Published in final edited form as:

Biochem Biophys Res Commun. 2009 November 13; 389(2): 257–264. doi:10.1016/j.bbrc.2009.08.143.

Tyrosine and serine phosphorylation regulate the conformation and subsequent threonine phosphorylation of the L1 cytoplasmic domain

Maxine M. Chen, Hyuma A. Leland, Chia-Yao Lee, and Steve Silletti*

Moores Cancer Center, University of California, San Diego, La Jolla, CA USA 92093

Abstract

Previously we identified threonine-1172 (T1172) in the cytoplasmic domain of the cell adhesion molecule L1 as phosphorylated in pancreatic cancer cells. Although both CKII- and PKC-blockade suppressed this modification, only CKII was capable of phosphorylating T1172 of a recombinant L1 cytoplasmic domain, suggesting the requirement for additional events to facilitate availability of T1172 to PKC. In this study, we demonstrate that the region around T1172 exists in distinct conformations based on both T1172 phosphorylation and the integrity of surrounding residues. We further demonstrate the role of membrane-proximal and membrane-distal residues in regulating cytoplasmic domain conformation, and that modification of 3 of the 4 tyrosines in the L1 cytoplasmic domain promote conformational changes that facilitate other events. In particular, phenylalanine-substitution of tyrosine-1151 or tyrosine-1229 promote opening up of the cytoplasmic domain in a manner that facilitates phosphorylation of the other 3 tyrosines, as well as phosphorylation of T1172 by PKC α . Importantly, we show that phosphorylation of serine-1181 is required for T1172 phosphorylation by CKII. These data define a specific role for secondary structure in regulating the availability of T1172 that facilitates phosphorylation by PKC.

Keywords

L1-CAM; CKII; PKC; phosphorylation; conformation; folding

Introduction

L1 is a type I transmembrane protein of the Ig superfamily that regulates active neural processes including cerebellar cell migration, neurite extension and axon guidance [1]. L1 is also expressed in human neuroectodermal tumors and monocytic leukemias [2], and L1 correlates with poor prognosis and advanced disease state in uterine/ovarian carcinomas [3], malignant cutaneous melanoma [4], and serous ovarian neoplasms [5]. L1's role in regulating processes associated with invasion make it well suited for use by an aggressive tumor. Indeed, stable ectopic expression of L1 in fibroblastic and melanoma cells induced MAP kinase activation and the expression of metastasis-associated genes promoting migration and invasion *in vitro* [6]. More recent work demonstrated that L1 is fully transforming and expressed at the invasive

*Corresponding Author: Moores UCSD Cancer Center, 3855 Health Sciences Dr. #0803, La Jolla, CA USA 92093-0803, ssilletti@ucsd.edu.

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tumor margin of colon cancers *in situ* [7], and that ectopic expression of L1 in colon cancer cells bestows a metastatic phenotype [8]. Importantly, the L1 cytoplasmic domain (L1-CD) was required for this effect.

The L1 cytoplasmic domain appears to be crucial for the proper functioning of this cell adhesion molecule, as it is highly conserved among species, and mutations cause severe neurological and developmental problems that collectively manifest as CRASH syndrome [9]. While cytoplasmic serine (S) and tyrosine (Y) phosphorylation events have been shown to regulate specific aspects of L1 function [2,11-13], little is known about threonine (T) phosphorylation of L1. Alanine replacement of both T1247 and S1248 in the L1-CD abrogated the L1-induced invasive phenotype of ovarian carcinoma cells [13]. This mutation, but not the mutation of S1248 alone attenuated L1-mediated erk activation and the concomitant expression of malignancy-associated L1-regulated gene products [6]. Interestingly, this double mutation did not impair L1 binding to RanBPM, a MAP kinase-activating protein that binds within the C-terminal 28 amino acids of L1 (aa1230-1257) [14], suggesting multiple mechanisms of erk regulation by L1. Although these data suggest that threonine phosphorylation might be important in regulating L1 function, the authors did not demonstrate T1247 phosphorylation of L1. Recently we demonstrated a novel threonine phosphorylation site in L1 (T1172), immediately N-terminal to the alternatively-spliced neuronal exon27 [15]. This residue exhibits steady-state saturated phosphorylation in pancreatic ductal adenocarcinoma cells, an event regulated by casein kinase II (CKII) and PKC. Although PKC-blockade suppressed T1172 phosphorylation in cells, purified active PKC preparations were incapable of phosphorylating recombinant L1-CD, suggesting either an indirect role for PKC in regulating this modification, or the requirement for additional factors to promote availability of the T1172 region of the molecule. To investigate this latter possibility, we utilized additional recombinant proteins to study the folding of the L1-CD in isolation, and the corresponding regulation of the conformational availability of the region surrounding T1172.

