

Complement Receptor 3 (CR3, Mac-1, Integrin $\alpha_M\beta_2$, CD11b/CD18) Is Required for Tyrosine Phosphorylation of Paxillin in Adherent and Nonadherent Neutrophils

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Abstract. Expression of the leukocyte (β_2) integrins is required for many functions of activated neutrophils (PMN), even when there is no recognized ligand for any β_2 integrin. To investigate the hypothesis that β_2 integrins may be involved in a signal transduction pathway related to cytoskeletal reorganization, we examined whether β_2 integrins have a role in tyrosine phosphorylation of the cytoskeletal protein paxillin. Treatment of PMN in suspension with phorbol esters, f-Met-Leu-Phe, and TNF- α resulted in paxillin tyrosine phosphorylation. However, treatment of β_2 -deficient (LAD) PMN failed to induce paxillin tyrosine phosphorylation. Normal PMN phosphorylated paxillin in response to adhesion to immune complexes, while the LAD PMN did not. Adhesion of phorbol ester activated-LAD PMN to the extracellular matrix proteins fibronectin, laminin, and vitronectin failed to induce paxillin tyrosine phosphorylation. Treatment of activated normal PMN with mAb directed against the β_2 integrin α chains demonstrated

that CR3 ($\alpha_M\beta_2$) was required for paxillin phosphorylation. Transfection of the cell line K562 with CR3 confirmed that CR3 ligation resulted in paxillin tyrosine phosphorylation. As a control, K562 transfected with CR2 (CD21) which bound equally avidly to the same complement C3-derived ligand (C3bi) as the CR3 transfectants, showed no enhanced tyrosine phosphorylation of paxillin upon receptor ligation. While both CR2 and CR3 transfectants showed efficient adhesion to a C3bi-coated surface, only the CR3 transfectants spread during adhesion and phosphorylated paxillin. Together these data demonstrate that CR3 is required for paxillin phosphorylation during activation of both adherent and nonadherent PMN. Even PMN activated in suspension or by adhesion to immune complexes, when no CR3 ligand is apparent, still require CR3 for a signal transduction pathway leading to paxillin tyrosine phosphorylation. This pathway is likely to be important for PMN function in inflammation and host defense.

CIRCULATING PMN are quiescent cells which became activated in response to a variety of soluble stimuli including cytokines, formyl-methionine-leucine-phenylalanine (fMLP),¹ and phorbol esters. After activation polymorphonuclear neutrophils (PMN) migrate to inflammatory sites, spread on extracellular matrix, activate the NADPH oxidase and arachidonate metabolism, and markedly increase the efficiency of ingestion of opsonized

particles. Many of these functions of activated PMN are dependent on the presence of complement receptor 3 (CR3), a member of the β_2 (CD18) integrin family, even when there is no known CR3 ligand present (20). However, the mechanism of involvement of CR3 in these diverse functions is incompletely understood. A common component of many CR3-dependent functions is the requirement for reorganization of the cytoskeleton. Thus, a testable hypothesis is that one essential role for CR3 in PMN activation is in a signal transduction pathway for cytoskeletal reorganization. A potential target for such a signal transduction event is the microfilament-associated protein paxillin (15, 26). Paxillin is a vinculin-binding protein which becomes tyrosine phosphorylated in fibroblasts after integrin ligation and which is present in focal contacts (7, 27). In addition, tyrosine phosphorylation of paxillin increases during IgG-mediated phagocytosis by macrophages and after bombesin activation of 3T3 cells (18, 34). Paxillin also binds to the SH3 domain

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1. *Abbreviations used in this paper:* AI, attachment index; CR3, complement receptor 3; fMLP, formyl-methionine-leucine-phenylalanine; LAD, leukocyte adhesion deficiency; PDBu, phorbol dibutyrate; PMN, polymorphonuclear neutrophils; TNF- α , tumor necrosis factor- α .

of the *src* family of tyrosine kinases, suggesting it may play a role in localizing signal transduction molecules to sites of organized cytoskeleton (30). We therefore hypothesized that paxillin phosphorylation may be associated with β_2 integrin-dependent signal transduction in PMN. In this work, we have investigated whether tyrosine phosphorylation of paxillin increases during PMN activation, and we have tested the hypothesis that β_2 integrins are required for this event.

Our results demonstrate that tyrosine phosphorylation of paxillin is induced by a variety of soluble and adhesive stimuli in PMN. In all cases, this phosphorylation is absent in PMN genetically deficient in β_2 integrins. Monoclonal antibodies against the various β_2 -associated α chains demonstrate that CR3, the most abundant β_2 integrin on PMN, is the essential integrin required for paxillin tyrosine phosphorylation. K562 cells transfected with CR3 confirm that CR3 ligation causes paxillin tyrosine phosphorylation. These data show that CR3 is required for paxillin phosphorylation in response to a broad range of adherent and soluble PMN activators and suggest that paxillin may be an important mediator of the many diverse functions of activated PMN for which CR3 is required.

Materials and Methods

Cells

PMN were purified from heparinized blood of healthy adult donors as described (19). PMN were pretreated with 5 mM diisopropyl fluorophosphate for 10 min at room temperature. Leukocyte adhesion deficiency (LAD) PMN were obtained from a patient followed at Baylor College of Medicine who has a complete absence of β_2 integrin expression (20). The LAD patient is a 15-yr-old female who showed no clinical evidence of infection at the times PMN were studied. K562 were grown in RPMI with 10% FCS in a 5% CO₂ environment.

cDNA Clones and Cell Transfection

The β_2 cDNA was isolated from a DMSO-differentiated HL60 cDNA library, which was the gift of Drs. Brian Volpp and William Nauseef (29), and cloned into pSFFV-neo (13). The α_M cDNA was the gift of Dr. Timothy Springer, Harvard Medical School (Boston, MA) (11). For transfection, the α_M cDNA was cloned into the stable expression vector pRc/RSV (Invitrogen, San Diego, CA). K562 were cotransfected with the α_M and β_2 cDNAs by electroporation at 225 V and 500 μ F using a gene pulser (Biorad Labs., Richmond, CA). Transfected cells were selected in 600 μ g/ml G418 (Geneticin, GIBCO BRL, Gaithersburg, MD) and a population with high level CR3 expression was derived using FACS. Control K562 were transfected with vector alone (pRc/RSV or pSFFV-neo) by an identical protocol. K562 were transfected with the CR2 cDNA in the pSFFV-neo vector as described (8).

