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

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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...
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 indodicarbocyanine Cy5 were obtained from Dharmacon (Lombard, IL). Humanized pRluc and GFP2 vectors were obtained from PerkinElmer Life Sciences. ERK1/2, MAPK14, MEK1, MEK2, and Src were obtained from Dharmacon (Lombard, IL). 5-iodotubercidin, ionomycin, LY294002, mastoparan, PD98059, protein kinase A (PKA), and protein kinase C (PKC) peptides, PP1, PP2, S282190, and U0126, were obtained from BioMol Research Laboratories (Plymouth Meeting, PA). Ac-E-YE-E, epidermal growth factor (EGF), erbstatin analog, NGF, and PAO were obtained from Sigma-Aldrich (St. Louis, MO). Anchorage-dependent cell lines (ND7 and HEK-293T) were obtained from the American Type Culture Collection (Manassas, VA). BAPTA-AM, genistein, 5-iodotubercidin, IGEPAL CA-630, sodium deoxycholate, pepstatin A, protein kinase A (PKA), and protein kinase C (PKC) peptides, PP1, PP2, S282190, and U0126, were obtained from BioMol Research Laboratories (Plymouth Meeting, PA). Anti-EGF, anti-EGF receptor, anti-phosphotyrosine, and horseradish peroxidase-conjugated sheep anti-mouse antibodies were generous gifts from Dr. C. Lagenaur (University of Pittsburgh, PA). Mouse anti-myc-epitope–tagged full-length wild-type L1-CAM and a carboxy-terminal chimeric construct (CHIM) encode unique BsrGI and AscI sites in the inter- vening region of L1-CAM. L1-CAM was expressed in Escherichia coli (induction of RNA) before ligation into the CHIM construct, or ND7 cells were harvested with trypsin-EDTA (0.05% trypsin, 0.02% EDTA; Invitrogen). The cell lysates were then pelleted at 10,000 g for 10 min at 4°C, washed with PBS, and resuspended in 0.5% SDS-PAGE, and transferred to nitrocellulose membrane. The membrane was blocked with 5% nonfat milk, incubated with rabbit polyclonal antibodies to L1-CAM antibody was used to detect the deletion of the carboxy-terminal 16 amino acids of L1-CAM, with rabbit anti-GFP antibody for 2 h at room temperature or 2 µg/ml anti-L1 antibody overnight at 4°C. Immunoblotting was performed using a mouse anti-myc antibody followed by a HRP-conjugated goat anti-rabbit antibody at a dilution of 1:5,000 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 µg/ml anti-GFP antibody for 2 h at room temperature or 2 µg/ml anti-L1 antibody overnight at 4°C. Densitometry of immunoblot films was carried out using a transilluminated flatbed 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 µM PD98059 and 100 ng/ml with EGF. For immunolocalization, cells were fixed with 4% paraformaldehyde in 0.12 M Pipes, 25 mM HEPES, 10 mM glucose, 0.1% (wt/vol) MgCl2, and 10 µg/ml aprotinin was added. The plates were then stained 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 band pass filter. The efficiency of energy transfer (EET) is determined by dividing acceptor emission intensity (GFP2) by donor emission intensity (Rluc). The results validating the proximity of GFP2 to Rluc 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-FIQYY or CHIM constructs, or ND7 cells were harvested with trypsin-EDTA and resuspended to a density of 6 × 106 cells/ml. Aliquots (5 µl) of cell suspensions were added to 100-mm cell culture dishes (Corning Life Sciences, Corning, NY) and incubated for 2 d and fixed with 4% paraformaldehyde in 0.12 M Pipes, 25 mM HEPES, 10 mM glucose, 0.1% (wt/vol) MgCl2, and 10 µg/ml aprotinin was added. The plates were allowed to incubate for 2 h (PD98059 and U0126) or 4 h (genistein). To each well, 10 µf of BluePlex substrate (final concentration of 5 µM; PerkinElmer Life Sciences) diluted in Dulbecco’s PBS containing 0.1% (wt/vol) Catc3, 0.1% (wt/vol) D-glucose, 0.1% (wt/vol) MgCl2, and 10 µg/ml aprotinin was added. The plates were then 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 band pass filter. The efficiency of energy transfer (EET) is determined by dividing acceptor emission intensity (GFP2) by donor emission intensity (Rluc). The results validating the proximity of GFP2 to Rluc 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.