Materials and Methods

Antibodies

α L1 C-terminus (C20) and α GST (110-218) pAbs were from Santa Cruz Biotechnology (Santa Cruz, CA). 2C2 was from Abcam (Cambridge, MA). α -phospho-T1172 (α P-T1172) and α -phospho-T1172-independent (α T1172-IND) pAbs were generated for us by ProSci, Inc. (Poway, CA). α -phospho-S/T-F pAb was from Cell Signaling (Beverly, MA).

Immunoblotting

Samples were separated by reducing SDS-PAGE, electroblotted to PVDF, sequentially incubated with primary and HRP-secondary antibody and visualized by ECL with PS-3 (Lumigen, Southfield, MI).

ELISA

GST-proteins were immobilized on 96-well plates and blocked with 0.5% gelatin prior to sequential incubation with primary and HRP-secondary antibodies. Antibody complexes were detected with the peroxidase substrate TMB. The reaction was stopped with 0.2N HCl and absorbance read at 450nm.

Kinase Assay

Purified active CKII α 2 was from Invitrogen (San Diego, CA). PKC isoforms were from Biomol/Enzo (Plymouth Meeting, PA). Proteins were coated and blocked as above and then incubated with CKII α 2 or PKC α , β 1, β 2, δ , or ϵ for 30 minutes at 30°C according to the

manufacturer's instructions for each preparation. L1 phosphorylation was assessed with α P-T1172. Histone-H1 activity control and was assessed with α phospho-S/T-F pAb.

Construction and Expression of L1 Fusion Proteins

pGEX neuronal L1 cytoplasmic domain Y to F mutant constructs were generously provided by W. Stallcup (The Burnham Institute, La Jolla, CA). Unless otherwise indicated, new recombinant L1 fusion proteins were generated by PCR using the appropriate primers (Table I). GST/L1¹¹⁴⁴⁻¹¹⁸⁶ was created by restriction digestion of pGEX-6P1/L1 nonneuronal¹¹⁴⁴⁻¹²⁵⁷ with *Stu*I (insert) and *Sma*I (3' on the vector) and religation. GST/L1¹¹⁴⁴⁻¹¹⁶⁸, GST/L1¹¹⁴⁴⁻¹¹⁷⁵ and GST/L1¹¹⁴⁴⁻¹¹⁷⁶ were created by PCR of pGEX-6P1/L1¹¹⁴⁴⁻¹²⁵⁷ using the 5'pGEX sequencing primer and appropriate reverse primer. Products were digested with *Eco*RI and inserted into pGEX-6P1. GST/L1¹¹⁶⁹⁻¹¹⁸⁶ was created by ligation of annealed and phosphorylated mini-exon primers (NN 1169-1186) into the *Eco*RI site of pGEX 6P-1. Site-directed mutagenesis was as described previously [16] using primers shown in Table I. All constructs were confirmed by dideoxy sequencing at the Moores UCSD Cancer Center DNA Sequencing Shared Resource. GST-fusion proteins were produced in BL21 bacteria as described [16] or in TKX1 bacteria with two-stage induction of GST protein and elk tyrosine kinase according to the manufacturers instructions (Stratagene, La Jolla, CA).

