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The Mechanism of Axon Growth

What We Have Learned From the Cell Adhesion Molecule L1

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Abstract

Cell adhesion molecules (CAMs) are not just an inert glue that mediates static cell-cell and cell-extracellular matrix (ECM) adhesion; instead, their adhesivity is dynamically controlled to enable a cell to migrate through complex environmental situations. Furthermore, cell migration requires distinct levels of CAM adhesivity in various subcellular regions. Recent studies on L1, a CAM in the immunoglobulin superfamily, demonstrate that cell adhesion can be spatially regulated by the polarized internalization and recycling of CAMs. This article examines the molecular mechanism of axon growth, with a particular focus on the role of L1 trafficking in the polarized adhesion and migration of neuronal growth cones.

Index Entries: L1; adhesion; growth cone; axon; endocytosis; recycling.

Introduction

One of the most fascinating types of cell motility is the migration of axons over long distances to their proper targets. During the period of axon elongation, the tip of the axon forms a specialized structure known as the growth cone *(1)*. The growth cone interacts with its environment via cell adhesion molecules (CAMs) and navigates the axon along the correct path. Thus far, three major classes of CAMs have been identified in the nervous system: integrins, cadherins, and CAMs of the immunoglobulin superfamily (IgCAMs). The importance of CAMs in axon-tract development in vivo has been best illustrated by the disease X-linked hydrocephalus in humans. Mutations of L1, an IgCAM, cause X-linked hydrocephalus, which is accompanied by hypoplasia of the major axon tracts, such as the corpus callosum and the corticospinal tract *(2–4)*. These malformations have also been

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found in two independent L1-knockout mouse lines *(5–8).* A more recent study demonstrated that L1-knockout mice have abnormal topographic mapping of retinal ganglion-cell axons to their targets in the superior colliculus *(9).*

L1 is a single-pass transmembrane protein expressed on developing and regenerating axons. Although the homophilic *trans*-binding of L1 molecules is their most common mode of action in promoting axon growth *(10,11),* a number of heterophilic partners that interact with L1 in *cis* or in *trans* have also been identified. For example, L1 presented as a culture substrate promotes axon growth via *trans*-interaction with $\alpha_v\beta_3$ integrin expressed on dorsal-root ganglion (DRG) neurons *(12).* The *cis*-interaction of NgCAM, a chick homolog of L1, with axonin-1 is involved in DRG axon growth on an NgCAM substrate *(13)* and probably in nociceptive sensory axon guidance in the spinal cord *(14).* Furthermore, *cis* and *trans* interactions of L1 with neuropilin-1 control axonal turning responses to semaphorin 3A *(15).* Although the extracellular binding events that involve L1 are exceedingly complex, even more mysterious are the intracellular molecular mechanisms that translate L1 binding into directed growth and guidance of the axon. However, recent revelations regarding L1 signaling and trafficking and its linkage with the cytoskeleton have provided substantial clues to the mystery. This article explains how the growth cone regulates L1 trafficking and L1-mediated adhesion in a way that is important for its migration.

Inside-Out Regulation of IgCAM-Mediated Adhesion

Cell motility requires the cell to have dynamically and spatially regulated interactions with its environment. These interactions are mediated by CAMs expressed on the migrating cell. For a cell to respond to changes in its environment, it must be able to regulate the function and/or expression of its CAMs. Furthermore, cell migration requires distinct levels of CAM adhesivity in different subcellular regions. Therefore, in response to external stimuli, CAM adhesivity must be controlled by region-specific signals from the inside to the outside of the cell. This type of signaling has been well-studied in the field of integrins *(16)* and cadherins *(17).* More recently, it has become evident that IgCAM-mediated adhesion can be controlled by at least four distinct mechanisms in an inside-out manner *(18):* the lateral oligomerization of CAMs, the internalization and recycling of CAMs, the proteolytic cleavage of the CAM ectodomain, and the transcriptional regulation of CAM expression (Fig. 1). Oligomerization and the internalization/recycling of CAMs are probably the major mechanisms of the inside-out regulation. The functional significance of the proteolytic cleavage of CAMs has been poorly understood, in the nervous system. The transcriptional regulation of CAM expression is much slower than the other post-transcriptional mechanisms, and cannot control cell adhesion in a region-specific manner at least in the short term.

