β2 Integrin-dependent Tyrosine Phosphorylation of Paxillin in Human Neutrophils Treated with Tumor Necrosis Factor

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Abstract. The focal adhesion protein paxillin undergoes tyrosine phosphorylation in response to signals mediated by integrins, neuropeptides and oncogene products, possibly via activation of the focal adhesion-associated kinase, p125^{FAK}. In the present work, tumor necrosis factor-α (TNF) stimulated tyrosine phosphorylation of paxillin in human neutrophils. Cell adhesion and participation of the β2 integrin CD18 were necessary, but not sufficient, for the response. Adherent neutrophils also tyrosine phosphorylated paxillin in response to phorbol ester, formylmethionyl-leucyl-phenylalanine and opsonized bacteria. In contrast, p125^{FAK} was constitutively tyrosine phosphorylated in a manner unaffected by adherence and/or TNF. Thus, cytokines and microbial products are among the stimuli that can induce the tyrosine phosphorylation of paxillin, and kinases other than p125^{FAK} may be responsible. This is the first identification of paxillin and p125^{FAK} in human cells and neutrophils, and one of the few identifications of a specific protein that undergoes tyrosine phosphorylation in response to any agonist in neutrophils or in response to TNF in any cell.

SURVIVAL of mammals depends on the ability of neutrophilic polymorphonuclear leukocytes (PMN)[1] to adhere to endothelium, emigrate into infected tissue, form abscesses, and kill bacteria. Key aspects of these responses are modeled by plating PMN on matrix protein-coated surfaces and stimulating the secretion of oxidants and proteases with inflammatory polypeptides (18, 21, 22). Among effective stimuli, tumor necrosis factor-α (TNF) has been most intensively studied (15, 18, 22).

It remains unclear how TNF elicits pleiotropic responses via receptors that embody no known enzymatic function (29, 35). Tyrosine phosphorylation was recently recognized as a signal induced by TNF (1, 8, 37). The only distance in which TNF-induced tyrosine phosphorylation has been shown to be critical for function is in β2 integrin-dependent responses of TNF-treated PMN, namely, reorganization of their actin cytoskeleton and secretion of large amounts of hydrogen peroxide (8). Neither the tyrosine kinases nor their sub-

strates involved in these responses have been characterized, beyond the localization of the most abundant tyrosine phosphoproteins to the region of the cells close to or in contract with the substrate (8). Indeed, only three tyrosine phosphoproteins have been identified in PMN treated with any stimulus: microtubule-associated protein-2 kinase or mitogen activated protein kinase (MAP kinase) (11), *rel* (6), and recently the tyrosine kinase p58^{fgr} (3). Likewise, only two tyrosine phosphoproteins have been identified in any cell treated with TNF: MAP kinase (34) and p58^{fgr} (3).

Here we study two leading candidates within focal adhesions for tyrosine kinase substrates involved in the adhesion-dependent, polypeptide-triggered secretory response of PMN. Paxillin is a 68-kD protein purified from chick embryo fibroblasts (31) which binds to vinculin (9, 31), v-crk (4), and the SH3 domain of c-src (36), localizes in focal adhesions (31, 32), and undergoes tyrosine phosphorylation during adhesion of fibroblasts (5), neuropeptide stimulation of 3T3 cells (39), phagocytosis by macrophages (12), and embryonic development of chicken and rat (30). Focal adhesion kinase (p125^{FAK}) is a 125-kD tyrosine kinase and c-src substrate (28) that is phosphorylated on tyrosine in response to adhesion of fibroblasts and may in turn phosphorylate paxillin (16). The present experiments identified both paxillin and p125^{FAK} in human PMN. Paxillin but not p125^{FAK} underwent tyrosine phosphorylation in response to TNF, phorbol ester (PMA), formyl-methionyl-leucyl-phenylalanine (fMLP) and opsonized bacteria, but only in adherent

1. *Abbreviations used in this paper:* DHCB, dihydrocytochalasin B; ECL, enhanced chemiluminescence; fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; KRPG, Krebs-Ringer phosphate buffer with glucose; LAD, leukocyte adhesion deficiency; MAP kinase, microtubule-associated protein-2 kinase or mitogen-activated protein kinase; PMA, phorbol myristate acetate; PMN, neutrophilic polymorphonuclear leukocytes; RT, room temperature; TNF, tumor necrosis factor-α.

