MAP Kinase Pathway–dependent Phosphorylation of the L1-CAM Ankyrin Binding Site Regulates Neuronal Growth

John D. Whittard, Takeshi Sakurai, Melanie R. Cassella, Mihaela Gazdciu, and Dan P. Felsenfeld

Department of Pharmacology and Biological Chemistry, Mt. Sinai School of Medicine, New York, NY 10029

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The growth of neuronal processes depends critically on the function of adhesion proteins that link extracellular ligands to the cytoskeleton. The neuronal adhesion protein L1-CAM serves as a receptor for nerve growth–promoting proteins, a process that is inhibited by the interaction between L1-CAM and the cytoskeleton adaptor ankyrin. Using a novel reporter based on intramolecular bioluminescence resonance energy transfer, we have determined that the MAP kinase pathway regulates the phosphorylation of the FIGQY motif in the adhesion protein L1-CAM and its interaction with ankyrin B. MAP kinase pathway inhibitors block L1-CAM–mediated neuronal growth. However, this blockade is partially rescued by inhibitors of L1-CAM–ankyrin binding. These results demonstrate that the MAP kinase pathway regulates L1-CAM–mediated nerve growth by modulating ankyrin binding, suggesting that nerve growth can be regulated at the level of individual receptors.

INTRODUCTION

Tyrosine phosphorylation plays an essential role in the regulation of adhesion-receptor function. Phosphorylation of adhesion receptors regulates not only protein structure but also receptor interactions with cytosolic binding partners, including signaling and structural proteins. L1-CAM, an adhesion protein originally identified in the nervous system, has been implicated in neural development, lymphocyte adhesion, and tumor-cell metastasis (Pancook *et al.*, 1997; Cohen *et al.*, 1998; Voura *et al.*, 2001; Gutwein *et al.*, 2005). The phosphorylation of the L1-CAM at conserved tyrosine residues in the cytoplasmic domain regulates L1-CAM interactions with the cytoskeleton. As these interactions directly modulate L1-CAM function in nerve growth and adhesion, identifying the kinase pathways that control L1-CAM phosphorylation is central to our understanding of the receptor's function.

L1-CAM, a member of the neuronal immunoglobulin superfamily, is essential in the growth and guidance of neurons in the developing vertebrate CNS (Hortsch, 2000). Mutations in the gene encoding L1-CAM in humans lead to a complex of developmental defects, including corpus callosum hypoplasia, mental retardation, and spastic paraplegia (Fransen *et al.*, 1995). Mice deficient in L1-CAM display specific guidance defects of descending cortico-spinal tract neurons where they cross the midline (Cohen *et al.*, 1998; Castellani *et al.*, 2000), again consistent with a role for L1-CAM in the guided growth of developing central neurons. L1-CAM binds to components of the cytoskeleton, including members of the ankyrin family of adaptor proteins (Davis and Bennett, 1994), members of the ezrin-radixin-moesin

(ERM) family (Dickson *et al.*, 2002) and components of the AP-2 clathrin complex (Kamiguchi *et al.*, 1998). L1-CAM interactions with ERM proteins regulate axon branching on L1-CAM substrates (Dickson *et al.*, 2002; Cheng *et al.*, 2005), whereas binding to AP-2 is necessary for L1-CAM endocytosis and some aspects of L1-CAM–mediated signaling (Schaefer *et al.*, 2002). In contrast, the binding of ankyrin to the L1-CAM cytoplasmic tail appears to regulate both adhesion and axon growth. Ankyrin has been suggested to play an essential role in L1-CAM–mediated growth cone initiation at the cell body (Nishimura *et al.*, 2003). However, ankyrin binding in the growing neurite plays an inhibitory role; reagents that block L1-CAM–ankyrin interactions increase the L1-CAM–dependent growth of neurons in culture (Gil *et al.*, 2003). Additionally, neurons expressing a truncated form of L1 that lacks the ankyrin binding site produce longer axons than neurons expressing full-length receptor, again supporting an inhibitory role for ankyrin binding in L1-mediated nerve growth (Cheng *et al.*, 2005). Finally, the binding of ankyrin G to the L1-family member neurofascin promotes neurofascin-mediated cell adhesion (Tuvia *et al.*, 1997). The anti-coordinate regulation of L1-mediated adhesion and nerve growth by ankyrin raises the possibility that ankyrin binding plays a critical role in regulation of L1-CAM function during development.

L1-CAM–ankyrin interactions are regulated by tyrosine phosphorylation at the conserved ankyrin binding site in the L1-cytoplasmic tail (comprised of the amino acid sequence FIGQY); tyrosine to histidine substitutions at this site inhibit L1-mediated recruitment of ankyrin to the cell membrane (Zhang *et al.*, 1998; Needham *et al.*, 2001; Gil *et al.*, 2003). Similarly, the activation of receptor-tyrosine kinases by their ligands drives indirectly the phosphorylation of the FIGQY tyrosine in vertebrate L1-family members and inhibits L1-CAM–ankyrin interactions, suggesting that phosphorylation plays a central role in the regulation of ankyrin binding to L1-CAM (Garver *et al.*, 1997; Gil *et al.*, 2003). In light of the inhibition of L1-CAM–mediated neuronal growth by ankyrin

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Address correspondence to: Dan P. Felsenfeld (dan.felsenfeld@mssm.edu).

binding, identifying the signaling pathways that regulate L1-CAM FIGQY phosphorylation and ankyrin binding may provide crucial insight into the function of L1-CAM in neuronal growth.

MATERIALS AND METHODS

Reagents

Rabbit anti-phosphotyrosine polyclonal and mouse anti-Src (clone GD11) monoclonal antibodies were obtained from Upstate Cell Signalling (Charlottesville, VA). Rabbit anti-ERK1 and MEK2 polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-GFP polyclonal and mouse anti-MEK1 monoclonal antibodies were obtained from Invitrogen (Carlsbad, CA). Rabbit anti-L1 polyclonal antibody was a gift from Carl Lagenaur (University of Pittsburgh, Pittsburgh, PA). Mouse anti-myc monoclonal antibody (mAb) was obtained from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Horseradish peroxidase (HRP)-conjugated sheep anti-mouse and donkey anti-rabbit antibodies were obtained from Amersham Biosciences (Piscataway, NJ). Donkey anti-mouse antibody conjugated to indocarbocyanine Cy3 and donkey anti-rabbit antibody conjugated to indocarbocyanine Cy5 were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Human embryonic kidney (HEK)-293 and rat pheochromocytoma (PC)12 cells were obtained from American Type Culture Collection (Manassas, VA). BAPTA-AM, genistein, 5-iodotubercidin, ionomycin, LY294002, mastoparan, PD98059, protein kinase A (PKA), and protein kinase C (PKC) peptides, PP1, PP2, SB202190, and U0126, were obtained from BioMol Research Laboratories (Plymouth Meeting, PA). Ac-Y-EEIE, epidermal growth factor (EGF), erbstatin analog, NGF, and PAO were obtained from Sigma-Aldrich (St. Louis, MO). The codon-humanized pRluc and GFP2 vectors were obtained from PerkinElmer Life Sciences (Boston, MA). The small interference RNAs (siRNAs) for Abl, ERK1/2, MAPK14, MEK1, MEK2, and Src were obtained from Dharmacon (SMARTpool siGENOME; Chicago, IL).