Oligomerization of the L1 family members (L1, neurofascin, NrCAM, and CHL1) is mediated via their cytoplasmic and extracellular domains. The cytoplasmic domain of the L1 family CAMs interacts with ankyrins (spectrin-binding proteins), which may lead to lateral oligomerization of the CAMs *(19).* Lateral oligomerization induced by this mechanism has been shown to enhance the homophilic *trans*-adhesion of neurofascin *(20).* The interaction of the L1 family CAMs with ankyrins is partly regulated by tyrosine phosphorylation/dephosphorylation in the CAM cytoplasmic domain *(21,22).* Alternatively, homophilic ligand binding to the L1 extracellular domain (L1ED) *in trans* may induce a conformational change that culminates in the oligomerization of the L1ED *(23)* and in increased ligand-binding affinity *(24).* Interestingly, the L1ED-mediated oligomerization is abolished by proteolytic cleavage within the third fibronectin type III repeat of the L1ED by plasmin *(23).* These findings indicate that L1 oligomerization is controlled by multiple inside-out mechanisms, including tyrosine kinases/phosphatases and

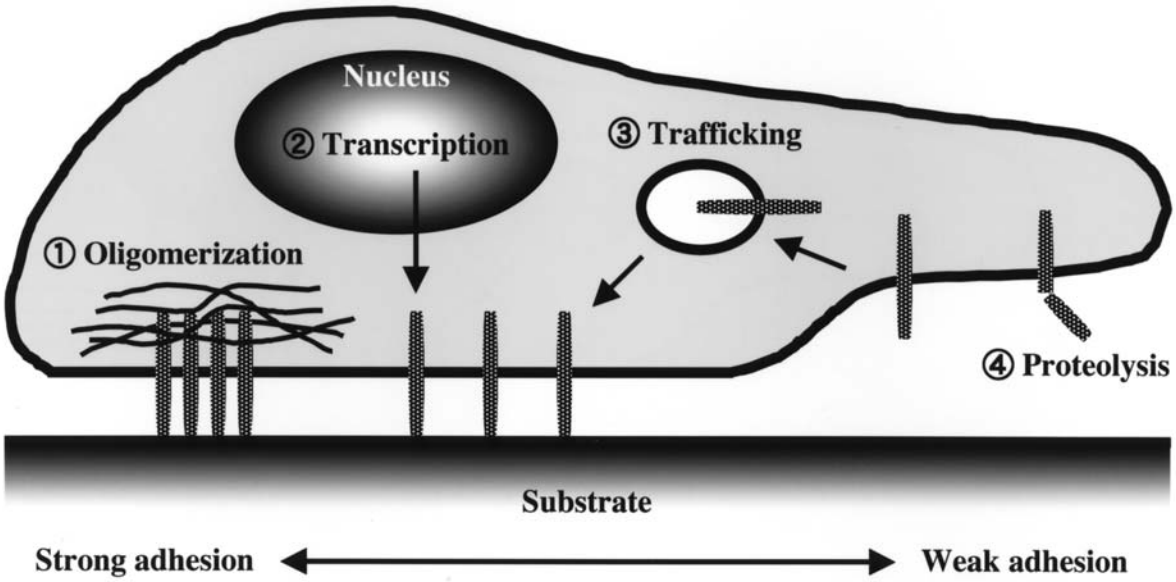

Fig. 1. Four distinct mechanisms involved in the inside-out regulation of IgCAM-mediated adhesion. **(1)** Cell adhesion is strengthened by the lateral oligomerization of CAMs induced, for example, by CAM-cytoskeletal interactions. Alternatively, the number of active CAMs on the cell surface that actually participate in cell adhesion is controlled by **(2)** transcriptional regulation of CAM expression, **(3)** internalization and recycling of CAMs, and **(4)** proteolytic cleavage of the CAM ectodomain. These four pathways cooperatively regulate cell adhesion both spatially and temporally.

