L-plastin is a calcium-regulated actin bundling protein expressed in leukocytes and some transformed cells, which is phosphorylated on serine in response to several different leukocyte-activating stimuli. Adhesion to immune complexes induced L-plastin phosphorylation in neutrophils, as did phagocytosis of IgG-opsonized particles, but insoluble immune complexes in suspension were very inefficient activators of L-plastin phosphorylation. Neutrophils express two IgG Fc receptors, the transmembrane FcγRII and the glycan phosphoinositol-linked FcγRIIIB. Use of monoclonal antibodies that distinguished the two Fc receptors demonstrated that FcγRII ligation was 100-fold more potent at signaling L-plastin phosphorylation than occupancy of FcγRIIIB. Depletion of intracellular calcium did not affect FcγRII-activated L-plastin phosphorylation, demonstrating that any potential regulation of plastin function by calcium did not affect its phosphorylation. Adhesion to immune complexes caused L-plastin to localize to podosomes, since it colocalized with actin to discrete, punctate Triton X-100-insoluble sites on the adherent neutrophil surface in a pattern indistinguishable from vinculin and α-actinin. Nonetheless, localization to podosomes was not required for L-plastin phosphorylation, since both neutrophils from a patient with leukocyte adhesion deficiency (CD18 deficiency) and neutrophils treated with anti-CD18 F(ab')2, which do not form podosomes upon adhesion to immune complexes, phosphorylated L-plastin normally. Indeed, L-plastin was normally phosphorylated in response to adhesion to immune complexes even when the actin cytoskeleton was disrupted with cytochalasin D. We conclude that efficient FcγRII-mediated phosphorylation of L-plastin requires cell adhesion but does not require IgG-induced rearrangements of the actin cytoskeleton. These data suggest a model in which plastin phosphorylation and localization to the actin cytoskeleton can act as two distinct mechanisms regulating L-plastin functions in neutrophils adherent to immune complexes.

Phagocytic cells such as neutrophils (PMN) and macrophages comprise an important component of host defense against infectious organisms. Phagocyte activation at sites of infection and inflammation is mediated by a variety of receptors, including IgG Fc receptors (FcγR). Ligation of FcγR in PMN by immune complexes leads to several effector events, such as degranulation, secretion of inflammatory cytokines and vasoactive lipids, phagocytosis, antibody-dependent cellular cytotoxicity, and the respiratory burst. Early events that occur after FcγR ligation include polymerization of actin, activation of tyrosine and serine/threonine kinases, and a rise in intracytoplasmic calcium concentration (1). The actin cytoskeletal rearrangement induced by IC is required for effector functions such as migration and phagocytosis (2). In addition, many molecules involved in signal transduction localize to the actin cytoskeleton, and some signal transduction pathways are dependent on an intact cytoskeleton. However, the mechanism by which FcγR ligation regulates actin assembly and the role of the actin cytoskeleton in FcγR-mediated signal transduction events are not understood.

Previous data have suggested that the calcium-regulated actin bundling protein L-plastin may be a candidate for integration of signal transduction cascades and actin cytoskeletal rearrangements in leukocytes (3). LPL is expressed exclusively in leukocytes and some transformed cells (4, 5). Its actin bundling capacity is inhibited by increases in calcium concentration in vitro within a physiologically relevant range (6, 7). The LPL homologue in yeast, SAC6, is required for efficient endocytosis and normal morphology (8, 9). Human LPL expression can rescue yeast with SAC6 mutations that cannot undergo endocytosis, suggesting strong evolutionary conservation of function in this gene family (10). LPL has been shown to localize to the phagocytic cup in phagocytosing macrophages and to punctate aggregates in adherent macrophages and PMN (3, 11, 12).

These data all suggest LPL is an important structural and regulatory component in the actin cytoskeleton. However, the role of LPL in microfilament assembly and disassembly is not understood. We have recently presented data suggesting a role for LPL in the inositol 1,4,5-trisphosphate-independent, cytoskeleton-dependent rise in [Ca2+]i, which occurs upon FcγR ligation (3). LPL is serine-phosphorylated in phagocytes in response to inflammatory cytokines, PMA, and chemotactic peptide stimulation, all of which also induce an increase in actin polymerization (13, 14). This suggests the possibility that LPL serine phosphorylation could be a mechanism to integrate signal transduction with cytoskeletal function. These data and the dependence of many FcγR-dependent responses in PMN on the cytoskeleton suggest the hypothesis that L-plastin phosphorylation is regulated by FcγR ligation.