Neurite Outgrowth Assays

Neurite outgrowth experiments were performed as described (Gil *et al.*, 2003) with slight modification. A 1-cm-diameter circle in a 35-mm Petri dish (Becton Dickinson, Franklin Lakes, NJ) was coated with poly-L-lysine (5 μ g/ml in phosphate-buffered saline [PBS]); Chemicon, Temecula, CA) for 1 h at room temperature. After several washes with PBS, the coated area was dried under the hood. Aliquots of 1 μ l of Ng-CAM (50 μ g/ml; Gil *et al.*, 2003) or laminin (30 μ g/ml, Becton Dickinson) were spotted on the coated area. Dishes were incubated for 1 h at room temperature, washed several times with PBS, and then blocked with 1% (wt/vol) bovine serum albumin. Cerebellar cells were prepared from P2-P4 mouse and plated on the prepared dishes in BME/B27/glucose/glutamine/Pen-Strep at a cell density of 3×10^5 cells/ml. Peptides and U0126 were diluted in dimethyl sulfoxide (10 mg/ml for peptides, 13 mM for U0126) and further diluted in media (final concentration 10 μ g/ml peptide; 10 μ M U0126) added to the cultures when cells were plated. Cultures were incubated for 2 d and fixed with 4% paraformaldehyde in 0.12 M phosphate buffer. Images were collected through CCD camera connected to a Zeiss Axiovert 100 inverted microscope (Thornwood, NY) and analyzed with NIH image.

Bioluminescence Resonance Energy Transfer (BRET) Construct Design

Bioluminescence resonance energy transfer (BRET) constructs were designed using vectors encoding *Renilla* luciferase and GFP2 (Sapphire GFP; Biosignal, PerkinElmer Life Sciences). Coding regions from each individual vector were copied by PCR with additional restriction sites, permitting their ligation into a single, concatenated coding region (GFP2:Rluc) between NotI and XhoI sites in a pcDNA3.1 Hygro (+) eukaryotic expression vector (Invitrogen). This chimeric construct (CHIM) encodes unique BsrGI and AclI sites in the intervening sequence. To create the reporter constructs from the CHIM construct, complementary oligonucleotides derived from the L1-CAM coding region were synthesized (Sigma Genosys) with the addition of a 5' overhang designed to generate a sticky end complementary to the BsrGI and AclI sites. The addition of the reporter insert resulted in the deletion of two amino acids (SG) at the interface between GFP2 and Rluc found in the CHIM construct. Before ligation into the CHIM construct, oligonucleotide pairs were mixed in equimolar concentrations, heated to 94°C (4 min), and allowed to cool slowly to room temperature, permitting the annealing of the complementary regions.

Calculations of Fluorescence Resonance Energy Transfer Efficiency

The relationship between Förster resonance energy transfer (FRET) efficiency (E) and donor-acceptor separation (r) is described by the equation $E = R_0^6 / (R_0^6 + r^6)$, where R_0 is the Förster distance at which transfer efficiency is

50% (Lakowicz, 1999). Changes in r resulting from a 24% change in E were calculated using $\Delta r/R_0 = [(1/0.76E) - 1]^{1/6} - [(1/E) - 1]^{1/6}$.

BRET

Near-confluent cultures of HEK-293 cells were harvested with trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA; Invitrogen) and resuspended to a density of 2.5×10^5 cells/ml. Aliquots (200 μ l) of cell suspensions were added to white 96-well culture plates (CulturPlate; PerkinElmer Life Sciences) and incubated for 12 h at 37°C. HEK-293 cells were transfected with either 0.1 μ g of DNA/well or 100 nM of siRNA/well using lipofectamine reagents (Lipofectamine Plus and Lipofectamine; Invitrogen) according to the manufacturer's instructions. After incubation of plates for either 48 h (DNA) or 72 h (siRNA) at 37°C the cells were washed once with warm DMEM without phenol red (Invitrogen), supplemented with 25 mM HEPES (Invitrogen). Transfected HEK-293 cells were treated with EGF for 15 min and inhibitors for 1 h (PD98059 and U0126) or 4 h (genistein). To each well, 10 μ l of DeepBlueC substrate (final concentration of 5 μ M; PerkinElmer Life Sciences) diluted in Dulbecco's PBS containing 0.1% (wt/vol) CaCl_2 , 0.1% (wt/vol) D-glucose, 0.1% (wt/vol) MgCl_2 , and 10 μ g/ml aprotinin was added. The plates were immediately counted using the Fusion Universal Microplate Analyzer (PerkinElmer Life Sciences). Bioluminescence resulting from Rluc emission was counted at 410 nm using a 370–450-nm band pass filter, and the energy transferred to GFP2 was counted at 515 nm using a 500–530-nm bandpass filter. The efficiency of energy transfer between Rluc and GFP2 is determined by dividing acceptor emission intensity (GFP2) by donor emission intensity (Rluc). The resulting values reflect the proximity of GFP2 to Rluc and are referred to as the BRET ratio. Results from BRET assays were normalized against values obtained from untreated cells transfected with the L1-BRET construct.

