raf-1 or B-Raf mutants (S621A or R89L) also produced insignificant inhibition of ERK2 phosphorylation (>1.2-fold).

Similarly, expression of FRNK (Schaller et al., 1993; Richardson and Parsons, 1996) or the autophosphorylation site mutant Fak(Y397F) (Schaller et al., 1994), which are dominant interfering inhibitors of p125^{fak}, did not affect L1-triggered HA–ERK2 activation (Fig. 4*B*), whereas under the same conditions they inhibited NCAM140-triggered MAPK activation in B35 cells (Schmid et al., 1999). Tyrosine phosphorylation of p125^{fak} was also not increased after L1 antibody triggering, as assessed by Western blotting with phosphotyrosine antibodies in Fak immunoprecipitates (data not shown).

pp60$^{c\text{-}src}$ mediates ERK phosphorylation induced by L1

Because neuronal process extension on purified L1 is regulated by the nonreceptor tyrosine kinase pp60$^{c\text{-}src}$ (Ignelzi et al., 1994), we investigated whether pp60$^{c\text{-}src}$ played a role in MAPK activation triggered by L1. B35-L1 neuroblastoma cells were cotransfected with HA–ERK2 and the dominant-negative c-Src(K295M) plasmid, which expresses a kinase-inactive form of pp60$^{c\text{-}src}$ because of mutation of an essential lysine residue (K295) in the ATP binding site (Twamley-Stein et al., 1993). Expression of the dominant-negative *src* plasmid inhibited ERK2 phosphorylation by ~70% in B35-L1 neuroblastoma cells treated with L1 antibodies (Fig. 5*A*). Moreover, endogenous ERK2 phosphorylation in cerebellar neurons from *src*-minus mice was 60% lower than that in wild-type neurons (Fig. 5*B*). The residual ERK2 phosphorylation in *src*-minus neurons and B35-L1 cells expressing dominant-negative Src suggested that there may an additional L1 pathway that is pp60$^{c\text{-}src}$ independent, in accord with the incomplete inhibition of L1-dependent neurite outgrowth in MEK-inhibited cells and by *src*-minus neurons (Ignelzi et al., 1994). L1 did not form a stable complex with pp60$^{c\text{-}src}$, because L1 and pp60$^{c\text{-}src}$ did not coimmunoprecipitate from Brij97-detergent extracts of B35-L1 cells with or without L1 clustering (data not shown) under the same conditions in which NCAM140 coimmunoprecipitated with the Src family kinase p59^{fyn} (Beggs et al., 1997).

