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The actin-bundling protein L-plastin supports T-cell motility and activation

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Summary

Tight regulation of actin dynamics is essential for T-cell trafficking and activation. Recent studies in human and murine T cells reveal that T-cell motility and full T-cell activation require the hematopoietic-specific, actin-bundling protein L-plastin. T cells lacking L-plastin do not form fully mature synapses and thus demonstrate reduced cytokine production and proliferation. Reduction or loss of L-plastin expression also reduces the velocity of T cells and impairs thymic egress and intranodal motility. While dispensable for proximal T-cell receptor and chemokine receptor signaling, L-plastin is critical to the later stages of synapse maturation and cellular polarization. Serine phosphorylation, calcium, and calmodulin binding regulate the bundling activity and localization of LPL following T-cell receptor and chemokine receptor engagement. However, the interaction between these regulatory domains and resulting changes in local control of actin cytoskeletal structures has not been fully elucidated. Circumstantial evidence suggests a function for L-plastin in either the formation or maintenance of integrin-associated adhesion structures. As L-plastin may be a target of the commonly used immunosuppressive agent dexamethasone, full elucidation of the regulation and function of L-plastin in T-cell biology may illuminate new pathways for clinically useful immunotherapeutics.

Keywords

L-plastin; actin cytoskeleton; immunological synapse; lamellipod; motility; T cells

Introduction

While first identified as an actin-regulatory protein specific to hematopoietic cells over 25 years ago (1, 2), the key role of L-plastin (LPL) in T-cell activation and motility has only recently been appreciated (3-11). A member of the α -actinin family, LPL stabilizes larger order f-actin structures by cross-linking parallel actin filaments into tightly approximated bundles (12, 13). Recent work, using short hairpin RNA (shRNA) technology to reduce expression of LPL in human T lymphocytes and in mice genetically deficient for LPL, has revealed that LPL is essential to later stages of T-cell activation and for T-cell motility. The upstream regulators and downstream effects of LPL have not yet been fully described. After a description of the structure and function of LPL, this review describes the currently known requirements for LPL in T-cell biology and speculates upon possible molecular interactions supported by LPL.

Actin cytoskeleton structures in T-cell motility and activation

The function of the actin-bundling protein L-plastin (LPL) can best be placed in context by first reviewing the rearrangements in the actin cytoskeleton essential for T-cell activation and motility (14-33). Actin polymerization and reorganization support the changes in cell morphology and polarization that enable directed migration and engagement with antigen-presenting cells (APCs) (34-38). Furthermore, actin filaments may serve as 'platforms' on which signaling complexes are constructed, and association with f-actin can anchor signaling complexes on the cell membrane (17, 39-42). Dynamic changes in f-actin are thus critical to effective immunity. LPL plays a critical role in modulating and shaping f-actin in T cells.

T cells are highly motile, with an average velocity of 8-15 $\mu\text{m}/\text{min}$ (43). Naive T cells constantly traffic into, through, and out of lymphoid tissues, scanning for cognate peptide displayed on resident APCs (38, 44-46). Continual patrolling of tissues enables the rapid recognition of pathogens or tumors and generation of an effective immune response. When stimulated by chemokines, T cells assume a polarized shape, often described as a 'hand mirror' (38, 47) (Fig. 1A). The stimulatory chemokine receptor is localized to the leading edge, or lamellipodia, while contractile elements that can propel the cell forward are localized to the rear edge of the cell, called the uropod (48). The establishment and maintenance of this polarized cell shape depends upon intact actin cytoskeletal architecture (47).

When a roaming T cell encounters cognate antigen on an APC, it stops and activates. Full T-cell activation requires the formation of a highly specialized contact site between the responding T cell and an APC, first described in 1998 (35, 36, 49, 50) (Fig. 1B). Termed an 'immunological synapse', this contact site organizes cell surface receptors and corresponding signaling proteins into central, peripheral, and distal supramolecular activation clusters (cSMAC, pSMAC, and dSMAC, respectively) (49-51). The TCR/CD3 complex accumulation in the center defines the area of the cSMAC, while the integrin leukocyte function-associated antigen-1 (LFA-1) localizes primarily to the pSMAC (35, 36). Larger molecules that would preclude tight approximation of cell membranes, such as CD43, are concentrated into the outer dSMAC (49). Generation of the synapse is an active process, with transport of signaling complexes into the cSMAC and pSMAC and exclusion of larger molecules that could interfere with the tight approximation of the opposing cell membranes (43). Actin polymerization and rearrangement is essential for the formation of the synapse and may provide the force required to segregate or move signaling components (36, 52-57).

The complex rearrangements of actin required for both motility and synapse formation are neatly summarized in two recent papers defining retrograde flow and myosin-mediated contraction during synapse generation (56, 57) (Fig. 1C). During motility, forward motion of a cell results from coordinated cycles of leading edge protrusion, adhesion, and then retraction (58). Two networks of actin, termed the lamellipodium and the lamellum, support these cycles (59). The underlying lamellum contains more stable actin networks, while more dynamic actin networks exist in the overlying lamellipodium (58, 59). F-actin polymerization in the lamellipodia drives flow of the actin network backwards until the actin network connects to an integrin-mediated adhesive site (Fig. 1C, step 1). Myosin II engages and pulls upon the actin, generating tension and inducing retraction of leading edge (Fig. 1C, steps 2, 3). Release of actin from the lamellipodial tip enables protrusion to begin anew (Fig. 1C, step 4), and a new adhesion site is generated (Fig. 1C, step 5). Cycles of protrusion, adhesion, retrograde actin flow, and myosin-mediated retraction enable forward motion of the cell (58). It has been suggested that T-cell synapse formation occurs in an analogous manner, with protrusion of cell membrane generated away from the site of APC contact,

with the TCR being ‘swept’ into the cSMAC by retrograde actin flow (60). A synapse is thus envisioned as a radially symmetric lamella and lamellipod wrapped around a central point (56, 57, 60, 61) (Fig. 1B).