the plasmin system. In addition to the plasmin-mediated proteolysis, the L1ED is cleaved at a membrane-proximal site by a member of the disintegrin-metalloproteinase (ADAM)-family proteases. The ADAM-mediated proteolysis is dependent on intracellular signaling, such as the Fyn and mitogen-activated protein kinase (MAPK) pathways *(25).*

How do internalization and recycling control CAM adhesivity? One obvious explanation is that the density of CAM molecules is spatially regulated by preferential internalization at one region of a cell, followed by recycling into a different region. Another possibility is that rapid internalization of CAMs decreases cell adhesion by shortening the dwell time of the CAMs on the cell surface. Early modeling studies suggested that after the *trans*-binding of CAMs, individual pairs of bound CAMs rapidly cycle between the bound and unbound states *(26).* Rapid internalization would cause CAMs to be quickly removed from the cell surface after *trans*-binding. Slow internalization would cause CAMs to remain on the cell surface for a long period so that they could be engaged in *trans*-binding multiple times before being removed from the cell surface. This possibility was supported by the recent observation that homophilic L1 adhesion is regulated by L1 endocytosis rates without affecting the amount of L1 expression on the cell surface *(27).* Inhibition of L1 endocytosis caused the L1-mediated cell aggregation rates to double. Therefore, endocytic CAM trafficking seems to regulate cell adhesion by changing the CAM density and its dwell time on the cell surface.

Endocytic L1 Trafficking in Neuronal Growth Cones

Growth-cone motility depends on cytoskeletal dynamics. The two major cytoskeletal components in a growth cone are actin filaments

(F-actin), which are predominantly located in the peripheral (P-) domain, and microtubules, which are predominantly in the central (C-) domain. Spatially localized actin polymerization/depolymerization and actin-myosin interactions generate a retrograde movement of F-actin *(28),* which is believed to function as a force-generating system to pull the growth cone forward *(29).* CAMs in the P-domain transmit this force by mechanically linking immobile extracellular ligands with the retrograde F-actin flow, leading to anterograde migration of the growth cone *(30).* The fate of the CAMs that are translocated into the C-domain by coupling to the F-actin flow was recently elucidated by revelations about the endocytic L1 trafficking in growth cones. L1 is preferentially internalized at the C-domain followed by centrifugal transport that is dependent on the dynamic ends of microtubules *(31).* After trafficking into the P-domain, L1 is reinserted into the plasma membrane of the leading edge *(31).* In this way, L1 is recycled from the C-domain to the leading front for reuse (Fig. 2). The CAMs can be viewed as the "feet" needed for a growth cone to crawl on a relevant substrate, and it is clearly economical to use the same CAM molecules for multiple forward steps.

Endocytic CAM trafficking plays another important role, in the spatial regulation of growth-cone adhesion. Forward translocation of the growth cone requires the CAM-cytoskeletal linkage as well as a gradient of adhesive interactions of the growth cone with its environment: strong adhesion at the growth cone's leading edge and weak adhesion at the C-domain *(32).* In this way, the cytoskeletal machinery is able to move the growth cone forward as attachments at its rear are released. In fact, my laboratory has shown that a growth cone migrating via an L1-dependent mechanism exhibits such a gradient of L1-mediated adhesion *(33).* The rate of L1-based axon growth has a positive correlation with both the amount of endocytosed L1 in the growth cone and the steepness of the gradient of L1 adhesion. Importantly, inhibition of L1 endocytosis in the growth cone attenuates L1-based axon growth and the gradient of L1 adhesion *(33).* These pieces of evidence indicate that the growth cone actively translocates L1 and probably other CAMs to create spatial asymmetry in its adhesive interactions with the environment, and that this spatial asymmetry is important for growth-cone migration.