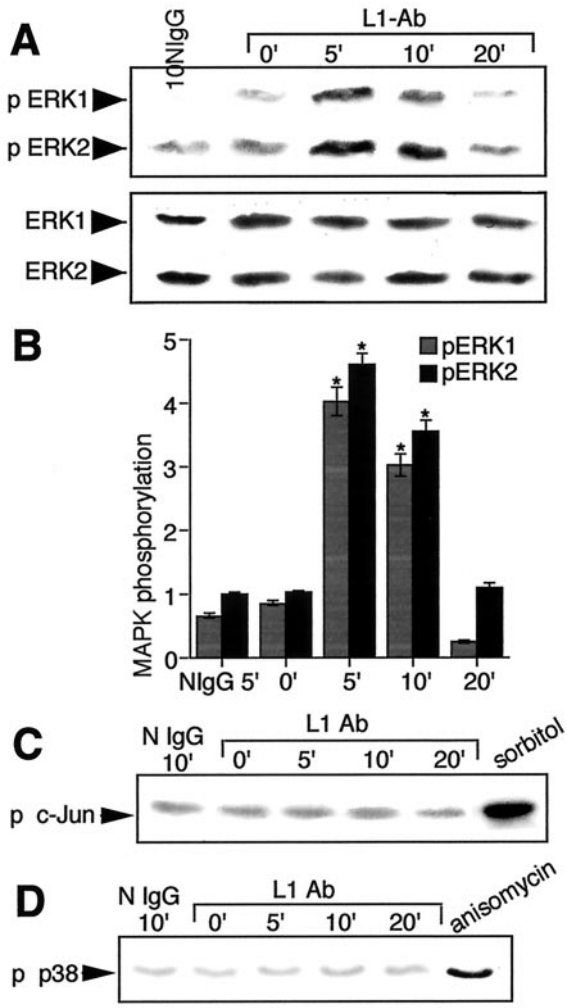

Figure 3. Clustering of L1 in B35 neuroblastoma cells transiently activates MAPK but not JNK or p38. *A, Top,* Rat B35 neuroblastoma cells stably expressing L1 (B35-L1) were incubated with either nonimmune mouse IgG or L1 mAb Neuro4 complexed with $F(ab')_2$ fragments of anti-mouse IgG for various times (5–20 min). Cell extracts were subjected to SDS-PAGE and immunoblotting with anti-active MAPK antibodies (*pERK1* and *pERK2*). *Bottom*, The same nitrocellulose filter was stripped and reblotted with an ERK antibody recognizing ERK1 and ERK2. This experiment was repeated three times with similar kinetics and extent of MAPK activation. *B,* Densitometric quantification of MAPK phosphorylation in the experiment shown in *A* is expressed in arbitrary units of ERK1 and ERK2 phosphorylation relative to that of the nonimmune IgG control. Error bars indicate SEs based on three experiments; an *asterisk* denotes statistical significance ($p < 0.05$). *C,* The same cell extracts shown in *A* were assayed for JNK activation by the JNK immune complex kinase assay. Autoradiography showed no increase in the phosphorylation of c-Jun(1–79) by JNK from L1-stimulated cells (*p c-Jun*). As a positive control, cells were incubated with 300 mM sorbitol for 15 min. This experiment was performed three times with similar results. *D,* The same cell extracts shown in *A* were subjected to SDS-PAGE and immunoblotting with anti-phospho p38 MAPK antibodies, which recognize phosphorylated/activated p38 (*p p38*). No increase in phosphorylation levels was seen. As a positive control, cells were incubated with 10 mM anisomycin for 10 min. This experiment was performed twice with similar results.

pp60$^{c\text{-}src}$ is required for dynamin-mediated endocytosis of L1

L1 has been shown to be endocytosed at the growth cone by a clathrin-mediated mechanism (Kamiguchi et al., 1998). To determine whether L1 endocytosis was mediated by pp60$^{c\text{-}src}$, B35-L1 cells were analyzed for L1 endocytosis by double immunofluorescence staining to visualize internalized and surface L1 with different fluorochromes. Cells were transfected with plasmids encoding wild-type Src, kinase-inactive Src(K295M), or dominant-negative dynamin(K44A) together with a plasmid expressing cyan fluorescent protein to identify transfected cells. To induce internalization of L1, we incubated living cells for 30 min at 37°C with the monoclonal antibody Neuro4, which is directed against an extracellular epitope of L1. Cells were fixed and stained for L1 on the cell surface with rhodamine-conjugated secondary antibodies. Cells were then permeabilized and stained for internalized L1 with FITC-conjugated secondary antibodies. Confocal microscopy revealed strong FITC labeling of internalized L1 antibody complexes in a punctuate, vesicular pattern throughout the cytosol both in cyan-positive cells (Fig. 6, *arrows, arrowheads*) expressing wild-type Src from a transfected plasmid (Fig. 6*A,E*) and in nontransfected cells expressing endogenous Src (Fig. 6*D,H*). This observation suggested that the amount of endogenous pp60$^{c\text{-}src}$ was not rate-limiting for endocytosis of L1 antibody complexes in B35-L1 cells. In striking contrast, cyan-positive cells (Fig. 6, *arrows, arrowheads*) in cultures transfected with dominant-negative Src(K95M) showed little L1 endocytosis (Fig. 6*B,F,C,G*), whereas cyan-negative cells serving as nontransfected controls within the same cultures exhibited prominent vesicular staining in the cytosol. Rhodamine staining for L1 on the cell surface appeared relatively unaltered by endocytosis, suggesting that the amount of internalized L1 was a relatively small portion of the total L1 expressed in B35-L1 cells. L1 endocytosis was not inhibited in B35-L1 cells treated with the MEK inhibitor PD98059 (50 μM) (Fig. 6*J,N*), in cells expressing PI3-kinase Δp85 (Fig. 6*K,O*), or in cells treated with the PI3-kinase inhibitor Ly294002 (20 μM), suggesting that PI3-kinase and MEK-MAPK did not contribute to the mechanism of endocytosis.

