Involvement of p90\textsuperscript{rsk} in Neurite Outgrowth Mediated by the Cell Adhesion Molecule L1*

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L1 is a neural cell adhesion molecule that has been shown to help guide nascent axons to their targets. This guidance is based on specific interactions of L1 with its binding partners and is likely to involve signaling cascades that alter cytoskeletal elements in response to these binding events. We have examined the phosphorylation of L1 and the role it may have in L1-directed neurite outgrowth. Cytosolic extracts from nerve growth factor-stimulated PC12 cells were fractionated by anion-exchange chromatography, and an activity was found that phosphorylated the cytoplasmic domain of L1. This activity was then assayed using a battery of L1-derived synthetic peptides. Based on these peptide assays and sequencing of radiolabeled L1 proteolytic fragments, the phosphorylation site was determined to be Ser\textsuperscript{1152}. Western blot analysis demonstrated that the L1 kinase activity from PC12 cells that phosphorylated this site was co-eluted with the S6 kinase, p90\textsuperscript{rsk}. Moreover, S6 kinase activity and p90\textsuperscript{rsk} immunoreactivity co-immunoprecipitate with L1 from brain, and metabolic labeling studies have demonstrated that Ser\textsuperscript{1152} is phosphorylated in vivo in the developing rat brain. The phosphorylation site is located in a region of high conservation between mammalian L1 sequences as well as L1-related molecules in vertebrates from fish to birds.

Recent evidence suggests a function for L1 beyond adhesion between two cell surfaces. When a growth cone migrating on laminin contacts L1, its morphology changes quickly, broadening and flattening even before the entire growth cone has moved onto the new substrate (Burden-Gulley et al., 1995). This suggests activation of a signal transduction cascade initiated by L1 contact that eventually affects the cytoskeleton. Further evidence for signal transduction cascades initiated by L1 comes from observations of changes in various intracellular second messenger systems upon activation of L1 by binding with soluble L1 or anti-L1 antibodies (Itoh et al., 1992; Schuch et al., 1989; Von Bohlen und Halbach et al., 1992; Williams et al., 1992).

L1 (Mos et al., 1988) (also termed NILE (Prince et al., 1989), 8D9 (Lemmon and McLoon, 1986), Ng-CAM (Grumet et al., 1984), G4 (Rathjen et al., 1987b)) is primarily expressed on projection axons of the central nervous system and peripheral nervous system, as well as on a few nonneuronal cell types, including Schwann cells and lymphocytes. It is a member of the immunoglobulin superfamily of adhesion molecules (Burden-Gulley and Lemmon, 1995), the extracellular domain of which is characterized by six immunoglobulin-like domains and five fibronectin-type III domains, and highly conserved transmembrane and cytoplasmic domains. The cytoplasmic domain is completely conserved in the known mammalian sequences, and two long stretches are perfectly conserved in the chick, comprising nearly 70% of the cytoplasmic domain (Hlavin and Lemmon, 1991). Two shorter sequences, one abutting the membrane and one 40 amino acids from the C terminus, are conserved, even in the Drosophila L1 homologue, neuroglian (Bieber et al., 1989). There are also two alternatively spliced exons that are present in neuronal L1 but not in L1 expressed in nonneuronal cells (Miura et al., 1991). The L1 molecule is both glycosylated and phosphorylated (Faisstner et al., 1984).

One possible mechanism for control of the signal transduction cascades initiated by L1 binding is the regulated phosphorylation of L1. We and others have described a number of kinase activities that coprecipitate with L1 immunoprecipitates (Sadoul et al., 1989; Wong et al., 1996). We have identified...
one of these as casein kinase II, which phosphorylates L1 at Ser1152. In this paper, we demonstrate that an S6 family kinase is also associated with L1.

The serine/threonine kinase, p90\textsuperscript{rsk}, was initially identified in the nervous system and has not been studied directly, but it is reported to be part of an NGF\textsuperscript{1}-inducible signaling cascade (Blenis, 1993). The role of p90\textsuperscript{rsk} in the nervous system has been the focus of much interest due to its ability to be phosphorylated and activated by the mitogen-associated protein kinases and is a component of this growth factor-sensitive signaling cascade (Blenis, 1993).

