

# Involvement of p90<sup>rsk</sup> in Neurite Outgrowth Mediated by the Cell Adhesion Molecule L1\*

(Received for publication, April 15, 1996)

Eric V. Wong<sup>‡</sup>, Andrew W. Schaefer<sup>‡</sup>, Gary Landreth<sup>‡§</sup>, and Vance Lemmon<sup>‡¶</sup>

From the Departments of <sup>‡</sup>Neurosciences and <sup>§</sup>Neurology, Case Western Reserve University, Cleveland, Ohio 44106-4975

**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<sup>1152</sup>. Western blot analysis demonstrated that the L1 kinase activity from PC12 cells that phosphorylated this site was co-eluted with the S6 kinase, p90<sup>rsk</sup>. Moreover, S6 kinase activity and p90<sup>rsk</sup> immunoreactivity co-immunoprecipitate with L1 from brain, and metabolic labeling studies have demonstrated that Ser<sup>1152</sup> 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. We performed studies to investigate the functional significance of this phosphorylation. Neurons were loaded with peptides that encompass the phosphorylation site, as well as the flanking regions, and their effects on neurite outgrowth were observed. The peptides, which include Ser<sup>1152</sup>, inhibit neurite outgrowth on L1 but not on a control substrate, laminin. A nonphosphorylatable peptide carrying a Ser to Ala mutation did not affect neurite outgrowth on either substrate. These data demonstrate that the membrane-proximal 15 amino acids of the cytoplasmic domain of L1 are important for neurite outgrowth on L1, and the interactions it mediates may be regulated by phosphorylation of Ser<sup>1152</sup>.**

strate-bound extracellular matrix molecules or cell surface molecules. Cell adhesion molecules are often involved in providing a suitable substrate upon which neurons can migrate or extend axons. L1 is a cell adhesion molecule that has been implicated in a variety of processes integral to the development of the nervous system, including neuronal migration (Lindner *et al.*, 1983), neurite outgrowth (Lagenaur and Lemmon, 1987), and axon fasciculation (Landmesser *et al.*, 1988; Stallcup and Beasley, 1985). Mutations in the L1 gene are linked to the human mental retardation diseases X-linked hydrocephalus and MASA (mental retardation, aphasia, shuffling gait, and adducted thumbs) syndrome (Rosenthal *et al.*, 1992; Wong *et al.*, 1995), in which defects of the corticospinal tract and corpus callosum are commonly found.

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 (Moos *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 (Faissner *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

The development of a functional nervous system depends, in part, on the ability of neurons to form the requisite specific connections with their targets. The guidance of axons through varied terrain and over relatively long distances is thought to be influenced by a variety of factors. These include physical channels and chemical signals, either diffusible factors or sub-

\* This work was supported by National Eye Institute Grant 5285 (to V. L.), NINDS, National Institutes of Health Grant 31987 (to G. L.), and National Science Foundation Grant IBN94-10433 (to G. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Neurosciences, Case Western Reserve University, 2109 Adelbert Rd., Cleveland, OH 44106-4975. Phone 216-368-3039; Fax: 216-368-4650; E-mail: vxl@po.cwru.edu.

one of these as casein kinase II, which phosphorylates L1 at Ser<sup>1181</sup>. In this paper, we demonstrate that an S6 family kinase is also associated with L1.

The serine/threonine kinase, p90<sup>rsk</sup>, was initially identified on the basis of its ability to phosphorylate the ribosomal 40 S subunit *in vitro*. This enzyme 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). The role of p90<sup>rsk</sup> in the nervous system has not been studied directly, but it is reported to be part of an NGF<sup>1</sup>-inducible signaling cascade in PC12 pheochromocytoma cells (Scimeca *et al.*, 1992). This paper describes the phosphorylation of a neural cell adhesion molecule, L1, by p90<sup>rsk</sup>. p90<sup>rsk</sup> associates with L1 at the membrane and phosphorylates L1 at Ser<sup>1152</sup>. This phosphorylation may regulate the interactions of L1 and intracellular signaling cascades or cytoskeletal elements involved in neurite outgrowth on specific substrates.

#### EXPERIMENTAL PROCEDURES

**Materials**—Protease inhibitors Pefabloc SC and aprotinin and horseradish peroxidase-conjugated goat anti-rabbit antibodies were from Boehringer Mannheim. Purified (>50%) p90<sup>rsk</sup> kinase was obtained from Upstate Biochemicals, Inc. (Lake Placid, NY). Anti-p90<sup>rsk</sup> polyclonal antibodies were purchased from Transduction Laboratories (Lexington, KY). [<sup>32</sup>P]H<sub>2</sub>PO<sub>4</sub> 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 (7S) 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 EGTA, 1 mM sodium orthovanadate, and 10 mM *p*-nitrophenyl phosphate (TEV-PNP) containing 0.32 M sucrose, 200 μM Pefabloc SC, and 100 μg/ml aprotinin. The homogenates were separated by ultracentrifugation on a discontinuous sucrose gradient (0.32 M–0.8 M–1.2 M) 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 45 min at 150,000 × *g* at 4 °C to remove insoluble material. The solubilized membrane fraction was then incubated for >4 h at 4 °C with Sepharose beads conjugated to a monoclonal anti-L1 antibody, mAb 74-5H7 (Lemmon *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<sup>2+</sup>-affinity chromatography using Ni-NTA agarose beads (Qiagen), using the manufacturer's protocols.