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1 The abbreviations used are: PMN, neutrophil (polymorphonuclear phagocyte); FcγR, IgG Fc receptor; LPL, L-plastin; IC, immune complex; IIc, insoluble immune complex; [Ca2+]i, intracellular calcium concentration; PMA, phorbol 12-myristate 13-acetate; FMLP, formyl-methionyl-leucyl-phenylalanine; BSA, bovine serum albumin; HSA, human serum albumin; HBSS, Hanks’ buffered saline solution; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; HLA, human leukocyte antigen; CR3, complement receptor 3; LAD, leukocyte adhesion deficiency; PIPES, 1,4-piperazinediethanesulfonic acid.
this work, we show that LPL becomes phosphorylated in PMN upon adhesion to IC-coated surfaces or phagocytic targets. FcγRII cross-linking was sufficient to induce LPL phosphorylation in adherent PMN, whereas FcγRII cross-linking only very inefficiently induced LPL phosphorylation. Stimulation of PMN with insoluble IC or by cross-linking FcγRII on cells in suspension was a poor stimulus for LPL phosphorylation. Because adhesion appeared to be important for FcγR-induced LPL phosphorylation, we investigated the localization of LPL in adherent PMN. Adhesion to IC, but not to BSA- or poly-lysine-coated surfaces, caused LPL to localize to the Triton-insoluble cytoskeleton in podosomes. Surprisingly, buffering of [Ca\(^{2+}\)]\(_i\) did not affect LPL phosphorylation or localization to the actin cytoskeleton. LPL phosphorylation did not require CR3, unlike many other adhesion-dependent events in PMN, whereas localization to podosomes was inhibited by blockade of CR3 with mAb. Furthermore, LPL phosphorylation was unaffected by inhibition of actin filament rearrangement with cytochalasin D. Therefore, FcγR-mediated induction of LPL phosphorylation was completely independent of its localization to the actin cytoskeleton, suggesting that LPL phosphorylation is regulated independently of its F-actin binding. These data suggest a model in which LPL phosphorylation and localization to the actin cytoskeleton can act as two distinct mechanisms regulating LPL function in PMN adherent to immune complexes.

**EXPERIMENTAL PROCEDURES**

Reagents—A 10× concentrated stock of HBSS, cytochalasin D, PMA, dimethyl sulfoxide, bovine serum albumin, rabbit anti-BSA polyclonal antisemur, poly-lysine, dextran T500, EGTA, protein A, human serum albumin, tissue culture plates, rhodamine phalloidin, and goat anti-mouse F(ab')\(_2\) were obtained as described (3, 12). Poly-L-lysine-coated surfaces were obtained as described (3, 15). [\(^{32}\)P]Phosphate (5 mCi/ml) was obtained from ICN Radiochemicals (Irvine, CA). Monoclonal Abs 3G8 (16), IV.3 (17), IB4 (18), and W6/32 (19) were purified as described (15). Anti-LPL mAb LPL 1.1 and LPL4A.1 were prepared as described previously (3) and used as tissue culture supernatant or purified IgG. Anti-vinculin and anti-α-actinin mAb and FITC-conjugated sheep anti-mouse IgG F(ab')\(_2\) were obtained from Sigma.

Preparation of PMN Suspensions—Human PMN were isolated from whole blood exactly as described (20). PMN were greater than 98% viable as indicated by the exclusion of trypan blue dye. Cells were suspended in HBSS with 1.0 mM Mg\(^{2+}\) and Ca\(^{2+}\) (HBSS-\(++\)) or 2.0 mM Mg\(^{2+}\) and 1 mM EGTA (HBSS-\(+\)EGTA) with 1% HSA.

Coating of Plates, Beads, and Coverslips with BSA and IC and Preparation of IIC—12-well tissue culture plates and 12-mm glass coverslips were coated with BSA-anti-BSA immune complexes exactly as described (3, 21) using various dilutions of anti-BSA antiserum to achieve different concentrations of IC on the surface. Tissue culture plates were coated with mAb using the protein A method as described (22) except the plates were blocked with 0.1% glycine, 1% HSA, pH 6.8, for 2 h at room temperature. 500 μl of PBS containing the concentration of mAb indicated in the figures was incubated in the wells for 6 h at 4°C and washed 2× prior to use with PBS. For IC and BSA-coated poly-styrene beads, 3 μm beads (Polysciences Inc., Warrington, PA) were incubated in 1 ml of 8% glutaraldehyde overnight at room temperature and then 0.1% BSA in PBS for 5 h, with mixing. The beads were washed twice and then incubated with 1 ml of 0.5 mM ethanamine overnight at room temperature. Beads were incubated with 1 ml of 1.5% rabbit anti-BSA in PBS for 4 h at room temperature and then washed twice to make IC-coated beads. IIC were made exactly as described (23).