Monoclonal Antibodies and Reagents

IB4 (anti-CD18, IgG2a), 944 (anti-CD11b, IgG2a), and 3.9 (anti-CD11c, IgG1) were the generous gifts of Dr. Sam Wright, Rockefeller University (New York, NY); Dr. Robert Todd, University of Michigan (Ann Arbor, MI); and Dr. Nancy Hogg, Imperial Cancer Research Fund (London, UK), respectively, (24, 25, 33). 10B5 (anti-CD11b, IgG1) was prepared as described (16). TSI-22 (anti-CD11a, IgG1) (10), W632 (anti-HLA I, IgG2a), and HB5 (anti-CR2) were from the American Type Culture Collection (Rockville, MD). Paxillin mAb was from Transduction Laboratories (Lexington, KY), and anti-phosphotyrosine mAb 4G10 was from UBI (Lake Placid, NY). PDBu and fMLP were from Sigma Chem. Co. (St. Louis, MO). Recombinant tumor necrosis factor- α (TNF- α) was from UBI (Lake Placid, NY).

Opsonized Particles

3- μ M latex beads (Polysciences Inc., Warrington, PA) were opsonized by rotating with 20 μ g/ml human IgM (Calbiochem, San Diego, CA) in 0.1 M carbonate buffer pH 9 for 1 h at 37°C. Beads were then washed three times with GVBS (veronal buffered saline with 0.1% gelatin, 0.15 mM Ca²⁺, and 1 mM Mg²⁺) and incubated with a 1:3 dilution of serum in this buffer for 1 h at 37°C. IgG- and C3bi-opsonized sheep red blood cells were prepared as described (16, 19). For assays of paxillin tyrosine phosphorylation 3×10^6 cells were incubated rocking at 37°C with 3×10^8 opsonized particles in HBSS⁺⁺ (1.5 mM Ca²⁺, 1.5 Mg²⁺) with 1% HSA for the indicated times. Attachment indices (AI) were determined by counting the number of particles attached per 100 PMN or K562 cells.

Cell Adhesion

Protein-coated surfaces were prepared by coating with poly-L-lysine as described (6), followed by BSA and anti-BSA for immune complex-coated surfaces (17), or 10 μ g/ml human IgM in PBS for 45 min at RT. Complement-coated surfaces were generated by incubating immune complex-coated surfaces or IgM-coated surfaces with a 1:5 dilution of serum in GVBS for 1 h at 37°C. Cells were incubated in HBSS⁺⁺ with 1% HSA at 37° for the indicated times. K562 adhesion, cell spreading, and paxillin phosphorylation were equivalent on either immune complex/complement-coated surfaces or IgM/complement-coated surfaces.

Cells in Suspension

3×10^6 PMN were suspended in polypropylene Eppendorf tubes in 1 ml HBSS⁺⁺ with 1% human serum albumin and incubated rocking at 30 cycles per minute at 37°C in room air. Under these conditions there was no evidence of cell adhesion (number of cells in suspension equal before and after incubation with or without TNF) or aggregation (assessed microscopically) after stimulation with TNF- α .

Immunoprecipitations and Immunoblotting

3×10^6 PMN or K562 were lysed per point in ice cold Tris-buffered saline, with 0.1% sodium azide (150 mM NaCl, 50 mM Tris-Cl, pH 7.5) containing 1% Triton X-100, 1% deoxycholate, 1 mM EGTA, 1 mM EDTA, 50 μ g/ml leupeptin, 50 μ g/ml aprotinin, 200 μ M sodium orthovanadate, and 1 mM H₂O₂ as described (3). For PMN, lysis buffer also contained 2 mM diisopropyl fluorophosphate. To determine paxillin phosphorylation after adhesion, adherent and nonadherent cells were combined by scraping adherent cells into lysis buffer and combining with the pellet of nonadherent cells obtained by centrifugation for 30 s at 2,000 g. Total cell lysates were rotated at 4°C for 30 min followed by centrifugation at 10,000 g for 10 min. Samples were then then rotated for 1 h at 4°C with paxillin mAb, followed by the addition of goat anti-mouse IgG Sepharose (Organon Teknika, West Chester, PA) and rotated for an additional hour.

Immunoprecipitates were washed three times in Tris-buffered saline with 0.1% Triton X-100, 0.1% deoxycholate, 0.1 mM EDTA, 25 μ g/ml leupeptin, 25 μ g/ml aprotinin, 200 μ M sodium orthovanadate, and 1 mM H₂O₂. Whole cell lysates were prepared by lysing 10^5 cells directly in sample buffer. Samples were heated for 5 min at 100°C in sample buffer with β -mercaptoethanol, separated on SDS-PAGE, and transferred electrophoretically to PVDF membrane (Millipore, Bedford, MA). Blots were stained with Coomassie blue, destained, and then blocked either overnight at 4°C or for 30 min at 37°C in blocking buffer (NaCl 150 mM, 10 mM Tris-Cl pH 8, 1% NP-40 and 2% BSA). Membranes were then incubated for 75 min at RT with primary antibody (anti-phosphotyrosine mAb 4G10 at 0.2 μ g/ml in blocking buffer), followed by washing in the same buffer without BSA. Blots were incubated with secondary antibody (HRP-conjugated anti-mouse IgG2b) (Caltag, San Francisco, CA) 1 μ g/ml in blocking buffer for 45 min at RT, washed, and then developed using an enhanced chemiluminescence kit (Amersham, UK). To determine total paxillin loading, blots were stripped as described (3), and probed as above using 0.2 μ g/ml paxillin mAb followed by 1:1,000 dilution of HRP-conjugated anti-mouse IgG (Cappel, Durham, NC).

Data Presentation

Anti-phosphotyrosine Western blots shown in the figures are of representative experiments. All experiments on normal PMN and K562 transfectants were performed 2–5 times with equivalent results.