**PC12 Cell Cytosolic Extracts**—PC12 cells (8–10 × 10<sup>7</sup> 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 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<sub>2</sub>, 2 mM MnCl<sub>2</sub>, 5 μM [<sup>32</sup>P]ATP (18 dpm/fmol), and the peptide substrate. L1CD was included at a final concentration of 1.5 μM. The reactions 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 radiolabeled substrates were visualized by autoradiography.

**Peptide Phosphorylation by p90<sup>rsk</sup>**—Lyophilized peptides were resuspended in water to make 10 or 20 mM stock solutions and used at a final concentration of 100 μM. These peptides were tested for the ability to act as a substrate for p90<sup>rsk</sup>. The reactions were carried out with 15 μl PC12 peak I or partially purified p90<sup>rsk</sup> (2 pg) in TEV-PNP buffer containing 10 mM MgCl<sub>2</sub>, 2 mM MnCl<sub>2</sub>, 5 μM [<sup>32</sup>P]ATP (18 dpm/fmol), and 100 μM 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 RKGSSS-NEPSSD, RKGSSSNEPSSD, and RKGAAANEPSSD, S6 peptide (RRRLSSLRA), myelin basic protein, Kemptide (LRRASLG), and syn-tide (PLARTSLVAGLPGKK)) 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<sub>2</sub>, 2 mM MnCl<sub>2</sub>, and 5 μM [<sup>32</sup>P]ATP (18 dpm/fmol). 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 μg) was added as a carrier, and the proteins were precipitated by incubation at 4 °C for 15 min and centrifuged. The supernatants were spotted in triplicate onto 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 membrane, 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<sup>rsk</sup> (amino acids 508–525 of rat p90<sup>rsk</sup>; Transduction Labs, Lexington, KY), were used at a concentration of 1 μg/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 20/PBS) for 1 h and visualized by chemiluminescence.

**In Vivo Labeling**—Two newborn Sprague-Dawley rat pups were anesthetized and injected intraventricularly with 5 mCi each of [<sup>32</sup>P]H<sub>2</sub>PO<sub>4</sub>. 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 solubilized in Triton X-100. The membrane extract was incubated for 4 h with anti-L1-conjugated beads. The beads were washed three times with TEV-PNP and resuspended 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<sub>r</sub> 200,000 full-length L1 and the M<sub>r</sub> 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 μg) was phosphorylated by 5 pg of partially purified p90<sup>rsk</sup> in TEV-PNP containing 10 mM MgCl<sub>2</sub>, 2 mM MnCl<sub>2</sub>, 5 μM [<sup>32</sup>P]ATP (45 dpm/fmol). 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<sub>18</sub> 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<sup>2+</sup>-/Mg<sup>2+</sup>-free PBS and preplated for 60 min to enrich for neurons. Approximately 5 × 10<sup>5</sup> cells were then electroporated in electroporation buffer (100 mM HEPES, 137 mM NaCl, 6 mM D-glucose, and 7 mM Na<sub>2</sub>HPO<sub>4</sub>) containing 250 μM FITC-dextran (average molecular weight, M<sub>r</sub> 4000) and 250 μM peptide at 800 V/cm and 500 microfarads (Bio-Rad Gene Pulser). The peptides tested were KRSK (KRSKGGKYAVKDKED), S/A<sup>1152</sup> (KRSKGGKYAVKDKED), and SCR

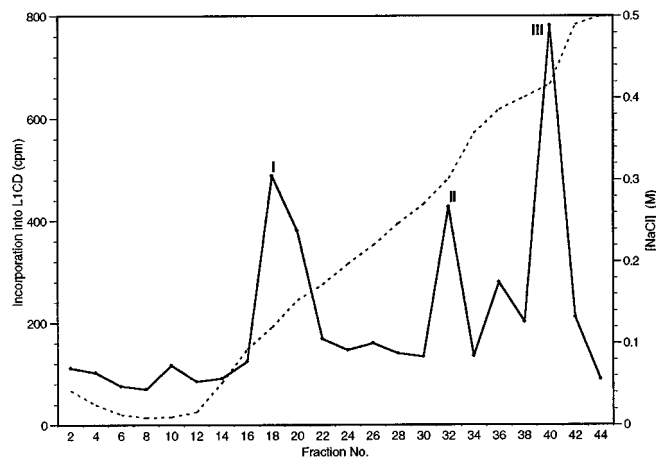
<sup>1</sup> The abbreviations used are: NGF, nerve growth factor; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; FITC, fluorescein isothiocyanate; CAM, cell adhesion molecule; CKII, casein kinase II; PBS, phosphate-buffered saline.