The Regulatory Mechanisms of L1 Endocytosis

The L1 family of CAMs has a highly conserved cytoplasmic domain that contains a tyrosine, followed by an alternatively spliced RSLE (Arg-Ser-Leu-Glu) sequence. The RSLE sequence of L1 is expressed in neurons, but not in other L1-expressing cells such as Schwann cells *(34).* The presence of RSLE results in a YRSL sequence, which conforms to the tyrosine-based sorting motif YxxØ, in which x is any amino acid and Ø is an amino acid with a bulky hydrophobic side chain *(35).* The tyrosine-based signals interact with the clathrin-associated adaptor AP-2, which is composed of four subunits (α, β1/β2, μ2, and σ2), resulting in endocytosis of the signal-bearing molecules via clathrin-coated pits and vesicles *(36,37).* We found that the Y^{1176}RSL sequence in the L1 cytoplasmic domain (L1CD) directly interacts with the μ2 chain of AP-2 *(38),* and that this interaction is required for the clathrin-mediated endocytosis of L1 *(27,33).* This finding suggests that the neuronal form of L1 is subject to the active internalization and recycling that are associated with the dynamic regulation of cell adhesion, and the RSLE-minus L1 on non-neuronal cells may serve as a less dynamic adhesive substrate. L1 endocytosis via clathrin-mediated pathways also requires the guanosine 5′ triphosphate (GTP)-binding protein dynamin *(38)* and the non-receptor tyrosine kinase Src *(39).* Dynamin is involved in the membrane fission event that results in the formation of clathrin-coated vesicles *(40).* Src is implicated in clathrin-mediated endocytosis via the phosphorylation of clathrin *(41)* and dynamin *(42).* Obviously, many other molecules participate in the