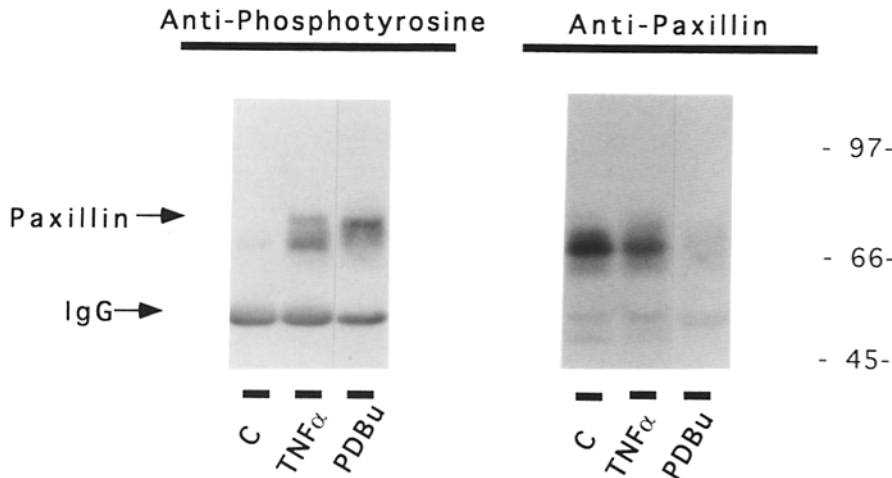
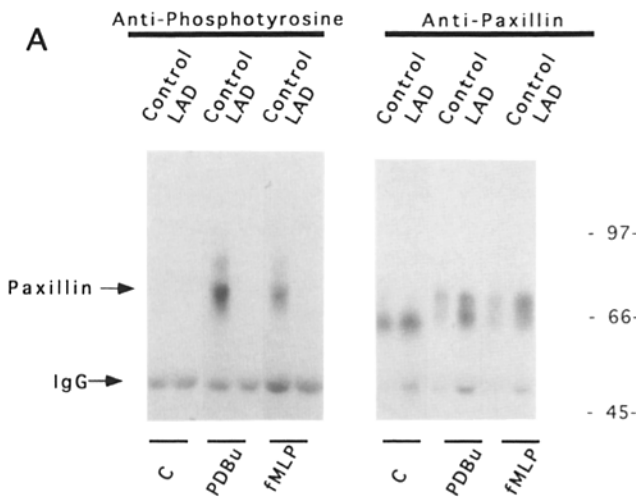


Figure 1. Tyrosine phosphorylation of paxillin in PMN. PMN in suspension were incubated with buffer (C), TNF- α (50 U/ml), or PDBu (15 ng/ml) for 25 min at 37°C. Paxillin immunoprecipitates were run on SDS-PAGE and immunoblotted with the anti-phosphotyrosine mAb 4G10 (*left panel*). PMN in suspension demonstrate tyrosine phosphorylation of paxillin after activation with TNF- α or phorbol esters. Reprobing of the same blot with anti-paxillin mAb is shown in the right panel. In each case, the entire sample was immunoprecipitated with anti-paxillin; and there is equivalent or greater paxillin in control lanes compared with activated samples. The consistent decrease in paxillin after phorbol ester or fMLP activation is probably due to proteolysis. Numbers to the right in each panel refer to M_r markers.



Results

Soluble PMN Activators Cause Paxillin Phosphorylation by a β_2 Integrin-dependent Mechanism

To determine whether paxillin was phosphorylated in response to activating stimuli in PMN, we assayed paxillin phosphorylation in unactivated PMN and in PMN treated with the phorbol ester phorbol dibutyrate (PDBu), the chemotactic peptide fMLP, and the cytokine TNF- α . All three stimuli activate PMN, as judged by increases in phagocytosis, adhesiveness, and oxygen metabolism (14, 20). All three activators caused a significant increase in tyrosine phosphorylation of paxillin (Figs. 1 and 2). Treatment of PMN from a patient with LAD, which lack all β_2 integrins, with either PDBu, fMLP, or TNF- α failed to induce tyrosine

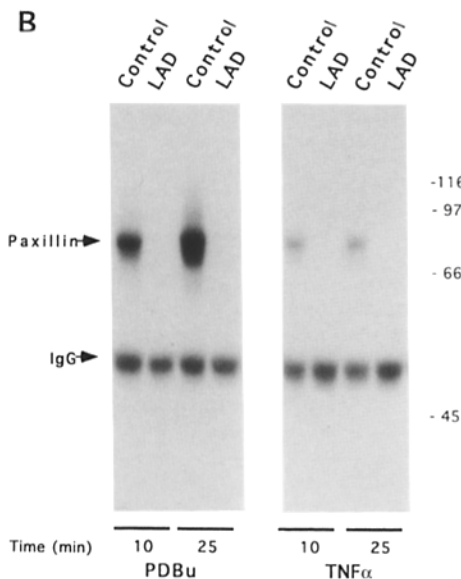


Figure 2. Paxillin phosphorylation is compared for normal and β_2 -deficient (*LAD*) PMN. (*A*) PMN in suspension were incubated with buffer, PDBu (15 ng/ml), or fMLP (10^{-6} M) for 25 min at 37°C. Paxillin immunoprecipitates were run on SDS-PAGE and immunoblotted with the anti-phosphotyrosine mAb 4G10. Enhanced tyrosine phosphorylation of paxillin in response to activation was seen in normal but not *LAD* PMN. Reprobing of the same blot with anti-paxillin mAb is shown on the right and demonstrates equivalent or greater paxillin loading from *LAD* PMN compared with controls. Activation results in a shift of paxillin to a higher molecular weight. This shift in size is independent of tyrosine phosphorylation and occurs equivalently in control and *LAD* PMN. (*B*) Control and β_2 -deficient (*LAD*) PMN in suspension were incubated for 10 or 25 min with PDBu (15 ng/ml) or TNF- α (50 U/ml). Paxillin immunoprecipitates were run on SDS-PAGE and phosphotyrosine immunoblotted. Again tyrosine phosphorylation of paxillin in response to activation was seen in normal but not *LAD* PMN. Paxillin phosphorylation increases markedly between 2 and 10 min after stimulation and remains sustained as long as the PMN are viable. There is some variability in the magnitude of tyrosine phosphorylation in response to each stimulus depending on the blood donor. On average, TNF gives less than half of the intensity of the PDBu response when developed with anti-PY.

phosphorylation of paxillin (Fig. 2). These data demonstrate that paxillin tyrosine phosphorylation occurs during PMN activation of nonadherent cells. This phosphorylation of paxillin requires expression of β_2 integrins. Immunoprecipitation of the activated cells with antiphosphotyrosine, followed by antipaxillin; and detection with antipaxillin demonstrated that <6% of the total pool of paxillin was detected as tyrosine phosphorylated by this method (data not shown). However, treatment of PMN with 100 μ M vanadate and 2 mM H_2O_2 , inhibitors of tyrosine phosphatases (21), markedly increased the proportion of tyrosine phosphorylated paxillin in the activated cells. These data suggest that the tyrosine phosphorylation of paxillin is tightly regulated by phosphatases in PMN.