Formation of the lamella and lamellipod and the polymerization of actin required for the retrograde actin flow are tightly regulated. Actin-binding proteins that characterize the lamellipodium include the Arp2/3 complex, cofilin, and θ -actinin (56-58). The Arp2/3 complex is also highly concentrated in the dSMAC (56). Myosin II localizes in both the lamellum and the pSMAC (56-58) and is essential for maintenance of lamella (59, 62). While analysis of the location and function of these regulatory molecules has illuminated several key aspects of the regulation of cell motility at the leading edge, many mechanistic questions remain. For instance, the analysis of lamellar formation in migrating chick fibroblasts has revealed that graded polarity actin bundles are critical to lamella (62), but the molecular requirements supporting the generation and maintenance of these bundles remain largely unexplored. After reviewing the structure, function, and requirement of LPL in T cells, this review explores possible mechanisms by which LPL fits into known models of actin remodeling during motility and synapse formation.

Structure and function of the actin-bundling protein L-plastin

L-plastin (leukocyte-plastin) is one of three plastin isoforms found in mammalian cells (1, 2, 63, 64). Plastins, also called fimbrin, are members of the θ -actinin family of actin-binding proteins. Plastins differ from other θ -actinin family members by encompassing two actin-binding domains in a single polypeptide chain. Plastin/fimbrin is widely conserved across all eukaryotic life forms, with mammalian plastin able to complement the yeast homolog SAC6 (65). The T-isoform of mammalian plastin is expressed in a broad tissue distribution, including muscle, skin, and neural tissue, and is thought to be expressed in all cells with replicative potential (63, 66). I-plastin expression is restricted to kidney and intestinal cells (2). L-plastin has been reported to be expressed only in hematopoietic cells and has been thought to be the only plastin isoform expressed in all immune cells (1, 2, 63, 64, 66, 67). Recent work from Immgen calls this specificity into question, as it appears that some lymph node stromal cells may express low levels of L-plastin, and some macrophages appear to upregulate T-plastin after stimulation (68). However, in T cells, L-plastin is the only isoform expressed (68). L-plastin is also ectopically upregulated in multiple cancer cells (reviewed in 13, 69, 70).

To understand the biochemical function of LPL, it is necessary to understand actin polymerization and remodeling. Actin filaments (f-actin) consist of polymerized globular actin (g-actin) subunits that self-associate in polarized, linear double helices (71, 72). One end of an actin filament is termed ‘barbed’, while the other is ‘pointed’, descriptions drawn from the electron micrographs of actin filaments decorated with myosin (73). At steady state, it is thought that polymerization occurs at the barbed end, while depolymerization occurs at the pointed end, a process known as treadmilling (74, 75). The length of an actin filament is determined by the ongoing rates of polymerization and depolymerization. The rate of actin polymerization and depolymerization can be regulated through ATP binding and hydrolysis (72). Each 43 kDa g-actin monomer binds ATP, and ATP-bound g-actin has a higher affinity for f-actin. After polymerization, the ATP is slowly hydrolyzed to ADP, and ADP-g-actin is more likely to dissociate from the actin-filament (72). A plethora of actin-binding proteins control actin dynamics, through g-actin sequestration, nucleation, ATP hydrolysis, nucleotide exchange, f-actin severing, capping, and bundling (24, 76-80). LPL is one of several bundling proteins (13).

The structure of LPL is outlined in Fig. 2A. Estimated at 66 kDa in length, the 627 amino acids of L-plastin contain an N-terminal regulatory ‘headpiece’, followed by four C-terminal

calponin homology (CH) domains. Each pair of CH domains creates an actin-binding domain. LPL is specific for β -actin; it did not appear to bind rabbit skeletal muscle α -actin or to chicken smooth muscle γ -actin (81). When folded, the two actin-binding domains of the single L-plastin molecule engage two separate actin filaments, bridging the filaments into bundles (Fig. 2B). LPL binds poorly to pre-existing actin filaments, and likely incorporates itself into actin bundles during active polymerization (82, 83). The structure of one of the two actin-binding domains of LPL bound to the side of an actin filament has been solved (84). While full-length LPL bound to f-actin has not yet been crystallized, the authors drew upon available structures of T-plastin and the *Arabidopsis thaliana* fimbrin core to complete a structural model of LPL cross-linking f-actin (85). Modeling of the interaction between LPL and f-actin revealed that binding of LPL to the side of a filament induces a conformational 'twist', closing the ATP-binding cleft of the g-actin monomer. Closure of the cleft increases the stability of ATP and delays hydrolysis to ADP. Thus, binding of LPL to f-actin stabilizes the polymerized filament, as well as inducing a conformational change by altering the twist and tilt of the filament. Incorporation of molecules of LPL during polymerization cross-links the actively elongating filaments into parallel arrays (82, 83) (Fig. 2B). The focus of research into the requirement for LPL in cellular structures has focused upon its bundling activity; the possibility that the conformational changes of f-actin induced by LPL binding may alter the binding affinity of f-actin for other actin-binding or signaling proteins has not been explored.

The N-terminal regulatory 'headpiece' of LPL contains serine phosphorylation sites, two calcium-binding EF-hand loops, and a consensus sequence for calmodulin binding (63, 86) (Fig. 2A). The bundling function of L-plastin has been shown to be regulated by both calcium binding and phosphorylation (81, 87). The calcium-dependence of T-cell actin bundling by L-plastin was first noted in 1992 (81). Investigators isolated LPL from Jurkat T cells and tested the binding and bundling of β -actin isolated from the same cells. Bundling was assessed through sedimentation and visualization under electron microscopy. Chelation of calcium through the addition of EGTA to the solution greatly increased the ability of LPL to bundle actin filaments. Through titration of the free calcium concentration, the authors determined that LPL bound f-actin at less than 10^{-7} M Ca^{2+} and not at more than 10^{-6} M Ca^{2+} (81). The intracellular T-cell concentration is estimated to vary between 50 nM and > 1 μM during activation (43). The experimentally defined range of calcium regulation of LPL binding to f-actin thus falls within the physiologically relevant ranges of T-cell activation. While calcium regulation of LPL binding to f-actin was clearly demonstrated in this *in vitro* work, *in vivo* correlates of direct calcium-mediated regulation of LPL during T-cell activation or motility have not yet been defined.