Western Blots and Immunoprecipitation

Near-confluent cultures of HEK-293 cells, stably transfected with either L1-FIGQY or CHIM constructs, or ND7 cells were harvested with trypsin-EDTA and resuspended to a density of 6×10^5 cells/ml. Aliquots (5 ml) of cell suspensions were added to 100-mm cell culture dishes (Corning Life Sciences, Corning, NY) and incubated for 12 h at 37°C. Stably transfected HEK-293 cells were treated with genistein for 4 h at 37°C and ND7 cells for 1 h with 100 μ M PD98059 and 15 min with 100 ng/ml NGF. Plates were washed with 5 ml of ice-cold PBS, and then cells were lysed with modified RIPA buffer (1% (wt/wt) IGEPAL CA-630, 1% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) SDS, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2, 2 mM EDTA, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 10 mM benzamide, 10 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 1 μ g/ml pepstatin at 4°C for 20 min and centrifuged at $15,000 \times g$ for 15 min at 4°C. The protein concentrations of the supernatants were determined by using the BCA protein assay (Pierce Chemical, Rockford, IL). The cell lysates were precleared with immobilized protein A (Pierce Chemical) for 3 h at 4°C. Immunoprecipitates were carried out with a rabbit anti-GFP or a rabbit anti-L1 polyclonal antibody and immobilized protein A beads overnight at 4°C. Alternatively, lysates were immunoprecipitated using agarose beads directly conjugated with rabbit anti-GFP (Vector Laboratories, Burlingame, CA). Beads were washed and resuspended in Laemmli buffer, analyzed by SDS-PAGE, and transferred to nitrocellulose membrane. The membrane was blocked, washed, and then incubated with 1 μ g/ml anti-phosphotyrosine antibody overnight at 4°C. The blot was then incubated with HRP-conjugated goat anti-rabbit antibody at a dilution of 1:5000 and then developed using the enhanced chemiluminescence system (SuperSignal West Pico chemiluminescent substrate; Pierce Chemical). Membranes were stripped using 0.2 M glycine-HCl (pH 2.5) and reprobed with 0.5 μ g/ml anti-GFP antibody for 2 h at room temperature or 2 μ g/ml anti-L1 antibody overnight at 4°C. Densitometry of immunoblot films was carried out using a transilluminated flat-bed scanner (Umax Powerlook 1100; Dallas, TX), calibrated using a series of neutral density filters scanned under identical conditions and analyzed using NIH Image J (National Institutes of Health, Bethesda, MD). Measurements were normalized to loading controls for each lane.

Immunofluorescence

HEK-293 cells were transfected with cDNA encoding an amino-terminal myc-epitope-tagged full-length wild-type L1-CAM and a carboxy-terminal GFP-tagged ankyrin B constructs using lipofectamine reagents. Transiently transfected HEK-293 cells were treated for 1 h with 100 μ M PD98059 and 100 ng/ml with EGF. For immunolocalization, cells were fixed for 10 min using 1% (wt/vol) paraformaldehyde in 60 mM Pipes, 25 mM HEPES, 10 mM EGTA, and 2 mM MgCl_2 . Staining was performed as described previously (Felsenfeld *et al.*, 1999). Briefly, ankyrin B was detected by indirect immunofluorescence using a rabbit anti-GFP polyclonal antibody and a donkey anti-rabbit antibody conjugated to indocarbocyanine Cy5. L1-CAM was detected by indirect immunofluorescence using a mouse anti-myc mAb and a donkey anti-mouse antibody conjugated to indocarbocyanine Cy3. Confocal micrographs were collected on an Olympus microscope (Melville, NY) using a 60 $\times$ objective at a plane intersecting cell–cell junctions.

Figure 1. Activation of the EGF-receptor modulates the BRET ratio of the L1-BRET construct. (A) Schematic diagram of the chimeric BRET constructs. The insert reporter domain is comprised of the indicated amino acid sequence for each construct. (B) Application of 100 ng/ml EGF significantly reduces the BRET ratio of the L1-BRET construct transiently transfected into HEK-293 cells (* $p < 0.01$). Subsequent results were normalized against values obtained from untreated cells transfected with the L1-BRET construct. (C and D) EGF reduces the BRET ratio of the L1-BRET construct in a dose- and time-dependent manner, respectively (* $p < 0.01$). The reduction in BRET ratio was maximal after 10 min and saturated at 10–20 ng/ml EGF. (E) Mutation of the FIGQY tyrosine to an aspartate, histidine, or phenylalanine residue abolishes the decrease in BRET ratio after stimulation of HEK-293 cells with 100 ng/ml EGF (* $p < 0.01$). In B–E, results shown are mean $\pm$ SD, $n = 5$. (F) Schematic diagram illustrating the inverse relationship between the BRET ratio of the L1-BRET construct and the phosphorylation state of the FIGQY sequence.

Images were analyzed using NIH ImageJ. Densitometry was performed using a 5-pixel-wide line scan normal to the interface between two L1-CAM-positive cells. Signal maximum for ankyrin staining at the junction between cells was determined at the position of the maximal L1-CAM staining to ensure that we were quantifying membrane rather than juxtamembrane staining. Minima were determined from the regions of the line overlapping the cytoplasm of either of the two cells. Membrane localization index was determined using the equation $\text{index} = \text{max}/(\text{max} - \text{min})$ as described (Gil *et al.*, 2003).

RESULTS

L1-BRET Reporter Activity Is Modulated by EGF-Receptor Activation and Depends on the FIGQY Tyrosine

To identify the signaling pathways that regulate L1-CAM phosphorylation at the FIGQY tyrosine, we have developed a novel, genetically encoded reporter based on intramolecular FRET. By concatenating a fluorescent donor and acceptor pair with an intervening kinase target, we can monitor small changes in conformation that accompany target phosphorylation. This approach has been used successfully in the past as a method to monitor the interaction of known kinase-substrate pairs, including both tyrosine and serine-threonine kinases (Miyawaki *et al.*, 1997; Zhang *et al.*, 2001; Wang *et al.*, 2005). However, unlike previous work where reporter constructs were designed based on known kinase targets, we are using this technique to identify kinase pathways based on a previously uncharacterized substrate sequence. This approach offers several distinct advantages over traditional biochemical methods, including the ability to monitor phosphorylation events in intact cells, avoiding artifacts associated with cell lysis. Additionally, genetically-encoded reporters can be targeted to distinct cellular compartments

allowing the localization of kinase-substrate interactions. Finally, the assay used in these experiments can be readily scaled to permit the evaluation of large numbers of experimental conditions as in the screening of chemical compound libraries.