EXPERIMENTAL PROCEDURES

Materials—Protease inhibitors Pefabloc SC and aprotinin and horseradish peroxidase-conjugated goat anti-rabbit antibodies were from Boehringer Mannheim. Purified p90\textsuperscript{rsk} lines were obtained from Upstate Biochemicals, Inc. (Lake Placid, NY). Anti-p90\textsuperscript{rsk} polyclonal antibodies were purchased from Transduction Laboratories (Lexington, KY). [\textsuperscript{32P}]H\textsubscript{3}PO\textsubscript{4} was purchased from ICN Biochemicals (Irvine, CA). Custom L1CD peptides were synthesized by Biosynthesis, Inc. (Lewisville, TX). Immobilon-P polyvinylidene difluoride membrane was from Millipore (Marlborough, MA). Renaissance enhanced chemiluminescent detection reagents were purchased from DuPont NEN. RPMI 1640 cell culture medium, fetal bovine serum, and NGF (75) g were purchased from Life Technologies, Inc. Other chemicals were purchased through Sigma.

L1 Immunoprecipitation—Brains from P7 Sprague-Dawley rat pups were homogenized in 20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM \(\beta\)-glycerophosphate (TEV-PNP buffer) containing 0.32 mM sucrose, 200 \(\mu\)M Pefabloc SC, and 100 \(\mu\)M aprotinin. The homogenates were separated by ultracentrifugation on a discontinuous sucrose gradient (0.32–1.8–12 mM) for 45 min at 60,000 g at 4 °C. The plasma membrane layer was solubilized in TEV-PNP containing 1% Triton X-100 and centrifuged. The supernatant was then incubated for 4–8 h at 4 °C with Sepharose beads conjugated to a monoclonal anti-L1 antibody, mAb 74-5SH7 (LeDoux et al., 1989). The beads were washed with TEV-PNP containing 1% Triton X-100, followed by TEV-PNP without detergent four times before use in kinase assays.

L1CD Preparation—The cytoplasmic domain of human L1, composed of residues 1144 to 1257, was cloned into the pQE13 bacterial expression vector (Qiagen) to produce a recombinant L1CD containing a hexahistidine epitope at the N terminus. This protein was expressed in Escherichia coli and L1CD purified from the bacteria by Ni\textsuperscript{2+}-affinity chromatography using Ni-NTA agarose beads (Qiagen), the manufacturer’s protocols.

PC12 Cell Cytosolic Extracts—PC12 cells (8–10 \(\times\) 10\textsuperscript{5} cells) were stimulated with NGF (50 ng/ml) for 30 min. The cells were then collected in TEV-PNP and lysed by sonication, followed by centrifugation for 30 min at 100,000 g at 4 °C. The supernatant was applied to a MonoQ HR5/5 anion-exchange column (Pharmacia Biotech Inc.) in TEV-PNP and developed with a 0–500 mM NaCl gradient. One-mL fractions were collected and stored at −80 °C.

Survey of L1CD Kinase Activities in PC12 Cells—Soluble proteins obtained from NGF-stimulated PC12 cells were fractionated by chromatography on a MonoQ HR 5/5 column, and the resulting fractions were assayed for kinase activity. Kinase assays contained 10 mM MgCl\textsubscript{2}, 2 mM MnCl\textsubscript{2}, 5 \(\mu\)M [\textsuperscript{32P}]ATP (18 dpm/\textsuperscript{pmol}), and the peptide substrate. L1CD immunoprecipitates or MonoQ fractions were incubated for 30 min at room temperature and stopped by the addition of sample buffer and boiling for 5 min. The reactions were then separated by SDS-PAGE (Laemmli, 1970). The solubilized substrates were visualized by autoradiography.

Peptide Phosphorylation by p90\textsuperscript{rsk}—Lyophilized peptides were resuspended in water to make 10 or 20 mM stock solutions and used at a final concentration of 100 \(\mu\)M. These peptides were tested for the ability to act as a substrate for p90\textsuperscript{rsk}. The reactions were carried out with 15 \(\mu\)M PC12 peptide (2 pg) in TEV-PNP buffer containing 0.2 mM MgCl\textsubscript{2}, 2 mM MnCl\textsubscript{2}, 5 \(\mu\)M [\textsuperscript{32P}]ATP (18 dpm/\textsuperscript{pmol}), and 100 \(\mu\)M peptide for 30 min at room temperature. The reactions were stopped by the addition of sample buffer and boiling for 5 min. The peptides were separated from other proteins in the reaction on a Tris-tricine SDS-PAGE system (Schagger and Von Jagow, 1987) modified with a 19–33% linear gradient resolving gel, and the radiolabeled peptides were visualized by autoradiography.

