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

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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 PKCα. 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.

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 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

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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. αL1 C-terminus (C20) and αGST (110–218) pAbs were from Santa Cruz Biotechnology (Santa Cruz, CA). 2C2 was from Abcam (Cambridge, MA). α-phospho-T1172 (αP-T1172) and α-phospho-T1172-independent (αT1172-IND) pAbs were

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′-ATGATGAAAGATGAGGCGTTCGGCGAGTACAGTG-3′ R 5′-CACTGTACTCGCCGAACGCCTCATCTTTCATCAT-3′
NN T1172E	F 5′-CTGTACTCGCCGAACTCCTCATCTTTCATCATC-3′ R 5′-GATGATGAAAGATGAGGAGTTCGGCGAGTACAG-3′
NN T1172F	F 5′-ATGAAAGATGAGTTCTTCGGCGAGTACAGT-3′ R 5′-ACTGTACTCGCCGAAGAACTCATCTTTCAT-3′
NN Y1176F	F 5′-TTCGGCGAGTTCAGTGACAACGAGGAGAAG-3′ R 5′-CTTCTCCTCGTTGTCACTGAACTCGCCGAA-3′
NN S1181A	F 5′-AGACCTTCGGCGAGTACGCTGACAACGAGGAGAAG-3′ R 5′-CTTCTCCTCGTTGTCAGCGTACTCGCCGAAGGTCT-3′
WT T1172A	F 5′-ATGAAAGATGAGGCCTTCGGCGAGTACAGG-3′ R 5′-CCTGTACTCGCCGAAGGCCTCATCTTTCAT-3′
WT T1172E	F 5′-ATGAAAGATGAGGAGTTCGGCGAGTACAGG-3′ R 5′-CCTGTACTCGCCGAACTCCTCATCTTTCAT-3′
WT T1172F	F 5′-ATGAAAGATGAGTTCTTCGGCGAGTACAGG-3′ R 5′-CCTGTACTCGCCGAAGAACTCATCTTTCAT-3′
WT S1181A	F 5′-AGGTCCCTGGAGGCTGACAACGAGGAGAAGG-3′ R 5′-CCTTCTCCTCGTTGTCAGCCTCCAGGGACCT-3′
Y1151A	F 5′-AGCAAGGGCGGCAAAGCATCAGTGAAGGATAAGGA-3′ R 5′-TCCTTATCCTTCACTGATGCTTTGCCGCCCTTGCT-3′
Y1151F	F 5′-AGCAAGGGCGGCAAATTCTCAGTGAAGGATAAGGA-3′ R 5′-TCCTTATCCTTCACTGAGAATTTGCCGCCCTTGCT-3′
Y1229A	F 5′-GTTCATTGGCCAGGCAAGTGGCAAGAAGGAG-3′ R 5′-CTCCTTCTTGCCACTTGCCTGGCCAATGAAC-3′
1238stop	F 5′-AAGAAGGAGAAGGAGGCGTGAGGGGGCAATGA-3′ R 5′-TCATTGCCCCCTCACGCCTCCTTCTCCTTCTT-3′
1248stop	F 5′-CAGGGGCCACTTAACCCATCAACCCTGCCG-3′ R 5′-CGGCAGGGTTGATGGGTTAAGTGGCCCCTG-3′
PCR	
Cyto (1144)	F 5′-CGAATTCAAGCGCAGCAAGGG-3'
Cyto (1147)	F 5′-GGGAATTCAAGGGCGGCAAAT-3
Cyto (1257)	R 5′-GGAATTCCTATTCTAGGGCC-3'
1168stop	R 5′-TGAATTCACGGTCGGGCCTCAG-3'
1175stop	R 5′-TGAATTCACTCGCCGAAGGTCT-3′
1176stop	R 5′-TGAATTCAGTACTCGCCGAAGGTCT-3′
Mini Exon	
NN 1169-1186	F 5′-AATTCGCCCGACCGATGAAAGATGAGACCTTC GGCGAGTACAGTGACAACGAGGAGAAGG-3′ R 5′-AATTCCTTCTCCTCGTTGTCACTGTACTCGCC GAAGGTCTCATCTTTCATCGGTCGGGCG-3′