To facilitate the use of our reporter in large-scale screens, we based our construct on a variant of FRET that uses a bioluminescent donor BRET2 (Angers *et al.*, 2000). A 12-aa region of the ankyrin binding domain of L1-CAM (QFNEDGSFIGQY) was inserted between the *Renilla* luciferase (RLuc) and modified green fluorescent (GFP2) coding regions (Figure 1A; L1-BRET). A construct, lacking the L1-CAM sequence, was also generated as a positive control (Figure 1A; CHIM). Stimulation of cells with EGF resulted in a significant 24% decrease in the BRET ratio of the L1-BRET construct expressed in HEK-293 cells ($p < 0.01$; Figure 1B). In contrast, there was no change in the BRET ratio in similarly-treated cells transfected with the control CHIM construct. Subsequent results are represented as the percent change in BRET efficiency with respect to untreated cells (%ΔBRET Ratio). Trials using longer inserts (25 aa) showed a similar response to EGF, though of lower amplitude (unpublished data). EGF stimulation reduced the BRET ratio of L1-BRET-transfected cells in a dose-dependent manner (10–20 ng/ml EGF producing near-maximal reductions; Figure 1C). In EGF-stimulated cells, the reduction in the BRET ratio was maximal at 10 min (Figure 1D) and recovered within 60 min, consistent with the transient nature of EGF receptor (EGF-R) signaling events (Marshall, 1995). Phosphotyrosine immunoblots revealed that EGF-R was activated after stimulation of HEK-293 cells with EGF, but not when cells were either serum-

Figure 2. Kinase-dependent phosphorylation of the FIGQY tyrosine is responsible for changes in the BRET ratio of the L1-BRET construct. (A) The tyrosine kinase inhibitor, genistein, reverses the decrease in BRET ratio after EGF stimulation of HEK-293 cells transfected with the L1-BRET construct (* $p < 0.01$). The dotted line represents the basal level of BRET without EGF stimulation. (B) Phosphotyrosine immunoblot of the L1-BRET reporter. HEK-293 cells, transiently transfected with the L1-FIGQY constructs, were treated with varying concentrations of genistein (indicated in the figure) in the presence and absence of EGF (100 ng/ml). Cell lysates were immunoprecipitated with anti-GFP-coated agarose beads and subsequently analyzed by immunoblotting to detect tyrosine phosphorylated BRET constructs (inset). Densitometry measurements show a more than threefold induction of phosphotyrosine in the presence of EGF over unstimulated controls (dashed line) that is progressively reduced to background levels by increasing concentrations of genistein. Blot is representative of four experiments. (C) The phosphotyrosine phosphatase inhibitor, PAO (1 μ M), decreases the BRET ratio of the L1-BRET construct ($p < 0.01$). (D) Conversely, the EGF receptor-associated kinase inhibitor, erbstatin analog, increases the BRET ratio of the L1-BRET construct in a dose-dependent manner (* $p < 0.01$; ** $p < 0.05$). (E and F) The inhibitors, PP1 and PP2, have no effect on the BRET ratio of the L1-BRET construct, suggesting that the L1-FIGQY sequence is not phosphorylated by Src-family of tyrosine kinases. In A and C–F, results shown are mean $\pm$ SD, $n = 5$.

starved or maintained in medium containing 10% (vol/vol) fetal bovine serum (unpublished data).

To evaluate the role of the terminal tyrosine in the EGF-stimulated change in the BRET ratio, we mutated the tyrosine to an aspartate (FIGQD), histidine (FIGQH), or phenylalanine (FIGQF) residue (Figure 1A). These constructs displayed no significant change in the BRET ratio after stimulation of transfected HEK-293 cells with EGF (Figure 1E). Interestingly, the relative BRET ratios for the FIGQD construct were significantly lower ($p < 0.01$) than that observed for the FIGQF construct in either untreated cells or cells stimulated with EGF. These results support the hypothesis that decreases in the BRET ratio of L1-BRET construct are governed by changes in charge caused by tyrosine phosphorylation of the L1-CAM insert (Figure 1F).

L1-BRET Energy Transfer Depends on Tyrosine Kinase Activity That Regulates the Phosphorylation of the FIGQY Reporter Domain

To characterize in more detail the signaling pathways that regulate L1-FIGQY phosphorylation, we examined the effects of a variety of tyrosine kinase and phosphatase inhibitors on L1-BRET activity. The decrease in the BRET ratio after EGF stimulation was inhibited and reversed by pre-treating cells with genistein, a broad-spectrum tyrosine kinase inhibitor (Figure 2A; Akiyama *et al.*, 1987), raising the ratio above that of untreated cells (dashed line) to a level indistinguishable from that of the chimeric CHIM construct. The ability of genistein to raise the BRET ratio of the reporter above its basal level suggests that the reporter is partially

phosphorylated in unstimulated cells. The negative control for genistein, genistin, had no effect (100 μ M genistin; $-1.7 \pm 4.5\%$). Treatment of transfected HEK-293 cells with phenylarsine oxide (PAO), a tyrosine phosphatase inhibitor (Garcia-Morales *et al.*, 1990), resulted in a significant decrease in the BRET ratio ($p < 0.01$; Figure 2C). By immunoblot analysis, the L1-BRET protein was tyrosine phosphorylated in HEK-293 cells in the presence of EGF, and phosphorylation was progressively inhibited when cells were pretreated with increasing concentrations of genistein (as indicated in figure; dashed line represents signal from untreated cells; Figure 2B). There was no change in phosphotyrosine levels detected in the CHIM construct in the presence or absence of genistein (unpublished data), consistent with the idea that phosphorylation of the FIGQY tyrosine is responsible for the changes observed in the spectrum of L1-BRET. Despite differences in the basal phosphorylation level, the similarity in the dose-response curves measured by either BRET assay or immunoblot suggests strongly that the L1-BRET reporter assay provides a quantitative measurement of FIGQY phosphorylation in live cells.

To begin to identify the specific signaling molecules involved in L1-FIGQY phosphorylation, we used a series of increasingly specific inhibitors directed at various kinase pathways. Because the evaluation of the BRET signal is carried out using a 96-well plate fluorimeter, it is possible to screen large libraries of compounds, permitting the rapid evaluation of many different small-molecule inhibitors. Inhibiting the EGF receptor-associated kinase with the erbstatin analog (methyl 2,5-dihydroxycinnamate; Umezawa *et al.*, 1990) increased the

