Central role of paxillin phosphorylation in regulation of LFA-1 integrins activity and lymphocyte migration

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Key words: lymphocytes, paxillin, actin, Rac1, RhoA, LFA-1, PKC-δ

Coordinated changes of actin cytoskeleton and cell adhesion accompany maturation of lymphoid cells, their migration through lymphoid organs and to sites of inflammation, as well as metastasis of transformed cells. Here we discuss the central role of the actin-regulating adaptor protein, paxillin, during lymphocyte transition from a polarized, motile cell phenotype to a round and immobile one with fully active LFA-1. In Baf3 murine pro-B lymphocytes, the former phenotype is induced by IL-3 that stimulates FAK-mediated phosphorylation of paxillin at tyrosines (Y) 31 and 118 and a consequent Rac1 activation. Rearrangements of actin cytoskeleton that lead to the cell’s acquisition of a spherical shape and LFA-1 activation are achieved upon activation of PKC-δ that binds and directly phosphorylates paxillin at threonine (T) 538 with consequent RhoA activation. This is accompanied by dephosphorylation of paxillin Y31/118 and by Rac1 inactivation. We propose a model of signaling cascades that reflects the interplay between the IL-3- and PKC-δ-mediated pathways.

Introduction

Lymphocyte migration plays a key role in a wide variety of physiological processes. During maturation of B- and T-lymphocyte precursors, the maturing cells must move to their appropriate location in lymph nodes, thymus and spleen in response to chemokines.1,2 During inflammatory responses, reactive lymphocytes must be recruited into the sites of inflammation, in response to “inflammatory interleukins,” such as IL-1, IL-3 and IL-6, and cytokines produced by other cells in these sites. When solid tumors, including lymphomas, metastasize and migrate from the initial site of malignant transformation to distant sites, they can be particularly challenging to treat.3 Lymphocyte migration is accompanied by a polarized redistribution of cytoskeletal proteins, chemo-attractant receptors, adhesion and signaling molecules.4,5 Adhesion molecules in such lymphocytes assemble into complexes at point contacts, structures that are similar to the focal adhesions of fibroblasts. In contrast to slowly migrating fibroblasts, rapidly migrating lymphoid cells form very few point contacts and have only a few visible integrin clusters. These clusters are usually short-lived and very dynamic. Because of their short time of interaction with the substrate, rapidly migrating lymphocytes only weakly adhere to the substrate. Arrest of cell migration is associated with rearrangements of the actin cytoskeleton and activation of integrins that lead to the formation of large and stable focal adhesions. These adhesions are usually less dynamic and, therefore, capable of firm attachment to the substrate. While rapid adhesion turnover requires activity of Focal Adhesion Kinase (FAK) and Src, stabilization of focal adhesions is usually accompanied by inactivation of these kinases.6,8 This is consistent with the notion that active FAK keeps RhoA in check and that depletion of FAK leads to RhoA activation.9

Activity of integrins, including (α, β) LFA-1 integrin is critical in mediating lymphocyte adhesion vs. migration.10 This surface receptor is selectively expressed on leukocytes. It recognizes and binds its ligands, intracellular adhesion molecules 1, 2 and 3 (ICAM-1, 2, 3).11 Leukocytes circulating in the bloodstream express inactive LFA-1 that is unable to bind to the ligands. Changes in LFA-1 activity occur during lymphocyte maturation, during the immune response, which often involves migration through tissues to sites of inflammation, as well as during metastasis of transformed cells. Under experimental conditions, stimulation of T-cell receptors or exposure to phorbol esters, pharmacological analogs of the endogenous PKC activator, Diacyl Glycerol (DAG), lead to LFA-1 activation. This phenomenon is also called “inside-out” signaling.