In Vitro Peptide Phosphorylation by L1-associated Kinases—Substrates for a variety of kinases (c-fos-derived peptides RKGSSEPPSS, RKGSSSNEPSS, and RKAAAESEPS, S6 peptide (RRRLSSLRA), myelin basic protein, Kemptide (LRRASLG), and syntide (PLARTSVGLPGK)) were phosphorylated by L1 immunoprecipitates in in vitro reactions consisting of L1 immunoprecipitates and 0.25 mM peptide in TEV-PNP buffer containing 10 mM MgCl\textsubscript{2}, 2 mM MnCl\textsubscript{2}, and 5 \(\mu\)M [\textsuperscript{32P}]ATP (18 dpm/\textsuperscript{pmol}). These reactions were incubated for 30 min at room temperature and stopped by the addition of trichloroacetic acid at a final concentration of 3.5%. Bovine serum albumin (10 \(\mu\)g) was added as a carrier, and the proteins were precipitated with 15% trichloroacetic acid. The supernatants were spotted in triplicate on P81 phosphocellulose paper (Glass et al., 1978). The P81 strips were washed four times in 75 mM phosphoric acid to remove unbound radioactive ATP. The labeling was assessed by Cerenkov counting in a Beckman LS750 scintillation counter.

Western Blot Analysis—L1 immunoprecipitates or MonoQ fractions from PC12 cell extracts were mixed with sample buffer and boiled for 5 min. The samples were then separated by SDS-PAGE. The proteins were electroblotted to Immobilon-P membranes, and the membrane was then blocked with 5% evaporated nonfat milk in PBS. The primary antibodies, rabbit polyclonals directed against the C terminus of p90\textsuperscript{rsk} (508–525 of rat p90\textsuperscript{rsk}, Transduction Labs, Lexington, KY), were used at a concentration of 1 \(\mu\)g/ml in 5% milk/0.05% Tween 20 in PBS. The membrane was incubated with primary antibody for 1 hour at room temperature with agitation and washed with 0.1% Tween 20 in PBS. The membrane was then probed with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:1000 in 5% milk/0.05% Tween 20PBS) for 1 h and visualized by chemiluminescence.

In Vivo Labeling—Two newborn Sprague-Dawley rat pups were anesthetized and injected intravenicularly with 5 \(\mu\)Ci each of [\textsuperscript{32P}]H\textsubscript{3}PO\textsubscript{4}. The pups were then incubated for 12 h in a humidified incubator at 35 °C. The pups were then anesthetized and sacrificed by decapitation. The brains were dissected and homogenized in TEV-PNP containing protease inhibitors as above, and the membranes were separated by centrifugation and precipitated in SDS-PAGE sample buffer and boiled. The sample was then separated by SDS-PAGE, transferred to Immobilon-P by electroblotting, and visualized by autoradiography. Bands representing the M, 200,000 full-length L1 and the M, 85,000 primary proteolytic breakdown product, which includes the cytoplasmic domain, were excised and digested in preparation for sequencing (see below).

Peptide Sequencing—Sequencing was done by Dr. Carol M. Beach at the University of Kentucky Macromolecular Structure Analysis Facility. L1CD (10 \(\mu\)g) was phosphorylated by 5 pg of partially purified p90\textsuperscript{rsk} in TEV-PNP containing 10 mM MgCl\textsubscript{2}, 2 mM MnCl\textsubscript{2}, 5 \(\mu\)M [\textsuperscript{32P}]ATP (45 dpm/\textsuperscript{pmol}). The sample was then digested with endoproteinase Asp-N for 18 h at 37 °C, and the resulting peptides were separated by HPLC on a C\textsubscript{18} reverse phase column. Collected fractions were analyzed for protein concentration and radioactivity. The fractions containing significant radioactivity were then sequenced with an ABI protein sequencer using covalent sequencing supports.