Table 1. Effects of pharmacological reagents on L1-BRET reporter ratio

Inhibitor	Effect	% Δ BRET ratio
Decreased ratio		
EGF	Activation of the EGFR	-22.02 ± 3.24
NGF	Activation of the NGFR	-19.23 ± 2.68
PAO	Tyrosine phosphatase inhibitor	-20.62 ± 3.22
Increased ratio		
Erbstatin analog	EGFR-associated tyrosine kinase inhibitor	24.93 ± 5.95
Genistein	Tyrosine kinase inhibitor	32.68 ± 5.21
PD98059	MEK inhibitor	32.14 ± 3.16
U0126	MEK inhibitor	37.54 ± 11.50
No change		
Ac-Y-EEIE	Src SH2 domain interaction inhibitor	-10.31 ± 7.23
BAPTA-AM	Calcium chelator	-1.93 ± 7.51
5-Iodotubercidin	ERK2 inhibitor	0.66 ± 4.65
Ionomycin	Calcium ionophore	4.14 ± 10.95
LY294002	PI3-kinase inhibitor	-1.40 ± 2.56
Mastoparan	GPCR activator	-5.71 ± 5.52
PKA Peptide	PKA inhibitor	1.30 ± 4.62
PKC Peptide	PKC inhibitor	0.85 ± 5.87
PP1	Src family tyrosine kinase inhibitor	1.16 ± 2.69
PP2	Src family tyrosine kinase inhibitor	-0.39 ± 5.01
SB-202190	p38 MAPK (α and β) inhibitor	-2.08 ± 3.71

BRET ratio of the L1-FIGQY construct in a dose-dependent manner (Figure 2D).

Members of the Src family of nonreceptor tyrosine kinases have been implicated in many signaling events downstream of receptor-tyrosine kinase activation. Additionally, Src has been shown to phosphorylate directly a tyrosine in the L1-CAM cytoplasmic tail located at the AP-2-binding site (YRSLE; Schaefer *et al.*, 2002). To examine the role of Src-family kinases in L1-FIGQY phosphorylation, we added the Src-family kinase inhibitors PP1 or PP2 (Hanke *et al.*, 1996) to cells expressing the L1-BRET reporter. Surprisingly, neither PP1 nor PP2 had any detectable effects on basal L1-BRET activity (Figure 2, E and F), suggesting that Src-family kinases are not involved in this process. Taken together, these results suggest strongly that the basal phosphorylation of L1-BRET depends on tyrosine kinase and phosphatase activity, independent of Src-kinase activation.

The MAP Kinase Pathway Regulates the Tyrosine Phosphorylation of L1-BRET

Using a similar approach, we evaluated a large number of other inhibitors directed at signaling pathways shown previously to lie downstream of EGF-receptor activation, many of which had no effect (Table 1). However, previous work has shown that components of the MAP kinase pathway, ERK and p90rsk, can phosphorylate directly serines located in the cytoplasmic domain of L1-CAM (Schaefer *et al.*, 1999). To investigate whether the MAP kinase signaling cascade is required for the phosphorylation of the FIGQY sequence, we examined the effect of two inhibitors of the MAP kinase kinase MEK1/2, PD98059 and U0126 (English and Cobb, 2002) on the BRET ratio of the L1-BRET construct transfected in HEK-293 cells. Both of the MEK inhibitors increased the BRET ratio of

the L1-BRET construct in a dose-dependent manner (Figure 3, A and B), whereas an inhibitor of the p38 MAP kinase pathway (SB-202190; Davies *et al.*, 2000) had no effect (Table 1). Together, these results suggest that phosphorylation of the FIGQY sequence is dependent on activation of the ERK1/2 MAP kinase signaling pathway in HEK-293 cells.

To confirm that the effects of MEK inhibitors were due to the tyrosine phosphorylation of FIGQY, we examined the effect of PD98059 and U0126 on the BRET ratio of the FIGQD, FIGQH, and FIGQF constructs. There was no significant change in the BRET ratio of the FIGQD, FIGQH, and FIGQF constructs after inhibition of transfected HEK-293 cells with MEK inhibitors (Figure 3C). To determine whether components of the MAP kinase cascade regulate the phosphorylation of the FIGQY sequence in other cell types and downstream of other RTKs, we transiently transfected the L1-BRET construct into ND7 (neuroblastoma-sensory neuron hybrid) and PC12 (pheochromocytoma) cells (Dunn *et al.*, 1991; Pang *et al.*, 1995). Activation of the NGF-R resulted in a decrease in the BRET ratio of the L1-BRET construct in ND7 cells (Table 1), whereas inhibition of tyrosine kinases with genistein resulted in an increase in the BRET ratio (200 μ M genistein; $18.7 \pm 1.8\%$). Similar to results with HEK-293 cells, there were also increases in the BRET ratio of the L1-FIGQY construct when ND7 or PC12 cells were treated with PD98059 (Figure 3, D and E), suggesting that a common signaling pathway is responsible for the basal phosphorylation of L1-FIGQY in different cell types. To test the involvement of the MAPK signaling cascade in the tyrosine phosphorylation of full-length L1-CAM, we examined the effect of MEK inhibitors on ND7 cells stimulated with NGF. NGF stimulation of ND7 cells resulted in an approximately twofold increase in the level of tyrosine phosphorylation of endogenous L1-CAM, consistent with previous results (Salton *et al.*, 1983). This change was inhibited by pretreatment of these cells with the MEK inhibitor U0126 (Figure 3F). Background signal in the unstimulated cells may reflect the phosphorylation of some or all of the other three tyrosines in the L1-CAM cytoplasmic domain. These results suggest that tyrosine phosphorylation of endogenously-expressed L1-CAM is dependent on the MAPK signaling pathway.

The use of pharmacological reagents to inhibit signaling pathways is limited by the selectivity of each compound for a particular enzyme. To address this limitation and to characterize in greater detail the regulation of FIGQY phosphorylation, we used siRNA-mediated knockdown to disrupt the expression of specific kinases in our cells. HEK-293 cells were cotransfected with the cDNA encoding the L1-BRET reporter and siRNA pools targeting specific kinases (Dharmacon). siRNA reagent, 100 nM, was sufficient to decrease expression of each kinase tested by as much as 10-fold, as detected by immunoblot (Figure 4Ai). Additionally, siRNA reagents were selective for their particular target at the concentrations used; treatment of cells with an siRNA pool targeting ERK1/2 had no detectable effect on the expression of either MEK1, MEK2, or Src (Figure 4Aii). As in previous experiments, treatment of cells with the MEK1/2 inhibitor U0126 increased significantly the BRET ratio compared with control (untreated) cells at 72 h after transfection. Reduction in the expression of either MEK 1 or MEK2 also increased the BRET ratio, although neither one alone modulated the BRET ratio to the extent seen with U0126 (Figure 4B). However, targeting of both MEK1 and MEK2 or ERK1/ERK2 was as effective as U0126 at increasing the BRET ratio (Figure 4B). Cells transfected with an siRNA pool modified to block assembly into a RISC complex (Dharmacon) were not distinguishable from cells transfected with BRET reporter alone