Statistics

Antibody binding differences were analyzed by two-tailed Students *t*-Test.

Results and Discussion

Generation of α L1 antibodies directed to the region around T1172

L1 contains two alternatively spliced small exons, one N-terminal and one in the CD. Both exons are present in the neuronal isoform, and absent from the nonneuronal isoform [17]. The cytoplasmic exon (exon27) encodes a four amino acid sequence (¹¹⁷⁷RSLE) that recapitulates a tyrosine-based motif (¹¹⁷⁶YRSLE) that is required for interaction of this isoform with the clathrin adapter AP2 μ 2 and the cytoskeletal linker ezrin, as well as sorting of neuronal L1 to the axonal growth cone [11,12,18]. Recently we demonstrated the phosphorylation of T1172, immediately N-terminal to the differentially spliced neuronal exon27 [15]. To investigate the regulation of T1172 phosphorylation, we had a phospho-specific pAb (α P-T1172) produced for us by ProSci, Inc. (Poway, CA). This antibody was generated using a phosphorylated 7aa antigen (KDEpT¹¹⁷²FGE) coupled to KLH through an N-terminal cysteine as immunogen (Fig. 1A). Rabbit serum was affinity depleted of non-phospho-T1172-dependent species on a nonphospho-KDETFGE column, and unbound antibody was further purified on a phospho-KDETFGE column. Antibody eluted from the non-phospho-T1172 column (α T1172-IND) recognizes the KDETFGE sequence in a manner independent of T1172 phosphorylation (Fig. 1B,D). The α P-T1172 species purified from the KDEpTFGE column demonstrates specific recognition of the phosphorylated peptide (Fig.1B,C).

Epitope specificities of α L1 antibodies demonstrate different folding parameters of the region around T1172

Previously we demonstrated that T1172 phosphorylation disrupts binding of the 2C2 mAb to L1 [15], and although 2C2 poorly recognizes the peptide immunogen used to produce our antibodies, the binding observed is indeed blocked by T1172 phosphorylation in this context as well (Fig.1B). This mAb was generated against T1172-unphosphorylated whole molecule of the L1 orthologue, Ng-CAM, purified from chicken brain membranes, and recognizes a region of the L1-CD conserved between Ng-CAM and L1, immediately N-terminal to the neuronal exon27, although it does not recognize the alternatively spliced ¹¹⁷⁷RSLE sequence

itself [15]; 2C2 recognizes neuronal and nonneuronal L1-CD¹¹⁴⁴⁻¹²⁵⁷ recombinant proteins almost equivalently and with high affinity (Fig.2A). Similarly, the α T1172-IND pAb binds both isoforms equivalently, but in a manner that exhibits considerable loss of binding at higher dilution (Fig.2B). Accordingly, the α T1172-IND pAb performs less well at low protein coating concentrations and requires considerably more protein to achieve saturation of binding (Fig. 2C).

Taken together, these data are consistent with differences in the presentation of the polypeptide encompassing T1172 in the context of a phosphorylated 7mer bound to a plate or linked to KLH through an N-terminal cysteine, versus an unphosphorylated 7mer in the context of the whole cytoplasmic sequence. Perhaps surprisingly, however, these data suggest that the alternatively-spliced ¹¹⁷⁷RSLE sequence immediately C-terminal to T1172 does not appreciably affect the presentation of the adjacent epitope. More importantly, while removal of the T1172 sidegroup via alanine-substitution had only partial affect on 2C2 binding by both immunoblot and ELISA, a size-enhancing (T1172F) or negative charge-imparting (T1172E) mutation at 1172 completely abrogated 2C2 binding in both formats (Fig.2D,E,G). These data are consistent with T1172 not being an integral component of the 2C2 epitope, but with the addition of a large, charged phosphate disrupting 2C2 binding. Since the α T1172-IND pAb was generated against phosphorylated immunogen, it is perhaps not surprising that the substitution of T1172 with E or F had considerably less dramatic effect on its recognition of the recombinant proteins, however alanine-substitution almost completely abrogated α T1172-IND binding in ELISA (Fig.2D,F), suggesting that the threonine side group stabilizes the presentation of the adjacent amino acids. Interestingly, while α T1172-IND recognized both L1 isoforms and the T1172E and T1172F mutants by ELISA, it was unable to recognize the nonneuronal CD (either wildtype or containing these mutations) by immunoblotting unless the adjacent Y1176 or S1181 residues were mutated, or if C-terminal amino acids were removed (i.e. L1¹¹⁴⁴⁻¹¹⁸⁶)(Fig.2G). However, Y1176F and S1181A mutations have no effect on α T1172-IND binding in ELISA (Fig.2H). Moreover, while truncation of the L1-CD at 1186 had a positive effect on α T1172-IND binding in some situations (Fig.2H), truncation at Y1176 consistently suppressed binding and further removal of Y1176 dramatically increased α T1172-IND binding (Fig.2I), further demonstrating regulation of the folding of the region around T1172 by downstream residues.