PMN with available β2 integrins and an intact actin-based cytoskeleton.

Materials and Methods

PMN

PMN were isolated from heparinized blood of normal human donors and from a child with profound leukocyte adhesion deficiency (LAD) whose PMN responses were previously described (21), using a one-step, modified Ficoll-Hypaque gradient (Neutrophil Isolation Medium, Cardinal Associates, Santa Fe, NM) as described (22). Erythrocytes were lysed by hypotonic shock and PMN resuspended in ice-cold Krebs-Ringer phosphate buffer with glucose (KRPG) (145 mM NaCl, 5.7 mM sodium phosphate, 4.86 mM KCl, 0.54 mM CaCl$_2$, 1.22 mM MgSO$_4$, 5.5 mM glucose, pH 7.35, mOsm 300-315). For adherent cells, PMN were plated at 4×10^6/ml in 5 ml in 100-mm Primaria™ dishes (Falcon Labware, Becton-Dickinson & Co., Oxnard, CA) precoated with 3 ml FBS (Hyclone Systems, Logan, UT) prewarmed to 37°C. After 15 min, stimuli and/or inhibitors were added. Pure recombinant human TNF was a gift of Genentech, Inc. (South San Francisco, CA). PMA, fMLP, and dihydrocytochalasin B (DHCB) were from Sigma Chemical Co. (St. Louis, MO). A clinical isolate of *Listeria monocytogenes* from New York Hospital was opsonized by incubation in 20% normal human serum for 1 h on ice, washed in PBS, and stored at -70°C. At the indicated times, the dishes were placed on ice, the incubation medium was aspirated, and the residual cells were lysed in 125 µl of solubilization buffer (10 mM Tris-HCl, pH 7.4, 1% SDS, 1 mM sodium vanadate, 0.1 mM sodium molybdate, 1 mM sodium pyrophosphate, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 5 mM diisopropyl fluorophosphate, and 5 µg/ml each of pepstatin A, leupeptin, aprotinin, and chymostatin). The lysate was immediately boiled for 5 min and then stored at -80°C until further analysis. For suspended cells, 1 ml of the same suspension of PMN was incubated in FBS-coated polypropylene tubes and agitated at 100 cycles/min; this was necessary to prevent adhesion of the cells to the walls of the tube or to each other. At the indicated times, the tubes were centrifuged at 16,000 g for 10 s at room temperature (RT) and the cell pellets lysed in solubilization buffer as described above. Where indicated, PMN (50 $\times$ 10^6/ml) were preincubated with 1 µg/ml of the specified antibodies for 30 min at 4°C with end-over-end rotation and then plated on FBS coated plates as above. Protein concentrations of lysates were determined by the Bradford or modified Lowry methods (Bio-Rad Laboratories, Richmond, CA).

Antibodies

Anti-phosphotyrosine mAb 5E2 (IgG2b) was a generous gift of B. M. Fendly (Genentech Inc., South San Francisco, CA) (7). Anti-p125^{FAK} mAb antibody 2A7 (mouse IgG$_1$ against chicken p125^{FAK}) was a generous gift of T. Parsons (University of Virginia, Charlottesville, VA) (14). Anti-paxillin mAb 349 (mouse IgG$_1$ against chicken paxillin), rabbit polyclonal anti-phosphotyrosine IgG and recombinant, HRP-conjugated anti-phosphotyrosine mouse mAb (RC20) were purchased from Transduction Laboratories (Lexington, KY). Anti-CD18 mAb IB4 (mouse IgG$_{2a}$) and its F(ab')$_2$ fragments were a generous gift of S. Wright (Rockefeller University, New York). Anti-CD16 (Fc receptor) mAb 3G8 (mouse IgG$_1$) was a kind gift of J. Unkeless (Mt. Sinai Medical School, New York). Normal mouse IgG was purchased from Pierce (Rockford, IL). Monoclonal anti-vinculin (hVIN-1-IgG$_1$) antibody was from Sigma Chemical Co.