Figure 3. Components of the MAPK pathway regulate the BRET ratio of the L1-BRET construct and phosphorylation of L1-CAM. (A and B) The MEK inhibitors, PD98059 and U0126, increase the BRET ratio of the L1-BRET construct transfected in HEK-293 cells in a dose-dependent manner ($*p < 0.01$; $**p < 0.05$). (C) Mutation of the FIGQY tyrosine to an aspartate, histidine, or phenylalanine residue abolishes the increase in BRET ratio after inhibition of MEK with 100 μ M of PD98059 ($*p < 0.01$). (D and E) The MEK inhibitor, PD98059, increases the BRET ratio of the L1-BRET construct transfected in both ND7 and PC12 cells in a dose-dependent manner ($*p < 0.01$; $**p < 0.05$). In A–E, results shown are mean $\pm$ SD, $n = 5$. (F) Tyrosine phosphorylation of endogenously expressed L1-CAM is dependent on the MAPK signaling pathway. NGF-stimulated ND7 cells were treated with or without 20 μ M U0126. Cell lysates were immunoprecipitated with an anti-L1-CAM antibody and subsequently analyzed by immunoblotting with an anti-phosphotyrosine antibody to detect phosphorylated L1-CAM (upper blot). The level of endogenously-phosphorylated L1-CAM was decreased after treatment of NGF-stimulated ND7 cells with the MEK inhibitor, U0126. The same membrane was stripped and reprobed with an antibody directed against L1-CAM as a loading control (lower blot).

(Figure 4C). Strikingly, inhibition of MAPK14/p38 or the tyrosine kinases Abl or Src had no effect on BRET levels, suggesting that these enzymes are not involved in the basal phosphorylation of the L1-FIGQY sequence. Together, these results suggest that the phosphorylation of the L1-FIGQY motif depends on the activity of the MAP kinase cascade.

Membrane Localization Does Not Affect BRET Reporter Function

Many kinase/ligand pairs depend on colocalization for specificity and/or activation. As the endogenous L1-CAM FIGQY sequence is normally anchored at the cytoplasmic membrane, one concern with the L1-BRET reporter design was its lack of membrane localization. To address this, we generated a myristoylated construct, including 25 residues of the L1-CAM cytoplasmic sequence (Figure 5C; myr-L1-BRET). This construct displayed localization to the plasma membrane when expressed in HEK-293 cells (unpublished data). Like L1-BRET, there was a significant decrease in BRET ratio of the myristoylated construct when HEK-293 cells were stimulated with EGF ($p < 0.01$; Figure 5A). Strikingly, myristoylated constructs that used a shorter, 12-aa insert displayed a constitutively high level of energy transfer (1.16 ± 0.006), similar to the chimeric construct (1.23 ± 0.019). However, because the myristoylation sequence is located at the amino-terminus of the GFP moiety, on the same face of the GFP domain as the reporter/linker domain (as predicted by crystal structure; Ormo *et al.*, 1996), it is likely that membrane attachment bends the L1-BRET construct, restricting phosphorylation-dependent conformational changes. To examine whether the membrane localiza-

tion altered the signaling pathway responsible for L1-BRET phosphorylation, we treated HEK-293 cells expressing both myr-L1-BRET and single-amino acid substitution constructs (myr-FIGQH, myr-FIGQF; Figure 5C) with U0126 (20 μ M). Consistent with the results seen with the soluble reporters, U0126 increased the BRET ratio to a level similar to that seen with the CHIM construct (Figure 5B). Constructs lacking the terminal tyrosine showed no change in energy transfer. Together, these results demonstrate that membrane localization is not required for the activity of the L1-BRET construct.

The MAP Kinase Pathway Regulates L1-CAM-mediated Neuronal Growth

Activation of the EGF-R inhibits L1-CAM-dependent ankyrin B recruitment to the plasma membrane (Gil *et al.*, 2003). To determine whether the MAP kinase pathway modulates this interaction, we examined the effects of MEK inhibition on ankyrin B recruitment to the plasma membrane after EGF stimulation. Treatment of transfected HEK-293 cells with EGF leads to a decrease in the level of ankyrin B recruited to the plasma membrane as described previously (Gil *et al.*, 2003). The decrease in the level of ankyrin B recruitment to the plasma membrane after EGF stimulation was reversed after the addition of PD98059 (Figure 6, A and B). These results suggest that components of the MAPK pathway regulate the phosphorylation of the FIGQY tyrosine in the context of full-length L1-CAM and as a consequence can modulate the membrane recruitment of ankyrin B. To examine directly the role of MAP kinase signaling in the regulation of L1-CAM function in situ, we cultured cerebellar granular neurons on substrates coated with the L1-CAM

Figure 4. RNAi-mediated inhibition of MEK1/2 or ERK1/2 expression modulates the BRET ratio of the L1-BRET construct. (Ai) HEK-293 cell extracts were analyzed by Western blot to determine the expression levels of each targeted enzyme (siRNA and antibody are indicated at left) at 72 h after siRNA transfection (100 nM). Endogenous protein levels were decreased after RNAi targeting of ERK1/2, MEK1, MEK2, or Src. (Aii) However, RNAi targeting of ERK1/2 (same cell extract as in Figure 2Ai; first row) did not affect endogenous protein levels of MEK1, MEK2 or Src (antibody indicated at right). (B) RNAi targeting of ERK1/2, MEK1, or MEK2 significantly increased the BRET ratio of the L1-BRET construct, though MEK1 or MEK2 alone was less effective. Combination of the MEK1 and MEK2 siRNAs had an additive effect, increasing the BRET ratio of the L1-BRET construct to levels similar to ERK1/2 or U0126 ($p < 0.01$ for all experimental conditions). (C) RNAi targeting of MAPK14/p38, Abl, or Src had no effect on the BRET ratio of the L1-BRET construct. Transfection of HEK-293 cells with control RISC-free siRNA also had no effect on the BRET ratio of the L1-BRET construct. Results shown are the mean $\pm$ SD of three independent experiments.

ligand Ng-CAM, a chick L1-CAM homolog. As MAP kinase pathway inhibitors block neuronal growth through both L1-CAM and other receptor families, MAP kinase activity