Peptide Inhibition of Neurite Outgrowth—Dorsal root ganglia were dissected from embryonic day 9 chicks and dissociated with 0.1% collagenase, 0.1% DNase in Ca\textsuperscript{2+}-Mg\textsuperscript{2+}-free PBS and preplated for 60 min to enrich for neurons. Approximately 5 \(\times\) 10\textsuperscript{5} cells were then electroporated in electroporation buffer (100 mM HEPES, 137 mM NaCl, 6 mM D-glucose, and 7 mM Na\textsubscript{2}HPO\textsubscript{4}) containing 250 \(\mu\)M FITC-dextran (average molecular weight, M, 4000) and 250 \(\mu\)M peptide at 800 \(\mu\)g/m of 500 microfarads (Bio-Rad Gene Pulser). The peptides tested were KRSK (KRSGKGYSKVDKKE), S/A1152 (KRSGKGYKAVDKKED), and SCR.

1 The abbreviations used are: NGF, nerve growth factor; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; FITC, fluorescein isothiocyanate; CAM, cell adhesion molecule; CK1\textsubscript{II}, casein kinase II; PBS, phosphate-buffered saline.
(KSGKSKKDGRGYVDE). The electroporated neurons were washed in dorsal root ganglia medium (RPMI 1640, 10% fetal bovine serum, and 20 ng/ml NGF), resuspended in 200 μl dorsal root ganglia medium, and plated on two 50-mm² spots each of L1 or laminin adsorbed to nitrocellulose-coated tissue culture dishes as described previously (Lagenaur and Lemmon, 1987). The neurons were incubated for 8 h at 37 °C, 6% CO₂ in a humidified incubator. They were then washed twice with warm medium to remove debris and unattached cells and observed by fluorescence and phase-contrast microscopy. Of the remaining attached cells, ~50% were loaded with FITC-dextran. Labeled cells in the central 20 mm² of each well were counted and assessed for neurite outgrowth.

**Statistical Analysis of Peptide Loading**—The data obtained from the peptide loading experiments described above were examined using a categorical analysis. In this approach, the proportion of loaded cells bearing neurites was examined using several factors: control (FITC-dextran loaded) versus experimental (FITC-dextran and peptide), L1 versus laminin, the dish the cells were on, and the spot the cells were in the dish. There are interactions between these factors resulting in a design of four factors and three interactions. However, terms involving the dish and spot factors are regarded as insignificant. The difference in the proportion of KRSK-treated neurons bearing neurites on L1 with respect to the other conditions was significant at p = 0.002 by χ² analysis.

**RESULTS**

**L1 Kinase Activity Is Found in PC12 Cell Extracts—**When stimulated by NGF, the PC12 rat pheochromocytoma cell line takes on many of the morphological and physiological properties of sympathetic neurons (Greene et al., 1987). L1 expression is increased upon NGF stimulation (Salton et al., 1983), and activation of L1 upon binding by either antibodies or soluble L1 has been demonstrated to affect levels of intracellular second messenger systems including pH, Ca²⁺, and inositol phosphates (Schuch et al., 1989). PC12 cells have also been shown to undergo L1-dependent neurite outgrowth (Williams et al., 1992). Cytosolic extracts from NGF-stimulated PC12 cells were, therefore, used as a potential source of L1 kinases. These extracts were fractionated by anion-exchange chromatography on a MonoQ column and assayed for their ability to phosphorylate bacte rially produced recombinant L1 cytoplasmic domain (L1CD, amino acids 1144–1257). Analysis of column fractions yielded three peaks of L1 phosphorylating activity (Fig. 1).

**To determine the site at which the kinase present in peak I phosphorylated L1, L1 cytoplasmic domain-derived synthetic peptides** (Fig. 2) were assayed by in vitro phosphorylation experiments, and L1CD phosphorylated by this kinase was sequenced. The synthetic peptides were designed to encompass each of the serines in the L1 cytoplasmic domain sequence, as well as several amino acids to each side of the serine to preserve potential kinase recognition sites. Fig. 3A is an autoradiograph of the results of phosphorylating the nine peptides in vitro with peak I and separating the results by SDS-PAGE. Of these peptides, only the KRSK peptide (KRSKGKYSVK-DKED, amino acids 1144–1158) was phosphorylated. This peptide is derived from the membrane-proximal 15 amino acids and is completely conserved between zebrafish, chick, and mammalian L1. The KRSK peptides contained two serines corresponding to Ser1146 and Ser1152. Ser to Ala mutations were introduced into each site to determine which site(s) were phosphorylated. These mutated peptides were assayed by in vitro kinase assays (Fig. 3B). The mutation of the first serine, Ser1146, caused decreased phosphorylation of the peptide, possibly by altering but not abolishing the recognition site. The peptide carrying a mutation of the second serine, Ser1152, was not phosphorylated. Based on this result, the phosphorylation site was suspected to be at Ser1152.