Immunoprecipitation

Cell lysates normalized for protein concentration were diluted to 0.1% SDS in modified RIPA buffer (10 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.5% Nonidet-P40, 150 mM NaCl, 1 mM sodium vanadate, 0.1 mM sodium molybdate, 1 mM sodium pyrophosphate, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 5 µg/ml each of pepstatin A, leupeptin, aprotinin, and chymostatin). Diluted lysates were incubated with mAbs at 1/100 dilution, except polyclonal anti-phosphotyrosine and anti-paxillin which were used at 1/200 dilution, at 4°C overnight with protein G-Sepharose beads (Pharmacia LKB, Uppsala, Sweden). Precipitated immune complexes were washed three times in modified RIPA buffer. The pellets were resuspended in SDS sample buffer and heated at 100°C for 10 min before analysis by SDS-PAGE.

Immunoblotting

Lysates separated by SDS-PAGE (17) were transferred eletrophoretically to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH). Where specified, non-reducing conditions were used so that IgG heavy chain would not lie close to the molecular mass region of paxillin since the secondary reagents used for western blot detect IgG heavy chain and interfere with paxillin detection. The membranes were incubated overnight at 4°C in blocking buffer (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 0.1% Triton X-100, 10% FBS) and then for 4 h at RT with one of the following: mAb 5E2 at 1.5 µg/ml; mAb 2A7, 1/100 dilution; anti-paxillin mAb, 1/1,000 dilution; or mAb RC20, 1/2,000 dilution, all in blocking buffer. Membranes were washed in the same buffer without serum, blocked again in 20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 0.1% Tween 20, 10% FBS for 30 min at RT, and incubated at RT for 45 min with HRP-conjugated IgG sheep anti-mouse IgG when appropriate (Amersham Corp., Arlington Heights, IL) in the same buffer. After further washing in the same buffer without serum, immunoblots were developed using an enhanced chemiluminescence (ECL) kit (Amersham Corp.). Where indicated the immunoblot films were subjected to densitometric analysis by scanning on a Mirror 800 transparency scanner (Mirror Technologies, Roseville, MN) and the resulting scan analyzed by computer with the NIH Image 1.52 software.

Immunohistochemistry

PMN (10^6 cells/ml) were plated on FBS-coated 13-mm diam glass coverslips in 12-well plates containing prewarmed reaction mixture. After 15 min to allow the cells to contact the coverslips, TNF (250 ng/ml) was added or cells were left untreated. After incubation for the indicated times at 37°C in air, the medium was gently aspirated and cells fixed with 1% paraformaldehyde in cacodylate buffer, pH 7.4 (75 mM sodium cacodylate, 0.72% sucrose), for 10 min at RT. The supernatant was then replaced with 3.7% formaldehyde in PBS for 10 min at RT. Cells were then washed with PBS and permeabilized with 0.05% Triton X-100 in PBS for 4 min at RT. Fixed cells were incubated with blocking buffer (PBS + 10% calf serum) for 1 h at RT and then for 1 h at 37°C with a 1/100 dilution rabbit anti-phosphotyrosine antibody and 1/100 dilution of mouse anti-vinculin in blocking buffer. Coverslips were then washed three times in PBS and incubated for 45 min at 37°C with rhodamine-conjugated goat IgG F(ab')$_2$ anti-rabbit IgG (γ and light chain) and fluorescein-conjugated goat IgG F(ab')$_2$ anti-mouse IgG (γ and light chain) antibodies (Tago Laboratories, Burlingame, CA) in blocking buffer. Coverslips were washed twice in PBS and once in distilled water, inverted, and mounted in 90% glycerol in water on a glass slide.

Results

Phosphotyrosine-containing Proteins Colocalize with Vinculin

Double staining established that the majority of immunoreactive phosphoprotein was not only confined near the adherent surface of PMN plated on FBS-coated glass (8) but was specifically localized in focal adhesions, as judged by its colocalization with vinculin in finger-like structures (Fig. 1).