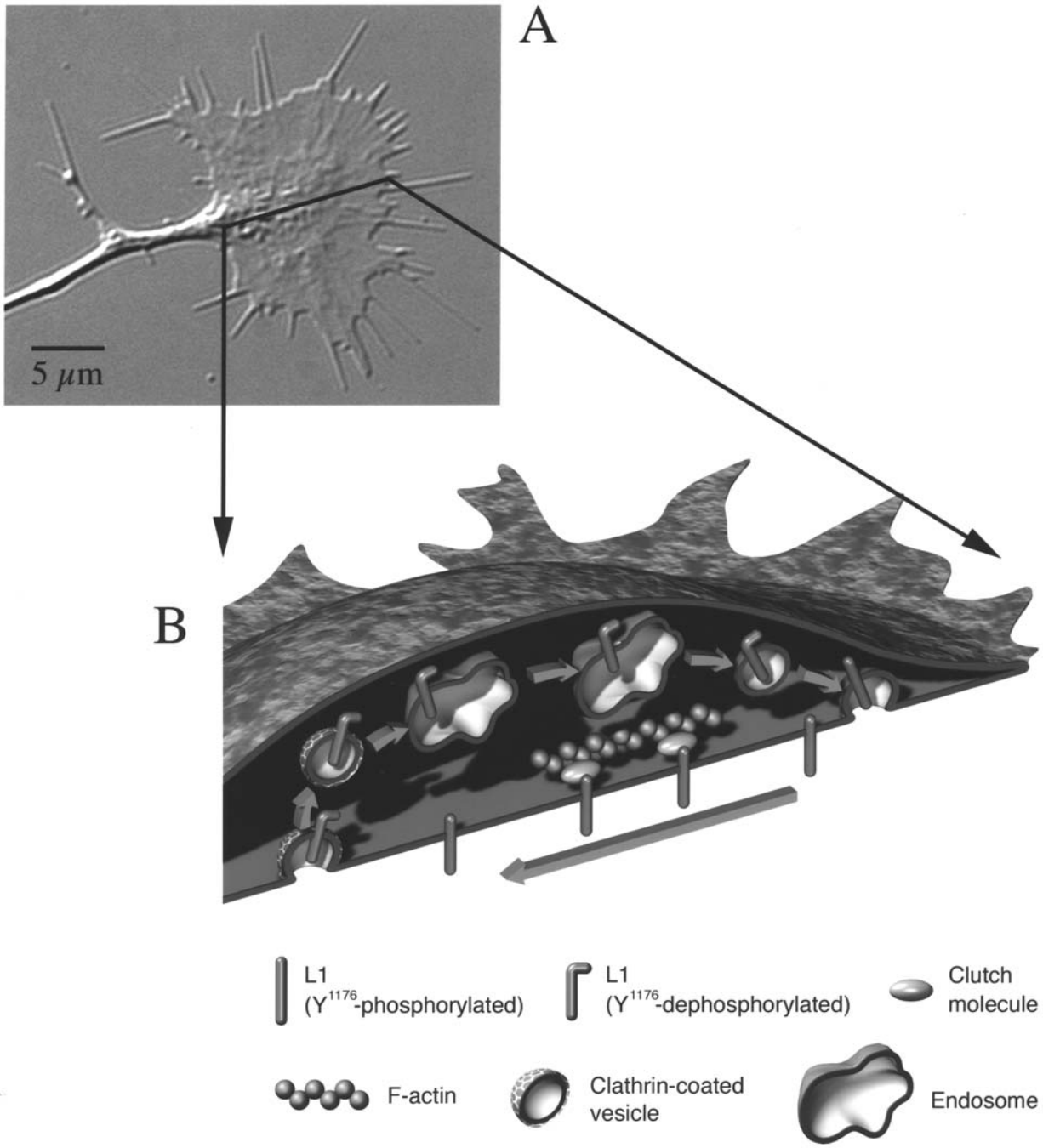

Fig. 2. **(A)** A differential interference contrast image of a chick DRG growth cone migrating on an L1 substrate. **(B)** A schematic view of the growth cone sectioned longitudinally, showing a model of L1 trafficking. Dephosphorylation of Y^{1176} in the L1CD triggers L1 internalization at the C-domain via clathrin-mediated pathways. Subsequently, the endocytosed L1 is transported into the P-domain through sorting and recycling endosomes. This process is dependent on the dynamic ends of microtubules (not shown in this figure). Next, the trafficking L1 is reinserted into the plasma membrane at the leading edge of the growth cone, probably after Y^{1176} is re-phosphorylated. Recycled L1 on the cell surface moves toward the C-domain by coupling with the retrograde F-actin flow via linker molecules. Reprinted with permission of the publisher from Kamiguchi H. and Lemmon V. (2000) *J. Neurosci.* **20,** 3676–3686.

process of L1 endocytosis. Preferential internalization of L1 at the C-domain of growth cones may be induced by the spatially restricted activation of the clathrin endocytic machinery and its associated molecules.