PKC α is capable of phosphorylating T1172 of L1 in vitro

The PhosphoMotif Finder program of the Human Protein Reference Database (<http://www.HPRD.org>) identified CKII and the PKC family as having a consensus motif appropriate for the region flanking T1172 (KDET¹¹⁷²FGE). Previously we demonstrated that CKII- and PKC-blockade caused a time-dependent dephosphorylation of T1172 in Panc1 cells [15]. Using differential inhibitor sensitivities we identified the potential PKC isoforms involved as a subset of conventional (α , β I) and novel (δ , ϵ) PKC isozymes. Unlike CKII, which phosphorylated T1172 of recombinant L1-CD¹¹⁴⁴⁻¹²⁵⁷ in vitro, none of these isoforms (or PKC- β II) were capable of phosphorylating T1172 in this protein [15]. To assess the capability of the implicated PKC isozymes to phosphorylate T1172 in the absence of constraints imposed by accessory sequences, we performed kinase assays with the L1 nonneuronal¹¹⁶⁹⁻¹¹⁸⁶ protein, or histoneH1 as control. Purified active PKC α promoted significant T1172 phosphorylation, while PKC β I, β II, δ and ϵ isozymes failed to phosphorylate T1172, as measured by lack of binding of the α P-T1172 pAb (Fig.3A). All kinases phosphorylated histoneH1 on the same plate however (Fig.3B), demonstrating that kinase activity *per se* is not responsible for the lack of T1172 phosphorylation observed. As expected, however, CKII promoted significant α P-T1172 pAb signal on L1¹¹⁶⁹⁻¹¹⁸⁶ (Fig.3A).

Phosphorylation of S1181 is required for T1172 phosphorylation by CKII in vitro

As we demonstrated previously [15], CKII phosphorylated both T1172 of the neuronal and nonneuronal L1-CD¹¹⁴⁴⁻¹²⁵⁷ (Fig.3C). As expected, alanine-substitution of T1172 abrogated phosphorylation of both L1 isoforms in this format. Unexpectedly, however, alanine-substitution of S1181 also completely abrogated T1172 phosphorylation (Fig.3C), demonstrating that CKII-dependent phosphorylation of S1181 must precede T1172 phosphorylation, likely by promoting alteration of protein conformation in the T1172 region as shown in Fig.2. Consistent with this proposition, pretreatment of L1¹¹⁴⁴⁻¹²⁵⁷ with CKII promoted PKC α -dependent phosphorylation of T1172 in both the full-length L1¹¹⁴⁴⁻¹²⁵⁷ and L1¹¹⁶⁹⁻¹¹⁸⁶ proteins (Fig.3D).