Immunofluorescent Staining—3×10\(^6\) PMN in HBSS were added to wells of a 24-well plate containing 12-mm glass coverslips (Fisher-coated with BSA or IC and incubated for 30 min at 37°C. The cells were extracted with cold Triton buffer (0.5% Triton X-100, 10 mM PIPES, 300 mM sucrose, 100 mM KCl, 3 mM MgCl\(_2\), 10 mM EGTA, pH 6.8) for 30 s, washed quickly with Triton buffer, and fixed for 20 min at room temperature with fixation buffer (25 mM PIPES, 50 mM KCl, 10 mM MgSO\(_4\), 5 mM EGTA, 3% paraformaldehyde, pH 7). Coverslips were washed once with PBS and once with protein solution (0.2% gelatin, 0.2% azide, 0.1% ovalbumin in PBS) and incubated with primary antibody (anti-plastin mAb LPL 1.1, anti-α-actinin, anti-vinculin or negative control mAb 6F2) overnight at 4°C. Coverslips were then washed with protein solution 3× and incubated with secondary antibody (fluorescein-conjugated sheep anti-mouse F(ab')\(_2\) for 1 h at room temperature, and again washed 2× with protein solution. For staining F-actin, the coverslips were incubated with rhodamine phaloidin in PBS (1:20) for 20 min at room temperature. Coverslips were mounted by washing twice in PBS and once in o-phenylenediamine dihydrochloride at 1 mg/ml in glycerol and mounted on glass slides.

LPL Phosphorylation—Purified PMN were suspended in HBSS at 50×10\(^6\)/ml and incubated for 1 h at 37°C in loading buffer (50 mM HEPES, 0.9% NaCl, 0.5 mM Ca\(^{2+}\), 1% dialyzed HSA, pH 7.4) to deplete phosphate. 5 mM MgCl\(_2\); [\(^{32}\)P]H\(_2\)PO\(_4\); was added; the cells were incubated for 1 h at 37°C, washed once with HBSS, and resuspended in HBSS-\(+\)EGTA with 1% HSA. 2.5×10\(^6\) cells were added to wells coated with BSA, IC, or mAb as described above, centrifuged for 1 min at 500 rpm, and incubated for 15 min or the time indicated in a 37°C water bath in room air. Alternatively, FMLP, PMA, or IC and BSA-coated polystyrene beads were added to cells in Eppendorf tubes and incubated in a 37°C water bath for the indicated times. The cells were lysed by adding 2× lysis buffer (1% Triton X-100, 1% deoxycholate, 150 mM NaCl, 100 mM NaF, 4 mM Na\(_2\)VO\(_4\), 10 mM disodium pyrophosphate, 20 mM HEPES, pH 8, with the protease inhibitors 2 mM diisofluorophosphate, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride) for 5 min on ice. The lysates were centrifuged in a microcentrifuge at high speed for 10 min. The supernatant was added to 40 μl of a 1:1 slurry of goat anti-mouse Sepharose and 2.5 μg anti-LPL mAb LPL 4A.1 and incubated on a rotator for 2 h at 4°C. The Sepharose was washed twice with 1× lysis buffer and twice with PBS, 0.05% Tween 20 and then boiled in SDS sample buffer for 2 min. The proteins were separated by SDS-PAGE, and phosphorylation was detected by autoradiography of the dried acrylamide gels. Phosphorylation was quantitated by densitometry of the exposed film using FL-4000 Imaging Software (Georgia Instruments, Atlanta, GA).

**RESULTS**

**LPL Is Phosphorylated in PMN Adherent to IC-coated Surfaces—**LPL is serine-phosphorylated in leukocytes in response to a variety of stimuli, including tumor necrosis factor, PMA, interleukin 1, and fMLP (13, 14, 24–26). In order to determine whether FcγR ligand induced LPL phosphorylation, LPL was immunoprecipitated from PMN loaded with [\(^{32}\)P]phosphate acid after stimulation with IC, and phosphorylation was detected by SDS-PAGE and autoradiography. Adhesion to surfaces coated with BSA and increasing dilutions of anti-BSA antiserum to form IC induced LPL phosphorylation in a dose-dependent manner (Fig. 1, A and B). Adhesion to BSA-coated surface without antibody did not induce LPL phosphorylation above base line. IC-coated beads also efficiently stimulated LPL phosphorylation, whereas BSA-coated beads had no effect (Fig. 1, C and D). The amount of LPL phosphorylation induced by IC-coated beads was maximal at a 100 bead/cell ratio. LPL phosphorylation in response to IC stimulation was quantitated by comparison with maximal phosphorylation induced by the known potent stimulus fMLP. The magnitude of LPL phosphorylation induced by adhesion to IC-coated plates and beads was 80–100% of that induced by the fMLP (Fig. 2A). In contrast, stimulation of PMN in suspension with insoluble IC only weakly induced LPL phosphorylation (Fig. 2A). Adhesion to optimal IC-coated plates or beads induced an average 16±7.1 and 13.7±5.3-fold peak increase (mean±S.D., n=5) in LPL phosphorylation above BSA control, respectively. IIC induced an average 3.2±2.9 (n=4)-fold increase in LPL phosphorylation which was significantly less than adhesion to IC-coated beads or plates (p<0.01). The difference between LPL phosphorylation induced by IC-coated beads or plates on one hand and IIC on the other was statistically significant (p<0.05) at 5 min and for all time points thereafter (Fig. 2A). Adhesion does not prime PMN for FcγR-induced LPL phosphorylation because insoluble IC added in suspension to cells adherent to BSA did not induce significant LPL phosphorylation (data not shown).