Bioluminescence Resonance Energy Transfer (BRET) Construct Design

Bioluminescence resonance energy transfer (BRET) constructs were designed using vectors encoding Renilla luciferase and GFP2 (Sapphire GFP; Biosig, PerkinElmer Life Sciences). Coding regions from each individual vector were PCR amplified with additional restriction sites, permitting their ligation into a single, concatenated coding region (GFP2:Rluc) between NotI and XhoI sites copied by PCR with additional restriction sites, permitting their ligation into the CHIM construct. Briefly, ankyrin B was detected by indirect immunofluorescence using a mouse anti-myc antibody followed by a HRP-conjugated goat anti-rabbit antibody at a dilution of 1:5,000. Briefly, ankyrin B was detected by indirect immunofluorescence using a mouse anti-myc antibody followed by a HRP-conjugated goat anti-rabbit antibody at a dilution of 1:5,000 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 µg/ml anti-GFP antibody for 2 h at room temperature or 2 µg/ml anti-L1 antibody overnight at 4°C.

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 = R0²/r² (R0² + r²), where R0 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 dR0 = [(1.076E) − 1]−1/6 − [(1/E) − 1]−1/6.
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:

\[
\text{index} = \frac{\text{max}(\text{membrane}) - \text{min}(\text{cytoplasm})}{\text{max}(\text{membrane}) + \text{min}(\text{cytoplasm})}
\]

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 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 ± 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.
Inhibitor, erbstatin analog, increases the BRET ratio of the L1-BRET construct in a dose-dependent manner (Figure 2A; Akiyama et al., 1987), raising the BRET ratio above its basal level suggests that the reporter is partially phosphorylated in unstimulated cells. The negative control for genistein, genistin, had no effect (100 μM genistin; −1.7 ± 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 phosphorylation of the FIGQY tyrosine (Figure 2D).

**FIGQY Reporter Domain**

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 μM genistin; −1.7 ± 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.

**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 pretreating 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 μM genistin; −1.7 ± 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.

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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 cascade 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).

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

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Effect</th>
<th>% Δ BRET ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decreased ratio</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGF</td>
<td>Activation of the EGFR</td>
<td>−22.02 ± 3.24</td>
</tr>
<tr>
<td>NGF</td>
<td>Activation of the NGFR</td>
<td>−19.23 ± 2.68</td>
</tr>
<tr>
<td>PAO</td>
<td>Tyrosine phosphatase inhibitor</td>
<td>−20.62 ± 3.22</td>
</tr>
<tr>
<td>Increased ratio</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erbstatin analog</td>
<td>EGFR-associated tyrosine kinase inhibitor</td>
<td>24.93 ± 5.95</td>
</tr>
<tr>
<td>Genistein</td>
<td>Tyrosine kinase inhibitor</td>
<td>32.68 ± 5.21</td>
</tr>
<tr>
<td>PD98059</td>
<td>MEK inhibitor</td>
<td>32.14 ± 3.16</td>
</tr>
<tr>
<td>U0126</td>
<td>MEK inhibitor</td>
<td>37.34 ± 11.50</td>
</tr>
<tr>
<td>No change</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ac-Y-EEIE</td>
<td>Src SH2 domain interaction inhibitor</td>
<td>−10.31 ± 7.23</td>
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<td>Calcium chelator</td>
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<td>0.66 ± 4.65</td>
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<td>4.14 ± 10.95</td>
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<td>Mastoparan</td>
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<tr>
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</tr>
<tr>
<td>PP2</td>
<td>Src family tyrosine kinase inhibitor</td>
<td>−0.39 ± 5.01</td>
</tr>
<tr>
<td>SB-202190</td>
<td>p38 MAPK (α and β) inhibitor</td>
<td>−2.08 ± 3.71</td>
</tr>
</tbody>
</table>

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 detectible 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 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 4Aii). 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 detectible 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 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.

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 μM genistein; 18.7 ± 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.
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 ($1.16 ^{\pm 0.006}$), similar to the chimeric construct ($1.23 ^{\pm 0.019}$). 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 localization 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 ($20 \mu M$). 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 MAPK 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...
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 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.
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 polyproline peptides suggest that small changes in peptide length (for peptides near the Förster distance $R_0$ of 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-

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 Ng-CAM or laminin substrates was 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 (Schafer 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 phosphatase directly serials 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 (Schafer 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-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 regulate neuronal growth at the level of individual adhesion receptors, a model that has important implications for axon guidance.

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