Dynamin GTPase plays a critical role in the formation of clathrin-coated vesicles during endocytosis of many receptors (Hinshaw and Schmid, 1995), as shown by the ability of dominant-negative dynamin(K44A) to block effectively the formation of endocytotic-coated vesicles (Damke et al., 1994). Dynamin-(K44A) also prevented L1 internalization in L1-B35 cells (Fig. 6*M*), confirming its role in internalizing L1 by a clathrin-mediated mechanism (Kamiguchi et al., 1998) in B35 cells. Although Rac has been shown to modulate receptor-mediated endocytosis in some systems (Lamaze et al., 1996), expression of dominant-negative Rac1(17N) in B35 cells did not affect L1 antibody-induced internalization (data not shown), suggesting that this small GTPase did not regulate L1 endocytosis. Importantly, the dynamin(K44A) mutant effectively inhibited L1 antibody-induced phosphorylation of HA–ERK2 in transfected B35-L1 cells (Fig. 5*A*), thus indicating that an intact endocytotic mechanism was necessary for L1 triggering of the MAPK cascade.

Taken together these results demonstrated that the dynamin-mediated internalization of L1 in B35-L1 cells depended on pp60$^{c\text{-}src}$ and that L1 endocytosis was required for activation of an MAPK cascade that regulated neurite outgrowth on L1.

DISCUSSION

Here we report that an MAPK-signaling pathway mediates neurite outgrowth on L1 and requires pp60$^{c\text{-}src}$-dependent endocytosis. This is the first demonstration of a neuronal function regulated by L1 via MAPK and of the role of pp60$^{c\text{-}src}$ in regulating