The serine phosphorylation site at serine 5 (S5) distinguishes LPL from I- and T-plastin. L-plastin was first recognized as a substrate of phosphorylation in T cells following interleukin-2 (IL-2) stimulation (88, 89). Constitutive phosphorylation of LPL correlated with IL-2-independent growth *in vitro*. Intriguingly, no further work has yet explored the relevance or requirement of LPL phosphorylation following IL-2 receptor engagement. Multiple additional stimulatory pathways have been found to stimulate S5 phosphorylation of LPL, including anti-CD3/CD28 and anti-CD3/CD2 cross-linking and CXCL12 stimulation of T cells, granulocyte-macrophage colony-stimulating factor (GM-CSF) treatment of eosinophils, and f-Met-Leu-Phe stimulation of neutrophils (3, 9, 90, 91). S5 phosphorylation of LPL has been reported to increase the binding of LPL to f-actin and to promote localization of LPL to sites of actin assembly (87). The kinases that phosphorylate S5 of LPL appear to vary by cell type and cell stimulus (9, 91-93). The S7 residue of LPL has also been reported to be phosphorylated in hematopoietic cells (86, 94). However, no physiological function or downstream correlate of S7 phosphorylation has been reported in any cell type.

Beyond direct actin bundling, LPL may participate in cellular processes by linking to other proteins critical to immune processes. A calmodulin-binding site in the N-terminal headpiece, localized between the calcium binding domains and the actin-binding domains, promotes the colocalization of LPL and calmodulin to the immunological synapse (6, 63). LPL has been demonstrated to directly interact with a granulocyte-specific protein, grancalcin (95, 96), and the microglia/macrophage-specific protein Iba1 (97), though the site(s) of binding to LPL are currently unmapped. In addition to containing the actin-binding domains, the C-terminal portion of LPL directly engages integrin β 1 chains (10, 98). As reviewed below, interactions of LPL with proteins other than f-actin are critical to the function LPL plays in T-cell activation. Whether similar interactions control the role of LPL in T-cell motility is as yet unexplored.

Requirement for LPL in T-cell activation

The first report of a role for LPL in T-cell activation came from Samstag and colleagues (99). An early publication in 1994 noted that serine phosphorylation of a 67 kDa protein, identified as LPL, was a correlate of T-cell costimulation with anti-CD3 plus anti-CD2 or cross-linking of CD4 or CD8 with anti-CD3 stimulation (99). Costimulation through anti-CD28 and anti-CD3 was also sufficient to induce strong phosphorylation of LPL at serine 5 (3). Signaling through CD3 or through CD2 or CD28 alone was insufficient to induce S5 phosphorylation. S5 phosphorylation was required for full activation of T cells. Knockdown of LPL, followed by re-expression of a mutant that cannot be phosphorylated on the serine 5 residue (S5A LPL), reduced T-cell activation as measured by upregulation of the activation markers CD25 and CD69 (3). Interestingly, internal expression levels of CD25 and CD69 were identical between T cells re-expressing wildtype (WT) LPL and S5A-LPL, implicating LPL S5 phosphorylation specifically in the transport of proteins to the cell surface. In support of this notion, the activation-induced downregulation of CD3 was not impaired in cells expressing S5A-LPL. Additionally, S5 phosphorylation was not required for correct localization of LPL to the immunological synapse. Whether S5 phosphorylation impacted T-cell proliferation or IL-2 production was not reported in this report (3).

Wabnitz *et al.* (6) followed this study by exploring the requirement for LPL in synapse formation. As reviewed above, responding T cells and APCs form a highly organized adhesion structure at the site of cell:cell contact. While TCR/CD3 engagement and costimulation are required to initiate synapse formation, LFA-1/ICAM-1 interactions guide the later stages of pSMAC maturation. LPL was predominantly localized in the pSMAC of human peripheral blood T cells (PBTs) conjugated to superantigen-loaded Raji B cells. Thus, the distribution of LPL in the synapse is similar to that of polymerized actin and LFA-1. When endogenous expression of LPL was reduced > 90% through short interfering RNA (siRNA), accumulation of polymerized actin in stimulated PBTs was reduced and the recruitment of LFA-1 to the pSMAC was impaired. By re-expressing mutant forms of LPL in human PBTs, the authors determined that both the N-terminal calmodulin binding site of LPL and the actin-binding domains were essential for its localization to the pSMAC, while the EF-hand loops and the S5 phosphorylation site were dispensable. Calmodulin was found to bind LPL in co-immunoprecipitation experiments. Interestingly, calcium inhibited the binding of calmodulin with LPL, as LPL binding to calmodulin was most evident when calcium was chelated with EGTA. Calmodulin also co-localized with LPL in the pSMAC. Antagonizing the function of calmodulin with the inhibitor W7 reduced recruitment of both LPL and LFA-1 into the pSMAC. Through time-lapse video microscopy, calmodulin binding was found to be dispensable for the initial translocation of LPL to a newly forming synapse but was essential for the prolonged retention of LPL at the contact site. Without the calmodulin binding site, LPL was 'lost' from the synapse.

Further analysis revealed that LPL co-immunoprecipitated with LFA-1. Reduction of LPL or loss of the LPL calmodulin binding site inhibited LFA-1 stabilization in the pSMAC and reduced the overall size of the synapse. Impaired synapse formation correlated with reduced proliferation of PBTs following activation stimulated by superantigen-pulsed APCs. Importantly, neither inhibition of calmodulin or knockdown of LPL prevented cSMAC formation downstream of CD3. Consistent with the proposition that LPL is dispensable for early CD3 signaling, knockdown of LPL did not impair the initial burst of calcium flux downstream of CD3 engagement, nor did LPL knockdown impair PBT proliferation stimulated by cross-linked anti-CD3 and anti-CD28 antibodies (6). Thus, the critical role for LPL during T-cell activation was found in the later stages of synapse formation.

An essential role for LPL in the stabilization or maintenance of the synapse was also found using mice deficient for LPL (7). Peripheral T cells isolated from mice with a germline deletion of LPL demonstrated impaired activation, as measured by reduced proliferation and IL-2 production, following stimulation with peptide-pulsed APCs or with plate-bound (immobilized) anti-CD3 and anti-CD28 antibodies. IL-2 production by LPL^{-/-} T cells was intact; however, if cells were stimulated with soluble antibodies (7), consistent with prior observations (6). Extensive analysis of early CD3 signaling pathways revealed no requirement for LPL in early tyrosine phosphorylation, calcium flux, or activation of ERK. However, spreading of LPL^{-/-} T cells on anti-CD3 coated coverslips was impaired, and the synapses of LPL^{-/-} T cells were smaller and contained less f-actin. Antigen-specific LPL^{-/-} T cells formed fewer conjugates with peptide-pulsed APCs. These impairments of late-stage T-cell engagement with APCs are the likely explanation of impaired proliferation and IL-2 production of stimulated LPL^{-/-} T cells (7).