Identification of Paxillin and p125^{FAK} in Human PMN and Assessment of Their Tyrosine Phosphorylation in Response to TNF

By immunoblot with anti-phosphotyrosine, the most readily detectable proteins to undergo tyrosine phosphorylation in adherent, TNF-treated PMN have apparent M_r ranging from 65,000 to 150,000 (8), spanning the sizes of focal adhesion proteins paxillin and p125^{FAK}. To determine if paxillin and p125^{FAK} are present in PMN as tyrosine phosphoproteins, PMN adherent to FBS-coated plates or in suspension were stimulated with TNF for 60 min and their lysates immunoprecipitated with anti-paxillin and anti-p125^{FAK} mAbs. Immunoprecipitates were divided in two equal portions and
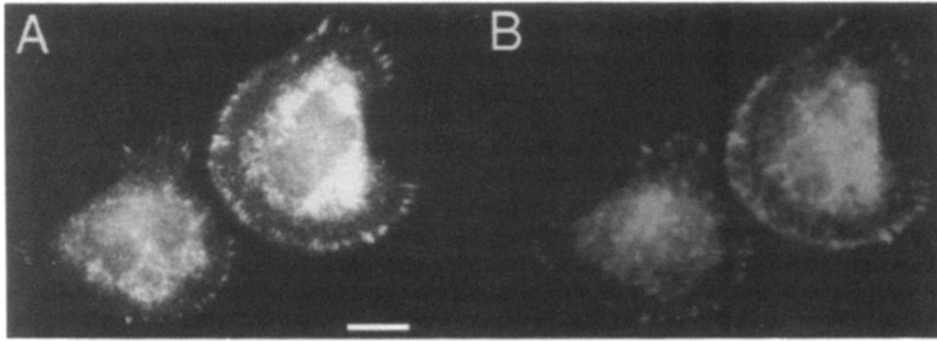
immunoblotted with anti-phosphotyrosine and either anti-paxillin or anti-p125^{FAK} mAbs. Paxillin was detected as a broad band of apparent molecular mass ~70 kD in a reducing SDS-PAGE immunoblot, as in other cells and species (5). Paxillin underwent tyrosine phosphorylation only in PMN that were both stimulated with TNF and allowed to adhere (Fig. 2 *A*). As little as 10 ng/ml TNF was effective (not shown). In contrast, while p125^{FAK} was detected in PMN and found to be tyrosine phosphorylated, its level of tyrosine phosphorylation was unaffected by adherence and/or TNF (Fig. 2 *B*). Blots probed with anti-paxillin mAb or anti-p125^{FAK} mAb showed that the same amounts of the test proteins were immunoprecipitated in each sample whether or not the cells were adherent or stimulated (not shown).
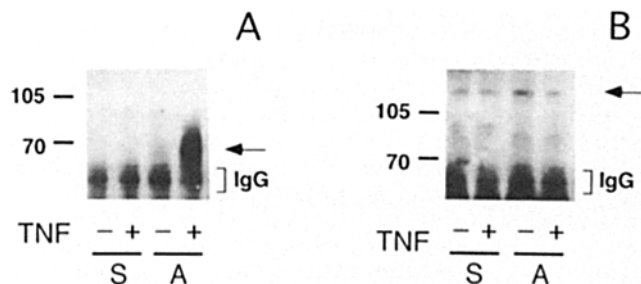
Role of Integrins

PMN from a patient with profound β2 integrin deficiency failed to tyrosine phosphorylate paxillin in response to TNF (Fig. 3). In normal PMN, paxillin phosphorylation in response to TNF was completely abolished by pretreatment with anti-CD18 mAb, both intact and as the F(ab')$_2$ fragment. Normal mouse IgG and a mAb binding to another abundant surface molecule (FcR) had no effect (Fig. 4).

Kinetics of TNF-induced, Adhesion-dependent Tyrosine Phosphorylation of Paxillin

Tyrosine phosphorylation of paxillin was first detected 15 min after stimulation of adherent PMN with TNF, peaked at 30 min and lasted at least 60 min. Again, tyrosine phosphorylation was dependent on adhesion to the serum-coated surface (Fig. 5). A blot of aliquots of the same immunopre-
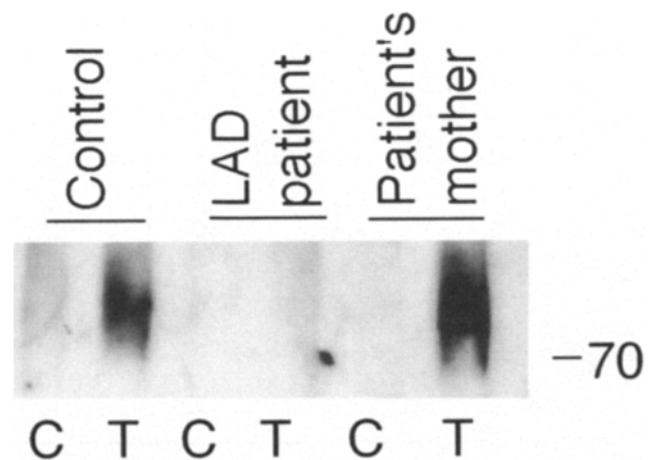