Tyrosine phosphorylation promotes unfolding of the L1 cytoplasmic domain

Using phenylalanine (F) substitutions to mimic the additional side group size of a phosphorylated tyrosine, we found that introduction of specific Y to F mutations in the L1-CD resulted in specific phosphorylation of other tyrosines by the broad-specificity tyrosine kinase elk when coexpressed during protein induction in TKX1 bacteria (Fig.4A,B). As such, membrane-proximal Y1151F and membrane-distal Y1229F mutations promoted phosphorylation of all three other tyrosines. In contrast, Y1176F promoted phosphorylation of only Y1211, while Y1211F did not promote phosphorylation of any other tyrosines. These data suggest that the L1-CD loops back onto itself in a manner regulated by sequences at the ends of the domain and disturbed by modification of the tyrosines near either end of the domain (Fig.4D). The CD begins with a stretch of basic amino acids (¹¹⁴⁴KRSK), and contains another stretch of basic residues (¹²³²KKEK) near the C-terminal end of the molecule. These sequences are similar to the dibasic motif (⁸⁴⁰RKHSKR) present in the third fibronectin-like repeat of the L1 extracellular domain that facilitates L1 homomultimerization. A database analysis demonstrated that similar sequences are present in numerous other multimerizing proteins (<http://blast.ncbi.nlm.nih.gov/Blast>). Therefore, we reasoned that these sequences at either end of the L1-CD might interact, thereby bringing the membrane-distal end of the protein into close proximity with the membrane-proximal end, a process that might then be destabilized by phosphorylation of the tyrosines at either end of the domain, but not those in the middle of the domain, consistent with the data shown in Fig.4A-C. If this were the case, one would expect that the appropriate mutations might promote breakdown of the L1-CD during production, since the wildtype L1¹¹⁴⁴⁻¹²⁵⁷ protein is very stable and does not exhibit significant breakdown, and since recombinant proteins produced in bacteria often exhibit breakdown when produced in units less than their entire domains, due largely to folding considerations. Accordingly, L1¹¹⁴⁴⁻¹²⁵⁷ harboring Y1151F, Y1176F and Y1229F mutations all exhibit considerable breakdown (Fig.4E). More importantly, removal of the C-terminal 20 amino acids, but not the C-terminal 10 amino acids promoted significant breakdown of the protein as well, and reciprocal truncation of the ¹¹⁴⁴KRSK sequence from the membrane-proximal end of the domain had a similar effect on protein breakdown, suggesting that these regions are involved in maintaining the tightly folded conformation of the L1-CD. Since these tyrosine modifications appear to cause such a dramatic change in the conformation of the L1-CD, we questioned whether they might promote availability of T1172 that could facilitate phosphorylation by PKC α . Indeed, treatment of the tyrosine-phosphorylated Y1151F mutant with PKC α promoted T1172 phosphorylation, which was not observed in the wildtype or Y1151A mutant (Fig.4F). However, this effect was dramatically less than that resulting from prior phosphorylation of S1181 by CKII, suggesting that this event may not represent a true regulatory mechanism.

Although the extracellular domain structure of L1 has been studied exhaustively (for review see [19]), the CD has been less studied from a structural point of view. Signaling studies have demonstrated the regulation of binding interactions with several members of the cytoskeletal,

endocytic and kinase families (for review see [20]) however only one other study has directly investigated the structure of the L1-CD. This study found that the L1-CD was primarily monomeric and largely disordered in solution, not exhibiting any strong tendency towards ordered secondary structure [21]. That said, the techniques utilized in this study only marginally detected the three short-strand structures predicted by the Neural Network Prediction program (<http://npsa-pbil.ibcp.fr/>), but were able to confirm the presence of β -strand in the ^{1214}S - ^{1220}N region, which is consistent with circular dichroism studies on the L1-related molecule neurofascin [22]. More importantly, size-exclusion chromatography actually demonstrated anomalous elution of the 14.4 KDa L1-CD, suggesting that the L1-CD in isolation may exhibit secondary structure that is somewhat labile under their assay conditions.