To confirm this finding, L1CD was phosphorylated by peak I in the presence of γ[32P]ATP and digested with endoproteinase Asp-N, which cleaves proteins on the N-terminal side of aspartic acid residues. The proteolytic fragments were separated by reverse-phase HPLC, and the radioactivity of the resulting fractions was assayed (Fig. 4A). There were two major peaks of radioactivity, exhibiting retention times of 32 and 65 min. Amino acid sequence analysis of these fractions, using covalent sequencing supports to allow tracking of the radiolabeled residue, demonstrated that the 65-min peak contained the fragment phosphorylated by CKII (amino acids 1170–1197; Wong et al., 1996), whereas the 32-min peak was composed of the fragments RSKGGKSVK (amino acids 1145–1154) and DQVQDSEARPMK (amino acids 1158–1169). The site of phosphorylation of the 32-min fragments was determined by assessing the elution of radioactivity from the sequencing reactions and indicated that the phosphorylation was at the eighth residue of the first fragment, corresponding to Ser1152 in the L1 sequence, in agreement with the synthetic peptide results.

S6 Kinase Activity Associates with L1—L1 has been demonstrated to associate with at least two distinct kinase activities in immunoprecipitates (Schuch et al., 1989). To determine the activity of these kinases, L1 immunoprecipitates were incubated with γ[32P]ATP and protein or synthetic peptide substrates for a variety of kinases. Among the substrates tested were c-Fos and Fos-derived peptides, PSSD and RKGS, myelin basic protein, S6 kinase, Kemptide, and syntide. Of the peptides tested, those which were most specific for S6 kinases, S6 kinase, and Kemptide, were most strongly phosphorylated (Fig. 5). To determine whether this phosphorylation was due to p90 kinase activity, rather than p70 or another S6 family kinase, Western blots of L1 immunoprecipitates from rat brain membrane preparations (Fig. 6B) were probed with...
anti-p90\textsuperscript{rsk} antibodies. The results demonstrated that p90\textsuperscript{rsk} immunoreactivity was associated with L1. Another S6 kinase, p70, was unable to phosphorylate L1CD in vitro (data not shown).

p90\textsuperscript{rsk} Phosphorylates L1CD—Previous work has demonstrated that the p90\textsuperscript{rsk} in NGF-stimulated PC12 cell extracts elutes from MonoQ columns within the same fractions as the L1-phosphorylating activity in PC12 peak 1 (Taylor et al., 1994). Western blot analysis of peak 1 confirmed the presence of p90\textsuperscript{rsk} (Fig. 6A), consistent with p90\textsuperscript{rsk} being the L1-phosphorylating kinase in this fraction. Since the PC12 fractions are heterogeneous, containing more than one kinase activity, a commercial preparation of p90\textsuperscript{rsk} was also used to phosphorylate L1CD in vitro to verify that p90\textsuperscript{rsk} can phosphorylate the L1 cytoplasmic domain. Proteolytic digestion and mapping of the phosphorylation site of p90\textsuperscript{rsk}-phosphorylated L1 demonstrated (in Fig. 4B) that there was strong phosphorylation of the fragment containing Ser\textsuperscript{1152} by p90\textsuperscript{rsk}. The p90\textsuperscript{rsk} also phosphorylated the KRSK peptide but not the other L1 synthetic peptides.