(KSGKSKKDRKGYVDE). The electroporated neurons were washed in dorsal root ganglia medium (RPMI 1640, 10% fetal bovine serum, and 20 ng/ml NGF), resuspended in 200  $\mu$ l dorsal root ganglia medium, and plated on two 50-mm<sup>2</sup> 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% CO<sub>2</sub> in a humidified incubator. They were then washed twice with warmed 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 mm<sup>2</sup> 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 on 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  $\chi^2$  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, Ca<sup>2+</sup>, 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 bacterially produced recombinant L1 cytoplasmic domain (L1CD, amino acids 1144–1257). Analysis of column frac-



**FIG. 1. PC12 extract characterization.** PC12 cytosolic extracts were separated on a MonoQ HR 5/5 anion exchange column, and 1-ml fractions collected. Fractions were incubated with L1CD and [<sup>32</sup>P]ATP to assay for L1-phosphorylating activity. The dotted line indicates salt concentration of the eluted fractions, and the solid line indicates incorporation of <sup>32</sup>P<sub>o</sub> into L1CD.

KRSKGGKYSVKDKEDTQVDSEARPMKDETFGEYRSLESNDNEEKAFGSSQPSLNGDIKPLGSDDSLADYGGSDVDVQFNEEDGSFIGQYSGKKEKEAAGNDSSGATSPINPAVALE  
 "KRSK" "SEAR" "RSLE" "VC11" "LADY" "VDV" "NED" "VC10" "SPIN"  
 KRSKGGKYSVKDKED QVDSEARPM FGEYRSLESNDNEE KAFGSSQPSLNGD DYGGSDVDVQ FIGQYSGKKEKE GGNDSSGATSPIN  
 SDDSLADY NEDGSFTGQ

**FIG. 2. L1 peptides.** Small (8 to 15 amino acids) peptides were synthesized for use in phosphorylation and function-blocking experiments. Each peptide contains at least one serine, and all serines of the L1 cytoplasmic domain are represented in this set of peptides.

tions yielded three peaks of L1 phosphorylating activity (Fig. 1). Peak III has been identified as casein kinase II and phosphorylates L1 at Ser<sup>1181</sup> (Wong *et al.*, 1996).

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 (KRSKGGKYSVK-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 Ser<sup>1146</sup> and Ser<sup>1152</sup>. 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, Ser<sup>1146</sup>, caused decreased phosphorylation of the peptide, possibly by altering but not abolishing the recognition site. The peptide carrying a mutation of the second serine, Ser<sup>1152</sup>, was not phosphorylated. Based on this result, the phosphorylation site was expected to be at Ser<sup>1152</sup>.

To confirm this finding, L1CD was phosphorylated by peak I in the presence of  $\gamma$ -[<sup>32</sup>P]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 assessed (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 RSKGGKYSVK (amino acids 1145–1154) and DTQVDSEARPMK (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 Ser<sup>1152</sup> 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 identity of these kinases, L1 immunoprecipitates were incubated with  $\gamma$ -[<sup>32</sup>P]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 RKGSSS, myelin basic protein, S6/rsk peptide, Kemptide, and syntide. Of the peptides tested, those which were most specific for S6 kinases, S6/rsk peptide and Kemptide, were most strongly phosphorylated (Fig. 5). To determine whether this phosphorylation was due to p90<sup>rsk</sup> activity, rather than p70 or another S6 family kinase, Western blots of L1 immunoprecipitates from rat brain membrane preparations (Fig. 6B) were probed with

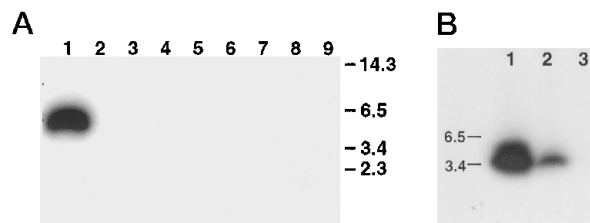


FIG. 3. **L1 peptide phosphorylation by PC12 peak 1.** *A*, autoradiograph of peak 1 phosphorylation of the peptides KRSK (Lane 1), SEAR (Lane 2), RSLE (Lane 3), VC11 (Lane 4), LADY (Lane 5), VDV (Lane 6), VC10 (Lane 7), NED (Lane 8), and SPIN (Lane 9), derived from the L1 cytoplasmic domain. *Right*, molecular weight markers in thousands. *B*, autoradiograph of KRSK (Lane 1), S/A<sup>1146</sup> (Lane 2), and S/A<sup>1152</sup> (Lane 3) peptides tested in phosphorylation reactions with peak 1. *Left*, molecular weight markers in thousands.

anti-p90<sup>rsk</sup> antibodies. The results demonstrated that p90<sup>rsk</sup> immunoreactivity was associated with L1. Another S6 kinase, p70, was unable to phosphorylate L1CD *in vitro* (data not shown).