Adhesion of PMN to IC-coated plates stimulated LPL phos-
phorylation with kinetics similar to soluble stimuli such as fMLP and PMA (Fig. 2B). Maximal phosphorylation was induced by 10 min and was sustained at levels slightly lower than peak levels for up to 40 min (Fig. 2, A and B).

LPL can be cleaved with trypsin into a “headpiece” containing the Ca\(^{2+}\) binding EF hand domains and a “tail” containing the actin binding regions. Trypsin cleavage of LPL immunoprecipitated from PMN stimulated with the monoclonal antibody LPL 4A.1 from cell lysates and analyzed by SDS-PAGE and autoradiography of the dried gels (B and D). Phosphorylation was quantitated by densitometry and plotted versus A, dilution of anti-BSA used to form immune complexes on the plates, or C, the bead/cell ratio. fMLP-induced LPL phosphorylation was used as an internal standard. IC-coated plates and IC-coated beads induced an average 16.4 ± 7.1- and 13.7 ± 5.3-fold increase in phosphorylation, respectively, above BSA controls (mean ± S.D., n = 5). Results depicted are from a single representative experiment.

Because Ca\(^{2+}\) is an important regulator of LPL actin-bundling activity in vitro, and the phosphorylation site(s) is located very near the Ca\(^{2+}\)-binding domains in the headpiece region, we tested the dependence of LPL phosphorylation on Ca\(^{2+}\). LPL phosphorylation in response to adhesion to IC was compared in Ca\(^{2+}\)-depleted and Ca\(^{2+}\)-replete PMN (Fig. 4). Incubation of PMN in buffer containing 2 mM Mg\(^{2+}\) and 1 mM EGTA depleted [Ca\(^{2+}\)] below 10 nM, and [Ca\(^{2+}\)] did not rise in response to fMLP, IIC, or ionomycin (data not shown and Ref. 27). LPL phosphorylation in PMN adherent to IC-coated surfaces was not affected by Ca\(^{2+}\) depletion (Fig. 4).

Fc\(\gamma RI\) is Necessary and Sufficient to Induce LPL Phosphorylation in Adherent PMN—Human PMN express Fc\(\gamma RI\) and the glycan phosphoinositol-linked Fc\(\gamma RI\)IB, both of which generate signals in response to IC ligation (15, 23, 28, 29). In order to determine the role of each Fc\(\gamma R\) in LPL phosphorylation initiation, we first used monoclonal antibodies to block ligand binding to either Fc\(\gamma RI\) or Fc\(\gamma RI\)IIB. Treatment of PMN with Fab fragments of ligand-blocking anti-Fc\(\gamma RI\) mAb IV.3 completely inhibited LPL phosphorylation in PMN adherent to IC-coated surfaces (Fig. 5). In contrast, F(ab\(^{\prime}\))\(_{2}\) fragments of the ligand-blocking anti-Fc\(\gamma RI\) mAb 3G8 only partially inhibited LPL phosphorylation, and the control monoclonal antibody anti-HLA had no effect on LPL phosphorylation in PMN adherent to IC-coated surfaces. Anti-Fc\(\gamma RI\) Fab had no effect on fMLP-induced LPL phosphorylation (data not shown) showing their specificity for IC-induced LPL phosphorylation. These results demonstrate that Fc\(\gamma RI\) is necessary to induce LPL phosphorylation in PMN adherent to IC-coated surfaces.