Figure 5. Localization of the L1-BRET construct to the membrane does not affect the function of the L1-BRET reporter. (A) Application of 100 ng/ml EGF significantly reduces the BRET ratio of an L1-BRET construct containing 25 residues of the L1 cytoplasmic sequence and a myristoylation site located upstream of the GFP coding region ($*p < 0.01$). (B) Mutation of the FIGQY tyrosine to a histidine or phenylalanine residue abolishes the increase in BRET ratio of the myristoylated construct after inhibition of MEK with 20 μ M U0126 ($*p < 0.01$). In A and B, results shown are mean $\pm$ SD, $n = 5$. (C) Schematic diagram of the myristoylated chimeric BRET constructs. The insert reporter domain is comprised of a myristoylated construct including the indicated aa sequence in the insert.

has been suggested to regulate pathways common to nerve growth in general (Perron and Bixby, 1999; Schmid *et al.*, 2000). To determine if L1-CAM function was itself modulated by MAP kinase activity, we grew neurons in the presence of both a MEK inhibitor (U0126) and a peptide AP-YF that inhibits L1-CAM interactions with ankyrin. Previous work has demonstrated that AP-YF stimulates L1-dependent neuronal growth (Gil *et al.*, 2003). The AP-YF sequence is based on the L1-FIGQY domain, suggesting that it serves as a competitive inhibitor of L1-ankyrin interactions. Therefore, we hypothesized that if MAP kinase lies upstream of L1-CAM phosphorylation, the addition of AP-YF should override the effects of U0126, blocking ankyrin binding independent of L1-FIGQY phosphorylation. As shown previously, the addition of AP-YF stimulates significantly L1-mediated neuronal growth compared with a scrambled, control peptide (AP-Scr; Figure 6C; white bars). Addition of U0126 reduces mean neurite length (gray bars). However, in the presence of AP-YF, neuronal growth was stimulated by almost twofold compared with neurons grown in the presence of a control peptide. Axon growth on laminin was inhibited by U0126 but was not rescued by AP-YF treatment. These results strongly suggest that the activity of the MAP kinase pathway regulates L1-CAM-mediated neuronal growth in an ankyrin-dependent manner.

Figure 6. The MAPK pathway regulates ankyrin B localization and L1-CAM-mediated neuronal growth. (A) Growth-factor inhibition of ankyrin B binding to L1-CAM is dependent on activation of the MAPK pathway. HEK-293 cells cotransfected with cDNAs encoding full-length myc-tagged L1-CAM and ankyrin-B:GFP were treated with 100 ng/ml EGF and/or 100 μM PD98059. L1-CAM (green) and ankyrin B (red) were visualized by indirect immunofluorescence using CY3 and CY5 antibodies, respectively. Fluorescent images were combined to determine colocalization (yellow). Bar, 10 μm. EGF leads to a decrease in the level of ankyrin B recruited to the plasma membrane of HEK-293 cells. The decrease in ankyrin B recruitment to the plasma membrane is reversed after the addition of the MEK inhibitor, PD98059. (B) Direct quantification of ankyrin B colocalization with L1-CAM at the cell membrane (as described; Gil *et al.*, 2003). In B, results shown are the mean ± SD of two independent experiments. (C) MAP kinase activity regulates L1-CAM-mediated neuronal growth in an ankyrin-dependent manner. Axon growth from cerebellar granular neurons cultured on either

DISCUSSION

L1-BRET Serves as a Reporter of L1-CAM FIGQY Phosphorylation

Using a novel reporter based on intramolecular BRET, we have provided evidence for the role of the MAP kinase pathway in the phosphorylation of the L1-CAM cytoplasmic domain at the conserved tyrosine residue located at the ankyrin binding site. Our reporter, based on the region of L1-CAM adjacent to this phosphorylation site, permits us to monitor changes in the phosphorylation level of this target sequence in the presence and absence of growth factors and pharmacological inhibitors and after the knockdown of specific kinases. This approach has allowed us to characterize the pathway that lies upstream of this tyrosine. Strikingly, several common tyrosine kinases that have been shown to phosphorylate other cell-surface glycoproteins, including Src-family kinases and Abl, do not appear to participate in the basal phosphorylation of the L1-CAM FIGQY tyrosine.

The genetically-encoded reporter construct used in these studies relies on phosphorylation-induced changes in the conformation of the L1-derived sequence that separates the BRET donor and acceptor. By avoiding the need to purify the phosphorylation target, this method is less sensitive to changes in kinase and phosphatase activity that accompany cell lysis. The slight differences between results obtained by immunoblot and BRET in response to varying concentrations of genistein (Figure 2, A and B) may reflect the increased sensitivity of the BRET assay. Additionally, BRET reporters provide information about the location of kinase-substrate interactions in the cell; by targeting the reporter to subcellular compartments, one can determine where the active kinase is distributed. The L1-BRET reporter appears to function equally well either in the cytosol or anchored to the inner leaflet of the plasma membrane, suggesting that the kinase in question is freely diffusing in the cytosol. Therefore, BRET-reporters are likely to provide a powerful method for evaluating kinase-substrate interactions in live cells.

Energy transfer is acutely sensitive to the separation and orientation of the donor and acceptor domains. However, the window of separation in which energy transfer occurs is fairly narrow, limited to 10–100 Å (Lakowicz, 1999). Studies using FRET to quantify the length of polypeptide peptides suggest that small changes in peptide length (for peptides near the Förster distance R_0 ; ~50 Å) can give rise to large changes in FRET efficiency (Stryer and Haugland, 1967; Schuler *et al.*, 2005). The L1-BRET reporter was designed based on the assumption that the L1-CAM cytoplasmic domain is largely lacking in secondary structure (based on structural studies of the L1-family member neurofascin; Zhang *et al.*, 1998). We estimated the maximum dimensions of the insert-based length of a fully extended peptide (3.63 Å per aa; 43.56 Å for the 12 amino acid FIGQY insert; Creighton, 1984). By starting near the critical distance for energy transfer, small changes in the conformation of the reporter insert are likely to yield the largest possible changes in the spectral profile of the reporter. Calculations based on the Förster equation (see *Materials and Methods*) suggest that the 24% decrease in FRET efficiency would require optimally only a 3.6 Å increase in the separation of donor and acceptor (assuming a Förster distance, R_0 of 50 Å). In addition to changes in donor-

Ng-CAM or laminin substrates were retarded by MAP kinase pathway inhibitors (10 μM U0126). However, growth on Ng-CAM was partially rescued by the addition of a peptide that inhibits L1-CAM-ankyrin interactions (AP-YF), whereas growth on laminin was unaffected by similar treatment.

acceptor separation, changes in donor-acceptor orientation may also modulate FRET efficiency (Lakowicz, 1999).