**p90<sup>rsk</sup> Phosphorylates L1CD**—Previous work has demonstrated that the p90<sup>rsk</sup> in NGF-stimulated PC12 cell extracts elutes from MonoQ columns within the same fractions as the L1-phosphorylating activity in PC12 peak I (Taylor *et al.*, 1994). Western blot analysis of peak I confirmed the presence of p90<sup>rsk</sup> (Fig. 6A), consistent with p90<sup>rsk</sup> 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<sup>rsk</sup> was also used to phosphorylate L1CD *in vitro* to verify that p90<sup>rsk</sup> can phosphorylate the L1 cytoplasmic domain. Proteolytic digestion and mapping of the phosphorylation site of p90<sup>rsk</sup>-phosphorylated L1 demonstrated (in Fig. 4B) that there was strong phosphorylation of the fragment containing Ser<sup>1152</sup> by p90<sup>rsk</sup>. The p90<sup>rsk</sup> also phosphorylated the KRSK peptide but not the other L1 synthetic peptides.

The L1 immunoprecipitates from rat brain were incubated with [ $\gamma$ -<sup>32</sup>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<sup>1152</sup> was strongly labeled, in agreement with the results from proteolytic fragment analysis of L1CD phosphorylated by PC12 peak I or p90<sup>rsk</sup> (Fig. 4, A and B).

Finally, to demonstrate the physiological relevance of phosphorylation of L1 at Ser<sup>1152</sup>, two newborn rats were injected intracranially with [<sup>32</sup>P]H<sub>3</sub>PO<sub>4</sub>, 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<sup>1152</sup> (Fig. 4D). These data demonstrate that L1 is phosphorylated *in vivo* at Ser<sup>1152</sup>, and p90<sup>rsk</sup> specifically phosphorylates L1 at this site.

**Effects of Peptide Containing p90<sup>rsk</sup> Phosphorylation Site on Neurite Outgrowth**—The site at which p90<sup>rsk</sup> phosphorylates L1, Ser<sup>1152</sup>, 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<sup>1152</sup> to regulate this function. To determine what

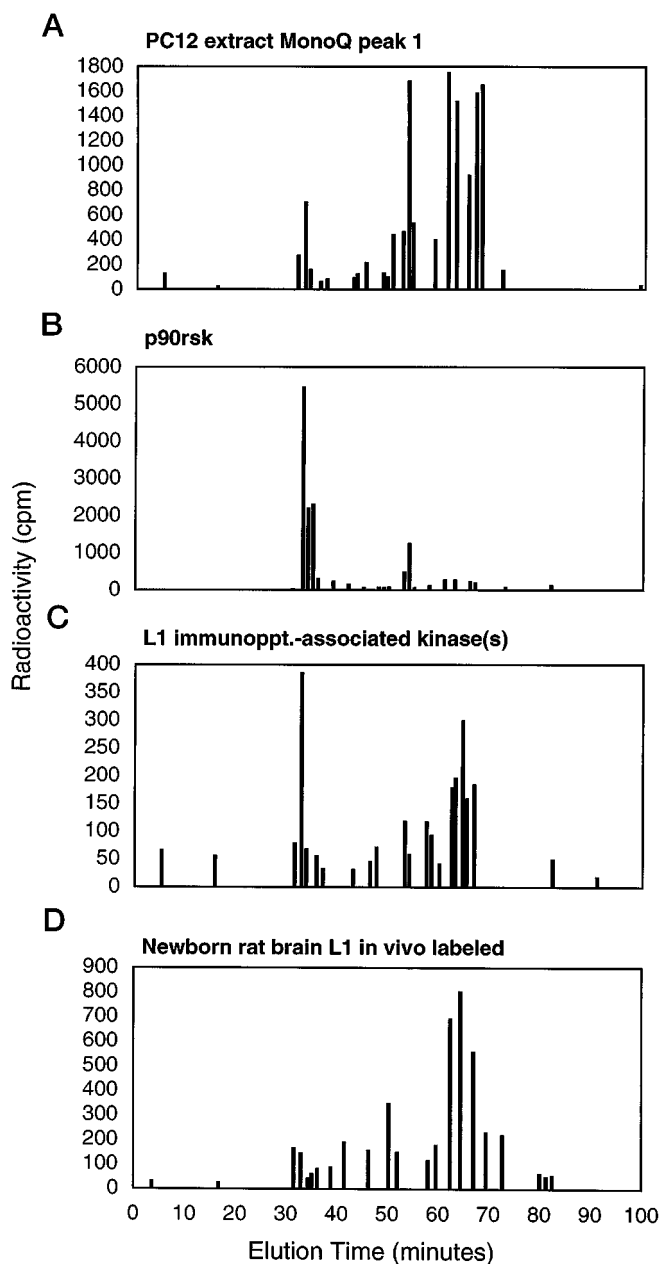


FIG. 4. **Proteolytic fragment analyses of phosphorylated L1.** L1CD was phosphorylated *in vitro* by PC12 peak 1 (A), p90<sup>rsk</sup> (B), and L1 immunoprecipitates from rat brain (C). Metabolically labeled L1 was obtained from newborn rats that were radiolabeled with <sup>32</sup>P *in vivo*, and the L1 immunoprecipitated from the brain. Proteolytic fragments of L1CD or L1 were obtained by digestion with endoproteinase Asp-N. The resulting fragments were separated by reverse-phase HPLC, and the eluted fractions were assayed for radioactivity (D). The peak at 32 min was sequenced.

