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A role for the actin-bundling protein L-plastin in the regulation of leukocyte integrin function

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ABSTRACT **Regulation of leukocyte integrin avidity is a crucial aspect of inflammation and immunity. The actin cytoskeleton has an important role in the regulation of integrin function, but the cytoskeletal proteins involved are largely unknown. Because inflammatory stimuli that activate integrin-mediated adhesion in human polymorphonuclear neutrophils (PMN) and monocytes cause phosphorylation of the actin-bundling protein L-plastin, we tested whether L-plastin phosphorylation was involved in integrin activation. L-plastin-derived peptides that included the phosphorylation site (Ser-5) rapidly induced leukocyte integrin-mediated adhesion when introduced into the cytosol of freshly isolated primary human PMN and monocytes. Substitution of Ala for Ser-5 abolished the ability of the peptide to induce adhesion. Peptide-induced adhesion was sensitive to pharmacologic inhibition of phosphoinositol 3-kinase and protein kinase C, but adhesion induced by a peptide containing a phosphoserine at position 5 was insensitive to inhibition. These data establish a novel role for L-plastin in the regulation of leukocyte adhesion and suggest that many signaling events implicated in integrin regulation act via induction of L-plastin phosphorylation.**

An important feature of polymorphonuclear neutrophils (PMN) is the ability to become activated rapidly at sites of inflammation. Recruitment of PMN into inflamed tissues and subsequent execution of essential effector functions require integrin-mediated cell–cell and cell–extracellular matrix adhesion (1, 2). The integrin $\alpha M\beta 2$ (Mac-1, CD11b/CD18) binds its ligand poorly in quiescent cells. Activation of $\alpha M\beta 2$ and reorganization of the actin cytoskeleton are both critical events for PMN migration into tissues and for the development of the effector phenotype (1, 2). Both protein kinase C (PKC) and phosphoinositol (PI) 3-kinase have been implicated in activation of $\alpha M\beta 2$-mediated adhesion (3–5), but the molecular mechanisms by which this event occurs are not well understood. The actin cytoskeleton is important for driving membrane remodeling during adhesion-dependent functions such as migration and phagocytosis (6, 7). The actin cytoskeleton also acts as a platform to bring together surface receptors, activatable enzymes, and substrates during signal transduction from a variety of receptors, including integrins (8–11). In addition, the actin cytoskeleton likely has an essential role in the activation of $\beta 2$ integrin-dependent adhesion (10, 12). Although several cytoskeletal proteins are known to bind integrin β-chain cytoplasmic tails (13, 14), the mechanism by which the cytoskeleton modulates integrin avidity for ligand is unknown.

L-plastin (LPL) is a leukocyte-specific actin-bundling protein that has been implicated in regulating PMN signal transduction (15). LPL is a member of the fimbrin family of actin-binding proteins characterized by two actin-binding domains and a headpiece region containing two EF hand-type calcium-binding domains (Fig. 1 and ref. 16). Calcium binding inhibits actin-bundling activity of plastins *in vitro* (17, 18), but the role of calcium in regulating LPL function in cells is not known. LPL is unique in the fimbrin family because it can become phosphorylated on serine in the headpiece region (19, 20), suggesting that phosphorylation may be a specific mechanism of regulating LPL function in leukocytes. A variety of inflammatory mediators that activate $\beta 2$ integrins such as chemokines, formylated bacterial peptides, cytokines, immune complexes, and phorbol 12-myristate 1-acetate (PMA), also induce LPL phosphorylation (19, 21–25). Despite its close association with activation of adhesion, LPL phosphorylation in PMN does not require $\beta 2$ integrin expression (24), suggesting that its serine phosphorylation may precede $\alpha M\beta 2$ activation. Thus, we hypothesized that LPL phosphorylation may have a role in regulating integrin-mediated adhesion in leukocytes.

In this paper, we directly test whether LPL phosphorylation is involved in $\alpha M\beta 2$ activation by treating PMN with a synthetic peptide (LPL*tat*) derived from the region of LPL containing the serine required for phosphorylation linked by its carboxyl terminus to a highly basic region of the HIV tat protein that enables peptide translocation across cell membranes (26, 27). We demonstrate a fundamental role for serine phosphorylation of the actin-bundling protein L-plastin in regulation of $\alpha M\beta 2$-dependent adhesion. Our data suggest that many of the signaling events that activate adhesion in leukocytes do so through LPL phosphorylation.

MATERIALS AND METHODS

Materials. Okadaic acid, wortmannin, and LY294002 were obtained from LC Services (Woburn, MA). Gö6976 was from Biomol (Plymouth Meeting, PA). The anti-$\beta 2$ mAb IB4 (28) and the anti-HLA mAb W6/32 (29) were prepared as described (30). The anti-$\beta 1$ mAb P1F6 (31) was provided by Dean Sheppard (University of California at San Francisco), and the anti-$\beta 5$ mAb P5D2 (32) was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). CBRM1/5 binds a neoepitope on activated $\alpha M\beta 2$ (33) and was kindly provided by Timothy Springer (Harvard Medical School). All other reagents were from sources as published previously (5, 24).