Impaired activation of LPL^{-/-} T cells had physiological correlates; LPL^{-/-} mice were more resistant to experimental autoimmune encephalitis (EAE). EAE is an autoimmune disorder induced in mice through immunization with the MOG peptide, a component of myelin basic protein. EAE models the human disease multiple sclerosis. LPL^{-/-} mice were less likely to succumb to EAE, and those mice that did develop EAE showed reduced clinical symptoms and fewer areas of demyelination on histological analysis. Reduced severity of EAE correlated with reduced proliferation and reduced production of interferon- γ (IFN- γ) and IL-17 by T cells recovered from MOG-challenged LPL^{-/-} mice and restimulated *in vitro*. Furthermore, the survival of skin allografts following discontinuation of immunosuppressive therapy was prolonged in LPL^{-/-} mice, correlating with reduced *in vitro* proliferation of LPL^{-/-} T cells in a mixed lymphocyte reaction. Thus, experiments in LPL^{-/-} mice confirmed an essential role for LPL in the formation of the immunological synapse. Loss of LPL resulted in reduced T-cell activation and amelioration of EAE and skin allograft rejection (7).

Impaired conjugate formation likely results in the failure to retain LPL^{-/-} T cells at the site of antigen presentation (11). Germinal center formation and T-dependent antibody formation has been recently reported to depend upon LPL (11). Transfer of transgenic LPL^{-/-} T cells into WT donors isolated a mild defect in T_H differentiation and a profound defect in the rapid population expansion of LPL^{-/-} T cells following antigen challenge. Somewhat surprisingly, the reduced numbers of responding LPL^{-/-} T cells did not correlate with observable decreased proliferation or with increased apoptosis. Instead, competitive transfer experiments revealed an early defect in the accumulation or retention of LPL^{-/-} T cells in the spleen following antigen challenge. The most parsimonious explanation of this decrease is that reduced conjugate formation between APCs and LPL^{-/-} T cells leads to reduced retention of antigen-specific T cells at sites of antigen presentation. Because fewer cells are retained early in the immune response, there is a severe competitive disadvantage in

generating sufficient numbers of responding T cells to sustain full germinal center formation (11).

A third method has been used to explore the requirement for LPL in T-cell activation. A recent paper has used novel nanobody technology to interfere with the function of L-plastin during T-cell activation. Taking advantage of the heavy-chain only antibodies generated by camelids (100), the authors created small 'nanobodies' in alpacas that bind to defined epitopes on LPL (101). One nanobody recognizes the N-terminal EF-hands of LPL, interfering with the regulatory 'headpiece'. Another nanobody recognizes an epitope that falls between the two actin-binding domains. Both nanobodies inhibit T-cell activation following TCR engagement, as measured by proliferation and IL-2 production. Reduced proliferation correlated with smaller T-cell synapses, as was seen in other work (7). Anti-LPL nanobodies also disrupted T-cell polarization towards the antigen-presenting cell and MTOC docking. Interestingly, there was no interference of ERK activation downstream of nanobody activity, confirming prior research demonstrating that early TCR signaling events remain intact in the absence of LPL, but later stage events are impaired (7). The authors noted direct interaction of LPL with phosphorylated LFA-1. Sequestration of unbound LPL by nanobodies interfered with LPL binding to LFA-1. Reduced levels of LPL available to bind LFA-1 were thought to result in impairment of synapse maturation and stabilization, thus impairing the outcomes of T-cell activation (10). This study confirms the findings of prior reports that used knockdown or knockout technology to reduced intracellular levels of LPL. More importantly, the authors demonstrate that targeting specific regions of LPL can inhibit T-cell activation, raising the possibility that LPL could be used in the future as a rational design target of novel immunotherapeutics (10).

The only discrepancy found in the above studies was the question of whether CD25 and CD69 upregulation required LPL. When the S5A mutant of LPL was overexpressed, CD3-stimulated upregulation of CD25 and CD69 was impaired (3). However, CD25 and CD69 upregulation was intact in activated T cells derived from LPL^{-/-} mice (7). It is possible that human and mouse T cells use LPL in slightly different ways, such that human cells require functional LPL for upregulation of activation markers while murine T cells do not. It seems more likely, however, that overexpression of an S5A LPL mutant may function as a dominant negative, thus generating a different phenotype from LPL-deficiency. Further experimentation will be required to resolve these possibilities.

Requirement for LPL in T-cell motility

In addition to an essential role in the stabilization or maintenance of the nascent immunological synapse, LPL has also been found to be essential in supporting T lymphocyte motility (4, 5, 9). LPL was identified as an essential component of T-cell chemokine-mediated signaling by an unbiased proteomics screen (4). In brief, the 'lipid raft' fractions of cell membranes were isolated from CCR6-expressing Jurkat T cells stimulated with CCL20 or left unstimulated. Cell lysates of stimulated or unstimulated Jurkat T cells were generated by mechanical homogenization in the presence of 1% Triton X-100. Lipid rafts were then defined as the portion of cell lysates found at the interface of the 5% and 30% sucrose layers following ultracentrifugation on a sucrose gradient. Proteins localized to the lipid raft fractions were identified using in-gel trypsin-mediated digestion, labeling with isobaric tags for absolute and relative quantitation (iTRAQ), and LC-MS/MS. Over 700 proteins in the lipid raft fractions were identified, 132 of which showed either increased or decreased association with lipid rafts following chemokine stimulation, with an increase of > 1.29-fold and a decrease of < 0.77-fold defined as significantly different. Coronin1A, LPL, and stress-induced phosphoprotein 1 (STIP1) were all recruited into lipid rafts after chemokine engagement and were selected for further analysis. Using siRNA technology,

LPL and STIP1 were found to be required for normal Jurkat T-cell transwell migration, while coronin1A was dispensable. As was seen in T-cell activation, LPL was not required for calcium flux or ERK phosphorylation following receptor engagement (4).