effects they would have on L1-mediated neurite outgrowth, the KRSK peptide (encompassing the first 15 residues of the L1 cytoplasmic domain) previously used in the *in vitro* kinase assays, the S/A<sup>1152</sup> 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

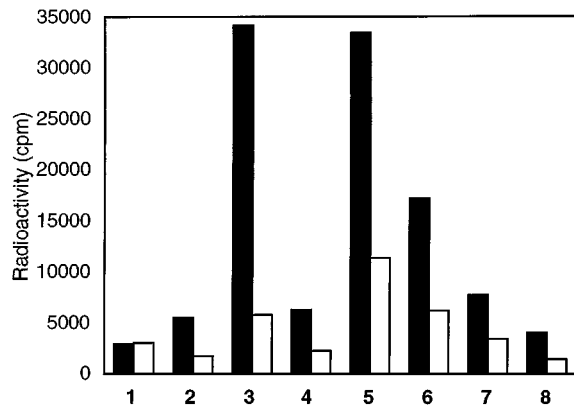


FIG. 5. **Substrate specificity of L1-associated kinases.** *In vitro* phosphorylation of various peptide substrates: column 1, PSSD; column 2, Fos; column 3, S6/rsk peptide; column 4, myelin basic protein; column 5, Kemptide; column 6, syntide; column 7, RKGSSS; and column 8, no peptide, by kinase activities coprecipitating with L1 immunoprecipitates on mAb 74-5H7-coated Sepharose beads (■) or rat brain membrane extracts adsorbed to uncoated Sepharose beads (□).

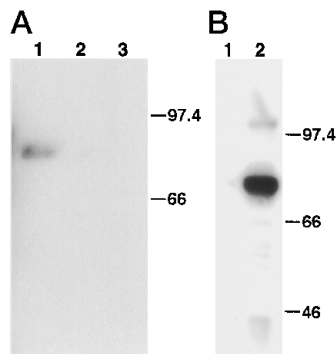


FIG. 6. **p90<sup>rsk</sup> Western blots.** *A*, peaks 1, 2, and 3 of PC12 extracts fractionated by anion-exchange chromatography in a MonoQ column were separated by SDS-PAGE, blotted onto Immobilon-P, and probed with a polyclonal rabbit anti-p90<sup>rsk</sup> antibody. *Right*, molecular weight markers 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.

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 and absence of KRSK peptide (250  $\mu$ M) 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/A<sup>1152</sup>, 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.