LFA-1 activation is accompanied by clustering of the receptors on the cell surface and change of their intramolecular conformation, referred to, respectively, as avidity and affinity changes.11,12 LFA-1 integrins bind to the actin cytoskeleton by the cytosolic domains of their α and β subunits.13 Receptors’ lateral movement requires temporary dislodgement from the actin cytoskeleton; therefore, actin destabilization often triggers LFA-1 clustering. LFA-1 clustering, even when caused by actin-disrupting drugs, is always accompanied by an increase of LFA-1 affinity. It has been proposed that during LFA-1 clustering, various signaling molecules come into contact with each other thus, resulting in a change of integrin conformation.14 Human LFA-1 receptors have three discrete conformational states, characterized by low, intermediate and high affinity, as measured by the
Paxillin, a highly phosphorylated adaptor molecule plays a key role in control of cells adhesion and migration. Binding of the Rap1-effector protein, RapL or talin to the cytosolic portion of the Rap1-effecter protein, RapL or talin to the cytosolic portion restricting the receptor’s mobility.12

edge of motile lymphocytes and that it activates LFA-1 without that RapL is also involved in LFA-1 recruitment to the leading skeleton matrix can also affect integrin mobility. It is thought partition of the receptors.”18 Specifically, the actin-based cytoskeleton, leaves the other mechanism intact.

Studies of receptors that, like LFA-1, possess transmembrane domains showed that their activity is regulated by two independent mechanisms, “actin meshwork confinement” and “dynamic partition of the receptors.”19 Specifically, the actin-based cytoskeleton provides mesh-like barriers responsible for the confinement of the receptors on the cell surface, and the receptors are recruited into lipid-rich membrane rafts during dynamic diffusion over the cell surface. These mechanisms are independent; therefore, disruption of one of them, by drugs that target cytoskeleton or lipid domains, leaves the other mechanism intact.

This review is based on the collective knowledge accumulated in the field of cell migration. In prior studies Katagiri and co-authors reported that in the IL-3-dependent murine pro-B-lymphocytic cell line, BaF3, three independent pathways regulate LFA-1 integrins activity.12 While H-Ras and Rac1 mediate LFA-1 activation through PI3-kinase, the pathway regulated by Rap1 is PI3-independent. The endogenous second messenger Diacyl Glycerol (DAG), or its pharmacological analog, TPA, activates LFA-1 integrins in a protein kinase C (PKC)-dependent fashion. We have further extended the understanding of “inside-out” signaling cascades leading to LFA-1 activation in BaF3. We suggest that phosphorylation of paxillin at two distinct sites, tyrosines 31/118 and threonine 538, initiated by the Ras-coupled IL-3 receptor or by activated PKC-δ, respectively, coordinates the signals from these three pathways (Fig. 1). While we concentrated on the studies of migration arrest and LFA-1 activation in BaF3,22-25 the described signaling cascades and related biological responses were also observed in other families of hematopoietic cells. All hematopoietic cell lines that we have assessed utilize at least some of the growth factor- or TPA-induced pathways we have described. In the IL-3-dependent pluripotent myeloblastoid cell line, 32D, the pathways induced by IL-3 and phorbol esters are fully preserved, as evident by paxillin phosphorylation status, LFA-1 activity and cell motility. Consistent with this, 32D is highly motile and undifferentiated in the presence of IL-3, whereas it adheres to ICAM-1 and undergoes macrophage differentiation in response to PKC-δ activation, thus, providing another in vitro example of PKC-δ/TT38 involvement in maturation of hematopoietic cells. What is more, M-CSF, a growth factor that is known to activate PKC-δ, is involved in macrophage differentiation of myeloblastoid cells.28

**Discussion**
GTPase, Rac1, a regulator of the actin cytoskeleton. Rac1, but not RhoA or cdc42, is responsible for actin cytoskeleton changes that produce a polarized and highly motile phenotype of Baf3. Consistently, actin inhibitors, but not tubulin inhibitors, disrupt this cell phenotype, immobilizing the cells. It is thought that actin provides barriers for transmembrane domain-containing integrins clusters, such as LFA-1 (“meshwork confinement”), thus contributing to regulation of their activity. Consistently with this role of actin, an actin-regulating protein, paxillin, forms “pockets” surrounding clusters of LFA-1 integrins, primarily at the leading edge of polarized Baf3. These LFA-1 clusters, however, only minimally adhere to their ligand, ICAM-1, facilitating a highly motile cell phenotype.