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Abbreviations: PMN, polymorphonuclear neutrophils; LPL, L-plastin; IC, immune complex; FCS, fetal calf serum; PMA, phorbol 12-myristate 1-acetate; fMLP, formylmethionylleucylphenylalanine; PKC, protein kinase C; PI, phosphatidylinositol; FITC, fluorescein isothiocyanate.

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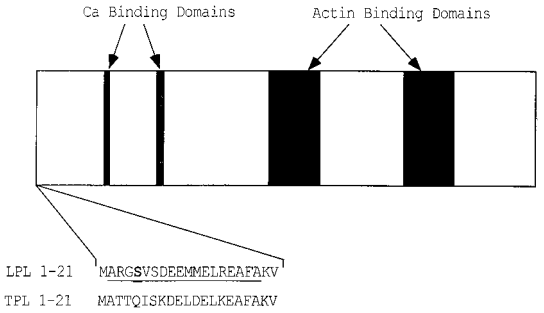

FIG. 1. The domain structure of the fimbrin family and the N-terminal amino acid sequence of LPL and TPL. The fimbrin family is characterized by two EF hand calcium-binding domains in the N-terminal headpiece region and two α-actinin-type actin-binding domains. The sequence of amino acids 1–21 of LPL and TPL is in brackets. The sequence used to derive the LPL*tat* peptide is underlined. Ser-5, which is critical for LPL phosphorylation, is in bold. Note that TPL, which has not been shown to be phosphorylated, has a Gln at position 5.

Peptide Synthesis. Peptide synthesis was carried out on a PE/ABD Peptide Synthesizer, Model 433 (Perkin–Elmer/Applied Biosystems), Foster City, CA) by using fluorenylmethoxycarbonyl-protected amino acids (Anaspec, San Jose, CA). Side-chain deprotection and cleavage of the peptide from the resin were performed with trifluoroacetic acid/anisole/dimethyl sulfide/ethanedithiol (9:0.0:0.25:0.25). Peptides were purified by RP-HPLC, and peptide identity was confirmed by mass spectrometry using an LCQ Iontrap (Finnigan-MAT, San Jose, CA). Fluorescein isothiocyanate (FITC)-conjugated peptides were obtained from Quality Control Biochemicals (Hopkington, MA).

Purification of Leukocytes and Adhesion Assays. Human PMN were purified as described from whole blood by dextran sedimentation and gradient centrifugation (34). Human monocytes were purified as described from whole blood by elutriation (35–37). Murine bone marrow PMN were harvested as described and purified by density gradient centrifugation (38). Ninety-six-well Immulon 2 plates were coated and adhesion assays were performed exactly as described (5). To assess tat peptide effects on adhesion, various concentrations of peptides suspended in $HBSS^{++}$ (Hanks' buffered salts solution with 1 mM Mg^{2+} and 1 mM Ca^{2+}) were added to the cells after allowing them to settle onto fetal calf serum (FCS)-coated wells for 7 min at room temperature. The cells were incubated at 37°C for the indicated time. The fluorescence (485-nm excitation, 530-nm emission wavelengths) was measured by using an fMax fluorescence plate reader (Molecular Devices) before and after washing twice with 150 μl PBS. Percent adhesion was calculated by dividing the fluorescence after washing by the fluorescence before washing. In preliminary experiments, fluorescence was shown to be linearly related to cell number (data not shown).

L-plastin phosphorylation. LPL phosphorylation was assessed in [^{32}P]phosphoric acid-loaded PMN exactly as described (24). To assess the effects of tat peptides on LPL phosphorylation, cells were added to FCS-coated wells and incubated for 7 min at room temperature to allow the cells to settle before adding peptides at the indicated final concentrations. LPL immunoprecipitates were subjected to SDS/PAGE, and phosphorylation was assayed by autoradiography of dried gels. Phosphorylation was quantitated by densitometry of the exposed film by using FL4000 Imaging Software (Georgia Instruments, Atlanta, GA).

To determine the site of LPL phosphorylation, HeLa cells were transfected with 2 μg LPL cDNA in pBSII-SK(+) (Stratagene) and 1 hr later were infected with a recombinant vaccinia virus encoding the T7 polymerase, exactly as described (39). After 6 hr, the HeLa cells were loaded with [^{32}P]phosphoric acid as above and then treated with 2 μM okadaic acid for 60 min at 37°C. LPL phosphorylation was assayed as above.