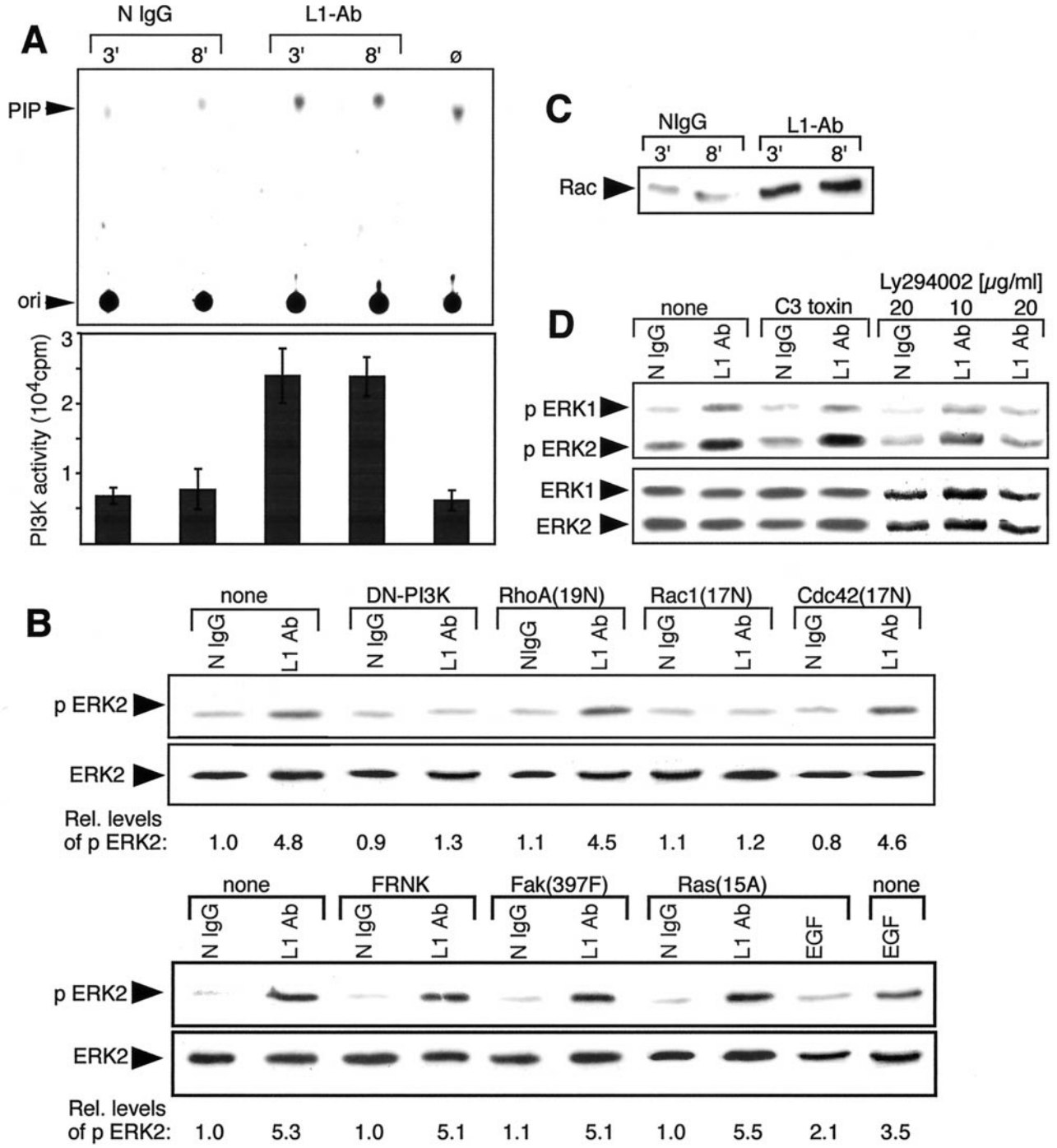

Figure 4. Dominant-negative mutants of PI3-kinase and Rac1 inhibit MAPK activation induced by L1 clustering in B35 neuroblastoma cells. *A,* B35-L1 cells were incubated with mouse IgG or the L1 monoclonal antibody Neuro4 complexed with $F(ab')_2$ fragments of anti-mouse IgG. Cell lysates (800 μg) were immunoprecipitated with anti-phosphotyrosine mAb (4G10), and the precipitates were incubated in an *in vitro* kinase reaction with phosphoinositides. Phospholipid products were separated by thin-layer chromatography (*top*). Error bars in the graph (*bottom*) indicate SEs based on three experiments; an *asterisk* denotes statistical significance ($p < 0.05$). *B, Top row*, B35-L1 cells transiently expressing HA–ERK2 and the dominant-negative (*DN*) mutants PI3-kinase (*PI3K*) Δp85, RhoA(19N), Rac1(17N), Cdc42(17N), FRNK, Fak(397F), or Ras(15A) were incubated with mouse IgG or the L1 monoclonal antibody Neuro4 complexed with $F(ab')_2$ fragments of anti-mouse IgG. As a control, cells were exposed to 100 ng/ml EGF for 5 min. Cell extracts were immunoprecipitated with anti-HA antibodies and then subjected to SDS-PAGE and immunoblotting with anti-active MAPK antibody (*p ERK2*). *Bottom row*, The same nitrocellulose filters were stripped and reblotted with an antibody recognizing ERK2 protein. The experiments were performed three times with similar results. *C,* B35-L1 cells were incubated with normal mouse IgG or the L1 monoclonal antibody Neuro4 complexed with $F(ab')_2$ fragments of anti-mouse IgG for 3 and 8 min. GTP-loaded Rac was pulled down from the cell lysates (500 μg) by the addition of RBD–GST fusion protein conjugated to Sepharose beads, and the relative levels of Rac–GTP were evaluated by SDS-PAGE and immunoblotting with anti-Rac1 antibodies. *D, Top*, B35-L1 cells were exposed to the C3 toxin Rho inhibitor or the Ly294002 PI3-kinase inhibitor as described in Materials and Methods and then incubated with normal mouse IgG or the L1 monoclonal antibody Neuro4 complexed with $F(ab')_2$ fragments of anti-mouse IgG for 7 min. Cell extracts were subjected to SDS-PAGE and immunoblotting with anti-active MAPK antibody (*p ERK2*). *Bottom*, The same nitrocellulose filters were stripped and reblotted with an antibody recognizing ERK2 protein. *ori*, Origin; *Rel*, relative.