In order to determine whether Fc\(\gamma RI\) ligation is sufficient to initiate LPL phosphorylation, LPL phosphorylation was determined in PMN adherent to surfaces coated with anti-Fc\(\gamma RI\), anti-Fc\(\gamma RI\)I, or the control antibody anti-HLA. Adhesion to anti-Fc\(\gamma RI\) efficiently induced LPL phosphorylation, whereas more than 100 times more anti-Fc\(\gamma RI\)I was necessary to induce LPL phosphorylation, and anti-HLA did not induce LPL phosphorylation (Fig. 6). Adhesion to anti-Fc\(\gamma RI\) (10 μg/ml coating concentration) induced an average 7.3 ± 1.7-fold increase (mean ± S.D.) in LPL phosphorylation above adhesion to the control anti-HLA. Adhesion anti-Fc\(\gamma RI\) at the same coating concentration induced significantly less (1.3 ± 0.1-fold increase) LPL phosphorylation (p < 0.005, n = 4). Differences in antibody binding to the plate did not account for the difference in LPL phosphorylation induced by anti-Fc\(\gamma RI\) and anti-Fc\(\gamma RI\)IIB, because at low concentrations more anti-Fc\(\gamma RI\) bound to the plate than anti-Fc\(\gamma RI\) as detected by enzyme-
linked immunosorbent assay (data not shown). LPL phosphorylation induced by adhesion to anti-FcγRIII was completely inhibited by pretreating PMN with anti-FcγRII Fab and only partially inhibited by anti-FcγRIII F(ab′)2 treatment (Fig. 7), suggesting that the anti-FcγRIII effect is dependent on FcγRII. Perhaps FcγRII interacts with the Fc domain of anti-FcγRIII, which may become available when very high concentrations of mAb are incubated with the protein A-coated plate. Crosslinking of FcγRII on PMN in suspension did not lead to significant LPL phosphorylation (data not shown), confirming the requirement for adhesion for LPL phosphorylation, as demonstrated above for IC.

LPL Localizes to Podosomes in PMN Adherent to IC-coated Surfaces—Fcγ Receptor-induced L-Plastin Phosphorylation in PMN

**Fig. 2.** Activation of PMN by adhesion to surface-bound IC but not IIC in suspension efficiently induces LPL phosphorylation with kinetics similar to fMLP- and PMA-induced LPL phosphorylation. A, purified PMN loaded with 32P were prepared as in Fig. 1 and were allowed to adhere to BSA- or BSA-anti-BSA IC-coated plates prepared using a 1:100 dilution of anti-BSA, or IC- or BSA-coated beads at a bead/cell ratio of 100:1, or stimulated with a 1:25 dilution of IIC prepared as described under “Materials and Methods” or a buffer control. B, PMN were stimulated with fMLP (40 ng/ml), or PMA (10 ng/ml), or buffer alone for the indicated time. Phosphorylation was detected by autoradiography of SDS-PAGE of LPL immunoprecipitates and quantitated by densitometry. To compare experiments, values were normalized to fMLP-induced LPL phosphorylation at 10 min (equals 1 density unit) as an internal standard for each experiment. The points plotted represent the mean ± S.E. from three samples. IIC-induced phosphorylation was significantly less than phosphorylation induced by IC-coated plates or beads at 5 min and all points thereafter (p < 0.05).

**Fig. 3.** Adhesion to IC-induced phosphorylation of the headpiece of LPL in PMN. Purified PMN loaded with 32P prepared as in Fig. 1 were allowed to adhere for 30 min at 37°C to BSA-anti-BSA IC-coated plates prepared as described under “Materials and Methods” using a 1:100 dilution of anti-BSA or stimulated with fMLP (40 ng/ml) or PMA (10 ng/ml). LPL was immunoprecipitated from cell lysates with LPL 4A.1. LPL was eluted from the washed immunoprecipitates using 10 mM EDTA. The eluted LPL was subjected to trypsin digestion (0.25 μg/ml) in 50 mM Tris buffer, pH 6.8, for 60 min at room temperature. The digested protein was subjected to SDS-PAGE and transferred to polyvinylidene difluoride. Western blot of the membrane using a mAb LPL 7.2 that recognizes the actin binding domain region of LPL revealed two bands at 65 and 55 kDa. N-terminal sequence analysis of the 55-kDa protein confirmed that it was the product of cleavage between the headpiece and actin binding domains (data not shown).