The L1 immunoprecipitates from rat brain were incubated with [γ-\textsuperscript{32}P]ATP and L1CD, and in vitro phosphorylation reactions were performed to determine the sites at which these L1-associated kinase activities could act. The radiolabeled L1 cytoplasmic domain was digested with endoproteinase Asp-N, the fragments were separated by HPLC, and the phosphorylation of the resulting fractions was evaluated (Fig. 4C). A peptide exhibiting a retention time of 32 min corresponding to phosphorylation at Ser\textsuperscript{1152} was strongly labeled, in agreement with the results from proteolytic fragment analysis of L1CD phosphorylated by PC12 peak 1 or p90\textsuperscript{rsk} (Fig. 4, A and B).

Finally, to demonstrate the physiological relevance of phosphorylation of L1 at Ser\textsuperscript{1152}, two newborn rats were injected intracranially with [γ-\textsuperscript{32}P]H\textsubscript{3}PO\textsubscript{4}, allowed to survive for 12 hours, and then sacrificed. The L1 was immunoprecipitated from the brains, proteolytically digested, and sites of in vivo phosphorylation were analyzed. Again, there was a peak of radioactivity eluting at 32 min from the HPLC, reflecting the phosphorylation of Ser\textsuperscript{1152} (Fig. 4D). These data demonstrate that L1 is phosphorylated in vivo at Ser\textsuperscript{1152}, and p90\textsuperscript{rsk} specifically phosphorylates L1 at this site.

Effects of Peptide Containing p90\textsuperscript{rsk} Phosphorylation Site on Neurite Outgrowth—The site at which p90\textsuperscript{rsk} phosphorylates L1, Ser\textsuperscript{1152}, is the ninth residue from the membrane in the cytoplasmic domain of L1. The membrane-proximal 40 residues of the cytoplasmic domain of L1 are completely conserved between the known mammalian and avian homologues of L1, and the first 10 residues are similar to L1-related molecules in mammals and Drosophila. This strong evolutionary conservation suggests a functional importance and a role for phosphorylation of Ser\textsuperscript{1152} to regulate this function. To determine what effects they would have on L1-mediated neurite outgrowth, the KR SK peptide (encompassing the first 15 residues of the L1 cytoplasmic domain) previously used in the in vitro kinase assays, the S/A\textsuperscript{1152} peptide, which substitutes an alanine for the phosphorylated serine, and SCR, a scrambled sequence peptide with identical amino acid composition to KRSK, were loaded by electroporation into dorsal root ganglion neurons, together with a fluorescent tracer, FITC-dextran. This tracer is of a similar molecular weight as the peptides, and the loss of the tracer should approximate passive loss of peptide from loaded cells. Although this would not take into account proteolytic degradation of the peptide, a small polypeptide tracer, FITC-labeled polylysine, was also retained by loaded cells at
the time of examination. The electroporation transiently permeabilizes the cells, resulting in uptake of the peptides and tracer molecules. These neurons were then plated on either laminin or L1 and incubated for 8 h, at which time the tracer was still present in loaded cells, and the cells loaded with only FITC-dextran had extended neurites (Fig. 7). The effect of the peptides was determined by measuring the proportion of labeled neurons bearing neurites and compared to the total number of labeled neurons. The results are summarized in Fig. 8. On laminin, approximately 30% of the labeled cells extended neurites in the presence of KRSK peptide (250 μM) loading. Thirty % of the FITC-loaded neurons on L1 bore neurites in the absence of KRSK peptide. However, only 20% of the FITC-labeled, KRSK-treated cells growing on L1 had neurites. Statistical analysis revealed that this reduction was significant at \( p < 0.002 \). These data indicate that the inhibition of neurite outgrowth is specific to L1-mediated interactions and not to the general mechanisms of neurite outgrowth. The unphosphorylatable variant peptide, S/A1152, possibly because it cannot compete for p90<sup>RSK</sup> phosphorylation, had no effect on the percentage of cells with neurites on either laminin or L1. Similarly, the scrambled sequence peptide had no effect on neurite outgrowth on either substrate.

**DISCUSSION**

L1 is a cell adhesion molecule of the immunoglobulin superfamily that binds to L1 molecules on opposing surfaces and to several other molecules as well (Brummendorf et al., 1993; Felsenfeld et al., 1994; Kuhn et al., 1991; Milev et al., 1994). Accumulating evidence suggests that L1 not only mediates adhesion but also acts as a receptor, transducing extracellular interactions into an intracellular second messenger cascade (Doherty and Walsh, 1994), leading ultimately to changes in the behavior of the neuron, influencing migration (Lindner et al., 1983), fasciculation (Stallcup and Beasley, 1985; Landmesser et al., 1988; Cervello et al., 1991), or axonal outgrowth (Lagenaur and Lemmon, 1987).