Figure 2. Effect of TNF and cell adherence on tyrosine phosphorylation of paxillin. PMN were plated on FBS-coated dishes (lanes marked *A*) or kept in suspension in FBS-coated tubes (lanes marked *S*) and stimulated with TNF (250 ng/ml) or left untreated for 60 min. Cell lysates (25 μg) were immunoprecipitated with anti-paxillin mAb and separated by reducing SDS-PAGE, transferred to a nitrocellulose membrane and probed with anti-phosphotyrosine mAb followed by ECL detection, using HRP-conjugated sheep anti-mouse IgG antibody. (*B*) Effect of TNF stimulation on tyrosine phosphorylation of p125^{FAK} in adherent and suspended PMN. PMN were prepared as in *A*. Cell lysates (25 μg) were immunoprecipitated with anti-p125^{FAK} mAb, separated by reducing SDS-PAGE, transferred to nitrocellulose and probed with anti-phosphotyrosine mAb, followed by ECL detection using HRP-conjugated sheep anti-mouse IgG antibody. The expected migrations of paxillin and p125^{FAK} are indicated, along with molecular mass markers in kD. IgG heavy chain from the immunoprecipitating antibodies was also detected by the HRP-conjugated sheep anti-mouse IgG secondary antibody.

Figure 3. Lack of paxillin phosphorylation in PMN from a patient with LAD. PMN were isolated form a normal donor, a child with LAD, and the patient's unaffected mother. PMN were plated on FBS-coated plates and stimulated with TNF (250 ng/ml) or left untreated for 60 min. Cell lysates (80 μg except *C* of the LAD patient, 20 μg) were immunoprecipitated with anti-paxillin mAb. Proteins were separated by nonreducing SDS-PAGE, transferred to nitrocellulose, and probed with HRP-conjugated anti-phosphotyrosine mAb followed by ECL detection. Molecular mass marker is indicated in kD.
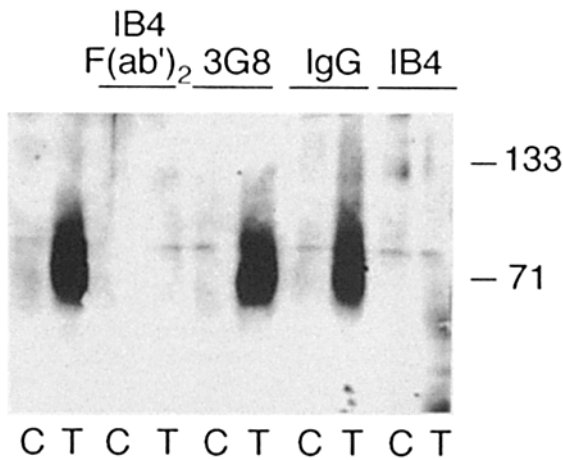
A

IP: anti-P~Tyr | anti-Paxillin

— 65

Amount loaded
(% of total
cellular paxillin)

20 10 5 2

C T C T   T T T T

30'   60'   60'

Figure 4. Block of paxillin phosphorylation by anti-CD18 antibodies. PMN were isolated and incubated at 4°C for 30 min with intact anti-CD18 mAb IB4 or its F(ab')₂ fragments, or as controls, normal mouse IgG or mAb 3G8 directed against Fc receptor. PMN were plated on FBS-coated plates and stimulated with TNF (250 ng/ml) or left untreated for 60 min. Cell lysates (125 μg) were immunoprecipitated with anti-paxillin mAb. Proteins were separated by nonreducing SDS-PAGE, transferred to nitrocellulose, and probed with HRP-conjugated anti-phosphotyrosine mAb followed by ECL detection. Molecular mass markers are indicated in kD.

cipitates probed with anti-paxillin confirmed that the same amounts of paxillin were precipitated in all cases (not shown). In experiments with different PMN donors, the time of initial phosphorylation of paxillin varied from 15–45 min, but always appeared to coincide with the onset of spreading of the PMN on the FBS-coated plates.