**Fig. 4.** A rise in \([Ca^{2+}]_i\) is not required for LPL phosphorylation in PMN adherent to IC-coated surfaces. Purified PMN loaded with 32P were suspended in HBSS with 2 mM MgCl₂ and either 1 mM EGTA or 1 mM CaCl₂ for 15 min and then were allowed to adhere for 30 min at 37°C to BSA or anti-BSA IC-coated plates prepared with 1:100 dilution of anti-BSA. PMA (10 ng/ml) was used for a positive control. LPL was immunoprecipitated from cell lysates with LPL 4A.1 and analyzed by SDS-PAGE. Phosphorylation was detected by autoradiography of dried gels and quantitated by densitometry. To compare experiments, LPL phosphorylation was normalized to PMA-induced LPL phosphorylation (equals 1 unit). Each bar represents the average ± S.E. of three samples from separate experiments. No significant differences in LPL phosphorylation induced by any stimulus were found between control and Ca²⁺-depleted PMN.
The plates. At the coating concentration of 10 

Distinct peripheral staining was ob-

PMN loaded with $^{32}$P prepared as in Fig. 1 were treated for 15 min at room temperature with buffer (no treatment), or 20 μg/ml of either Fab fragments of anti-FcRII mAb IV.3 or (Fab')$_2$ fragments of anti-FcRII 3G8 or F(ab')$_2$, the control mAb anti-HLA W6/32. The cells were then allowed to adhere to plates coated with BSA or BSA-anti-BSA IC prepared with a 1:3000 dilution of anti-BSA for 30 min at 37°C. LPL was immunoprecipitated for cell lysates with the mAb LPL 4A.1. Phosphorylation was detected by autoradiography of dried SDS-PAGE gels and quantitated by densitometry and normalized to IC-induced phosphorylation in buffer alone. Each bar represents the average ± S.E. of three samples from separate experiments. Anti-FcRII and anti-FcRIII both significantly inhibited IC-induced LPL phosphorylation (p < 0.05). Anti-FcRII inhibited LPL phosphorylation significantly more than anti-FcRIII (p < 0.05).

Fed receptor-induced L-Plastin Phosphorylation in PMN

Fig. 5. FcγRII is required for induction of LPL phosphorylation in PMN by adhesion to IC-coated surfaces. Purified PMN loaded with $^{32}$P prepared as in Fig. 1 were treated for 15 min at room temperature with buffer (no treatment), or 20 μg/ml of either Fab fragments of anti-FcRII mAb IV.3 or (Fab')$_2$ fragments of anti-FcRII 3G8, or F(ab')$_2$, the control mAb anti-HLA W6/32. The cells were then allowed to adhere to plates coated with BSA or BSA-anti-BSA IC prepared with a 1:3000 dilution of anti-BSA for 30 min at 37°C. LPL was immunoprecipitated for cell lysates with the mAb LPL 4A.1. Phosphorylation was detected by autoradiography of dried SDS-PAGE gels and quantitated by densitometry and normalized to IC-induced phosphorylation in buffer alone. Each bar represents the average ± S.E. of three samples from separate experiments. Anti-FcRII and anti-FcRIII both significantly inhibited IC-induced LPL phosphorylation (p < 0.05). Anti-FcRII inhibited LPL phosphorylation significantly more than anti-FcRIII (p < 0.05).

FIG. 6. Adhesion via FcγRII is sufficient for induction of LPL phosphorylation in PMN. Purified PMN loaded with $^{32}$P were prepared as in Fig. 1, and adhered to IC-coated surfaces for 20 min at 37°C. LPL was immunoprecipitated with LPL 4A.1 and analyzed by SDS-PAGE. Phosphorylation was detected by autoradiography of dried SDS-PAGE gels and quantitated by densitometry. Results are from a single representative experiment.

Adhesion via FcγRII is sufficient for induction of LPL phosphorylation in PMN. In contrast, adhesion to BSA (Fig. 8) or poly-L-lysine (data not shown) did not cause LPL to localize to the Triton-insoluble cytoskeleton. Depletion of [Ca$^{2+}$], by preincubation of cells in EGTA did not affect the distribution of LPL in PMN adherent to IC (data not shown).

Cytoskeleton in PMN, we stained adherent PMN with a mAb (LPL.11) raised against recombinant LPL. Adhesion to IC-coated surfaces caused LPL to localize to the Triton-insoluble cytoskeleton in punctate aggregates distributed throughout the adherent surface (Fig. 8). Distinct peripheral staining was observed in some cells, especially at the leading edge of polarized PMN. In contrast, adhesion to BSA (Fig. 8) or poly-L-lysine (data not shown) did not cause LPL to localize to the Triton-insoluble cytoskeleton. Depletion of [Ca$^{2+}$], by preincubation of cells in EGTA did not affect the distribution of LPL in PMN adherent to IC (data not shown).