The morphology of growth cones from chick retinal ganglion cell neurons differs radically depending upon the substrate on which they are growing (Payne et al., 1992). When growth cones migrating on laminin first encounter L1, there are significant morphological changes in the growth cone within 1 min (Burden-Gulley et al., 1995). This change is reflected in the redistribution of cytoskeletal components (Burden-Gulley and Lemmon, 1996) and is consistent with the idea that L1 binding triggers an intracellular signal that leads to cytoskeletal rearrangement (Atashi et al., 1992). In addition to generating signals via L1-L1 binding, L1 may also act as a signal transducing

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**FIG. 5.** *Substrate specificity of L1-associated kinases.* In vitro phosphorylation of various peptide substrates: column 1, PSSD; column 2, Fos; column 3, S6rsk peptide; column 4, myelin basic protein; column 5, Kemptide; column 6, syntide; column 7, RKGS; and column 8, no peptide, by kinase activities coprecipitating with L1 immunoprecipitates on mAb 74–5H7-coated Sepharose beads (●) or rat brain membrane extracts adsorbed to uncoated Sepharose beads (□).

**FIG. 6.** *p90<sup>RSK</sup> Western blots.* A, peaks 1, 2, and 3 of PC12 extracts fractionated by anion-exchange chromatography in a MonoQ column were separated by SDS-PAGE, blotted onto Immobilon-P, and probed with a polyclonal rabbit anti-p90<sup>RSK</sup> antibody. Right, molecular weight markers in thousands. B, an anti-p90<sup>RSK</sup> antibody was used to probe rat brain membrane proteins adsorbed to Sepharose beads (1) or anti-L1 (2; Mab 74–5H7) conjugated beads. Right, molecular weight markers in thousands.

**FIG. 7.** *Peptide inhibition of neurite outgrowth.* Dissociated chick (E9) dorsal root ganglia neurons were electroporated with FITC-dextran only (A, B, E, and F) or in combination with KRSK (C, D, G, and H). The cells were plated on either L1 (A, E, C, and G) or laminin (B, D, F, and H). Arrows indicate cells that were dye-loaded as observed by fluorescence microscopy. Bar, 10 μm.
FIG. 8. Inhibition of neurite outgrowth. Dissociated embryonic (E9) chick dorsal root ganglion neurons were electroporated with FITC-dextran only or in combination with either KRSK, S/A1152, or SCR. The cells were plated on either L1 or laminin. Bars, S.E.M.

receptor for other adhesion molecules. Although axonin-1 homophilic interactions are sufficient for adhesion (Rader et al., 1993), neurite outgrowth involving axonin-1 requires an interaction with the chick L1 homologue, Ng-CAM (Kuhn et al., 1991). Similarly, TAG-1, the mammalian homologue of axonin-1, interacts with L1 to produce neurite outgrowth (Felsenfeld et al., 1994). In these situations, L1/Ng-CAM could act as a signal transducing receptor for TAG-1/axonin-1 in TAG-1/axonin-1 directed neurite outgrowth, since these glycosyl/phosphatidylinositol-linked molecules do not have direct communication inside the cell.

Several different second messenger systems may be involved in L1-mediated signaling, as evidenced by reports of changes in intracellular Ca$^{2+}$, p38, and inositol phosphates upon activation of L1 in a variety of cell types (Schuch et al., 1989; Von Bohlen und Halbach et al., 1992). Recently, Ca$^{2+}$ signaling was linked to Ng-CAM expression during neuronal migration in bird forebrain (Goldman et al., 1996). Doherty and Walsh (1994) have advanced the idea that activation of a variety of cell adhesion molecules, including L1, leads to activation of the fibroblast growth factor receptor and subsequently to an arachidonic acid second messenger cascade (Doherty and Walsh, 1994). This cascade involves generation of diacylglycerol by phospholipase C, conversion to arachidonic acid by diacylglycerol lipase, and calcium influx through L- and N-type channels (Doherty et al., 1994; Williams et al., 1994a, 1994b). The nonreceptor tyrosine kinase Src has also been implicated in neurite outgrowth on L1: neurons from src-knockout mice have a diminished capacity to extend neurites on an L1 substrate (Ignelzi et al., 1994).