Estimation of the Fraction of Paxillin Undergoing Tyrosine Phosphorylation

PMN on FBS-coated plates were stimulated with TNF for 30 or 60 min or left untreated for the same times. Lysates
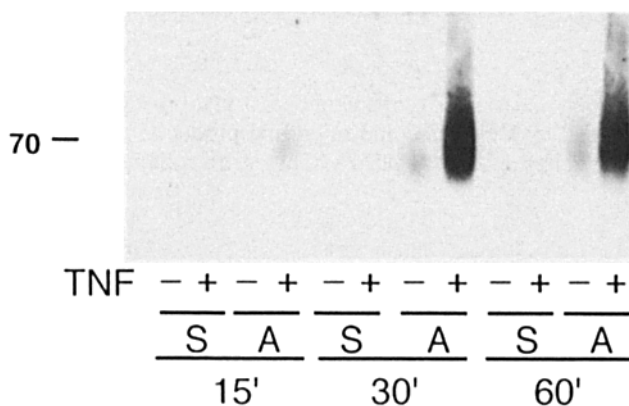

Figure 5. Time course of TNF-stimulated tyrosine phosphorylation of paxillin. PMN were plated on FBS-coated plates (*A*) or kept in suspension (*S*) and stimulated with TNF (250 ng/ml) or left untreated for the indicated times. Cell lysates (300 μg) were immunoprecipitated with anti-paxillin mAb. Proteins were separated by nonreducing SDS-PAGE, transferred to nitrocellulose and probed with HRP-conjugated anti-phosphotyrosine mAb followed by ECL detection. Molecular mass marker is indicated in kD.
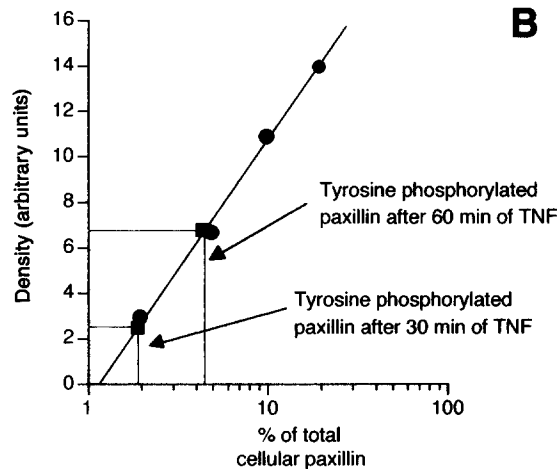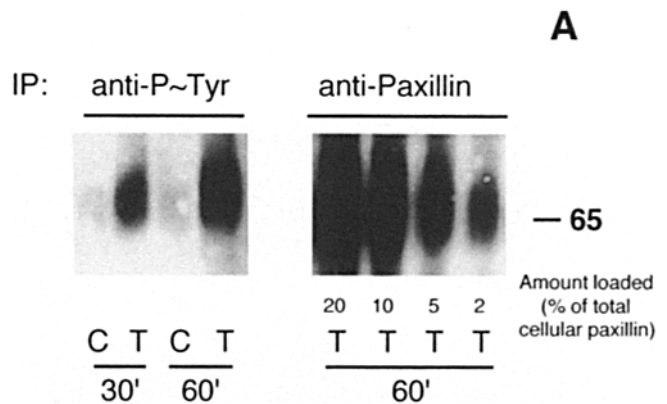

B

Figure 6. Proportion of paxillin undergoing tyrosine phosphorylation. (*A*) PMN were plated on FBS-coated dishes and stimulated with TNF (*T*) (250 ng/ml) or left untreated (control, *C*) for the indicated times. Cell lysates (350 μg) were immunoprecipitated with anti-phosphotyrosine polyclonal Ab. The supernatants were immunoprecipitated again with anti-paxillin mAb. Different amounts (as indicated) of the total immunoprecipitated paxillin were loaded in each lane. The samples were subjected to nonreducing SDS-PAGE and the separated proteins transferred to nitrocellulose and probed with anti-paxillin followed by ECL detection. Molecular mass marker is indicated in kD. (*B*). The bands present in the immunoblot from *A* were subjected to densitometry and the density (relative units) plotted against the fraction of total immunoprecipitated paxillin loaded in each lane. The regression function (r^2 = 0.993) from the anti-paxillin immunoprecipitates (●) was used to calculate the amount of paxillin present in the anti-phosphotyrosine immunoprecipitates (◆).