Phosphorylation of IC-induced LPL was not dependent on CR3 and does not require localization to podosomes—Many adhesion-dependent events in PMN are dependent on the β$_2$ integrin CR3 (Mac-1, CD11b/CD18). FcγγR-induced paxillin phosphorylation and leukotriene B4 synthesis require CR3, and PMN will de-adhere from IC-coated surfaces in the absence of CR3 (21, 32). The much increased efficiency of LPL phosphorylation by IC in adherent PMN suggested a possible role for CR3 in signaling to activate the LPL kinase. To determine whether CR3 was required for IC-induced LPL phosphorylation, we compared PMN from a patient with leukocyte adhesion deficiency which does not express CR3 and PMN treated with F(ab')$_2$ of the anti-CD18 mAb IB4 to normal PMN. As previously reported (32), adhesion of normal PMN, PMN treated with anti-CD18 F(ab')$_2$, and LAD PMN to IC-coated surfaces was equivalent at 10 min. However, by 20 min PMN treated with anti-CD18 F(ab')$_2$ and LAD PMN released from the surface (32). Even at times when adhesion was quantitatively equivalent, treatment with anti-CD18 inhibited podosome formation in PMN adherent to IC-coated surfaces (Fig. 9). Despite these differences in adhesion between normal PMN and LAD PMN, adhesion to IC-coated surfaces is sufficient for induction of LPL phosphorylation in PMN.
and LAD or mAb-treated PMN, LPL phosphorylation induced by adhesion to IC-coated surfaces was equivalent (Fig. 10). This was true even at 20 min, when the LAD and F(ab')2-treated cells had de-adhered from the IC-coated surface (data not shown). These results show that CR3 is not required for IC-induced LPL phosphorylation and that de-adhesion of LAD PMN from IC-coated surfaces is not associated with dephosphorylation of LPL. Finally, localization to podosomes is not required for phosphorylation, since PMN treated with anti-CD18 does not form podosomes on IC but does phosphorylate LPL as well as untreated cells.

Cytochalasin D Does Not Inhibit LPL Phosphorylation—In order to further investigate the role of F-actin association in the phosphorylation of LPL, we treated PMN with cytochalasin D to inhibit actin polymerization and then allowed the cells to adhere to IC-coated surfaces. Treatment with 25 μM cytochalasin D to inhibit actin polymerization completely inhibited PMN spreading on IC-coated surfaces but had no effect on LPL phosphorylation (Fig. 11). This result suggests that actin polymerization and an intact actin cytoskeleton are not required for the induction of LPL phosphorylation. Taken together with the independence of phosphorylation from sustained adhesion or localization to podosomes, these data indicate that LPL phosphorylation is independent of interaction with the actin cytoskeleton.

DISCUSSION

Rapid rearrangement of the actin cytoskeleton occurs after stimulation of PMN with inflammatory mediators, chemotactic peptides, and immune complexes (33–36). It has long been known that actin polymerization is required for PMN motility and phagocytosis (2). This is not surprising, since the actin cytoskeleton is thought to provide the mechanical force for these processes. Recently, it has become increasingly clear that actin polymerization also is required for appropriate activation of some signal transduction pathways and that signaling cascades can assemble by interaction of key components with cytoskeletal elements (3, 37, 38). Focal adhesions are aggregates of actin and actin-binding proteins at the plasma membrane that are sites of signal transduction in non-leukocytes (39). Many signal transduction molecules, including Src family kinases, paxillin, focal adhesion kinase, phosphoinositide 3-kinase, and PLCγ, localize to the actin cytoskeleton in focal adhesions (37, 38). While motile leukocytes often do not make fully formed focal contacts, these cells do demonstrate punctate sites of F-actin accumulation on the membrane, called podosomes. Podosomes, like focal adhesions, are sites of accumulation of tyrosine-phosphorylated proteins (40), suggesting that they also can be sites for interaction of signaling molecules. In PMN, inhibition of actin polymerization with cytochalasin D inhibits the rise in [Ca2+]i induced by cross-linking FcγR with immune complexes (3). Thus, the actin cytoskeleton is not only a regulatable scaffold that controls the shape and motility of the cell but also an important site of convergence for elements of diverse signal transduction cascades. We have begun an attempt to characterize the molecular basis for cytoskeleton-dependent signal transduction and effector functions in PMN. Recent experiments in our lab suggest that a cytoskeletal protein, LPL, is an important component in the FcγR-initiated rise in [Ca2+]i induced by immune complexes in PMN (3).