Our studies utilize recombinant L1-CD linked to GST, which may provide a support not available for the CD studied in solution and in absence of the transmembrane and ectodomain sequences of L1. Indeed, we previously observed that S/T phosphorylation of the L1-CD regulated L1 ectodomain conformation and subsequent shedding in pancreatic cancer cells [15], supporting a role for the interplay between these domains in maintaining structural integrity of the molecule. Thus, our findings with immobilized L1-CD are not necessarily in disagreement with those of Tyukhtenko et al. [21], but rather extend the interpretation of those findings with respect to the immobilized and/or membrane-anchored scenario. Indeed, in support of our proposition that the L1-CD exhibits some degree of binding back onto itself, it should be noted that both juxtamembrane residues and residues at the C-terminus are involved in L1 binding to the actin-linker ezrin [21].

The role of L1 cytoplasmic phosphorylation has dramatic implications for the regulation of L1 biology. For example, endocytosis of the L1 neuronal isoform is regulated by phosphorylation/dephosphorylation of Y1176 through modulation of L1 interaction with the clathrin adapter protein AP2 μ 2 [11], and CKII-dependent phosphorylation of S1181 directly adjacent to this region has been shown to regulate trafficking of the internalized neuronal L1 isoform and subsequent axon growth [23]. Additionally, leucine substitution of S1181 enhanced neurite outgrowth and migration [12], further suggesting S1181 is a regulator of neuronal phenotype. The relevance of S1181 to nonneuronal L1 is unclear however. We have found that CKII exhibits a preference for T1172 of the nonneuronal isoform of L1 [15](Fig3C). CKII phosphorylated S1181 of both constructs, as shown previously by α P-S1181 pAb reactivity [15] as well as the current dependence of T1172 phosphorylation on the integrity of S1181 in both isoforms (Fig.3D). Thus the preferential phosphorylation of S1181 versus T1172 by CKII in neuronal L1 may represent a regulatory mechanism based on the alternatively spliced small exon ($^{1177}\text{RSLE}$) that separates these residues and recapitulates the tyrosine-based motif ($^{1176}\text{YRSLE}$) required for sorting of neuronal L1 to the axonal growth cone [10]. Indeed, Faissner et al. [24] demonstrated that L1 in cerebellar cell cultures is only phosphorylated on serine. This is consistent with the fact that we did not previously detect T1172 phosphorylation in nerves in situ, nor neurally-differentiated PC12 cells [15]. More importantly, ^{1173}F is involved in L1-AP2 μ 2 binding, therefore T1172 phosphorylation may not take place in cells that express the neuronal isoform, or this event may regulate neuronal L1-AP2 μ 2 interactions.

Our data suggest that CKII may be most relevant to S1181 phosphorylation, and that PKC may be responsible for the direct catalytic modification of T1172 in cells. PKC has been implicated in L1 biology previously; PKC activation induces L1 ectodomain shedding [25,26], a process that regulates epithelial cell migration [27]. The relevance of this event to neuronal isoform-expressing cells is unclear, however. Indeed, we previously found that although the broad-spectrum S/T kinase inhibitor staurosporine caused T1172 dephosphorylation in both Panc1 pancreatic and M21 melanoma cells, neither CKII nor PKC (nor combined) blockade affected T1172 phosphorylation in M21 cells [15]. Since M21 cells express PKC α and CKII, the involvement of a different kinase by these cells is likely a regulatory difference and not merely

compensation for a lacking kinase. Importantly, T1172 phosphorylation/dephosphorylation occurs with similar kinetics in both M21 and Panc1 cells, suggesting that T1172 is likely an important mediator of L1 biology in melanoma cells as well.

In summary, we propose a model in which L1-CD folding reflects L1-CD phosphorylation state, with specific reference to the availability of T1172. We further demonstrate the requirement for prior phosphorylation of S1181 by CKII to facilitate T1172 phosphorylation. While we recognize the limitations of these studies utilizing recombinant proteins, these data are consistent with our previous observations [15] and the published literature, and given the nuances of L1 biology and the diverse approaches used in the various studies, we do not expect all findings to be absolute. As such, the techniques employed previously have continued to advance our understanding of the regions of L1 involved in protein-protein interactions. Indeed, Tyukhtenko et al. [21] recently identified a previously unknown region of the L1-CD responsible for ezrin interactions, and L1-induced MAP kinase activity is regulated in two different ways by the same region of the L1-CD [14,15]. We do, however feel that the findings of this study are an important stepping-stone in the further delineation of L1 activity.