Because PMN activation with PDBu and fMLP leads to β_2 integrin-dependent homotypic PMN aggregation, we considered the possibility that PMN aggregation initiates a β_2 integrin-dependent signal transduction pathway. However, TNF- α is reported not to induce PMN aggregation (2), and we observed no aggregation microscopically, yet TNF- α does stimulate paxillin phosphorylation (Fig. 1). Similarly, when PMN were diluted 50-fold before PDBu stimulation, no aggregation was observed, but paxillin phosphorylation was unaffected (data not shown). These data disassociate stable PMN aggregation from β_2 integrin-dependent paxillin phosphorylation.

Tyrosine Phosphorylation of Paxillin in Adherent PMN Requires β_2 Integrins

To test whether adhesion was a stimulus for paxillin phosphorylation in PMN we used immune complex- or extracel-

lular matrix protein-coated surfaces. Immune complex-coated surfaces were tested initially since both normal and LAD PMN can adhere and spread on this surface (1, 17). After adhesion to immune complexes, normal PMN showed a marked increase in paxillin tyrosine phosphorylation, but LAD PMN did not (Fig. 3). Thus, in PMN, Fc gamma receptor-mediated activation of paxillin phosphorylation requires CD18. However, not all tyrosine phosphorylation induced by FcR ligation requires expression of β_2 integrins. The increase in tyrosine phosphorylated proteins seen in a whole cell lysate after adhesion to immune complexes was almost indistinguishable between LAD and normal PMN (Fig. 3).

In mouse macrophages, interaction with IgG-opsonized particles has been shown to induce paxillin tyrosine phosphorylation (18). However, despite avid binding of IgG-opsonized particles by human PMN, neither control nor LAD PMN demonstrated paxillin phosphorylation in response to this stimulus (Fig. 3).

Adhesion to extracellular matrix-coated surfaces also was tested as a stimulus for paxillin tyrosine phosphorylation. Since PMN adhere poorly to integrin ligands in the absence of activation (5), PMN were treated with PDBu before adhesion to fibronectin, laminin, and vitronectin. In no case did adhesion, even after phorbol stimulation lead to paxillin phosphorylation in LAD PMN (Fig. 3). Although LAD PMN demonstrate a β_2 integrin-independent binding to laminin through $\alpha_6\beta_1$ (4), this is not sufficient to induce paxillin phosphorylation. Thus, paxillin phosphorylation in PMN in response to both adherent and nonadherent stimuli is dependent on β_2 integrins.

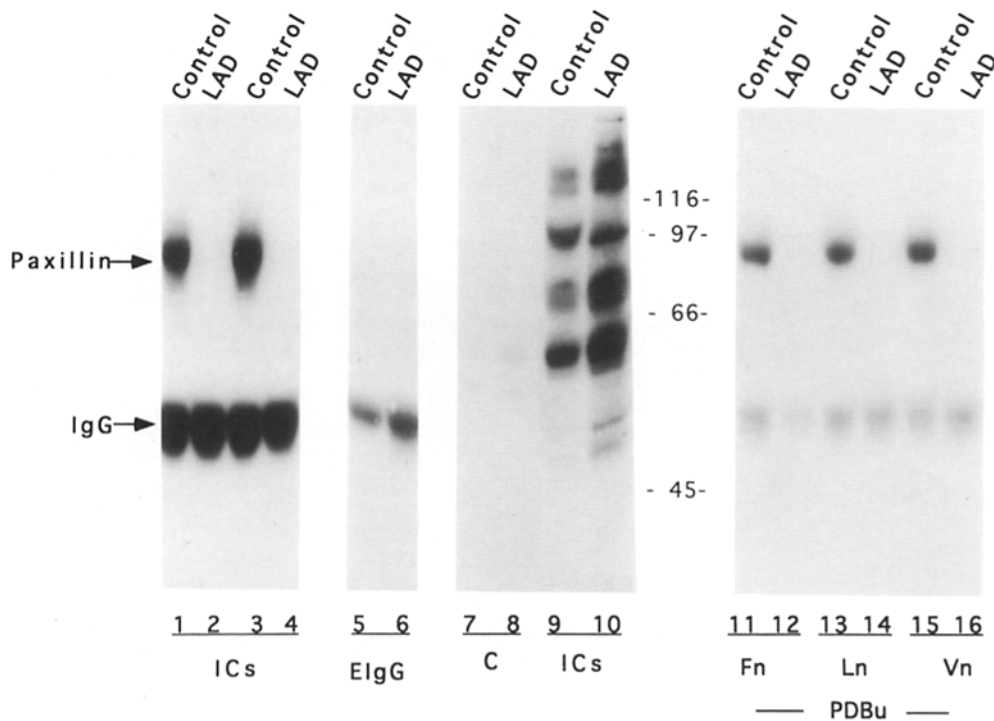


Figure 3. PMN tyrosine phosphorylation of paxillin in response to IgG and adhesion. Control and β_2 -deficient (LAD) PMN were adhered to immune complex-coated surfaces for 10 min (lanes 1 and 2) or 25 min (lanes 3 and 4), followed by paxillin immunoprecipitation and phosphotyrosine immunoblotting. Control PMN show a marked tyrosine phosphorylation of paxillin after adhesion to an immune complex-coated surface, while β_2 -deficient PMN (LAD) failed to phosphorylate paxillin. (Lanes 5 and 6) PMN were incubated for 25 min in suspension with IgG-opsonized sheep red blood cells. Neither control nor β_2 -deficient PMN (LAD) phosphorylated paxillin in response to Fc γ R ligation in suspension. (Lanes 7 and 10) PMN were incubated for 25 min in suspension (C) or adherent to immune com-

plexes (ICs). The whole cell lysate was run on SDS-PAGE and phosphotyrosine immunoblotted. Adhesion to an immune complex-coated surface induced tyrosine phosphorylation of numerous proteins in both control and LAD PMN. (Lanes 11-16) Control and LAD PMN were stimulated with PDBu and allowed to adhere to fibronectin-, laminin-, or vitronectin-coated surfaces for 25 min. Paxillin immunoprecipitates were immunoblotted with anti-phosphotyrosine mAb. LAD PMN failed to phosphorylate paxillin in response to PDBu stimulation or adhesion to these extracellular matrix protein-coated surfaces. M_r markers were as in Fig. 1.

