Ubiquitination within the membrane-proximal ezrin-radixin-moesin (ERM)-binding region of the L1 cell adhesion molecule

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The dynamic turnover of the L1 cell adhesion molecule to and from the plasma membrane that is mediated through exo- and endocytic trafficking is central to axon outgrowth. Although the ubiquitination of L1 in response to incubation with an L1 antibody that mimics L1-L1 homophilic binding has been previously shown, the endocytic trafficking pathway of the ubiquitinated L1 destined for degradation is yet unclear. I have recently shown that the ubiquitinated L1 is endocytosed by Rabex-5, which is an ubiquitin-binding protein and guanine nucleotide exchange factor for Rab5, into early endosomes from the plasma membrane. Here, I speculate on the putative ubiquitination site within the membrane-proximal ezrin-binding motif in the cytoplasmic domain of L1 and discuss the regulatory role of this motif in the competition between ubiquitination and the binding of ezrin prior to L1 internalization.

Membrane trafficking of L1, an immunoglobulin (Ig) superfamily cell adhesion molecule (CAM), is implicated in the polarized adhesion and migration of growth cones. The insertion of L1 into the peripheral region of the growth cone is thought to generate traction that pulls the growth cone components forward, while the removal of L1 by clathrin-mediated endocytosis in the rear may facilitate translocation.[1] Thus, coordinated exo-and endocytic trafficking and the site of L1 insertion and retrieval in the plasma membrane are major factors that determine the motility of the growth cone.

L1 consists of six Ig-like domains that are followed by five fibronectin type-III repeats, a transmembrane sequence and a cytoplasmic domain. Interactions of L1 with ligands through its extracellular domain support axon outgrowth and cell migration via recruitment of signaling molecules, endocytic trafficking proteins and cytoskeletal proteins to the cytoplasmic domain of L1.[2] Importantly, the regulation of these molecular interactions that modulate the activity of L1 is coupled with phosphorylation/dephosphorylation in the cytoplasmic domain of L1. In addition to this type of posttranslational modification, it has recently become clear that ubiquitination in the cytoplasmic domain of CAMs, including those in the Ig superfamily, plays a crucial role for the internalization of ubiquitinated CAMs into endosomes from the plasma membrane.[3-6] Because 2 distinct regions in the cytoplasmic domain of L1 interact with ezrin/radixin/moesin (ERM) proteins that organize the cortical cytoskeleton by linking filamentous actin to the apical membrane of cells,[7] one might suppose that the cytoplasmic domain of L1 might be regulated by posttranslational modification to dissociate L1 from ERM proteins prior to its internalization. Although tyrosine phosphorylation at Y1151 that is mediated by Src-family kinases seems to be involved in the modulation of the interaction with ezrin,[8] it remains unclear how the interactions of L1 with ERM proteins are regulated prior to the endocytosis of L1.

In a previous study, I focused on the molecular machinery underlying the endocytic trafficking pathway of L1 into endosomal/lysosomal compartments, which controls the expression of L1 on the cell surface upon incubation with a L1 antibody.[6] In order to clarify the impact of ubiquitination on the endocytosis of L1, I constructed a ubiquitination-deficient mutant of L1, $L1^{K11R}$, in which 11 Lys residues, two of which constitute a putative ERM-binding motif (**Fig. 1A**), were substituted with Arg residues, because ubiquitination occurs predominantly at the ε-amino group of the lysine residue within a substrate. Indeed, I confirmed that ubiquitination was abrogated in the absence of the 11 Lys residues.[6] Furthermore, the targeting of the $L1^{K11R}$ mutant into lysosomes was dramatically reduced compared with that of the control $L1^{WT}$ because the $L1^{K11R}$ mutant was not able to interact with Rabex-5.[6] Given the involvement of ubiquitination in DM-GRASP, another Ig superfamily CAM, in retinal ganglion cell axon navigation,[4] it would be interesting to determine whether the $L1^{K11R}$ mutant affects cell adhesion and cell migration.