LPL is a member of the plastin family of actin-bundling proteins that is expressed exclusively in leukocytes and some transformed cells. The other members of the plastin family are i-plastin, expressed in intestinal epithelial cells and kidney, and t-plastin, expressed in most other cells (5, 41). Members of this family have two calcium-binding domains of the EF-hand type and two α-actinin-type actin-binding domains. LPL actin bundling activity has been shown to be negatively regulated by calcium in vitro (6, 7). It is clear that members of the plastin family are critical components of the actin cytoskeleton, because mutation of the yeast homologue, SAC6, causes defects of endocytosis and abnormal morphology that can be rescued with human i- and t- but not i-plastin (10). This suggests considerable functional conservation of the plastin family from yeast to man. However, the different expression patterns among the plastins in higher eukaryotes suggest that distinct functions have evolved among members of the family. The data suggesting that LPL is a component of the FcγR-initiated increase in [Ca2+]i, induced by IC in PMN potentially links a highly conserved cytoskeletal protein to signal transduction in leukocytes.

LPL is unique within the plastin family because it can be phosphorylated. The function of LPL serine phosphorylation is not known. In adherent macrophages stimulated with PMA, the majority of phosphorylated LPL is associated with the Triton-insoluble cytoskeleton, suggesting that phosphorylation may be involved in regulation of LPL association with the actin cytoskeleton (11). Soluble stimuli such as interleukin 2, fMLP,
tumor necrosis factor-\(\alpha\), lipopolysaccharide, and PMA, which induce LPL phosphorylation all increase actin polymerization. We have found that in PMN, ligation of Fc\(\gamma\)R by IC-coated surfaces such as beads or plates efficiently induces LPL phosphorylation with kinetics similar to these soluble stimuli. The kinetics of phosphorylation are slow in response to all these stimuli, with no detectable phosphorylation at 2 min and a peak at 10 min, implying that LPL phosphorylation is a relatively late event, possibly requiring other, more immediate signaling events.

While PMN express two distinct receptors for IgG, Fc\(\gamma\)RII alone is both necessary and sufficient for induction of LPL phosphorylation by IC in adherent PMN. In contrast to PMA or fMLP, cross-linking of Fc\(\gamma\)RII on PMN in suspension with insoluble IC or with anti-Fc\(\gamma\)R mAb and anti-mouse IgG was not sufficient to induce significant LPL phosphorylation. This was not a result of diminished receptor occupancy by Fc receptor ligands in solution, because use of saturating concentrations of IV.3, so that all receptors were occupied, did not lead to LPL phosphorylation in nonadherent cells, even with cross-linking by a secondary antibody. The fact that Fc\(\gamma\)R-induced phosphorylation of LPL is dependent upon adhesion to a surface suggests that the signals initiated by IgG presented on a surface are different from those initiated by IgG complexes in suspension. While this also is true for leukotriene B\(\_\) synthesis, respiratory burst activation, and other effects of IgG stimulation (32, 42), the precise differences between adhesion and fluid phase presentation of immune complexes remain unknown. Some of these effects undoubtedly involve CR3, the major PMN adhesive integrin (21, 32), but plastin phosphorylation was equivalent in normal and LAD PMN, demonstrating that CR3 is not involved in this signaling. That signals initiated by surface-bound IgG are different from an IIC stimulus in suspension suggest that the processes of phagocytosis or adhesion to surfaces generates signals required for the complex regulation of the actin cytoskeleton that are not generated by IIC...
stimulation. This is consistent with the zipper hypothesis of phagocytosis, in which sequential engagement of multiple FcR by an opsonized target is required for ingestion. Ligation of even a very large subset of receptors at a single point on the plasma membrane is not sufficient to trigger phagocytosis (43). The kinetics and adhesion requirement of LPL phosphorylation suggest that this cascade is independent of Fgr activation by FcR ligation, rather than the temporally limited receptor engagement that occurs with IIC binding or FcRII capping. Similarity to zipperping makes LPL phosphorylation an excellent candidate for involvement in the phagocytic process.

Although adhesion is required for efficient LPL phosphorylation, a number of distinct lines of evidence suggest that the actin cytoskeleton rearrangements that accompany adhesion are not CR3-deficient and anti-CD18 mAb-treated PMN, which adhere abnormally, do not polarize and do not form podosomes still phosphorylate LPL normally. Even cytochalasin, which entirely blocks F-actin depolymerization in response to FcRII ligation, had no effect on LPL phosphorylation. This suggests that this signal transduction pathway initiated by PMN adhesion to IC actually does not depend on cytoskeletal rearrangements and that LPL localization to podosomes may be regulated quite independently of its phosphorylation. Preliminary data suggest that LPL phosphorylation is not inhibited by genestein or herbimycin, suggesting that this cascade is independent of Fgr activation by FcRII ligation.2 Thus, we hypothesize a tyrosine kinase- and actin cytoskeleton-independent signaling cascade specifically initiated by PMN adhesion to surface-associated IC. This pathway is likely to play a key role in the regulation of phagocytosis and of PMN responses to deposited immune complexes in a variety of inflammatory diseases.

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