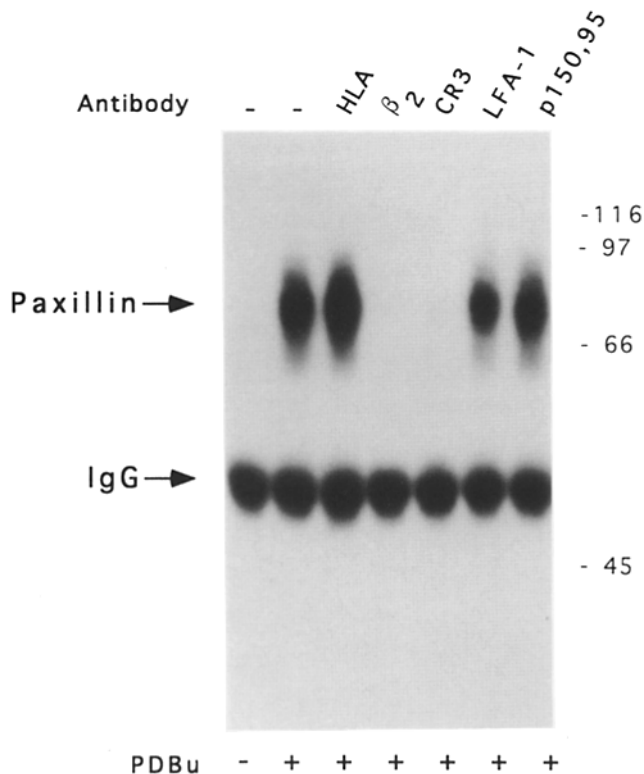


Figure 4. Monoclonal antibody inhibition of paxillin tyrosine phosphorylation. PMN in suspension were incubated \pm PDBu (15 ng/ml) in the presence of mAb against the different β_2 integrins or HLA class I as a control. Paxillin was immunoprecipitated and phosphotyrosine detected as in Fig. 1. mAb against the β_2 chain (IB4) and the CR3 α chain (944) inhibited paxillin tyrosine phosphorylation. In contrast, control mAb anti-HLA (W632), anti-LFA-1 α chain mAb (TSI-22), and anti-p150, 95 α chain mAb (3.9) failed to significantly inhibit paxillin tyrosine phosphorylation.

CR3 ($\alpha_M\beta_2$) Expression Is Necessary for Tyrosine Phosphorylation of Paxillin in PMN

LAD PMN lack all β_2 integrins. To determine whether any single one of the three β_2 integrins was necessary for paxillin phosphorylation in response to PMN activation, normal PMN were incubated with inhibitory antibodies to α_L , α_M , and α_X , and to the common β_2 chain before activation with PDBu. mAb directed against α_M and β_2 resulted in almost complete inhibition of phorbol ester-induced paxillin tyrosine phosphorylation (Fig. 4). In contrast mAb against α_L and α_X had no effect on paxillin tyrosine phosphorylation. These data suggest that $\alpha_M\beta_2$ (CR3) is required for paxillin phosphorylation during PMN activation.

Ligation of CR3 Leads to Paxillin Tyrosine Phosphorylation in Transfected K562 Cells

To determine whether CR3 was sufficient for paxillin tyrosine phosphorylation, we made stable transfectants in K562 with this integrin (KCR3). K562 express only the integrin $\alpha_5\beta_1$ endogenously, and in particular express no β_2 integrins (23). As a control, we also transfected K562 with complement receptor 2 (CD21), a nonintegrin receptor which recognizes a ligand, the complement component C3bi, in common with CR3 (8) (KCR2). The KCR3 and KCR2 each expressed high levels of the transfected receptor (Fig. 5). Both KCR3 and KCR2 adhered avidly and equally to a complement-coated surface, with 124 KCR3 adhered/HPF, 143 KCR2 adhered/HPF, and <10 vector-transfected K562 adhered/HPF. The KCR3 showed significant spreading and extension of cellular processes after adhesion (Fig. 6 A). In contrast, the majority of the CR2-K562 remained rounded and failed to spread on the complement-coated surface (Fig. 6 B). Paxillin tyrosine phosphorylation was assessed as well in the adherent transfectants. In KCR3, adhesion to a

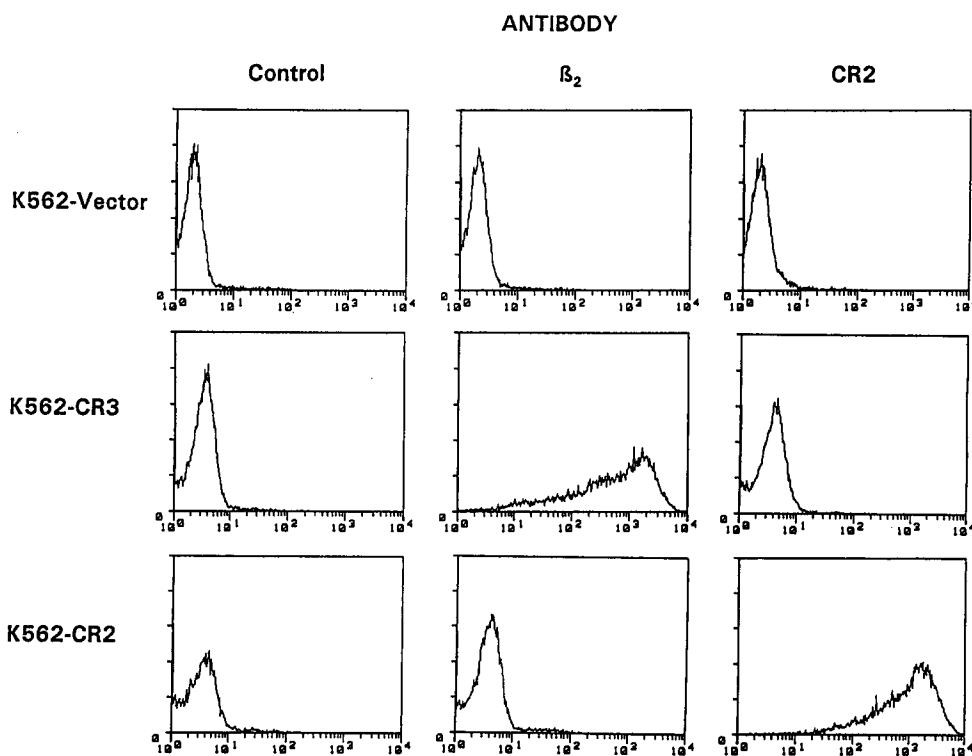


Figure 5. Expression of CR3 and CR2 on transfected K562 cells. Transfected K562 cells were incubated with either control mAb (MOPC IgG1), anti- β_2 mAb IB4, or anti-CR2 mAb HB5 and analyzed by fluorescent flow cytometry. CR3 α chain mAb 10B5 (16) stained K562-CR3 equivalently to the β_2 mAb (data not shown).