Uptake of Fluorescent Peptides. Purified PMN were suspended at 1×10^6 cells/ml in HBSS (Hanks' buffered salts solution without Mg^{2+} or Ca^{2+}) and incubated with FITC-conjugated peptides (100 μM) for 10 min at 37°C. The cells then were washed five times with HBSS. Cellular fluorescence was measured by flow cytometry. Remaining extracellular fluorescence was quenched by adding 0.1% trypan blue dye before measuring fluorescence. This concentration of trypan blue was shown to quench the extracellular fluorescence of fluorescent beads bound to PMN and K562 cells (40) and of PMN stained with FITC-conjugated primary antibodies (unpublished data).

RESULTS

LPL Phosphorylation Correlates with αMβ2 Activation. Two distinct, proximal signaling pathways exist for activation of αMβ2 in PMN, an FcγR-initiated PI 3-kinase-dependent pathway, and a G protein-linked receptor-induced PI 3-kinase-independent pathway (5). These stimuli also induce LPL phosphorylation in PMN (24). We tested whether or not LPL phosphorylation is regulated by these signaling pathways. Sustained adhesion to immune complex (IC)-coated surfaces, which requires αMβ2 activation (5), was abrogated by two different inhibitors of PI 3-kinase with distinct mechanisms of action, wortmannin and LY294002 (Fig. 2*A*). In contrast, formylmethionylleucylphenylalanine (fMLP)- and PMA-induced adhesion was insensitive to wortmannin and LY294002. Like adhesion, LPL phosphorylation induced by adhesion to IC was inhibited by wortmannin and LY294002, whereas fMLP- and PMA-induced LPL phosphorylation was wortmannin- and LY294002-insensitive (Fig. 2*B*). The IC_{50} for inhibition of LPL phosphorylation by wortmannin was 5 nM (data not shown), similar to the IC_{50} for inhibition of sustained adhesion to IC (5). fMLP-, but not IC- or PMA-induced adhesion and LPL phosphorylation were abolished by 2 μg/ml pertussis toxin (data not shown). These data demonstrate that the pathways that regulate αMβ2 activation in response to a variety of agonists also control LPL phosphorylation.

The phosphorylation site of LPL has been localized to the N-terminal 12 aa that include two serine residues (Fig. 1 and ref. 19). A genetic approach was adopted to map more precisely the phosphorylation site, substituting Ala residues for Ser-5 or Ser-7. Three mutants, S5A, S7A, and S5A/S7A, were expressed in HeLa cells by using a vaccinia virus transient expression system. LPL phosphorylation was enhanced by treatment with the phosphatase inhibitor okadaic acid, which we have shown previously to enhance LPL phosphorylation in PMN (data not shown). Wild-type LPL and the S7A mutant were phosphorylated in okadaic acid-treated HeLa cells, whereas the S5A and S5A/S7A mutants were not (Fig. 2*C Upper*). Differences in the amount of phosphorylation were not a result of differences in expression of the various proteins (Fig. 2*C Lower*). These data demonstrate that Ser-5 is necessary for LPL phosphorylation in HeLa cells, but do not rule out the possibility of Ser-7 also being phosphorylated, with a requirement for Ser-5 to achieve Ser-7 phosphorylation. Although it is possible that the mechanism of LPL phosphorylation is different in HeLa cells and leukocytes, okadaic acid enhancement of LPL phosphorylation in both HeLa cells and PMN and localization of the phosphorylation site to the N-terminal region of LPL containing Ser-5 and Ser-7 in murine macrophages and HeLa cells strongly suggest that the requirement for Ser-5, discovered in HeLa cells, is generalizable to leukocytes.