Acknowledgments

Grant support: S.Silletti is an American Cancer Society Research Scholar supported by ACS RSG-05-116-01-CSM and NIH grants CA130104 and CA109956.

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Figure 1. Production of antibodies specific to phosphorylated T1172 (α P-T1172) or independent of T1172 phosphorylation (α T1172-IND)

(A) Diagram of immunization and purification scheme. (B) ELISA (1:1,000) of α P-T1172 and α T1172-IND reactivity with phospho- and non-phospho-peptides with reference to 2C2. (C) Titration of α P-T1172 against phospho- and non-phospho-peptides. (D). Titration of α T1172-IND reactivity with phospho- and non-phosphopeptides.

Figure 2. The region around T1172 adopts different conformations dependent upon T1172 modification and the integrity of adjacent residues

(A-C) ELISA titrations of 2C2 and αT1172-IND reactivity with GST-proteins encoding the neuronal and nonneuronal L1-CDs (1144-1257), corrected for αGST. (D-E). ELISA of 2C2 and αT1172-IND reactivity with the wildtype nonneuronal L1-CD, or T1172A, T1172E and T1172F mutants. (G) Immunoblot using 2C2, αT1172-IND or αGST. (H,I) ELISA of 2C2 and αT1172-IND reactivity with the indicated point- or truncation-mutants. (I, inset) 2C2 immunoblot against truncation mutants.

Figure 3. Phosphorylation of T1172 by CKII or PKCα requires prior phosphorylation of S1181 by CKII

(A) ELISA of αT1172-IND reactivity with GST-L1 nonneuronal¹¹⁶⁹⁻¹¹⁸⁶ treated with the indicated PKC isoforms or CKII. Signal was corrected for αGST. (B) Activity of PKC preparations on histoneH1. (C) CKII phosphorylation of neuronal and nonneuronal L1-CD¹¹⁴⁴⁻¹²⁵⁷ harboring T1172A or S1181A mutations. (D) PKCα phosphorylation of neuronal and nonneuronal L1-CD¹¹⁴⁴⁻¹²⁵⁷ after pretreatment with CKII.

Figure 4. Tyrosine phosphorylation promotes extension of the L1 cytoplasmic domain
(A) Phosphotyrosine ELISA of recombinant proteins produced in TKX1 bacteria. **(B)** Phosphotyrosine immunoblot of the indicated proteins produced in TKX1 bacteria. α L1cyto (C20) pAb, control. NIH Image densitometric quantitation of phosphotyrosine signal corrected for C20 is shown. **(C)** Tabulation of the effect of tyrosine modification on subsequent tyrosine phosphorylation in TKX1 bacteria. **(D)** Schematic of L1-CD conformational regulation by tyrosine phosphorylation. **(E)** α GST immunoblot of recombinant proteins. **(F)** α P-T1172 ELISA of proteins produced in TKX1 bacteria treated with PKC α . HistoneH1 shown for reference.

Table 1
Primers used in the construction of recombinant L1 proteins

The L1-CD sequence and corresponding regions encoded by recombinant proteins and amino acids of interest (bold, underlined) are shown for reference. TM, transmembrane sequence.