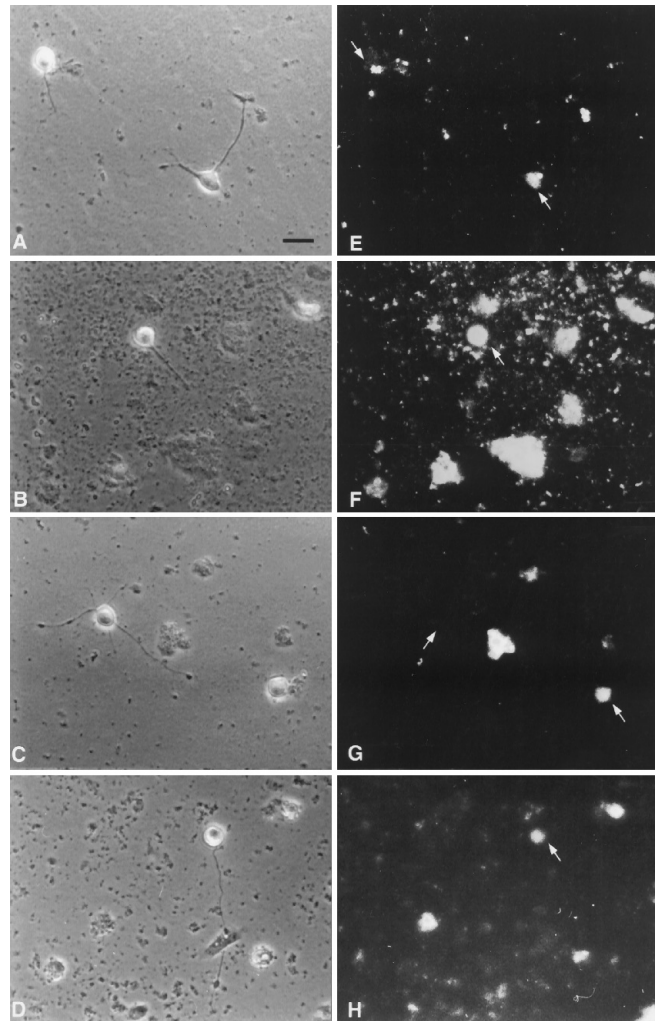


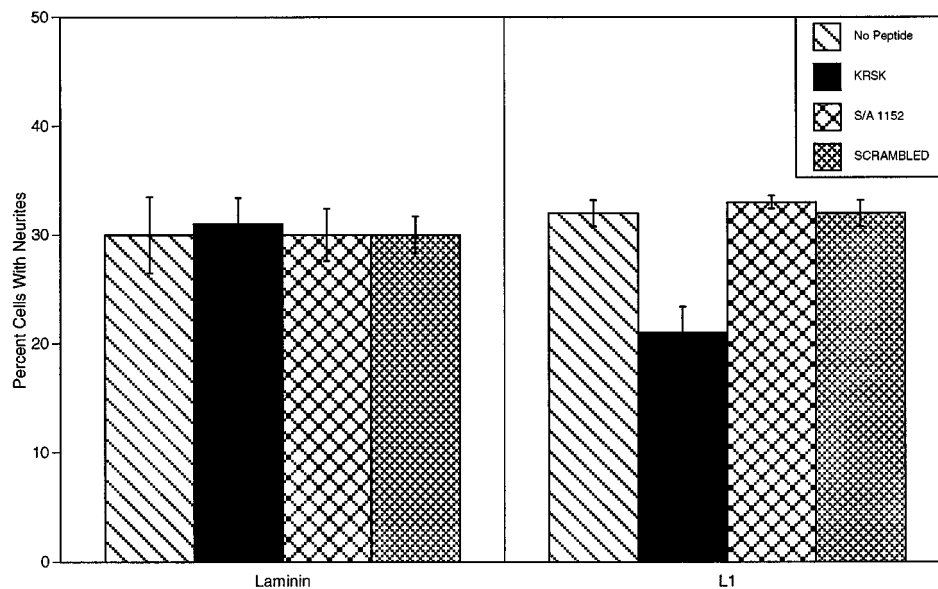
FIG. 7. **Peptide inhibition of neurite outgrowth.** Dissociated chick (E9) dorsal root ganglia neurons were electroporated with FITC-dextran only (*A*, *B*, *E*, and *F*) or in combination with KRSK (*C*, *D*, *G*, and *H*). The cells were plated on either L1 (*A*, *E*, *C*, and *G*) or laminin (*B*, *D*, *F*, and *H*). Arrows indicate cells that were dye-loaded as observed by fluorescence microscopy. Bar, 10  $\mu$ m.

## 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

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/A<sup>1152</sup>, 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 glycosylphosphatidylinositol-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<sup>2+</sup>, pH, 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<sup>2+</sup> 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 $\gamma$ , 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.*, 1991) and phosphorylated (Faissner *et al.*, 1984) in the cytoplasmic domain. The phosphorylation suggested a potential mechanism by which L1 activity could be modulated. L1 has been found to be associated with a number of kinases (Sadoul *et al.*, 1989), including casein kinase II (Wong *et al.*, 1996). We have previously shown that CKII can phosphorylate L1 on Ser<sup>1181</sup> and that it is associated with L1. Continuation of the search for L1 kinases revealed p90<sup>rsk</sup>, which also coprecipitates with L1 from rat brain membrane preparations.

p90<sup>rsk</sup> is well described in several systems (Blenis, 1993; Erikson, 1991) and can be activated when it is phosphorylated by mitogen-associated protein kinase kinases. The p90<sup>rsk</sup> ki-

nase is composed of two kinase domains, an N-terminal cGMP-dependent kinase-like domain and a C-terminal domain bearing resemblance to the catalytic domains of phosphorylase *b* and Ca<sup>2+</sup>/calmodulin kinases (Alcorta *et al.*, 1989). Recent data suggest that the N-terminal catalytic domain mediates substrate phosphorylation, whereas the C-terminal domain is involved in autophosphorylation (Bjorbaek *et al.*, 1995). There is no clear consensus recognition site for p90<sup>rsk</sup>, but like the cGMP- and cAMP-dependent kinases, there is a general requirement for basic residues in the vicinity of the target serine or threonine. The residues around Ser<sup>1152</sup> do not fit the RXXS consensus found in several p90<sup>rsk</sup> target sites, but the serine is bracketed by arginine residues in the form RXXSR and may represent a novel site for p90<sup>rsk</sup> phosphorylation.

The discovery of p90<sup>rsk</sup> began with a search for protein kinases that inducibly phosphorylate the S6 protein (Erikson, 1991; Novak-Hofer and Thomas, 1984; Sturgill and Wu, 1991). Thus, it was expected to be involved in mitogen-stimulated pathways. However, several other functions have now been 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 (Jurivich *et al.*, 1991), and neuronal differentiation of PC12 cells (Scimeca *et al.*, 1992). The mitogen-activated pathways leading to p90<sup>rsk</sup> 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-associated protein kinases, and p90<sup>rsk</sup> (Blenis, 1993). Activation of such a cascade has been described upon binding of IgM on B lymphocytes (Tordai *et al.*, 1994). Although p90<sup>rsk</sup> 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<sup>rsk</sup> in growth cone particle preparations purified from rat brain (data not shown).