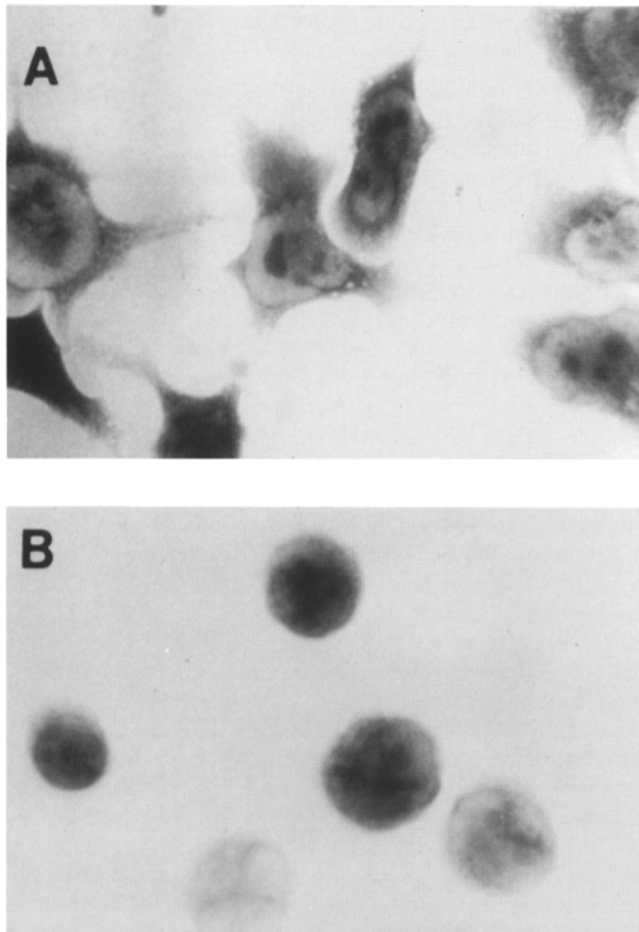


Figure 6. Adhesion of KCR3 and KCR2 to C3bi-coated surface. CR3- (A) and CR2- (B) transfected K562 cells were allowed to adhere to complement-coated surfaces for 30 min, followed by Giemsa staining. Despite avid adhesion of both transfectants to the surface, there was a marked morphologic difference in the adherent cells. K562 transfected with vector alone did not adhere to the C3bi-coated surface (not shown).

complement-coated surface was associated with tyrosine phosphorylation of paxillin, while there was no paxillin phosphorylation in the adherent KCR2 (Fig. 7). Thus, CR3 ligation is sufficient to induce paxillin tyrosine phosphorylation in K562 cells and is associated with cell spreading on a complement-coated surface.

Phorbol Esters Are Not Sufficient to Induce Paxillin Phosphorylation in Nonadherent K562

Since paxillin was phosphorylated after phorbol ester treatment of nonadherent PMN, we examined whether phorbol esters could cause paxillin phosphorylation in transfected K562 cells in suspension (Fig. 8). KCR3 in suspension either with or without phorbol ester treatment showed no paxillin tyrosine phosphorylation. Next, we tested whether ligand binding by nonadherent cells could stimulate paxillin phosphorylation. Both KCR2 and KCR3 were constitutively active for binding of 3 μ m diameter beads coated with C3bi (AI: KCR2 = 1369 ± 185 , KCR3 = 950 ± 155 , mean \pm SEM). Addition of this CR3 ligand to cells in suspension in-

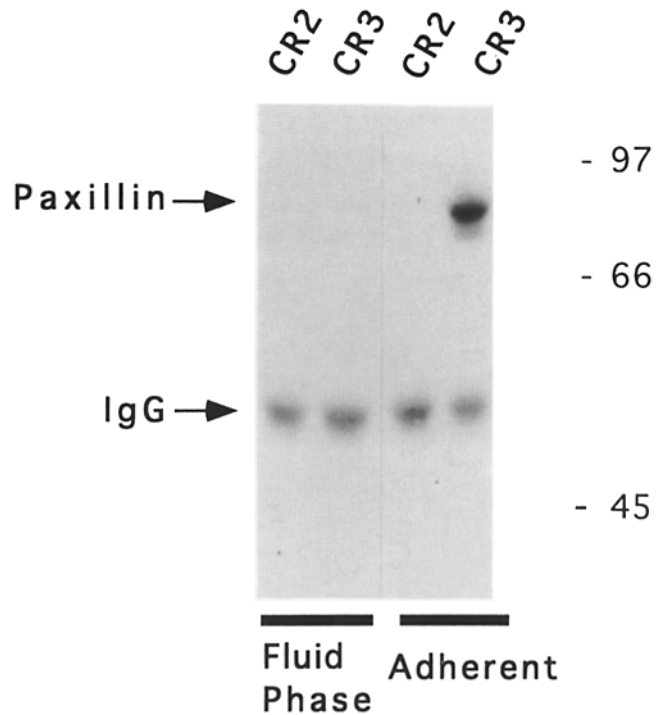


Figure 7. Paxillin tyrosine phosphorylation in transfectants. CR2- and CR3-transfected K562 cells were incubated for 30 min in suspension or allowed to adhere to a complement-coated surface, followed by paxillin immunoprecipitation and phosphotyrosine immunoblotting. Tyrosine phosphorylation of paxillin was not detected in either transfectant in suspension. Tyrosine phosphorylation of paxillin was apparent in the CR3 but not the CR2 transfectant in response to adhesion to a complement-coated surface. Equivalent paxillin loading in each lane was confirmed by reblotting with anti-paxillin mAb (data not shown). *M_r* markers are as in Fig. 1.

duced tyrosine phosphorylation of paxillin in KCR3 (Fig. 8). C3bi-bead binding in combination with phorbol ester treatment led to a further increase in paxillin phosphorylation in KCR3. In contrast KCR2 failed to phosphorylate paxillin even with the combination of ligand and phorbol ester treatment. Thus, there is a CR3-dependent mechanism for paxillin tyrosine phosphorylation in both adherent and a nonadherent K562. In contrast to PMN, which required only phorbol ester treatment to phosphorylate paxillin, the K562 transfectants required the addition of a CR3 ligand to induce paxillin phosphorylation.