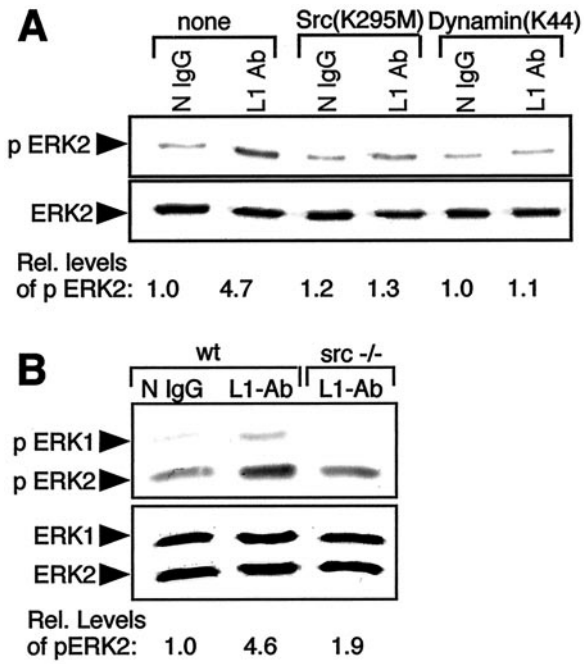

Figure 5. Dominant-negative mutants of pp60$^{c\text{-}src}$ and dynamin inhibit MAPK activation induced by L1 clustering in B35 neuroblastoma cells. *A, Top*, B35-L1 cells transiently expressing HA–ERK2 and the dominant-negative mutants c-Src(K295M) or dynamin (K44A) were incubated for 7 min with mouse IgG or the L1 monoclonal antibody Neuro4 complexed with F(ab′)$_2$ fragments of anti-mouse IgG. Cell extracts in NP-40 lysis buffer were immunoprecipitated with anti-HA antibodies and then subjected to SDS-PAGE and immunoblotting with anti-active MAPK antibody (*p ERK2*). *Bottom*, The same nitrocellulose filters were stripped and reblotted with an ERK antibody recognizing ERK2 protein. The experiments were performed twice with similar results. *B, Top,* Cerebellar neurons from wild-type mice (*wt*) or *src*-minus mice *(src−/−)* were incubated with mouse IgG or the L1 monoclonal antibody Neuro4 complexed with F(ab′)$_2$ fragments of anti-mouse IgG. Buffers of the cell extracts were subjected to SDS-PAGE and immunoblotting with anti-active MAPK Ab (*p ERK2*). *Bottom*, The same nitrocellulose filters were stripped and reblotted with an ERK antibody recognizing ERK2. The experiments were performed twice with similar results.

dynamin-mediated endocytosis of L1 as an essential initial step in MAPK activation. Key intermediates on the L1–MAPK-signaling pathway were identified to be the small GTPase Rac and PI3-kinase. Because ERKs activate gene expression via phosphorylation of the transcription factors Elk-1 (Marais et al., 1993) and CRE-binding protein (Ginty et al., 1994), these results raise the possibility that neurite outgrowth may rely on signaling events in the growth cone and nucleus. The proposed signaling pathway is outlined in Figure 7.

Results of this study demonstrate the requirement of an intact MEK–ERK pathway in regulating neurite outgrowth by cerebellar neurons on an L1 substrate. ERK phosphorylation is widely used for neurite outgrowth in response to pleiotropic axon growth/guidance cues, which include L1m NCAM (Schmid et al., 1999), the extracellular matrix (Ihara et al., 1997), and neurotrophins (Xia et al., 1995; Riccio et al., 1997). L1 signaling to MAPK may also function in synaptic plasticity as suggested from antibody perturbation experiments (Luthi et al., 1994), because ERKs are phosphorylated in neurons during β-adrenergic and serotonergic stimulation (Koch et al., 1994; Michael et al., 1998) and in hippocampal long-term potentiation (LTP) (English and Sweatt, 1997). The extent of ERK phosphorylation triggered by L1 (four- to fivefold) approximated that induced by NCAM (Schmid et al., 1999), NGF (Riccio et al., 1997), integrins (Chen et al., 1994), and LTP (English and Sweatt, 1997), underscoring its potential physiological relevance. By transducing signals that converge within the MAPK pathway, adhesion molecules, growth factors, and neurotransmitters may coordinately regulate the growth or branching of axons and dendrites. While our work was in progress MAPK was shown to be activated in L1-transfected NIH3T3 cells dependent on endocytosis (Schaefer et al., 1999). Our studies in neural cells are in accord with this report but go further by demonstrating a physiological role for MAPK in L1-dependent neurite outgrowth and by identifying pp60$^{c\text{-}src}$, Rac, and PI3-kinase as key signaling intermediates in the pathway.