Ser<sup>1152</sup>, the site of p90<sup>rsk</sup> 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 ankyrin-binding glycoprotein (Davis *et al.*, 1993). The 10 membrane-proximal intracellular residues of Nr-CAM, neurofascin, and ankyrin-binding glycoprotein are identical to L1 except Ser<sup>1152</sup>, 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/A<sup>1152</sup> 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 p90<sup>rsk</sup> 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 Ser<sup>1152</sup> 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 p90<sup>rsk</sup>, the substrate site of which is Ser<sup>1152</sup>. Disruption of interactions between L1 and p90<sup>rsk</sup> or other proteins in the vicinity of Ser<sup>1152</sup> 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 acutely regulated. However, p90<sup>rsk</sup> has previously been well studied as part of an extracellularly initiated signal transduction cascade. Therefore, in contrast to CKII, p90<sup>rsk</sup> 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.

**Acknowledgments**—We are grateful for the assistance of Dr. Carol M. Beach of the University of Kentucky Macromolecular Structure Analysis Facility who performed the sequencing of the L1 fragments and to Dr. Paul A. Thompson of the Department of Psychiatry at Case Western Reserve University for statistical analysis of our data.

## REFERENCES

- Alcorta, D. A., Crews, C. M., Sweet, L. J., Bankston, L., Jones, S. W., and Erikson, R. L. (1989) *Mol. Cell. Biol.* **9**, 3850–3859
- Atashi, J. R., Klinz, S. G., Ingraham, C. A., Matten, W. T., Schachner, M., and Maness, P. F. (1992) *Neuron* **8**, 831–842
- Bieber, A. J., Snow, P. M., Hortsch, M., Patel, N. H., Jacobs, J. R., Traquina, Z. R., Schilling, J., and Goodman, C. S. (1989) *Cell* **59**, 447–460
- Bjorbaek, C., Zhao, Y., and Moller, D. E. (1995) *J. Biol. Chem.* **270**, 18848–18852
- Blenis, J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 5889–5892
- Brummendorf, T., Hubert, M., Treubert, U., Leuschner, R., Tarnok, A., and Rathjen, F. G. (1993) *Neuron* **10**, 711–727
- Burden-Gulley, S. M., and Lemmon, V. (1995) *Semin. Dev. Biol.* **6**, 79–87
- Burden-Gulley, S. M., and Lemmon, V. (1996) *Cell Motil. Cytoskeleton*, in press
- Burden-Gulley, S. M., Payne, H. R., and Lemmon, V. (1995) *J. Neurosci.* **15**, 4370–4381
- Calvo, V., Bierer, B. E., and Vik, T. A. (1992) *Eur. J. Immunol.* **22**, 457–462
- Cervello, M., Lemmon, V., Landreth, G., and Rutishauser, U. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 10548–10552
- Chen, R. H., Sarnecki, C., and Blenis, J. (1992) *Mol. Cell. Biol.* **12**, 915–927
- Davis, J. Q., and Bennett, V. (1994) *J. Biol. Chem.* **269**, 27163–27166
- Davis, J. Q., McLaughlin, T., and Bennett, V. (1993) *J. Cell Biol.* **121**, 121–133
- Doherty, P., and Walsh, F. S. (1994) *Curr. Opin. Neurobiol.* **4**, 49–56
- Doherty, P., Furness, J., Williams, E. J., and Walsh, F. S. (1994) *J. Neurochem.* **62**, 2124–2131
- Erikson, R. (1991) *J. Biol. Chem.* **266**, 6007–6010
- Faissner, A., Kruse, J., Nieke, J., and Schachner, M. (1984) *Dev. Brain Res.* **15**, 69–82
- Felsenfeld, D. P., Hynes, M. A., Skoler, K. M., Furley, A. J., and Jessell, T. M. (1994) *Neuron* **12**, 675–690
- Glass, D. B., Masaracchia, R. A., Feramisco, J. R., and Kemp, B. E. (1978) *Anal. Biochem.* **87**, 566–575
- Goldman, S. A., Williams, S., Barami, K., Lemmon, V., and Nedergaard, M. (1996) *Mol. Cell. Neurosci.* **7**, 29–45
- Greene, L., Aletta, J., Rukenstein, A., and Green, S. (1987) *Methods Enzymol.* **147**, 207–216
- Grumet, M., Hoffman, S., and Edelman, G. M. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 267–271