A more detailed analysis of the role of LPL in T-cell migration and the physiological consequences of LPL deficiency were explored using LPL^{-/-} mice (5). LPL^{-/-} mice were crossed to two different transgenic TCR models, n3.L2 and OT-1. The n3.L2 TCR recognizes a peptide derived from the d allele of hemoglobin bound to I-E^k. T cells expressing n3.L2 are thus selected into the CD4⁺ compartment (102). T cells expressing OT-1 recognize the SIINFEKL peptide derived from ovalbumin bound to class I (K^b), and are thus selected into the CD8⁺ compartment. There was a threefold increase in the number of n3.L2⁺ CD4SP thymocytes in n3.L2 LPL^{-/-} mice and a twofold increase in the number of OT-1⁺ CD8SP thymocytes in OT-1 LPL^{-/-} mice. The SP thymocytes in LPL^{-/-} mice were predominantly CD62L^{high}CD69^{low}CD24^{very low}, consistent with the presence of mature thymocytes that should have emigrated from the thymus. Intrathymic injections of FITC confirmed delayed thymocyte egress in LPL^{-/-} mice, and analysis of mixed bone marrow chimeras confirmed that WT thymocytes held a competitive advantage over LPL^{-/-} thymocytes during thymic egress.

Delayed thymic egress was likely due to impaired motility of LPL^{-/-} thymocytes, as transwell migration experiments identified a cell-intrinsic requirement for LPL in migration of T cells towards the chemoattractants CCL19 and sphingosine-1-phosphate. Two-photon microscopy revealed an intranodal migration defect of LPL^{-/-} OT-1 T cells. Intranodal velocity and motility of LPL^{-/-} T cells was significantly reduced. The meandering index, a measure of the degree to which a cell's movement varies from a straight line, was slightly but not significantly reduced in the absence of LPL. As seen in studies of TCR signaling, LPL was not required for initial chemokine receptor engagement or signaling. CCR7-mediated Rac activation, and initial f-actin polymerization occurred in LPL^{-/-} T cells as it did in WT T cells. Furthermore, chemokine costimulation resulted in increased T cell:APC conjugate formation and CD69 upregulation (5). Chemokine-mediated enhancement of T cell:APC conjugate formation relies on inside-out signaling that upregulates the affinity of the integrin LFA-1 (103). The increased affinity of LFA-1 enhances adhesion between T cells and APCs (103). Notably, the baseline rate of LPL^{-/-} T cell:APC conjugate formation was reduced as was previously seen (7). Despite apparently intact early chemokine signal transduction, the polarization of LPL^{-/-} T cells was impaired (5). Five minutes after stimulation with CCL19, far fewer LPL^{-/-} T cells demonstrated polarization of the chemokine receptor CCR7 to the leading edge of the cell, and after an hour, far fewer LPL^{-/-} T cells demonstrated clear uropod formation. Thus, LPL was shown to be dispensable for early chemokine signaling but essential for the downstream establishment of cytoskeletal polarization required for efficient migration. In the absence of LPL, thymic egress was disrupted, revealing a physiological requirement for LPL in T-cell migration (5).

The major findings of our 2010 report (5) have been confirmed and expanded upon in human T cells using siRNA technology (9). CXCL12 stimulation of human CD3⁺ T cells induced S5 phosphorylation of LPL and localization of LPL to lamellipodia. PKC inhibitors Bisindolymaleimide I, Ro-31-8220, and Go6976 substantially reduced S5 phosphorylation of LPL, while inhibitors of PKA (H89), PI3K (wortmannin, LY294002), tyrosine kinases (genistein), p38 (SB203580), and ERK (PD95089) had minimal effect on S5 phosphorylation of LPL following CXCL12 stimulation. No role for PKC α , PKC β , PKC γ , PKC δ , PKC ϵ or PKC ζ in the phosphorylation of LPL at S5 was found using siRNA knockdown of the individual kinases or combinations (/ , / , / , /) tested. However, pharmacological inhibition of PKC δ was found to prevent the phosphorylation of LPL at S5 in a dose-dependent manner; siRNA-mediated targeting of PKC δ did not reduce

PKC expression satisfactorily. Thus, PKC appears to be the dominant PKC isoform recruited to phosphorylate LPL following chemokine stimulation (9). Similar studies have not yet been performed to determine whether S5 phosphorylation of LPL is regulated in a similar manner downstream of TCR engagement.

Human T cells in which LPL expression was reduced by 75-95% through siRNA targeting demonstrated markedly impaired polarization following stimulation with CXCL12 (9). As found in murine LPL^{-/-} T cells (5), initial Rac activation following chemokine receptor stimulation did not require LPL in human T cells (9). However, Freeley *et al.* (9) found that Rac activity persisted in T cells in which LPL expression had been reduced following chemokine stimulation. Akt phosphorylation was reduced but ERK activation was unaltered in T cells treated with LPL siRNA and stimulated with CXCL12 (9). Diminished polarization and disrupted Rac activity correlated with reduced transwell migration of T cells treated with anti-LPL siRNA, consistent with prior reports (4, 5). Live cell imaging experiments revealed reduced velocity and increased persistence of T cells with reduced levels of LPL plated on immobilized ICAM-1 and CXCL12 (9). Interestingly, the velocity of anti-LPL siRNA-treated T cells was also reduced when plated on the integrin ligands VCAM-1 and fibronectin, but increased persistence was not always seen. Thus, the integrin involved may alter the function of LPL during T-cell migration. Additionally, LPL was not required for the chemokine-mediated upregulation of LFA-1 affinity, as assessed by ICAM-1 binding and a static adhesion assay (9), a finding consistent with intact chemokine-mediated co-stimulation of LPL^{-/-} T cells (5). Finally, LPL appeared to be dispensable for transendothelial migration (TEM), though the authors did not examine whether reduction of LPL expression shifted TEM from a paracellular to a transcellular route (9).

The above studies clearly identify an essential role for LPL during T-cell polarization and migration, but many questions remain. It is not yet clear whether LPL itself polarizes following chemokine stimulation. In human T cells, LPL appeared to be polarized to the lamellipodia following CXCL12 ligation (9), while specific localization of LPL to the lamellipod of murine T cells stimulated with CCL19 was not described (5). Phosphorylated LPL was reported to also be localized to the lamellipod in human T cells. While the authors clearly demonstrate S5 phosphorylation after chemokine stimulation, they do not explore whether S5 phosphorylation is essential to the function of LPL during lymphocyte migration (9). Finally, it is curious that deficiency of LPL led to diminished thymic egress but not to reduced TEM (5, 9).

A unified view of LPL in T cells—required for integrin-mediated adhesion complexes?