Primer Type	Primer Sequence
Mutagenesis	
NN T1172A	F 5'-ATGATGAAAGATGAGGCGTTTCGGCGAGTACAGTG-3' R 5'-CACTGTACTCGCCGAACGCCTCATCTTTCATCAT-3'
NN T1172E	F 5'-CTGTACTCGCCGAACCTCATCTTTCATCATC-3' R 5'-GATGATGAAAGATGAGGAGTTCGGCGAGTACAG-3'
NN T1172F	F 5'-ATGAAAGATGAGTTCTTCGGCGAGTACAGT-3' R 5'-ACTGTACTCGCCGAAGAAGTCTATCTTTCAT-3'
NN Y1176F	F 5'-TTCGGCGAGTTTCAGTGACAACGAGGAGAAG-3' R 5'-CTTCTCCTCGTTGTCACTGAACTCGCCGAA-3'
NN S1181A	F 5'-AGACCTTCGGCGAGTACGCTGACAACGAGGAGAAG-3' R 5'-CTTCTCCTCGTTGTCACTGAGCGTACTCGCCGAAGGTCT-3'
WT T1172A	F 5'-ATGAAAGATGAGGCGTTTCGGCGAGTACAGG-3' R 5'-CCTGTACTCGCCGAAGGCCTCATCTTTCAT-3'
WT T1172E	F 5'-ATGAAAGATGAGGAGTTCGGCGAGTACAGG-3' R 5'-CCTGTACTCGCCGAACCTCATCTTTCAT-3'
WT T1172F	F 5'-ATGAAAGATGAGTTCTTCGGCGAGTACAGG-3' R 5'-CCTGTACTCGCCGAAGAAGTCTATCTTTCAT-3'
WT S1181A	F 5'-AGGTCCCTGGAGGCTGACAACGAGGAGAAGG-3' R 5'-CCTTCTCCTCGTTGTCACTGAGGCGCCGACCT-3'
Y1151A	F 5'-AGCAAGGGCGGCAAAGCATCAGTGAAGGATAAGGA-3' R 5'-TCCTTATCCTTCACTGATGCTTGCCGCCCTTGCT-3'
Y1151F	F 5'-AGCAAGGGCGGCAAATTCTCAGTGAAGGATAAGGA-3' R 5'-TCCTTATCCTTCACTGAGAATTGCGGCCCTTGCT-3'
Y1229A	F 5'-GTTTCATTGGCCAGGCAAGTGCAAGAAGGAG-3' R 5'-CTCCTTCTTGCCACTTGCCCTGGCCAATGAAC-3'
I238stop	F 5'-AAGAAGGAGAAGGAGGCGTGAGGGGGCAATGA-3' R 5'-TCATTGCCCCCTCACGCCTCCTTCTCCTTCTT-3'
I248stop	F 5'-CAGGGGCCACTTAACCCATCAACCCGCGCG-3' R 5'-CGGCAGGGTTGATGGGTTAAGTGGCCCTG-3'
PCR	
Cyto (1144)	F 5'-CGAATTCAAGCGCAGCAAGGG-3'
Cyto (1147)	F 5'-GGGAATTCAAGGGCGGCAAAT-3
Cyto (1257)	R 5'-GGAATTCTATTCTAGGGCC-3'
I168stop	R 5'-TGAATTCACGGTCGGGCCTCAG-3'
I175stop	R 5'-TGAATTCACGCGCCGAAGGTCT-3'
I176stop	R 5'-TGAATTCAGTACTCGCCGAAGGTCT-3'
Mini Exon	
NN 1169-1186	F 5'-AATTCGCCCCGACCGATGAAAGATGAGACCTTCGGCGAGTACAGTGACAACGAGGAGAAGG-3' R 5'-AATTCCTTCTCCTCGTTGTCACTGTACTCGCCGAAGGTCTCATCTTTCATCGGTTCGGGCG-3'
<p>TM 1177 RSLE</p> <p> -1144 KRSKGGK<u>Y</u>SVKDKEDTQVDSEARPNKDE<u>TFGEY</u>SDNEEK-----AGGNDSSGATSPINPAVALE¹²⁵⁷</p> <p> -----1144-1257----- </p> <p> -----1147-1257----- </p> <p> -----1144-1186----- </p> <p> -----1144-1176----- </p> <p> -----1144-1175----- </p> <p> -----1144-1168----- </p> <p> -----1169-1186--- </p> <p> -----1144-1247----- </p> <p> -----1144-1237----- </p>	