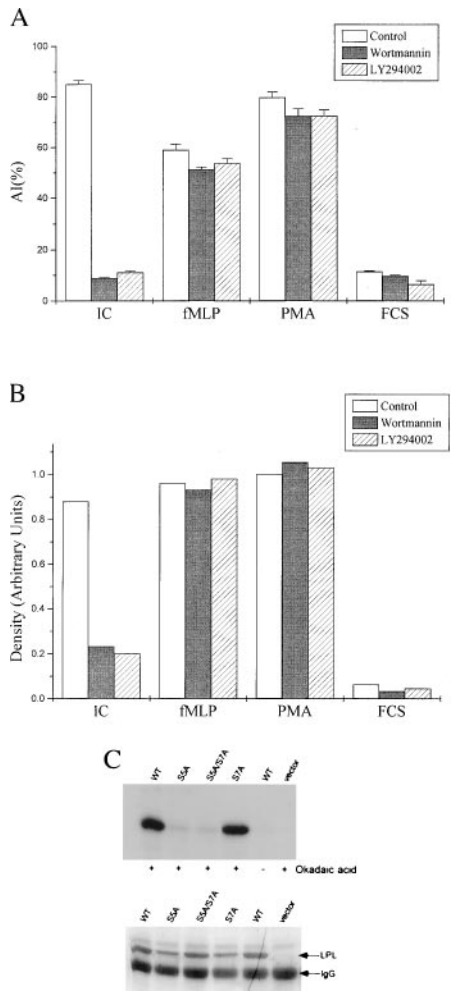

FIG. 2. The same proximal pathways that activate $\alpha M\beta 2$-mediated adhesion induce phosphorylation of serine 5 of LPL. (*A*) PMN loaded with the fluorophore calcein were treated with control buffer, wortmannin (100 nM), or LY294002 (25 μM) before measurement of adhesion to IC (30 min) or fMLP- or PMA-induced adhesion to FCS (3 min). The data are the mean ± SE of triplicate wells, reported as attachment index (AI), which is the percentage of cells that remain adherent after washing. Wortmannin and LY294002 significantly inhibited adhesion to IC, but did not affect fMLP- or PMA-stimulated adhesion to FCS (<0.05). The data are representative of three separate experiments. (*B*) PMN loaded with [^{32}P]phosphoric acid were treated with control DMSO, wortmannin (100 nM), or LY294002 (25 μM) and allowed to adhere to plates coated with IC or FCS or stimulated in suspension with fMLP (100 nM) or PMA (50 ng/ml) for 20 min at 37°C. LPL was immunoprecipitated and phosphorylation was quantitated by densitometry of autoradiograms. Data are normalized to maximal LPL phosphorylation induced by PMA. Each point represents the average of two separate experiments. (*C*) LPL or S5A, S7A, or S5A/S7A mutants of LPL were expressed in HeLa cells. After 6 hr of infection, the cells were loaded with [^{32}P]phosphoric acid and treated with buffer control or the serine phosphatase inhibitor okadaic acid (1 μM) for 30 min at 37°C. LPL phosphorylation (*C Upper*) was assayed as in *B*. Loading of LPL was assessed by Coomassie blue stain (*C Lower*). Results are representative of three separate experiments.

LPL N-Terminal Peptide Induces Adhesion in PMN. To determine whether the N-terminal region of LPL containing the phosphorylation site was sufficient to regulate adhesion in PMN, we treated PMN with peptides corresponding to amino acids 2–19 of LPL (Fig. 1) linked at the carboxyl terminus to a highly basic peptide derived from the HIV tat protein (LPL*tat*, Table 1). This tat sequence is sufficient to enable translocation of peptides across cell membranes (26, 27). The LPL*tat* peptide rapidly induced adhesion of freshly isolated PMN to FCS-coated surfaces (<10 min, data not shown), with maximum adhesion at a peptide concentration of 100 μM (Fig. 3*A*). Control peptides in which the *tat* sequence was absent (LPL, Table 1) or present at the amino terminus (*tat*LPL, Table 1) did not induce PMN adhesion (Fig. 3 *A* and *B*). Maximal adhesion induced by LPL*tat* was almost equivalent to that stimulated by an optimal dose of PMA for PMN (Fig. 3*B*) and identical to PMA-stimulated adhesion for freshly isolated peripheral blood monocytes (Fig. 3*C*). Adhesion to FCS-coated surfaces is mediated by $\alpha M\beta 2$ (5) and requires integrin activation as determined by expression of the "activation" neoepitope on $\alpha M\beta 2$ recognized by the CBRM1/5 mAb (3, 5). LPL*tat*-stimulated adhesion to FCS was inhibited by antibody to $\beta 2$, by CBRM1/5, and by cytochalasin D (Fig. 3*D*), demonstrating that LPL*tat* induced adhesion by activating $\alpha M\beta 2$. Although LPL*tat* could induce adhesion of normal mouse PMN, it did not induce adhesion of $\beta 2$ integrin-deficient (41) PMN (Fig. 3*E*). LPL*tat* did not affect PMN adhesion to immune complexes or PMA-stimulated adhesion to FCS (Fig. 3 *B* and *C*, and data not shown). Furthermore, LPL*tat* treatment of PMN did not activate the respiratory burst or induce an increase in intracellular calcium concentration (data not shown), demonstrating that, although activating adhesion, the peptide did not induce general PMN activation.

To determine the specificity of LPL*tat* activation of adhesion, the effects of several additional peptides were tested on PMN (Fig. 3*B*) and monocyte (Fig. 3*C*) adhesion. A peptide derived from the homologous amino-terminal amino acids of the closely related actin-bundling protein T-plastin (TPL*tat*, Table 1), which has Gln rather than Ser at the fourth position (Fig. 1 and ref. 42), did not induce adhesion in PMN or monocytes. A peptide in which amino acids 2–19 of LPL were scrambled before addition of the tat sequence (SCR*tat*, Table 1) also did not induce adhesion in PMN and only minimally induced adhesion in monocytes.