From a synthesis of the above studies, it becomes clear that LPL plays little if any role in early TCR or chemokine receptor-mediated signaling events. Whether using murine T cells from LPL^{-/-} mice or human T cells in which LPL expression levels have been reduced through nanobody or siRNA targeting, neither TCR nor chemokine receptor proximal signaling required LPL, nor did LPL appear to be required for the upregulation of integrin affinity triggered by chemokine or TCR-engagement, a signaling process termed integrin ‘inside-out’ signaling. Freeley *et al.* (9) confirmed CXCL12-mediated upregulation of LFA-1 affinity in human T cells with reduced LPL expression through ICAM-1 binding and plate-bound adhesion assays, and Morley *et al.* (5) demonstrated intact chemokine-mediated costimulation, which is dependent upon chemokine-induced changes in LFA-1 affinity. LPL thus appears to be dispensable for integrin inside-out signaling. However, LPL appears to be essential in downstream, later-stage events, such as cell polarization or maturation of the immune synapse—a role consistent with the hypothesis that LPL may function in the maintenance or maturation of integrin-mediated adhesion events. To properly explicate a

postulated role for LPL in integrin-mediated adhesion events, we now briefly review the structure and function of integrin complexes.

Integrins are transmembrane heterodimers that function primarily to promote cell adhesion, either to other cells or to extracellular matrix proteins (104, 105). The primary integrin expressed by T cells is $\alpha_L\beta_2$, LFA-1. Integrins undergo remarkable conformational changes in response to either chemoattractant or other activating signaling. In the low affinity state, the heterodimer is bent acutely towards the cell membrane (105). Activation through cellular inside-out signaling induces a conformational unfolding of the integrin, shifting it to a conformation with intermediate affinity for its ligand. Full activation, or a shift to a high affinity conformation, occurs when the α and β chains of the integrin 'open up'. The high affinity state is best stabilized by binding of the integrin to its ligand and by association of the cytoplasmic tail of the bound integrin to the actin cytoskeleton. Anchorage to f-actin and engagement of integrin ligand exerts sufficient force across the integrin heterodimer to maintain the high affinity conformation (105). Cytoskeletal proteins that are essential to the integrin inside-out signaling include talin-1 and kindlin-3 (106-109). As discussed above, currently available evidence suggests that LPL is dispensable for inside-out signaling.

Clustering of integrins also enhances integrin affinity (110, 111). Clustering is defined as the lateral assembly of multiple integrin heterodimers into larger-order structures at sites of cell adhesion (110). In adherent, predominantly sessile cells, such as fibroblasts, these sites of cell adhesion form well-defined, highly stable structures called focal adhesions. In migratory cells like T cells, these sites of integrin adhesion turn over rapidly and may not contain the same macromolecular structures that characterize classic fibroblast focal adhesions. Regardless of the term used to describe the site of adhesion, integrin clustering is essential for both immune synapse formation and *in vivo* T-cell trafficking (38, 46, 112-114). Integrin-rich microclusters sweep signaling components towards the cSMAC during synapse formation, and integrin-rich adhesion sites can provide traction during some forms of T-cell motility. Formation of integrin clusters requires at least talin, immobilized ECM, and generation of the phosphoinositide PIP_2 , while f-actin is dispensable (110, 111, 115, 116). While LPL has been shown to be required for the accumulation of LFA-1 to the synapse during T-cell activation (6), a detailed molecular analysis of a potential requirement for LPL in early integrin clustering has not been reported, and whether LPL participates in integrin clustering remains an open question. In addition to increasing integrin affinity, integrin clusters generate a positive feedback loop that promotes cell spreading and enables the generation of signaling complexes (116).

Following a shift to the high-affinity state and clustering of subunits, integrins generate adhesion complexes that link the integrin to f-actin structures and generate outside-in signals. Integrin outside-in signal transduction regulates multiple cell processes critical for T-cell activation and motility, including changes in cell shape, polarity, survival, and proliferation (105). Outside-in signaling requires a variety of intracellular signaling intermediaries, including heterotrimeric G proteins, src-family kinases, Syk-family kinases, monomeric small G-proteins such as Rac, Rho and cdc42, and focal adhesion kinase (FAK)/Pyk2 (117). The earliest identified signaling event following integrin engagement is activation of the G_{13} subunit of heterotrimeric G proteins (117), which interacts directly with α_1 and α_3 integrin chains. In the absence of G_{13} , src family kinases are not activated downstream of integrin ligation, and cells fail to spread on integrin ligands. Following G_{13} activation, src family kinases such as Fyn, Hck, Fgr, and Src itself, are recruited and activated (118). Src family kinases, in turn, regulate the activation of the small monomeric G proteins Rho and Rac, likely through the activation of guanine nucleotide exchange factors Vav1, Vav2, and DOCK180 (119-122). These signaling complexes also recruit Syk family kinases and FAK and Pyk2, along with cytoskeletal proteins such as vinculin and paxillin

(105, 123-125). In B cells, total levels of Pyk2 and Pyk2 phosphorylation are reduced in the absence of LPL (126), suggesting a possible function for LPL in linking integrins to downstream signaling moieties. While a similar requirement for LPL in the maintenance of Pyk2 protein expression has not been observed in T cells, work to determine whether LPL participates in integrin outside-in signaling in T cells is ongoing (11).

While integrin ligation and signaling is undoubtedly essential to the normal migration of T cells, how integrins exert force during migration has not been fully determined. Another possible mechanism by which LPL supports T-cell motility and activation would be the linkage of integrin-mediated adhesion sites and the bundled actin filaments that support the lamella (58). As noted in the introduction above, cell protrusion at the leading edge of a cell requires both a dorsal lamellipod and a ventral lamella (56, 58, 62) (Fig. 1C). Integrin-mediated adhesion sites in the lamella provide the structure against which traction forces are exerted by myosin upon the lamellipodia. LPL could serve as link between the integrin tail and lamellar bundled f-actin. In support of this possibility, reduction of LPL expression in T cells impaired the cycling of Rac activity required for lamellipodia extension (9). Integrin-mediated cell spreading and retraction occurs through the generation of dynamic waves of f-actin modulated by the small GTP-binding proteins Rho and Rac (122, 127, 128). Reduction of LPL expression resulted in sustained Rac activity, disrupting lamellipodia formation (9). Further experimentation would be required to determine whether LPL is essential for maintenance of lamellar actin and anchoring of lamella at sites of integrin adhesion.