I constructed various L1 mutants shown in **Figure 1A** and determined the ubiquitination levels for each mutant by comparing the mutant with wild-type L1 ($L1^{WT}$, **Fig. 1B**). Surprisingly, the
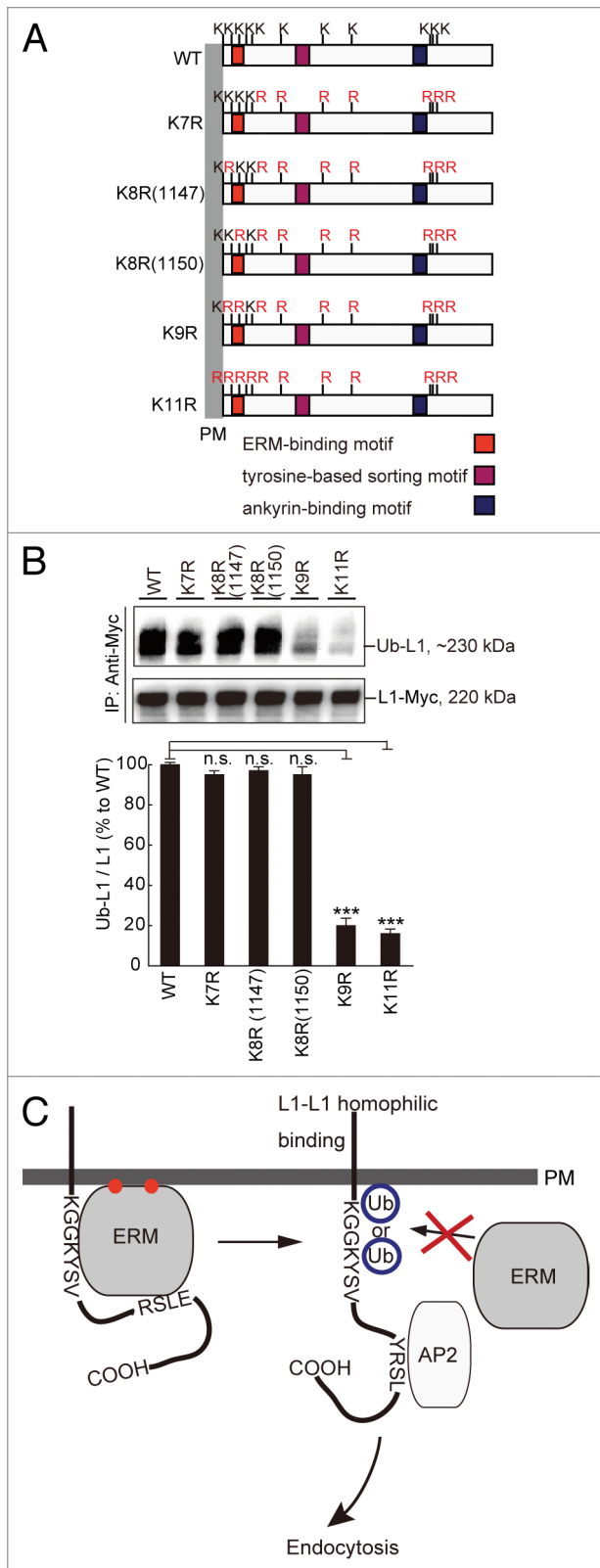
Figure 1. Schematic representation of the L1 cytoplasmic domain. (**A**) Red, putative ezrin-binding motif (KGGKYSV); purple, tyrosine-based AP-2 binding motif (YRSLE); blue, ankyrin-binding motif (FIGQY). The red characters indicate the sites of point mutations that were used in this study. PM, plasma membrane. (**B**) In cells expressing the indicated plasmids, lysis, immunoprecipitation and immunoblot analyses were performed as indicated (upper panel). Bars represent the relative densitometric values of Ub-L1/L1 (lower). Experiments were repeated three times, and the data are displayed as the mean ± standard error of the mean (SEM). ***, $p < 0.001$. (**C**) The proposed molecular mechanism underlying the ubiquitin-dependent internalization of L1 upon L1-L1 homophilic interaction. (Left) The ezrin protein binds to the juxtamembrane region (KGGKYSV) and the RSLE region of the L1 cytoplasmic domain, and this interaction is involved in traction-force generation. Ezrin/radixin/moesin (ERM) activation requires PtdIns(4,5)P_2 binding (red circle). (Right) In contrast, the ubiquitination within the membrane-proximal region masks this binding site, resulting in the dissociation of the ubiquitinated L1 from the actin network beneath the plasma membrane upon L1-homophilic binding.

involved in the ubiquitination. In order to further address which Lys residue within the ERM-binding motif was ubiquitinated, L1$^{K8R(1147)}$ and L1$^{K8R(1150)}$ mutants that were each mutated at the ERM-binding motif were constructed and their ubiquitination levels were determined. However, the ubiquitination levels for each mutant were unchanged and were compatible to those of L1^{WT} (**Fig. 1B**), suggesting that either Lys residue and/or both of Lys residues within the ERM-binding motif were ubiquitinated by an unknown ubiquitin-ligase (E3) enzyme and that this was presumably mediated through multimonoubiquitination.

Taken together with the previous results that ezrin did not coimmunoprecipitate with L1 in the cells overexpressing HA-tagged ubiquitin but did in control cells,[6] the results shown here support the hypothesis that ubiquitination within the ERM-binding motif reduced the proposed interactions with ezrin and thereby facilitated L1 endocytosis (**Fig. 1C**). Intriguingly, it has recently been reported that the ubiquitination of the juxtamembrane domain of E-cadherin inhibits p120-catenin binding and targets E-cadherin for degradation.[9] Thus, ubiquitination in the cytoplasmic domain of CAMs might have a dual role: sorting signals to the lysosomes and regulating protein-protein interactions, making it possible to internalize the ubiquitinated CAMs from the plasma membrane that is crowded with proteins linked to the cytoskeleton.

Given the causative role of L1 in severe neurological syndromes, such as X-linked hydrocephalus, and the hypoplasia of major axon tracts when the L1 gene is mutated,[10] as well as its contribution to a variety of metastatic cancers,[11] further experiments are required for the identification of the E3 ubiquitin ligase and the deubiquitinating enzyme for L1 in order to gain insight into the molecular machinery underlying the regulation of the cell surface pool of L1.

ubiquitination of the L1^{K9R} mutant in which Lys was substituted with Arg, including in the ERM-binding motif, was drastically reduced by 80–90% compared with L1^{WT}, whereas the ubiquitination of the L1^{K7R} mutant was not affected (**Fig. 1B**), indicating that the Lys residues within the ERM-binding motif might be

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