Alternatively, a post-translational modification in the L1CD could be responsible for the region-specific endocytosis of L1. This possibility has recently been investigated by Lemmon's group *(43)*. The L1CD is subject to phosphorylation in vivo at Y^{1176}, the critical tyrosine residue for AP-2 binding. Phosphorylation of Y^{1176} prevents the L1CD from interacting with AP-2, thereby preventing the clathrin-mediated endocytosis of L1. Most of the L1 molecules on the cell surface are phosphorylated at Y^{1176}, and their dephosphorylation can be induced by L1-mediated cell-cell contact or the crosslinking of L1. In growth cones, Y^{1176}-dephosphorylated L1 is found in vesicle-like structures, further supporting the theory that L1 endocytosis is triggered by Y^{1176} dephosphorylation. Src phosphorylates Y^{1176} in vitro, suggesting that Src plays dual and opposing roles in the regulation of L1 endocytosis: Src is required for L1 endocytosis most likely by phosphorylating the clathrin endocytic machinery, but may prevent L1 endocytosis through phosphorylation of the L1CD. Because Src family kinases are localized to lipid rafts/detergent-resistant membranes (DRMs) *(44,45)*, L1 phosphorylation by Src is likely to depend on whether the L1 is associated with these membrane microdomains. If so, it is possible that lipid rafts/DRMs in the growth-cone membrane are involved in the spatial regulation of L1 endocytosis. In support of this idea, my laboratory demonstrated that approx 50% of L1 molecules are expressed within DRMs in neurons, and that the L1-dependent migration of a growth cone requires DRMs in its P-domain *(46)*. DRMs in the C-domain are barely involved in growth-cone migration. It is intriguing to speculate that, in the P-domain, the localization of L1 to DRMs allows for its Y^{1176} phosphorylation by Src, which prevents its ectopic endocytosis. In contrast, L1 becomes dephosphorylated at Y^{1176} in non-DRM regions in the C-domain, leading to its clathrin-mediated endocytosis.

In addition to internalization from the plasma membrane, there are several points at which the endocytosed L1 must follow the correct pathway if it is to be recycled to the growth-cone leading edge. An example of such a "choice point" is at sorting endosomes, where most of the endocytosed L1 should be sorted into recycling endosomes but not into late endosomes/lysosomes. However, some of the L1 molecules should be sorted into late endosomes/lysosomes to be degraded. Therefore, growth cones must have regulatory mechanisms to select the correct endocytic pathway for L1 molecules. This regulation is likely to be mediated by cytosolic molecules that recognize the L1CD and influence membrane sorting. The regulatory mechanisms have not yet been investigated, but one possibility is that the pathway of L1 trafficking is regulated by the phosphorylation state of the L1CD, especially by the phosphorylation on S^{1181} by casein kinase II *(47)*. S^{1181} lies within a cluster of acidic amino acids in the L1CD; this cluster conforms to a sorting signal that can be linked to the clathrin-sorting machinery via PACS-1, a cytosolic protein involved in controlling the correct subcellular localization of membrane proteins *(48,49)*.

The Role of L1 Endocytosis in Outside-In Signal Transduction

Endocytic L1 trafficking plays a role in the inside-out regulation of cell adhesion as well as in outside-in signal transduction. Studies from several different groups showed that outside-in signals generated by distinct CAMs—such as L1, NCAM, *N*-cadherin, and laminin receptors—converge on MAPK activation and stimulate axon growth *(39,50–53)*. For example, the clustering of L1 on the cell surface activates the MAPK pathway via phosphatidylinositol 3-kinase and the small GTPase Rac1, and the MAPK activation is involved in L1-stimulated axon growth *(39)*. Remarkably, the clathrin-mediated endocytosis of L1 is required for the full activation of MAPKs by L1 clustering *(51)*.

This may be an important mechanism for restricting activated MAPKs to specific subcellular locations. In fact, MAPKs activated by L1 clustering do not colocalize with the clustered L1 on the cell surface, but instead colocalize with the clustered L1 that has been internalized and sorted into early endosomes *(51).* Because the L1CD can be phosphorylated by at least two MAPK cascade components, including ERK2 *(51,54),* one possible role of the MAPKs that are activated in a spatially restricted manner is to control the endocytic pathway of L1 at a choice point.

Conclusions

Recent findings on L1 functions in growth cones have provided a framework for understanding the molecular mechanisms that are the foundation of axon growth. It is likely that this framework can be applied to axon growth stimulated by other CAMs, although their trafficking and interactions with the cytoskeleton clearly involve distinct molecules and regulatory mechanisms. A very important question in this field is how the growth cone controls CAM behavior in a coordinated and spatially defined manner. Application of the latest technologies for precise imaging and the manipulation of molecular functions in living cells will help to answer this question.

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