An important finding of our work is the requirement for pp60$^{c\text{-}src}$ in dynamin-dependent endocytosis of L1 and activation of MAPKs in neural cells. These results provide a mechanistic understanding of the inhibition of L1-dependent neurite outgrowth observed in *src*-minus neuronal cultures (Ignelzi et al., 1994). Our studies suggest (1) that pp60$^{c\text{-}src}$ acts as a gatekeeper of the MAPK cascade by regulating L1 endocytosis and (2) that endocytosis and MAPK activation are needed for neurite growth on L1. Dynamin-mediated endocytosis is also an early step in MAPK activation induced by EGF (Vieira et al., 1996), serotonin 5-HT1A (Della Rocca et al., 1999), insulin-like growth factor I (Chow et al., 1998), lysophosphatidic acid (Luttrell et al., 1997), and β_2-adrenergic agonists (Daaka et al., 1998). Although only L1 and the β_2-adrenergic receptor have been shown to depend on pp60$^{c\text{-}src}$ for endocytosis and MAPK activation (Ahn et al., 1999), it is conceivable that Src family kinases may participate in endocytosis/signaling by other receptors. It remains to be determined whether L1 clustering activates pp60$^{c\text{-}src}$ by dephosphorylating Tyr 527 in the Src C-terminal domain (Bjorge et al., 1996) possibly via tyrosine phosphatase PTP-α (Ponniah et al., 1999). A role for tyrosine phosphatases in L1 signaling is consistent with the ability of L1 antibodies to induce dephosphorylation of tyrosine in growth cone proteins (Atashi et al., 1992; Klinz et al., 1995). Because neurite outgrowth by cerebellar neurons on L1 was not totally suppressed by MEK inhibitors or in *src*-minus neurons (Ignelzi et al., 1994), there may be an additional Src/MAPK-independent mechanism for L1-dependent neurite growth. For example, neurite growth responses in common to L1, NCAM, and N-cadherin have been shown to occur via the basic fibroblast growth factor receptor, phospholipase C, and production of arachidonic acid (Saffell et al., 1997). It should be noted that because different cell types (cerebellar neurons and B35 neuroblastoma cells) were used to measure neurite outgrowth and endocytosis, the results do not formally prove that endocytosis of L1 is required for the regulation of neurite outgrowth. Other than pp60$^{c\text{-}src}$ it is not clear whether components of the L1–MAPK cascade identified in B35 cells are also deployed for signal transduction and neurite outgrowth in cerebellar neurons and other neuronal cell types. Nonetheless, the finding that pp60$^{c\text{-}src}$ was needed for ERK activation in both cell types and that deletion of the *src* gene causes inhibition of neurite outgrowth in cerebellar neurons (Ignelzi et al., 1994) supports the possibility that Src-dependent endocytosis mediates neurite outgrowth on L1.

L1 signaling, as delineated in B35 cells, differed from the prototypical Ras–MAPK cascade used by NCAM and integrins (Schlaepfer and Hunter, 1997; Schmid et al., 1999) in its independence from Ras and Fak. However, Ras-independent (Howe and Juliano, 1998) and FAK-independent signaling pathways are