We can extend our understanding of the signaling cascades induced by IL-3 by considering the experiments made on the 32D cell line. Phosphorylated paxillin tyrosines 31 and 118 recruit the SH2-containing protein, CRKL. By complexing with the guanine nucleotide exchange factor, C3G, CRKL activates the small GTPase, Rap1. Binding of the Rap1-effector protein, RapL, to the cytosolic domain of the αι subunit of LFA-1 contributes to LFA-1 activation. It is thought that RapL activates integrins without abolishing their mobility on the cell surface and, therefore, can occur in highly motile cells.

In an attempt to integrate the results from Baf3 and 32D cell lines, we proposed that IL-3, Ras, FAK, paxillin phosphorylated on its tyrosines 31/118, Rac1 and PI-3 are components of the pathway that mediate a motile phenotype with partially active LFA-1 integrins. Phosphorylated tyrosines 31/118 of paxillin also mediate PL-3-independent Rap1 signaling that probably also contributes to this cell phenotype. It is worth noting that the pathway regulated by phosphorylated Y31/118 of paxillin and Rac1 target the actin cytoskeleton rather than LFA-1 itself as is suggested by the distribution of paxillin “pockets” around clusters of LFA-1 receptors and their activation is probably merely the consequence of their clustering as was also suggested by Lub and co-authors. Therefore, the described pathway may contribute to activation of a variety of integrins. We also suggest that pathways that lead to FAK-dependent phosphorylation of paxillin Y31/118 and initiated by IL-3/Ras in Baf3 and 32D, may also be activated by other Ras-coupled receptors, as we observed in several cell lines of hematopoietic origin and fibroblasts.

What signaling cascades could be activated by paxillin phosphorylated at Y31/118 to mediate the described cell phenotype? Most of the studies published on paxillin Y31/118 were made on adherent cells such as fibroblasts, but the results still may be relevant to the lymphocytic cells we study. It is thought that activated FAK mediates phosphorylation of paxillin Y31/118 by Src tyrosine kinase. These phosphorylated tyrosines stabilize paxillin’s interaction with Src itself and recruit CSK, a Src inhibitor, thus providing rapid regulation of Src activity.

Phosphorylated Y118 provides a docking site for Erk kinase that stabilizes binding of FAK or its chiefly hematopoietic homolog, Pyk2, to paxillin and activates Rac1. In adherent cells phosphorylated paxillin Y31/118 recruits CRKII that, in a complex with C3G, activates Rap1. Finally, phosphorylated paxillin Y31 and Y118 are recognized by the SH2 domain of p120RhoGAP, thereby displacing p190RhoGAP, a RhoA inhibitor, RhoA suppression prevents formation of large and non-dynamic integrin clusters. In summary, phosphorylation of paxillin at Y31/118 affects multiple parallel pathways that result in activation of Rac1 and Rap1 and inhibition of RhoA.

While phosphorylated tyrosines Y31/118 of paxillin are the major regulators of Rac-1, this small GTP-ase can be regulated by alternative pathways. For instance, a molecular complex consisting of p21 GTPase-activated kinase (PAK), PIX (a guanine nucleotide exchange factor) and p95PKL (paxillin-kinase linker) binds to the LD4 domain of paxillin, leading to Rac1 activation.

In addition, p130Cas mediates Rac1 and Rap1 activation through recruitment of DOCK180.