To determine the role of Ser-5, the amino acid required for LPL phosphorylation, in activation of $\alpha M\beta 2$ mediated adhesion by LPL*tat*, we examined the effects of a peptide identical to LPL*tat* except for a substitution of Ala for Ser at this critical position (S5A*tat*, Table 1). The S5A*tat* peptide failed to induce adhesion of either PMN (Fig. 3 *A* and *B*) or monocytes (Fig. 3*C*) except at the highest concentration of peptide tested (200 μM). At 200 μM, all tat peptides induced adhesion that was no longer inhibited by anti-$\beta 2$ antibodies (data not shown), likely because of membrane perturbations by the tat sequence. These data demonstrate a requirement for Ser-5 in integrin activation by LPL*tat*. In contrast, a peptide with a S7A mutation (S7A*tat*, Table 1) retained partial ability to activate adhesion. Moreover, a peptide with a phosphorylated Ser at amino acid 5 of LPL (S5PO$_4$*tat*) was fully active for induction of adhesion. None of the peptides that failed to induce adhesion were inhibitory to PMA-stimulated adhesion, suggesting that the peptides did not have nonspecific inhibitory effects on PMN function.

To determine whether the inactive tat peptides were able to cross the leukocyte plasma membrane equivalently to LPL*tat* peptides, LPL*tat* and S5A*tat* were directly fluoresceinated and intracellular concentrations were compared by measuring PMN fluorescence after incubation with the peptides. FITC-LPL*tat* peptide retains the ability to induce adhesion, whereas the FITC-S5A*tat* is inactive (data not shown). After extensive washing of PMN, residual potential fluorescence of extracellular or surface-bound peptide was quenched by addition of trypan blue. As shown in Fig. 3*F*, the intracellular concentrations of the active and inactive tat peptides were identical. Thus, the failure of S5A*tat* to activate adhesion was not a result of inability to enter the cell cytoplasm.

Role of Phosphoinositol 3-Kinase and PKC in LPLtat-Induced Adhesion. PI 3-kinase and PKC both have a role in the activation of $\alpha M\beta 2$ avidity by a variety of physiologic stimuli

Table 1. Sequence of LPL tat peptides

LPL amino acid 2–19	ARG**S**VSDEEMMELREAFA
LPL	ARG**S**VSDEEMMELREAFA
*tat*LPL	*YGRKKRRQRRRG*ARG**S**VSDEEMMELREAFA
LPL*tat*	ARG**S**VSDEEMMELREAFA*YGRKKRRQRRRG*
SCR*tat*	AGDESEMEFVMASALRRE*YGRKKRRQRRRG*
TPL*tat*	ATTQISKDELDELKEAFA*YGRKKRRQRRRG*
S5A*tat*	ARG**A**VSDEEMMELREAFA*YGRKKRRQRRRG*
S5PO4*tat*	ARG**S**VSDEEMMELREAFA*YGRKKRRQRRRG*
S7A*tat*	ARG**S**VaDEEMMELREAFA*YGRKKRRQRRRG*

One-letter amino acid codes are used. HIV tat (amino acid 47–57) sequence is in italics. The serine corresponding to LPL S5 is in bold. Phosphorylated amino acid is underlined. Mutated amino acid in S7A*tat* is lowercased.

(Fig. 4*A* and refs. 3–5). PI 3-kinase and PKC also are involved in LPL phosphorylation in response to activation stimuli (Fig. 4*B*). To determine whether or not LPL*tat* activation of adhesion showed the same dependence on these kinases, we determined the effects of the PI 3-kinase inhibitors wortmannin and LY294002, and the calcium-dependent PKC inhibitor Gö6976, on PMN adhesion (Fig. 4*A*). Both PI 3-kinase and the PKC inhibitor blocked LPL*tat*- and S7A*tat*-induced adhesion. However, adhesion induced by the constitutively phosphorylated peptide S5PO$_4$*tat* was unaffected by any of the inhibitors. This suggested that the roles for PI 3-kinase and PKC in the activation of $\alpha M\beta 2$-mediated adhesion depends, at least in part, on their activation of LPL phosphorylation.

To determine whether phosphorylation of endogenous LPL was required for LPL*tat*-mediated adhesion, we examined the phosphorylation state of LPL after PMN treatment with activating and control peptides. As shown in Fig. 4*B*, the adhesion-activating peptides LPL*tat*, S5PO$_4$*tat*, and S7A*tat* all induced phosphorylation of endogenous LPL, whereas the control peptides S5A*tat* and TPL*tat* did not. Phosphorylation of endogenous LPL in response to activating peptides was inhibited both by PI 3-kinase inhibitors and PKC inhibitors. Thus, although S5PO$_4$*tat* induction of LPL phosphorylation was sensitive to inhibition by wortmannin, LY294002, and Gö6976, its activation of adhesion was not, demonstrating that peptide-induced adhesion did not require phosphorylation of endogenous LPL. No peptide affected PMA-induced phosphorylation of LPL (data not shown).