- Grumet, M., Mauro, V., Burgoon, M. P., Edelman, G. M., and Cunningham, B. A. (1991) *J. Cell Biol.* **113**, 1399–1412
- Hlavin, M. L., and Lemmon, V. (1991) *Genomics* **11**, 416–423
- Ignelzi, M. A., Jr., Miller, D. R., Soriano, P., and Maness, P. F. (1994) *Neuron* **12**, 873–884
- Itoh, K., Kawamura, H., and Asou, H. (1992) *Brain Res.* **580**, 233–240
- Jurivich, D. A., Chung, J., and Blenis, J. (1991) *J. Cell. Physiol.* **148**, 252–259
- Kuhn, T. B., Stoeckli, E. T., Condrau, M. A., Rathjen, F. G., and Sonderegger, P. (1991) *J. Cell Biol.* **115**, 1113–1126
- Laemmli, U. K. (1970) *Nature* **227**, 680–685
- Lagenaur, C., and Lemmon, V. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 7753–7757
- Landmesser, L., Dahm, L., Schultz, K., and Rutishauser, U. (1988) *Dev. Biol.* **130**, 645–670
- Lemmon, V., and McLoon, S. (1986) *J. Neurosci.* **6**, 2987–2994
- Lemmon, V., Farr, K., and Lagenaur, C. (1989) *Neuron* **2**, 1597–1603
- Lindner, J., Rathjen, F. G., and Schachner, M. (1983) *Nature* **305**, 427–430
- Milev, P., Friedlander, D. R., Sakuri, T., Karthikeyan, L., Flad, M., Margolis, R. K., Grumet, M., and Margolis, R. U. (1994) *J. Cell Biol.* **127**, 1703–1715
- Miura, M., Kobayashi, M., Asou, H., and Uyemura, K. (1991) *FEBS Lett.* **289**, 91–95
- Moos, M., Tacke, R., Scherer, H., Teplow, D., Fruh, K., and Schachner, M. (1988) *Nature* **334**, 701–703
- Novak-Hofer, I., and Thomas, G. (1984) *J. Biol. Chem.* **259**, 5995–6000
- Papkoﬀ, J., Chen, R. H., Blenis, J., and Forsman, J. (1994) *Mol. Cell. Biol.* **14**, 463–472
- Payne, H. R., Burden, S. M., and Lemmon, V. (1992) *Cell Motil. Cytoskeleton* **21**, 65–73
- Prince, J. T., Milona, N., and Stallcup, W. B. (1989) *J. Neurosci.* **9**, 1825–1834
- Rader, C., Stoeckli, E. T., Ziegler, U., Osterwalder, T., Kunz, B., and Sonderegger, P. (1993) *Eur. J. Biochem.* **215**, 133–141
- Rathjen, F. G., Wolff, J. M., Chang, S., Bonhoeffer, F., and Raper, J. (1987a) *Cell* **51**, 841–849
- Rathjen, F. G., Wolff, J. M., Frank, R., Bonhoeffer, F., and Rutishauser, U. (1987b) *J. Cell Biol.* **104**, 343–353
- Rosenthal, A., Jouet, M., and Kenrick, S. (1992) *Nat. Genet.* **2**, 107–112
- Sadoul, R., Kirchoﬀ, F., and Schachner, M. (1989) *J. Neurochem.* **53**, 1471–1478
- Salton, S. R. J., Shelanski, M. L., and Greene, L. A. (1983) *J. Neurosci.* **3**, 2420–2430
- Schagger, H., and Von Jagow, G. (1987) *Anal. Biochem.* **166**, 368–379
- Schuch, U., Lohse, M. J., and Schachner, M. (1989) *Neuron* **3**, 13–20
- Scimeca, J. C., Nguyen, T. T., Filloux, C., and Van Obberghen, E. (1992) *J. Biol. Chem.* **267**, 17369–17374
- Stallcup, W. B., and Beasley, L. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 1276–1280
- Sturgill, T. W., and Wu, J. (1991) *Biochim. Biophys. Acta* **1092**, 350–357
- Taylor, L., Swanson, K., Kerigan, J., Mobley, W., and Landreth, G. (1994) *J. Biol. Chem.* **269**, 308–318
- Tordai, A., Franklin, R. A., Patel, H., Gardner, A. M., Johnson, G. L., and Gelfand, E. W. (1994) *J. Biol. Chem.* **269**, 7538–7543
- Von Bohlen und Halbach, F., Taylor, J., and Schachner, M. (1992) *Eur. J. Neurosci.* **4**, 896–909
- Williams, E. J., Doherty, P., Turner, G., Reid, R. A., Hemperly, J. J., and Walsh, F. S. (1992) *J. Cell Biol.* **119**, 883–892
- Williams, E. J., Furness, J., Walsh, F. S., and Doherty, P. (1994a) *Neuron* **13**, 583–594
- Williams, E. J., Walsh, F. S., and Doherty, P. (1994b) *J. Neurochem.* **62**, 1231–1234
- Wong, E. V., Kenrick, S., Willems, P., and Lemmon, V. (1995) *Trends Neurosci.* **18**, 168–172
- Wong, E. V., Schaefer, A. W., Landreth, G., and Lemmon, V. (1996) *J. Neurochem.* **66**, 779–786