PKC-δ phosphorylates paxillin at T538, resulting in actin depolymerization and maximal LFA-1 activation. We have shown that one of the PKC isoforms expressed in Baf3 cells, PKC-δ, when activated by DAG or its pharmacological analog, TPA, directly binds and phosphorylates paxillin within its LIM4 domain at T538. Within a minute of PKC-δ activation, a partial depolymerization of actin could be observed, which results in a loss of cell polarization, redistribution of paxillin “pockets” containing the LFA-1 clusters, and an increase of LFA-1 affinity. Such cells firmly adhere to the ICAM-1 ligand and become immobile. It is well known that integrin activation is a function of RhoA. In fact, TPA-induced LFA-1 activation in other lymphoid cells has been shown to be mediated by RhoA. In Baf3, LFA-1 activation is preventable by pre-treatment with C3 toxin, a RhoA inhibitor, suggesting RhoA involvement. LFA-1 activation can be induced by replacement of the endogenous paxillin with T538E paxillin, a phosphomimetic mutant. Figure 2 shows clusters of LFA-1 integrins located within paxillin “pockets” in such cells. Our findings confirm and extend the report of Katagiri et al. about the existence of another pathway in Baf3 that is initiated by activated PKC-δ and paxillin phosphorylated at T538 and leads to RhoA activation and further increase of LFA-1 activity. In fact, LFA-1 activation in all cell lines we assessed always coincides with the level of paxillin phosphorylation on T538. Again, because the PKC-δ-mediated pathway primarily targets actin cytoskeleton rather than LFA-1 integrins themselves, it may well be involved in activation of some other integrins.

Phosphorylation of paxillin at T538 by PKC-δ is accompanied by dephosphorylation of paxillin Y31/118 and inhibition of Rac1. To assess the biological role of this dephosphorylation, a phosphoinhibitory paxillin mutant with alanine substitution of Y31/118 was expressed in Baf3 cells. This mutant inactivates the effect produced by either IL-3 deprivation or cytochalasin D, which cause FAK inactivation and dephosphorylation of paxillin Y31/118, leading to disruption of Rac1 signaling.

It should be noted that the described transition from the Rac1-to RhoA-mediated signaling cascade in Baf3 cells is accompanied by a transient depolymerization of actin. The status of the actin cytoskeleton during such a transition may be controlled by the
of active FAK, is a prominent target of PKC-δ. This PKC isoform phosphorylates Src kinase at serine 12, which inhibits Src activity in some cell lines. Besides paxillin, Src binds, phosphorylates and stimulates the activity of p190RhoGAP, probably by interfering with p120GAP binding. Inactivation of Src by PKC-δ would inactivate p190RhoGAP, thus, contributing to RhoA activation. Thus, we propose that in BaF3 cells both Src S12 and paxillin T538 are direct targets of activated PKC-δ, and their phosphorylation contributes to switching from Rac1-mediated to RhoA-mediated signaling. The depolymerization of the actin cytoskeleton that follows TPA addition would further inactivate FAK.

We can only speculate how phosphorylation of paxillin’s T538 may affect downstream signaling. Several proteins bind paxillin within its LIM4 domain, e.g., tubulin and PTP-PEST, and both of them could be affected by phosphorylation of T538. Our earliest experiments showed that tubulin is not a driving force for morphologic changes observed in TPA-treated BaF3 cells. The potential effect on PTP-PEST, however, is especially interesting. While paxillin is not a direct target of PTP-PEST, this phosphatase can dephosphorylate FAK, bringing dephosphorylation of paxillin’s Y31/118 and Rac1 inhibition. P130CAS and Shc are other prominent targets of the PTP-PEST phosphatase. Similar to paxillin, phosphorylated P130CAS is involved in activation of Rac1 and Rap1. Hence, dephosphorylation of P130CAS would enhance the effects produced by dephosphorylated paxillin. Dephosphorylation of Shc would inactivate Ras downstream signaling, thus inactivating Rac1. Ras normally counteracts the RhoA pathway, thus, its inactivation also contributes to RhoA activation. Finally, Rac1GEF, VAV1 and p190RhoGAP appear to be direct targets of PTP-PEST, and their dephosphorylation would inactivate Rac1 and activate RhoA. Thus, PTP-PEST effects await direct experimental proof.

**Summary and Perspective**

In this report we discussed the signal transduction cascades behind the dynamic and coordinated changes of paxillin phosphorylation and actin cytoskeleton, on the one hand, and LFA-1 integrin activity, on the other. We proposed a model of signaling cascades that may be involved in regulation of transition from a polarized, motile lymphocyte phenotype to an adherent one. These events are constantly occurring in lymphoid organs, tissues and blood vessels. Therefore, appreciation of these mechanisms is essential for the understanding of immune system function.

**Acknowledgments**

This research was supported in part by the Intramural Research Program of the NIH and the National Cancer Institute.
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