DISCUSSION

The actin cytoskeleton plays a fundamental role during integrin-mediated adhesion (8–13, 43). Integrins interact with the

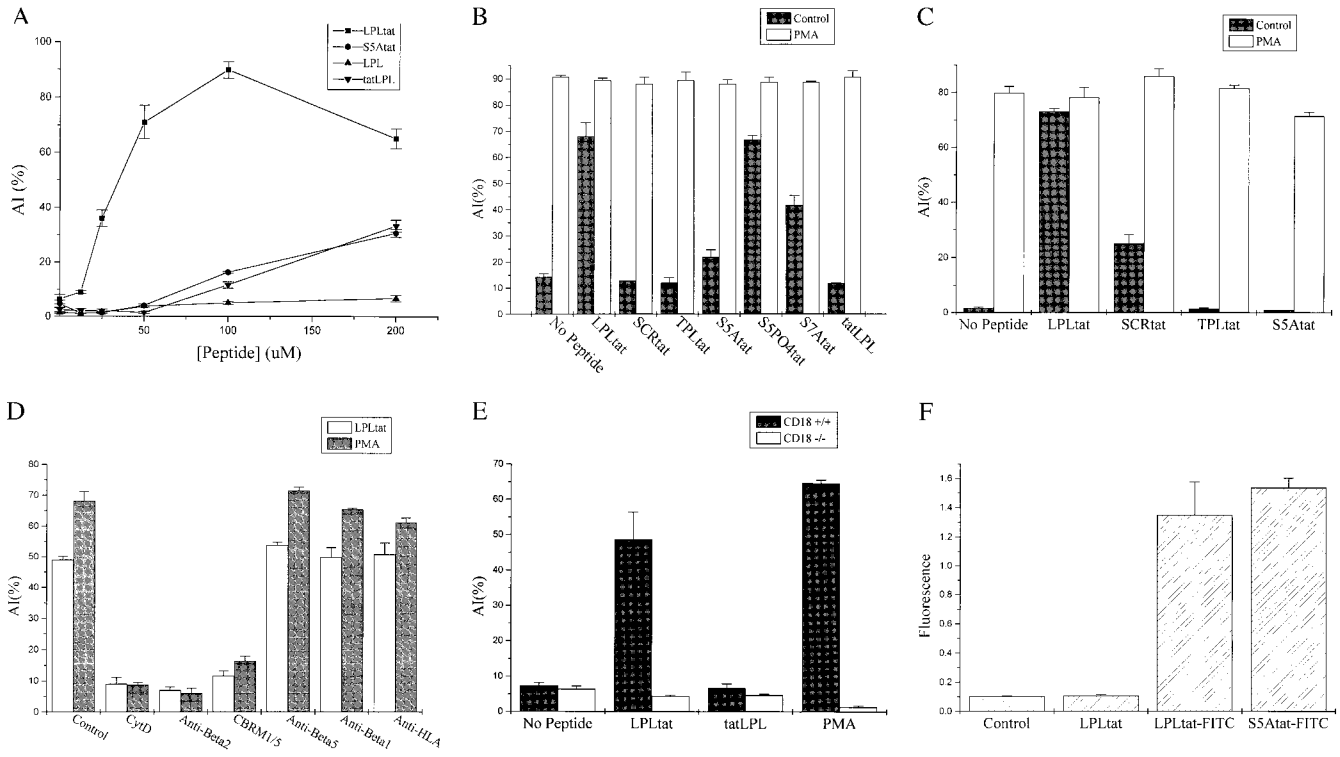

FIG. 3. Cell-permeant peptides from the amino terminus of LPL activate leukocyte integrin-mediated adhesion. To measure peptide effects on adhesion, purified PMN (*A, B, D,* and *E*) or monocytes (*C*) loaded with calcein were added to microtiter-plate wells coated with FCS followed by the addition of peptides with or without PMA (50 ng/ml) and incubated for 15 min at 37°C. (*A*) Peptide dose response. (*B* and *C*) One hundred micromoler of various peptides. The data are the mean ± SE of triplicate wells. Results are representative of at least three separate experiments. (*D*) Effects of cytochlasin D (10 μg/ml), anti-β2 F(ab′)2 mAb IB4, CBRM1/5, or the control anti-β1, anti-β5, or anti-HLA mAbs (all 20 μg/ml) on LPLtat-induced adhesion. (*E*) Adhesion of purified murine bone marrow PMN from wt (+/+) or CD18 (β2 integrin)-deficient (−/−) mice. (*F*) PMN were incubated with FITC-conjugated peptides and washed, and total fluorescence was quantitated by flow cytometry after quenching the extracellular fluorescence with the addition of 0.1% trypan blue dye. Data are presented as the mean ± SE fluorescence for three separate experiments.