Adhesion Augments the Ability of C3bi to Induce Paxillin Phosphorylation in PMN

Since a CR3 ligand induced paxillin phosphorylation in nonadherent KCR3, we tested whether this also was true in PMN. Unlike KCR3, which are constitutively active for ligand binding, resting PMN express CR3 which is not in an optimal conformation for C3bi binding (28, 32). Cell activation by either phorbol esters or fMLP induces a higher CR3 affinity for C3bi-coated targets and causes membrane expression of an internal pool of CR3. Since these activation stimuli alone induce paxillin phosphorylation in PMN, we used Mn^{2+} to test the effect of C3bi binding by PMN on paxillin tyrosine phosphorylation. Addition of Mn^{2+} to the

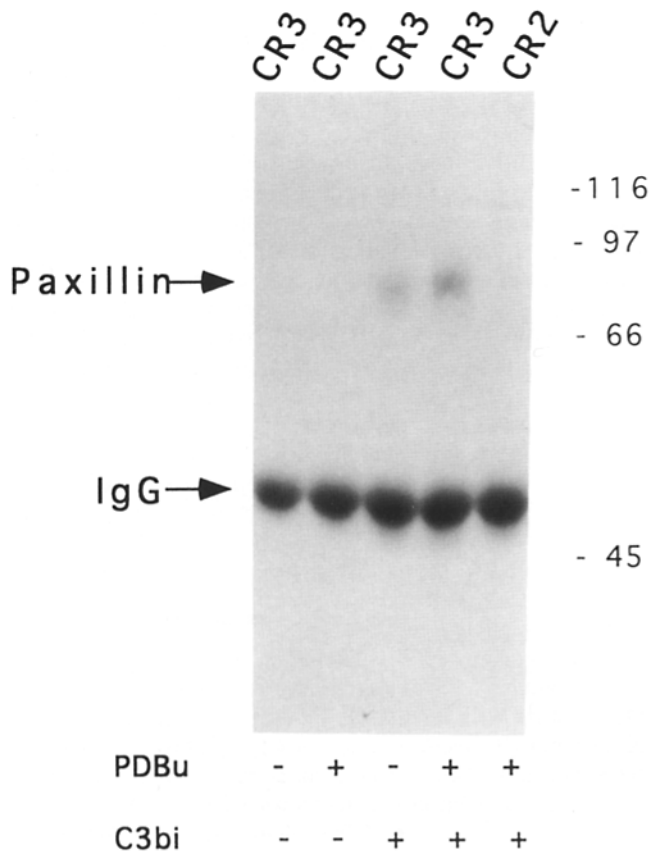


Figure 8. Tyrosine phosphorylation of paxillin in transfectants binding C3bi-beads. CR3- or CR2-transfected K562 cells were incubated in suspension \pm PDBu (30 ng/ml) and \pm C3bi-coated beads. Paxillin immunoprecipitates were phosphotyrosine immunoblotted. The CR3-transfected cells phosphorylated paxillin in response to C3bi-bead binding. This paxillin phosphorylation was further enhanced by the combination of C3bi-bead binding and phorbol ester treatment of the CR3-transfected cells. The CR2-transfected cells failed to phosphorylate paxillin even in response to the combination of ligand binding and phorbol esters. M_r markers are as in Fig. 1.

buffer has been described to augment ligand binding by other integrin molecules by inducing a conformational change (12). Similarly, Mn^{2+} markedly augmented C3bi-particle binding by PMN without enhancing receptor expression (C3bi-particle attachment: Baseline AI = 180 ± 11 ; with Mn^{2+} = 1335 ± 162 , mean \pm SEM). PMN in suspension were incubated with C3bi-coated targets, with or without addition of Mn^{2+} to the buffer. Binding of C3bi by nonadherent PMN induced minimal paxillin tyrosine phosphorylation, whether or not Mn^{2+} was present in the buffer (Fig. 9). In contrast, adhesion of PMN to a C3bi-coated surface resulted in detectable paxillin tyrosine phosphorylation, which was enhanced by the addition of Mn^{2+} . However, tyrosine phosphorylation of paxillin in these experiments was much less than after phorbol ester activation of PMN, suggesting that there are additional activation steps involved in efficient paxillin tyrosine phosphorylation beyond ligand binding to CR3.

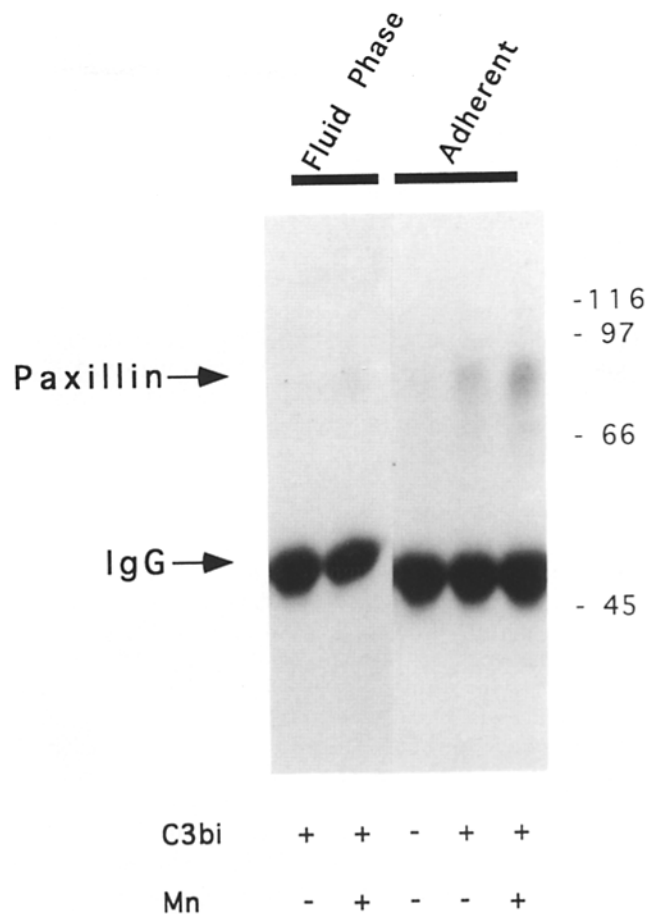


Figure 9. Paxillin tyrosine phosphorylation in unactivated PMN in response to C3bi ligand. PMN were incubated for 25 min in suspension with C3bi-opsonized particles (AI = 1294), or adherent to a complement-coated surface, with or without 250 μ M Mn^{2+} . Control cells were adherent to an albumin-coated surface. Paxillin immunoprecipitates and immunoblots were prepared as in Fig. 1. Cells in suspension showed minimal paxillin tyrosine phosphorylation. Cells adherent to the complement-coated surfaces demonstrated greater paxillin phosphorylation than cells in suspension. Mn^{2+} enhanced the paxillin phosphorylation slightly.