On the other hand, relatively little is known about the factors that may regulate the functions of L1 in activating such signaling systems. L1 is both alternatively spliced (Miura et al., 1984) in the cytoplasmic domain. The phosphorylation suggested a potential mechanism by which L1 activity could be modulated. L1 has a diminished capacity to extend neurites on an L1 substrate (Ignelzi et al., 1994).

The nonreceptor tyrosine kinase Src has also been implicated in neurite outgrowth on L1: neurons from src-knockout mice have a diminished capacity to extend neurites on an L1 substrate (Ignelzi et al., 1994). In these systems, Src activity is linked to this kinase. Among these are insulin-regulated glycogen metabolism, platelet (Papkoff et al., 1994) and T-cell activation (Calvo et al., 1992), stress responses (Jurvich et al., 1991), and neuronal differentiation of PC12 cells (Socci et al., 1992). The mitogen-activated pathways leading to p90$^{rsk}$ stimulation involve activation of a receptor tyrosine kinase, followed by sequential activation of Raf, MEK (mitogen-associated protein kinase kinase), erk-1 and erk-2 mitogen-activated protein kinases, and p90$^{rsk}$ (Blenis, 1993). Activation of such a cascade has been described upon binding of IgM on B lymphocytes (Tordai et al., 1994). Although p90$^{rsk}$ is considered a cytosolic protein, which when activated can translocate to the nucleus, it is also found in membrane fractions (Chen et al., 1992), and we have recently found p90$^{rsk}$ in growth cone particle preparations purified from rat brain (data not shown).

Ser$^{1152}$, the site of p90$^{rsk}$ action, is located nine amino acids from the membrane, within one of the most highly conserved regions of the molecule. Of note, this serine is conserved only in the closest homologues of L1 and is not in related members of the L1 group of immunoglobulin superfamily adhesion molecules, including the chick proteins Nr-CAM (Grumet et al., 1991) and neurofascin (Rathjen et al., 1987a) or the rat ankryin-binding glycoprotein (Davis et al., 1993). The 10 membrane-proximal intracellular residues of Nr-CAM, neurofascin, and ankryin-binding glycoprotein are identical to L1 except Ser$^{1152}$, which is changed to a proline residue, implying an
important function for this region. The presence of the serine at residue 1152 only in L1 may allow its functional regulation by phosphorylation. The KRSK peptide inhibition studies described here show that perturbation of interactions with this region of the L1 cytoplasmic domain disturbs L1-mediated neurite outgrowth. There is a 33% decrease in the percentage of cells bearing neurites when loaded with the KRSK peptide compared to tracer dye alone or scrambled sequence peptide. Interestingly, the S/A1152 peptide, which is a nonphosphorylatable KRSK peptide, has no significant effect by this measure, indicating that phosphorylation of KRSK is involved in this inhibition of neurite outgrowth. One mechanism by which the inhibition may take place is by competitive inhibition of p90rsk phosphorylation of L1, preventing it from undergoing phosphorylation-dependent conformational changes or protein-protein interactions. Another possible mechanism is that phosphorylation of the KRSK peptide allows it to interact with some other protein, which normally interacts with L1 only when Ser1152 is phosphorylated. Although the putative protein interaction is not known, it is unlikely to be the recently described ankyrin-L1 interaction (Davis and Bennett, 1994), which has been mapped to a region between residues 1200–1230.

The data presented here indicate that L1 is associated with and phosphorylated by the S6 kinase p90rsk, the substrate site of which is Ser1152. Disruption of interactions between L1 and p90rsk or other proteins in the vicinity of Ser1152 has a significant deleterious effect on neurite outgrowth. One of the initial hypotheses in searching for L1 kinases was that they may transiently alter L1 function. The first L1 kinase we found, CKII, is generally in a constitutively active state and unlikely to be regulated. However, p90rsk has previously been well studied as part of an extracellularly initiated signal transduction cascade. Therefore, in contrast to CKII, p90rsk could be involved in a transient change in the phosphorylation state of L1 and consequently lead to changes in the conformational and functional state of L1 that determine the distinct morphological and motile characteristics of neurite outgrowth on L1.

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