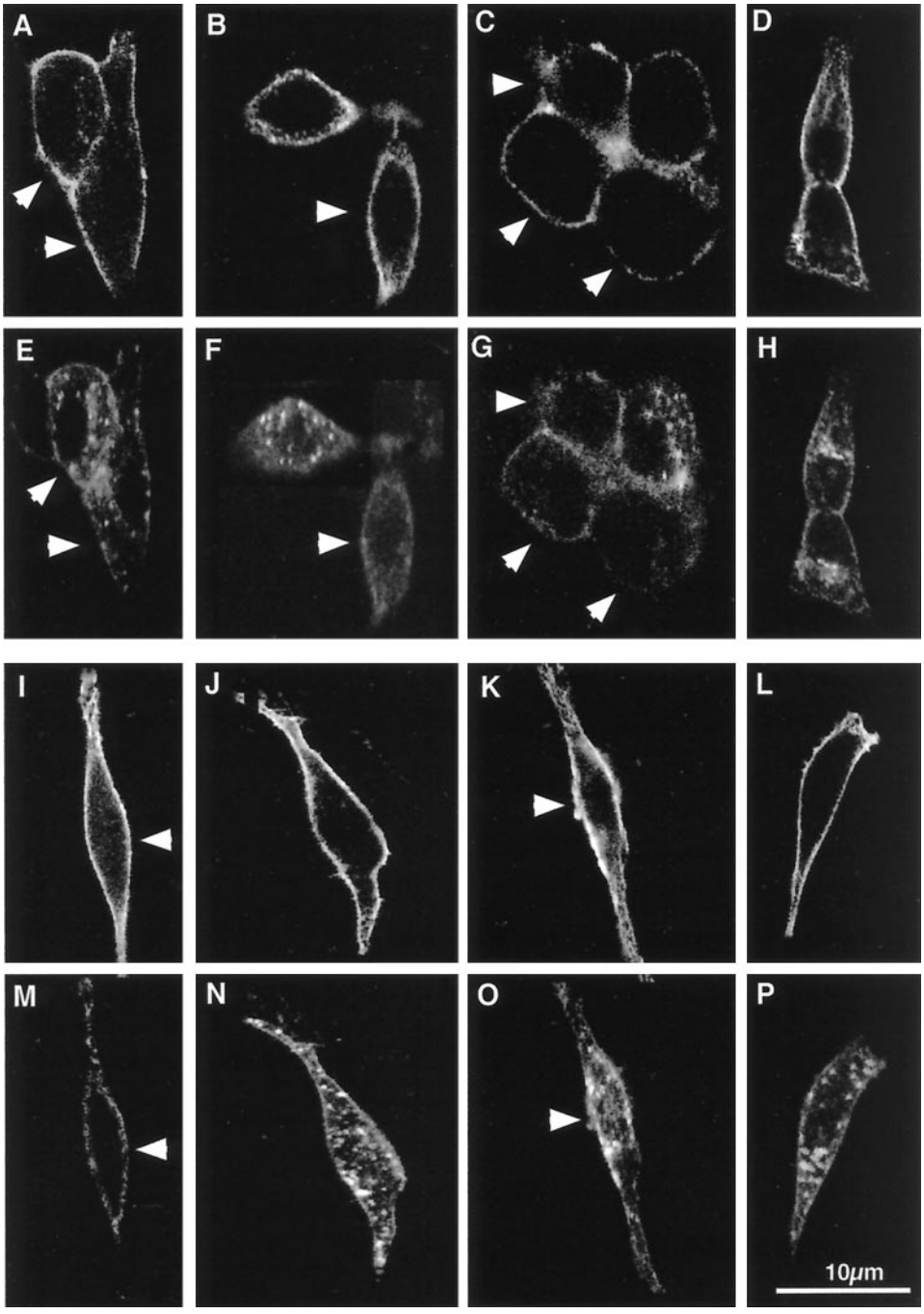

Figure 6. Endocytosis of L1 antibody complexes requires pp60$^{c\text{-}src}$ and dynamin. Endocytosis of L1 antibody complexes induced with Neuro4 monoclonal antibodies against an extracellular epitope of L1 was visualized in transfected B35-L1 neuroblastoma cells by double immunofluorescence staining for L1 on the cell surface (rhodamine) and internalized L1 (FITC) using confocal microscopy as described in Materials and Methods. B35-L1 cells were cotransfected with one of the pcDNA3 plasmids listed below together with equimolar amounts of a plasmid expressing enhanced cyan fluorescent protein (ECFP) as a marker for transfected cells. Arrows and *arrowheads* denote ECFP-positive cells. *Images* show representative cells from all experiments. *A*, *E,* Wild-type c-Src. *B*, *F, C*, *G,* Dominant-negative Src(K295M). *D*, *H,* No transfection. *I*, *M,* Dominant-negative dynamin-(K44A). *J*, *N,* Treatment with MEK inhibitor PD98059 (50 μM). *K*, *O,* Dominant-negative PI3-kinase (Δp85). *L*, *P,* Treatment with PI3-kinase inhibitor Ly294002 (20 μM). *A—D* and *I–L* show L1 on the cell surface (rhodamine); *E–H* and *M–P* show internalized L1 (FITC). Scale bar, 10 μm.

used for some forms of integrin signaling (Lin et al., 1997; Miranti et al., 1998). FAK-independent signaling in platelets shares with the L1 pathway the involvement of pp60$^{c\text{-}src}$, Rac, and PI3-kinase (Miranti et al., 1998). Although we have not found β1-integrins stably associated with L1, a role for specific β-integrin subtypes in L1–MAPK signaling remains open, because L1 has been shown to interact functionally with integrins for neurite outgrowth and cell migration on L1, mediated in part by an RGD sequence in the sixth Ig domain (Montgomery et al., 1996; Yip et al., 1998).

Neuronal growth cones display distinctive morphologies on L1, NCAM, and laminin (Payne et al., 1992; Abosch and Lagenaur, 1993; Burden-Gulley et al., 1995, 1997); yet each substrate can activate MAPKs, raising the question of how specificity arises. Activation of different signaling intermediates on the MAPK pathway may allow neurons to respond to extracellular cues in distinct ways. In B35 cells, L1 signals to MAPK via pp60$^{c\text{-}src}$, PI3-kinase, and Rac, whereas NCAM140 signals to MAPK via Fyn, FAK, Ras, and Rho (Schmid et al., 1999). Nonetheless, the involvement of Rac in L1 signaling is consistent with the lamellipodial morphology displayed by growth cones of retinal ganglion cells on L1 (Payne et al., 1992; Burden-Gulley et al., 1995). Activated Rho family GTPases differentially modulate actin cytoarchitecture (Nobes and Hall, 1995). Rac generates lamellipodia by inducing actin depolymerization via LIM kinase-1 (Arber et al., 1998; Yang et al., 1998), Rho induces actin stress fibers and growth cone collapse (Kozma et al., 1997), and Cdc42 elicits filopodia via LIM kinase-2 (Sumi et al., 1999). PI3-kinase may