We cannot preclude the possibility that our reporter serves as a phosphorylation-dependent binding site for another protein in the cytosol. However, it is unlikely that the binding partner is ankyrin itself, as ankyrin has a footprint that is considerably larger than our insert (as much as 37 aa; Zhang *et al.*, 1998). Additionally, efforts to coprecipitate L1-BRET with other proteins have failed to reveal any stable interactions (J. D. Whittard, unpublished results). Therefore, we conclude that the phosphorylation of the tyrosine in the L1-BRET insert leads to changes in the separation and/or orientation of the donor and acceptor moieties in our reporter construct after phosphorylation, perhaps due to changes in charge.

The capacity of PD98059 and U0126 to inhibit L1-FIGQY phosphorylation suggests that the MAP kinase cascade comprises an integral component of the pathway that regulates L1-CAM phosphorylation. Although these inhibitors target the MAP kinase kinases MEK1/2, which have dual threonine/tyrosine kinase activity, MEK1/2, to date, is only known to phosphorylate ERK1/2, suggesting that these kinases are tightly linked (Raman and Cobb, 2003). Consistent with this idea, reduction in the expression of either MEK1/2 or ERK1/2 inhibits FIGQY phosphorylation, suggesting that the direct kinase lies downstream of the MAP kinase pathway. Although other Src-family kinases may be involved in this pathway, pharmacological and siRNA data suggest that they are not involved in the basal phosphorylation of the L1 FIGQY motif. On the basis of the size of the L1-BRET insert, we infer that the footprint of the kinase is restricted to the 11 aa upstream of the target tyrosine. Additionally, the kinase in question does not depend on membrane localization for its activity, because both soluble and membrane-linked reporters respond in an indistinguishable manner.

MAP Kinase Pathway Activity and the Regulation of L1-CAM-mediated Nerve Growth and Adhesion

Although we have focused on the tyrosine phosphorylation of the L1-CAM cytoplasmic tail at the FIGQY motif that mediates ankyrin binding, MAP kinase signaling has also been implicated in L1-CAM function as a receptor for nerve growth-promoting signals (Schaefer *et al.*, 1999; Loers *et al.*, 2005). Additionally, the MAP kinase pathway has been implicated in L1-mediated neuroprotection (Loers *et al.*, 2005). Several components of the MAP kinase cascade phosphorylate directly serines in the L1-CAM-cytoplasmic domain. These include two serines adjacent to the FIGQY domain that are direct targets for ERK2 phosphorylation. Although the role of these serines in L1-CAM-cytoskeleton interactions is not known, L1-CAM cross-linking and internalization have been directly implicated in the activation of ERK1/2 (Schaefer *et al.*, 1999; Schmid *et al.*, 2000). Moreover, inhibitors of the MAP kinase cascade retard L1-stimulated neuronal growth (Schmid *et al.*, 2000), consistent with the model that L1-CAM functions as a receptor, propagating ligand-activated signals to downstream targets which effect neuronal growth (Figure 7A). In contrast, the work presented here suggests that L1-CAM is an effector of neuronal growth and is itself a target of components of the MAP kinase cascade (Figure 7B). Both ankyrin-dependent and ankyrin-independent pathways are likely to operate in parallel, a conclusion supported by the incomplete rescue of neuronal growth by the AP-YF peptide after U0126 treatment (Figure 6C). Previous work has shown that ankyrin binding to L1-CAM inhibits L1-mediated traction-force generation and neuronal growth (Gil *et al.*, 2003; Cheng *et al.*, 2005). The identification of the MAP kinase pathway as a regulator of L1-FIGQY phosphorylation, L1-

Figure 7. Schematic diagram illustrating two possible mechanisms for L1-CAM function in neuronal growth. (A) Previous work has suggested that activation of L1-CAM by extracellular ligands leads to the MAP kinase-dependent stimulation of neuronal growth. + and – signs indicate the effect of each interaction on neuronal growth. Inhibitors of MEK1/2 block L1-CAM-stimulated neuronal growth, suggesting that L1-CAM activation lies upstream of this enzyme. (B) Work presented here suggests that L1-CAM depends on MAP kinase activity to regulate L1-CAM binding to ankyrin, an interaction that inhibits L1-CAM-mediated nerve growth. Although inhibitors of MEK1/2 block L1-CAM-mediated neuronal growth, that outgrowth is restored by the inhibitory peptide AP-YF that blocks L1-ankyrin interactions. Together, these results suggest that L1-CAM serves as both a transducer of nerve-growth-promoting signals and as an effector of neuronal growth.

ankyrin B interactions and L1-mediated neuronal growth reinforces the central role of ankyrin B binding in the regulated growth of neurons on L1-CAM ligands. The ability of L1-CAM to serve as both an activator and a target of MAP kinase pathway activity raises the possibility that L1-CAM functions as part of an autocrine loop, activating itself through MAP kinase after extracellular ligand activation. In this respect, L1-CAM may function as a motility receptor, displaying ligand-dependent regulation of traction force generation in a manner similar to integrins (Sheetz *et al.*, 1998).

Together, these results point to a MAP kinase pathway-dependent regulation of L1-CAM phosphorylation at the FIGQY tyrosine. Additionally, these results demonstrate the usefulness of intramolecular BRET reporters as the basis for blind screens for identifying kinases based on target protein sequences. The MAP kinase pathway has long been associated with the regulation of neuronal growth in general, and L1-stimulated neuronal growth in particular. The results

presented here suggest that L1-CAM is itself a target of MAK kinase regulation. The activity of L1-CAM in neuronal growth, a process inhibited by ankyrin binding, is therefore subject to the same inside-out regulation as the adhesive activity of integrins (Law *et al.*, 1996; Hortsch *et al.*, 1998). As ankyrin binding has also been implicated in promoting vertebrate L1-family member adhesive capacity (Tuvia *et al.*, 1997), these results suggest that the MAP kinase cascade may play a central role in the switch from process growth to static adhesion seen during neural development, an idea supported by the increase in ankyrinB-L1-CAM colocalization in postnatal rat brain (Mintz *et al.*, 2003). Moreover, in light of the high level of sequence conservation among L1 family members at the FIGQY motif, the MAP kinase cascade may regulate L1 family-ankyrin interactions in other contexts where this interaction plays a critical role, including the clustering of voltage-gated Na⁺ channels at the Nodes of Ranvier and the axon initial segment (Zhou *et al.*, 1998; Sherman *et al.*, 2005). Finally, these observations raise the possibility that localized changes in MAP kinase activity could regulate neuronal growth at the level of individual adhesion receptors, a model that has important implications for axon guidance.

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