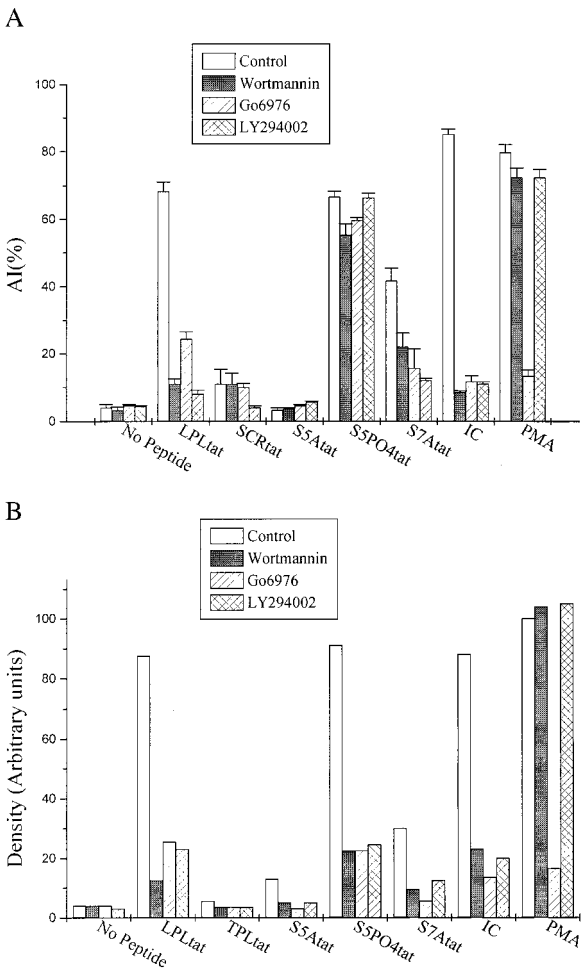

FIG. 4. PI 3-kinase and PKC activity is required for LPL*tat*, but not S5PO$_{4tat}$*-induced adhesion.* (*A*) Adhesion of PMN treated with DMSO control, wortmannin (100 nM), Gö6976 (50 nM), or LY294002 (25 μM) in response to cell permeant peptides. PMN were allowed to adhere to IC-coated surfaces or the indicated peptides (100 μM) or PMA (50 ng/ml) were added to PMN after allowing the cells to settle on a FCS-coated surface. Each point represents the mean ± SE for triplicate wells. Results are representative of at least three separate experiments. (*B*) Phosphorylation of endogenous LPL in response to cell-permeant peptides. PMN were treated with DMSO control, wortmannin (100 nM), Gö6976 (100 nM), or LY294002 (25 μM) and then were allowed to adhere to IC or were allowed to settle on an FCS-coated surface before the addition of peptides (100 μM) or PMA (50 ng/ml). LPL phosphorylation was assessed as in Fig. 2. Data are normalized to maximal phosphorylation induced by PMA (50 ng/ml) and represent the average of two separate experiments.

actin cytoskeleton via association of their cytoplasmic tails with actin-binding proteins such as α-actinin, talin, vinculin, and filamin (13, 14). High concentrations of cytochalasin D inhibit adhesion by disrupting the cytoskeletal structures necessary to form adhesive contacts with the substrate. The actin cytoskeleton is important in integrin biology not only for its function in the post-ligand-binding events necessary for adhesion, but also for its active role in regulating the state of integrin avidity (10, 12). Recent evidence suggests that the actin cytoskeleton restricts lateral mobility of β2 integrins within the membrane of unactivated cells (12). Low doses of cytochalasin D share with PMA the ability to increase the lateral mobility of β2 integrins and induce adhesion in a variety of leukocyte cells types (refs. 10 and 12 and our unpublished data). These data suggest that release of integrins from cytoskeletal constraints is an important step in activating adhesion, perhaps by allowing receptor clustering to occur, thereby increasing cell avidity for a ligand-coated substrate. This hypothesis implies that there are specific actin cytoskeletal structures, presumably plasma membrane-associated, that restrict integrin diffusion in unactivated leukocytes. To test this hypothesis further requires a better understanding of the molecular nature of the structures involved in restriction of integrin diffusion.