Integrin release is also critical to effective cell movement, as adhesion sites are disassembled and detached from the underlying substrate as a cell moves forward (47). Internalization of integrin complexes via endocytosis is one mechanism by which adhesive sites are disassembled (47, 129). Integrin endocytosis may occur through clathrin-dependent or – independent endosomal pathways. Internalized integrins are either recycled to the cell surface, where they become incorporated into new sites of adhesion, or they are degraded in lysosomes. A third possible model for the function of LPL in cell motility would thus be as a participant in the endocytic recycling of integrins. Evidence for a possible role of LPL in recycling includes the observation that LPL can bind directly to Rab5, a GTP-binding protein critical to endocytic machinery (130). Furthermore, the yeast homolog of LPL, fimbrin SAC6, is essential in yeast endocytosis and polarization (131). This model would predict that LPL-deficient cells would demonstrate defects in the disassembly and internalization of adhered integrins, prolonging the half-life of adhesive sites and thus slowing cell migration.

Available evidence suggests that LPL most likely plays a role in the late stages of integrin-mediated f-actin remodeling required for sustained T cell:APC contacts and for directed migration. The available evidence, however, is all circumstantial, and whether LPL is required for active integrin outside-in signaling, for linkage of integrins to lamellar f-actin, or for the disassembly and endocytic recycling of integrin complexes remains to be experimentally defined. These non-mutually exclusive models provide testable predictions, and further experiments to elucidate the role of LPL will also illuminate current questions in cell motility and synapse formation, such as how lamellar and lamellipodial actin structures are separated and maintained.

LPL in other cell types

A speculated role for LPL in modulating integrin-mediated adhesion complexes, by any of the above mechanisms, is consistent with studies of the function of LPL in other cell types. Analysis of the requirement for LPL in B-cell motility and development revealed that LPL is essential for integrin-mediated enhancement of chemokine-stimulated transwell migration,

but dispensable for chemokine-mediated upregulation of integrin affinity (126). This requirement for LPL in integrin-mediated enhancement of migration correlated with a loss of expression of the integrin-associated kinase, Pyk2, and disruption of Pyk2 phosphorylation following chemokine and IgM stimulation (126). LPL was isolated in a phosphoproteomics screen of eosinophils stimulated with GM-CSF (91). The phosphorylation site was again identified as S5. LPL co-immunoprecipitated with the integrin-associated protein paxillin and the actin-binding protein cofilin in eosinophils, irrespective of stimulation. The overexpression of a phosphorylated peptide derived from the N-terminus of LPL (amino acids 2–19) enhanced the expression of the integrin α_2 and promoted chemotaxis to eotaxin *in vitro* (91). LPL was found in an unbiased screen to be upregulated in a cell line that expressed constitutively activated integrins (132). Finally, LPL has been demonstrated to directly bind to the cytoplasmic tail of β_1 integrins (98). Thus, observations in other cell types support a model in which LPL interacts with integrins to regulate T-cell motility and activation. The function of LPL in other cell types, including cancer cells, has been extensively reviewed elsewhere (13, 70, 133, 134).

Applications: LPL as a potential target of immunomodulators

Because LPL is restricted in expression to cells of the immune system and because of evident physiological requirements for LPL in T-cell activation and motility, LPL may be a good target of immunomodulation. Recent work has demonstrated that CD3/CD28-stimulated and CD3/CD2-stimulated S5 phosphorylation of LPL is inhibited by the immunosuppressive agent dexamethasone (8). Consistent with a requirement for S5-phosphorylated LPL during synapse formation, dexamethasone treatment impaired the recruitment of both CD3 and LFA-1 to the site of T cell:APC conjugation. Re-expression of S5E LPL, a phosphomimetic, rescued LFA-1 recruitment and synapse formation in the presence of dexamethasone (8). While steroids have broad applicability in the treatment of autoimmune disorders, they have numerous effects on multiple tissues and severe side effects that can limit their long-term utility. A mechanistic understanding of how steroids exert immunosuppression has been lacking. If LPL is a major target in T cells of steroids, this work raises the exciting possibility of designing a ‘smarter steroid’, an immunosuppressive agent that reduces T-cell activation and ameliorates T-cell-mediated autoimmunity without the severe side effects of hyperinsulinemia, adipose deposition, and psychological manifestations that can accompany prolonged steroid use.

Support for targeting LPL with novel immunomodulators comes from a recent analysis of the compound Sch.414319 (135). Sch.414319 was found to ameliorate both EAE and a model of autoimmune arthritis in male Lewis rats. This compound belongs to a class of triaryl bis-sulfones that selectively antagonize the cannabinoid CB₂ receptor. The CB₂ receptor has been implicated in immune cell signaling and migration. Treatment of animals with Sch.414319 reduced the migration of inflammatory cells into the sites of antigen challenge *in vivo*. Phosphoproteome analysis of human peripheral blood mononuclear cells treated with Sch.414319 revealed correlative reduction of LPL phosphorylation (135). Pharmacological manipulation of LPL phosphorylation phenocopies the migration defects seen in models of LPL-deficiency. LPL could thus be targeted to treat human autoimmune disorders in which inhibition of T-cell trafficking has been shown to be therapeutic, such as multiple sclerosis and inflammatory bowel disease (136, 137).

Open questions

Multiple studies from several independent groups have established LPL as a critical regulator of T-cell activation and motility (3–11). Altered phosphorylation, reduced expression, or impaired ability to interact with calmodulin disrupts the function of LPL in

immune synapse formation and stabilization. Defects in synapse formation reduce T-cell activation (3, 6-8, 10). Physiological consequences of loss or inhibition of LPL include reduced formation of T-follicular helper cells, impaired antibody generation, resistance to EAE, and increased preservation of skin grafts (7, 11). Loss or reduced expression of LPL also impairs T-cell motility and trafficking, with impairments of thymic egress and intranodal motility (4, 5, 9). While these studies clearly define LPL as an important molecular regulator of T-cell biology, many mechanistic questions remain to be answered.