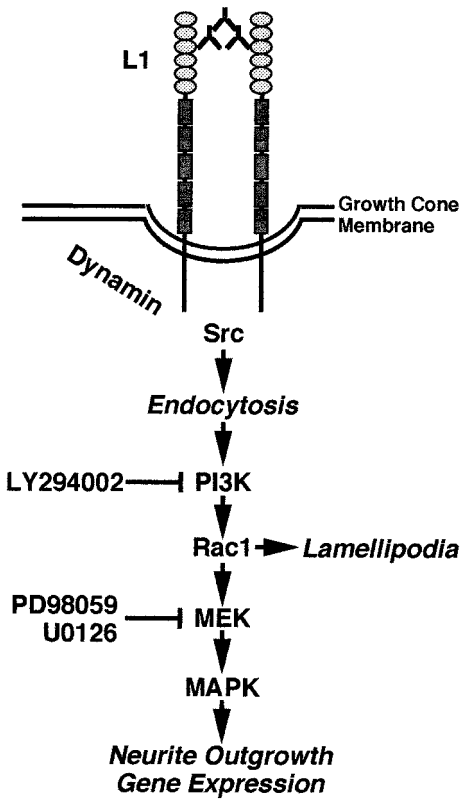

Figure 7. Proposed L1-signaling pathway. Cross-linking of L1 molecules on the growth cone membrane is proposed to induce dynamin-mediated endocytosis of L1 via the Src tyrosine kinase, leading to initiation of an intracellular signal transduction cascade involving the sequential activation of PI3-kinase, Rac, MEK, and MAPK. Inhibitors of PI3-kinase (Ly294002) and MEK (PD98059 and U0126) are shown. It is suggested that Rac activation in the growth cone leads to cytoskeletal changes resulting in lamellipodia and that MAPK may have nuclear effects on gene expression, both of which may be needed for neurite outgrowth.

promote Rac activation via the binding of phosphoinositide products to the plextrin homology domain of Rac–GEFs (Han et al., 1998; Nimnual et al., 1998). PI3-kinase is an important determinant of growth cone guidance, required for the turning of *Xenopus* growth cones toward a netrin gradient (Hong et al., 1999).

The ability of L1 to phosphorylate ERKs via Rac could occur by cross-cascade stimulation of MEK. Rac typically activates the JNK pathway (Crespo et al., 1996) but is an upstream regulator of p21-activated kinase, which directly phosphorylates MEK and stimulates ERK phosphorylation in a Ras-independent manner (Frost et al., 1997). Such cross-talk allows pathways for Rac activation to cooperate with prototypical Ras–Raf–MEK–MAPK pathways (Frost et al., 1997). In this way, NCAM or neurotrophin signaling via Ras may cooperate with L1 signaling via Rac for MAPK activation and neurite outgrowth. Although we found no evidence of c-Raf 1 or B-Raf in L1 signaling, basal levels of a Raf isoform might participate.

The ability of L1 clustering to activate PI3-kinase also supports a role for L1 in neuronal survival. PI3-kinase promotes neuronal survival and blocks apoptosis in response to neurotrophins and insulin-like growth factor I via the serine/threonine kinase Akt (Ashcroft et al., 1999). Although Akt was not examined in our studies, a role for L1 in neuronal survival is consistent with a 30% reduction in the number of hippocampal neurons in L1 knock-out mice (Demyanenko et al., 1999) and with the ability of L1 to enhance the survival of dopaminergic neurons (Hulley et al., 1998) and to prevent apoptotic death of cerebellar and hippocampal neurons in culture (Chen et al., 1999).

Src-mediated endocytosis of L1 has the potential for not only local actin cytoskeletal rearrangements in the growth cone but also nuclear transcriptional control. Via Rac and PI3-kinase, L1 could cause actin rearrangements to form lamellipodial extensions needed for neurite elongation. Endocytosis of L1 might also serve to release the growth cone from attachment to the substratum, facilitating its forward migration. L1 in endocytic vesicles would be oriented with its C terminal projecting toward the cytoplasm, enabling downstream signaling molecules in the growth cone to be recruited and/or activated, a scenario consistent with the observation that activated ERK colocalizes with L1 in endocytosed vesicles of L1-transfected fibroblasts (Schaefer et al., 1999). L1-signaling complexes might also be transported from the growth cone to the cell body, as shown for internalized NGF–Trk receptor complexes in pheochromocytoma 12 cells (Riccio et al., 1997). There dually phosphorylated ERKs would be in position to translocate into the nucleus, where they may induce transcription of the genes needed for neuronal process extension or cell survival.

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