Discussion

This study demonstrates a critical role for the β_2 integrin CR3 for tyrosine phosphorylation of the cytoskeletal protein paxillin in both nonadherent and adherent PMN. Studies in LAD PMN, and normal PMN treated with various mAb, all demonstrate a requirement for β_2 integrins, specifically for CR3. Studies in transfected cells show that CR3 ligation leads to paxillin phosphorylation, while equally avid engagement of CR2 with the same ligand does not. These results demonstrate that fluid phase activators of paxillin phosphorylation, such as phorbol esters, fMLP, and TNF- α all work through a mechanism which requires the plasma membrane integrin, rather than through activation of an exclusively intracellular signal transduction pathway.

These data in PMN and the transfected K562 cells lead us to the hypothesis that maximal paxillin tyrosine phosphorylation in PMN is a two step process requiring ligand binding

by CR3 in addition to an intracellular signal(s) which can be provided by phorbol esters, fMLP, TNF- α , or adhesion to a C3bi-coated surface. This hypothesis would explain the complete absence of paxillin tyrosine phosphorylation in β_2 -deficient PMN. Interestingly, for PMN in suspension, only the addition of the activating stimulus is required for paxillin tyrosine phosphorylation, while KCR3 require addition of an exogenous CR3 ligand as well. This suggests that PMN may contain an endogenous CR3 ligand. Since PMN aggregate in response to phorbol esters and fMLP, it is likely that CR3 recognizes another plasma membrane protein on PMN which may be serving as the ligand. However, TNF- α induces paxillin phosphorylation and does not cause PMN aggregation (2). Therefore it is possible that transient cell-cell or cell-substrate interactions may be sufficient to provide CR3 ligation, or that activated CR3 may recognize a ligand on the same cell.

For both PMN and KCR3, the activating stimulus can be supplied by adhesion to a ligand-coated surface. Thus, adhesion provides additional signals to the cell beyond integrin ligation. The fact that phorbol esters mimic this role for adhesion in both PMN and KCR3 suggests the possibility that adhesion may activate a necessary serine/threonine protein kinase which is not activated by integrin ligation. This conclusion is in accord with the discoveries that pp125^{FAK} phosphorylation in platelets also requires a stimulus beyond integrin ligation (22) and that focal contact formation in fibroblasts requires protein kinase C activation in addition to integrin ligation (31). This two signal model for activation of paxillin phosphorylation with CR3 ligation also is consistent with the observation that adhesion of unactivated PMN to extracellular matrix proteins is necessary but not sufficient for tyrosine phosphorylation of several unidentified proteins (14). In blood cells such as platelets and PMN, the additional step of activating the integrin receptor from its nonavid state to one which will bind ligand also is required. Presumably this is an additional protection in vivo against the consequences of intravascular PMN or platelet aggregation and activation.

Adhesion of PMN to an immune complex-coated surface stimulates paxillin tyrosine phosphorylation, while particulate immune complexes presented to cells in suspension do not. Like the other PMN activators, the immune complex-coated surface can only lead to paxillin tyrosine phosphorylation when the PMN express β_2 integrins. Tyrosine phosphorylation of several other proteins in response to adhesion to immune complexes is unaffected by the absence of these integrins. This observation suggests the hypothesis that paxillin tyrosine phosphorylation may be required for some functions which occur after PMN Fc γ R ligation which have been shown to require β_2 integrin expression. These functions include IgG-stimulated LTB₄ production, sustained spreading on immune complexes, and maximal IgG-mediated phagocytosis after PMN activation with phorbol esters, fMLP, TNF- α , and extracellular matrix proteins (17, 20). The kinetics of paxillin phosphorylation parallel these functions of activated PMN. In contrast, thioglycolate-elicited murine macrophages apparently stimulate tyrosine phosphorylation of paxillin directly through ligation of the transmembrane form of Fc γ RIII, a receptor which PMN lack (18). This alternative pathway to tyrosine phosphorylation of paxillin could explain the independence from β_2 integrins of IgG-mediated phagocytosis in these cells.

Whether the tyrosine phosphorylation of paxillin is directly related to function in PMN and other cells is unknown. There is a strong correlation between cell spreading with paxillin tyrosine phosphorylation in several cell types (7, 26). This is well illustrated by comparison of KCR2 and KCR3 in this study, since the two transfectants adhere equivalently to a C3bi-coated surface, but only in the KCR3 was paxillin phosphorylation and spreading observed. A much higher proportion of paxillin (20–30%) becomes phosphorylated in early embryonic cells and transformed fibroblasts than has been reported for other cytoskeletal proteins such as talin and vinculin (1%) (15, 26). Our results indicate that in PMN only a small proportion of total paxillin is phosphorylated at any given time. Since paxillin tyrosine phosphorylation has been associated with the presence of focal contacts, it is possible that the lower level of paxillin tyrosine phosphorylation in PMN reflects their rapid motility when activated. When PMN are activated in the presence of phosphatase inhibitors there is a marked increase in the proportion of paxillin which remains tyrosine phosphorylated, suggesting that paxillin tyrosine phosphorylation in PMN may be tightly regulated by phosphatase activity. Differences in level of paxillin tyrosine phosphorylation in different cells may depend on the balance between tyrosine kinase and phosphatase activity under the conditions studied. Inhibition of fibroblast tyrosine kinase activity inhibits both paxillin phosphorylation and focal contact formation suggesting a functional association (7). In mouse macrophages, paxillin localizes to the site of IgG FcR aggregation and phagocytosis. In the macrophages tyrosine kinase inhibitors block both phagocytosis and paxillin phosphorylation (18). In our experiments in PMN and K562, the tyrosine kinase inhibitors genistein, herbimycin, an erbstatin analogue, and tyrphostin failed to inhibit paxillin phosphorylation completely (Graham, I. L., and E. Brown, unpublished). Thus, even indirect tests of whether the phosphorylation itself is necessary for function were not possible. Since paxillin is known to provide a binding site for the SH3 domains of *src* family tyrosine kinases, it is also possible that the localization of paxillin to sites of organized cytoskeleton could serve a role in signal transduction which is independent of its tyrosine phosphorylation (30).

In summary, CR3 is required for signal transduction involving paxillin phosphorylation after activation of adherent and nonadherent PMN. This may imply either that CR3 is directly involved in activation of a tyrosine kinase, or alternatively, that CR3 is required for localization of paxillin to a site of tyrosine kinase activity. Identification of the tyrosine kinase involved in paxillin tyrosine phosphorylation in PMN may aid in differentiating these possibilities. It is intriguing to speculate that CR3-dependent tyrosine phosphorylation of paxillin may be part of a signal transduction pathway leading to the transition of PMN from a functionally inactive to active state. If so, activation of this tyrosine kinase represents a key step in the inflammatory process.

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