As discussed at length above, circumstantial evidence suggests a role for LPL in the modulation of integrin-mediated adhesion complexes during synapse formation and cell motility. LPL directly binds to the cytoplasmic tail of $\beta 1$ integrins, and disruption of binding to integrins disrupts the function of LPL in synapse formation (6, 10, 98). However, deficiency of LPL leads to impaired intranodal motility (5), cell movement that is thought to be largely integrin-independent (138-140). It may be that LPL functions independently of integrin ligation under some circumstances, or it may be that the degree to or mechanism by which LPL participates in lymphocyte motility may depend upon the mode of motility employed. T cells have been described to move in at least two different modes (45, 141-143). One mode of T-cell motility is termed 'walking' and one 'sliding'. In the walking mode of movement, observed on low adhesion strength surfaces, T cells generated multiple sequential sites of cell adhesion that were not well connected. On higher adhesion strength surfaces (higher levels of ICAM-1), T cells moved by sliding, a pattern that resembled fibroblast migration (141). Analysis of human T cells in which LPL expression was reduced revealed increased persistence of cell movement when cells were engaged with $\beta 2$ integrin (LFA-1) but not with $\beta 1$ integrin (VCAM-1) (9). Further analysis of T cells moving under different conditions and employing different integrin ligands will be required to define the precise roles and requirements of LPL in T-cell motility.

Much additional work is required to elucidate the interplay of the multiple regulatory domains of LPL. While the serine 5 residue of LPL is clearly phosphorylated following TCR and chemokine receptor engagement, the function of this phosphorylation has not been fully defined. In transfected Vero cells, phosphorylation of LPL at S5 increased its actin-bundling activity (87), but this has not yet been shown in cell types in which LPL is normally expressed. Furthermore, while S5A mutations alter TCR signaling (3, 8), no analysis of an S5A mutant has yet been shown in T-cell motility. Whether serine phosphorylation of LPL creates binding sites for additional proteins in signaling complexes is largely unexplored.

The kinases that phosphorylate LPL have not been completely defined. Inhibition of PKC resulted in reduced serine phosphorylation of LPL following CXCL12 stimulation in human T cells (9). However, whether LPL is phosphorylated by PKC downstream of TCR engagement has not been examined. As PKC β II, PKC δ , PKA isoforms, and other kinases have been implicated in the regulation of LPL phosphorylation in other cell types (91-93, 144), it is by no means certain the same kinase that phosphorylates LPL downstream of chemokine receptors will be the same as that downstream of TCR engagement. Whether integrin ligation induces or reduces phosphorylation has not been determined.

While emphasis has been placed on the increased affinity for f-actin following S5 phosphorylation of LPL (87), little research has been done to unravel how LPL is dissociated from actin bundles. As calcium binding has been shown to reduce the affinity of LPL for actin *in vitro* (81), it is tempting to speculate that localized calcium fluxes at sites of adhesion may promote LPL dissociation and release of adhesion. However, this has not been experimentally explored. In fact, no comprehensive structure-function analysis of LPL has yet been performed. While individual investigators have detailed regulation of LPL through S5 phosphorylation, calcium binding, calmodulin binding, μ -calpain cleavage, and direct

integrin binding (3, 6, 8, 10, 81, 87, 98), there is as yet no comprehensive understanding of how these multiple regulatory mechanisms interact during T-cell activation or motility.

LPL may function in T-cell biology in roles beyond those that have been described. LPL was first identified in T cells as a substrate of phosphorylation following IL-2 signaling, and constitutive phosphorylation of LPL was found to correlate with IL-2-independent growth in culture (88, 89). No further work has yet been done to determine whether phosphorylation of LPL is sufficient to confer survival in the absence of growth factors. Furthermore, no role for LPL in the ‘stopping’ of T cells after antigen engagement or in symmetry-breaking, which occurs when a T cell disengages from an APC, has yet been explored (28, 45, 51, 145). Exploring other functions for LPL during T-cell stopping or symmetry-breaking could illuminate open questions in how T-cell polarity is initiated, maintained, and then disrupted.

An improved understanding of the function of LPL in T-cell biology could provide critical insights into human health and disease. Human immunodeficiency syndromes can result from mutations in actin-regulatory proteins, such as Wiskott-Aldrich syndrome protein, DOCK8, Rac2, and coronin (146-158). One of the first reports of LPL in human patients noted an alternatively expressed form (1). Whether allelic forms of or loss of LPL contribute to human immunodeficiency syndromes has not been investigated. As LPL may be targeted by dexamethasone and other novel immunosuppressants, and as interference with LPL reduces the symptomatology of EAE and prolongs the half-life of skin grafts, LPL may be a viable target of pharmacological immunomodulation. The key roles of LPL in T-cell activation and motility reviewed here demonstrate that further examination of LPL is well warranted.

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Fig. 1. Actin dynamics are critical to T-cell motility and activation

(A). Motile T cells assume a polarized shape, with a lamellipod at the leading edge and a uropod at the trailing edge. Two networks of f-actin create the lamellipod and the lamella, with greater stability of bundles and filaments in the lamella and more rapid turnover of branching chains in the lamellipod. Contractile elements, such as the motor protein myosin II b (MIIB), are concentrated in the uropod. Arrow represents direction of retrograde actin flow. (B). The immunological synapse has been described as a radially symmetrical lamella/lamellipod, with actin flow and regulatory molecules similar to those found in a migrating cell. The dSMAC is analogous to the lamellipod (LP), the pSMAC to the lamella (LM) and the cSMAC as an actin-depleted area where signaling is terminated. This conceptual framework provides an explanation for the segregation of cell surface receptors and signaling molecules observed in the synapse and for the critical role of f-actin in forming and maintaining the synapse. (C). Proposed model for force generation and forward motion during cell movement, drawn from Giannone *et al.* (58). Step 1. Retrograde actin polymerization in the lamellipod (yellow) extends the actin network until it connects with a site of adhesion anchored in the lamella. Step 2. Contractile force generated by myosin (MII) pulls the lamellipodial actin, generating tension. Step 3. Tension on f-actin bends the lamellipod, retracting the edge, and a new site of adhesion forms. Step 4. The tip of the lamellipodia f-actin breaks off. Step 5. The cycle restarts.

Fig. 2. Structure and function of LPL

(A). LPL is a 66 kDa protein encompassing an N-terminal regulatory 'headpiece' followed by two ABDs. A serine residue at position 5 is heavily phosphorylated following external stimulation. Two EF-hand loops create a putative calcium-binding domain, and a calmodulin-binding domain (Cal) has been described. LPL contains 4 calponin homology (CH) domains, with each pair creating an ABD. (B). LPL intercalates between actin filaments as polymerization occurs, stabilizing and bundling f-actin. ATP binding by G-actin enables polymerization, which occurs more quickly at the barbed end. Slow hydrolysis of ATP to ADP results in depolymerization, more likely to occur at the pointed end. Diagrams are similar to those previously published (134).