We now have demonstrated that cell-permeant peptides from the amino terminus of LPL rapidly activate leukocyte integrin-mediated adhesion. Our results suggest that LPL may be a critical component of the cytoskeletal restriction of integrin diffusion in inactivated cells. Thus, in leukocytes, LPL-actin bundles may be essential components of the cortical cytoskeletal structures that constrain the integrin from free diffusion, and introduction of the LPL peptides might interfere with these membrane-associated actin-LPL bundles. This hypothesis would require that the amino terminus of LPL, which is not part of the actin-binding domains, nonetheless affects actin binding, and data from Matsudaira's laboratory suggests that the amino-terminal domain does indeed regulate actin binding by members of the fimbrin family (Paul Matsudaira, personal communication). Moreover, calcium binding to the LPL headpiece region causes a conformational change that inhibits actin-bundling activity *in vitro* (17, 18), consistent with a role for the amino terminus in regulating actin binding. There is evidence that LPL also may regulate intermediate filament assembly (44). Thus, the role of LPL in integrin activation may be significantly more complex than simple restriction of diffusion. For example, LPL peptides may affect the association of proteins other than LPL with the actin cytoskeleton or the activity of kinases or phosphatases that regulate integrin activation and cytoskeletal organization, thus leading indirectly to the assembly of cytoskeletal structures that strengthen integrin-mediated adhesion.

Several studies have implicated PKC and PI 3-kinase in the activation of leukocyte integrins (3–5, 45). Our data that PKC and PI 3-kinase inhibitors block LPL*tat*-induced adhesion are consistent with a fundamental role for these signaling molecules in induction of integrin-mediated adhesion. Both PI 3-kinase and PKC are critical components of phosphorylation cascades, and our data strongly suggest a critical role for phosphorylation of Ser-5 in LPLtat-induced activation of leukocyte adhesion. Because Ser-5 is required for both integrin activation and LPL phosphorylation, it is likely that it is the major phosphorylation site. This is consistent with the finding that the peptide that contains a phosphorylated Ser-5 is active for the induction of adhesion. Importantly, adhesion activated by the constitutively phosphorylated peptide S5PO$_4$*tat* is insensitive to inhibition of PKC and PI 3-kinase. Thus, the role of these signaling molecules in LPL*tat*-induced adhesion is likely to stimulate a pathway that leads to phosphorylation of the LPL*tat* peptide. This raises the possibility that many of the signaling pathways so far described to affect leukocyte integrin avidity may exert their effects through a mechanism dependent on the phosphorylation of LPL.

Understanding the function and regulation of Ser-5 phosphorylation is central to evaluating the hypothesis that LPL is a critical component of leukocyte integrin-mediated adhesion. Unfortunately, nothing is known about the biochemistry or cell biology of this modification. There is evidence against a direct role for PKC or protein kinase A in LPL phosphorylation (21, 22), and we have not been able to demonstrate direct LPL phosphorylation *in vitro* by protein kinase B or PKCδ, downstream effectors of PI 3-kinase, or by any other isoform of PKC (data not shown). Although phosphorylated LPL may be concentrated in the insoluble cytoskeleton of adherent macrophages (20), no distinct interactions have been demonstrated for the phosphorylated form of the molecule. Because phosphorylation of endogenous LPL is not required for S5PO$_4$*tat* induction of adhesion, our data strongly imply that phosphor-

ylation induces novel interactions of the peptide that contribute to integrin-mediated adhesion.

Inhibition of LPL*tat*- but not S5PO$_4$*tat*-induced integrin activation by wortmannin, LY294002, and Gö6976 implies that LPL*tat* must be phosphorylated to be active. This, in turn, implies that in purified PMN there is a basally active pathway that leads to extensive phosphorylation of LPL*tat*, but not endogenous LPL. This is likely because the intracellular concentration of LPL*tat* is higher than native LPL, the peptide Ser is a better kinase or poorer phosphatase substrate than the endogenous LPL Ser, the LPL*tat* peptide has a broader kinase or phosphatase specificity than endogenous LPL, or a combination of these factors leads to enhanced peptide phosphorylation. Why LPL*tat* induces LPL phosphorylation rather than acting as a competitive inhibitor is not known but raises the possibility of a positive feedback loop, suggesting that LPL phosphorylation may be an amplification step in the activation of leukocyte adhesion. The inability of *tat*LPL to induce integrin activation or phosphorylation of native LPL demonstrates that an amino-terminal location of the critical Ser is required for LPL modulation of integrin activation. This requirement may reflect an inhibitory effect of the amino-terminal tat sequence on the efficiency of *tat*LPL phosphorylation or on the ability of the phosphopeptide to activate the subsequent steps necessary for integrin-mediated adhesion.

In summary, we have demonstrated a mechanism for leukocyte integrin activation initiated by cell-permeant analogs of the amino terminus of the actin-bundling protein L-plastin. The activation signal apparently requires phosphorylation of the peptide, but does not involve global activation of the leukocyte, because neither the respiratory burst nor release of Ca^{2+} from intracellular stores is activated by this mechanism. These data suggest a novel cytoskeleton-dependent regulation of leukocyte integrin function involving LPL phosphorylation.

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