were immunoprecipitated with polyclonal anti-phosphotyrosine to separate paxillin that was tyrosine-phosphorylated from paxillin that was not. The supernatants from these samples were immunoprecipitated again using anti-paxillin mAb to collect the remaining paxillin. Both immunoprecipitates were divided in different proportions (see Fig. 6 *A* legend) and immunoblotted with anti-paxillin or anti-phosphotyrosine. Paxillin was present in the antiphosphotyrosine immunoprecipitates after 30 min of TNF treatment and in even greater amount after 60 min. Very little paxillin was present in anti-phosphotyrosine immunoprecipitates of control cells. However, the majority of total cellular paxillin was present in the supernatants of the anti-phosphotyrosine immunopre-

cipitates. Densitometric analysis of the immunoblots (Fig. 6 B) showed that the immunoblot ECL reaction was linear with respect to the logarithm of cell protein in the range of interest (2–20% of total non-tyrosine phosphorylated cellular paxillin). The regression function from the anti-paxillin immunoprecipitates was used to calculate the amount of paxillin present in the anti-phosphotyrosine immunoprecipitates. By this analysis, 2% of total cellular paxillin was tyrosine phosphorylated after 30 min and 4.5% was tyrosine phosphorylated after 1 h of treatment of adherent PMN with TNF. The efficacy of anti-phosphotyrosine and anti-paxillin immunoprecipitation was confirmed by the absence of tyrosine-phosphorylated paxillin in the paxillin immunoprecipitates and by the absence of tyrosine phosphorylated proteins and paxillin in the final supernatants (not shown).

Paxillin Undergoes a Posttranslational Modification Different from Tyrosine Phosphorylation

In the same experiments paxillin immunoprecipitates depleted of tyrosine-phosphorylated paxillin from control or TNF-stimulated cells were also analyzed. Immunoblots with anti-paxillin (Fig. 7) showed that the apparent molecular weight of the non-tyrosine phosphorylated paxillin increased from 51 kD (on nonreducing SDS-PAGE) in untreated cells to 58 kD in TNF-stimulated cells after 30 min, and to 63 kD after 60 min of stimulation. This demonstrated the occurrence of a time-dependent, TNF-induced posttranslational modification(s) of paxillin different from tyrosine phosphorylation.

Role of the Actin-based Cytoskeleton

Dihydrocytochalasin B blocks the barbed end of actin filaments and inhibits both spreading and the respiratory burst in TNF-stimulated PMN (18, 22). Accordingly, adherent PMN were stimulated with TNF in the presence or absence of DHCB or left untreated for different lengths of time. Cells were lysed and samples immunoprecipitated with anti-paxillin mAb. Equal aliquots of the immunoprecipitates were blotted with anti-paxillin or anti-phosphotyrosine mAbs. DHCB blocked completely the tyrosine phosphorylation of paxillin after 45 min of TNF stimulation. After 60 min only a very small amount of tyrosine phosphorylation was present, a marked reduction compared to cells treated with TNF but not with DHCB. Immunoblot of the same samples with

Figure 8. Effect of DHCB on tyrosine phosphorylation of paxillin. PMN were plated on FBS-coated dishes and stimulated with TNF (250 ng/ml) in the presence or absence of DHCB (10 μg/ml) or left untreated for the indicated times. Cell lysates (150 μg) were immunoprecipitated with anti-paxillin mAb, separated by nonreducing SDS-PAGE, transferred to nitrocellulose, and probed with HRP-conjugated anti-phosphotyorsine mAb or anti-paxillin mAb followed by ECL detection. Molecular mass markers are indicated in kD.

anti-paxillin showed that similar amounts were immunoprecipitated in all cases (Fig. 8).

Response to Other Stimuli

Phorbol ester, a potent activator of protein kinase C, formyl-methionyl-leucyl-phenylalanine, a chemotactic peptide released from Escherichia coli, and serum-coated Listeria monocytogenes all caused PMN to tyrosine phosphorylate paxillin. Although PMA stimulated less tyrosine phosphorylation of paxillin than TNF, PMA caused a greater upshift in paxillin's apparent molecular mass (Fig. 9).

Discussion

Tyrosine phosphorylation is essential for human PMN adherent to model biological surfaces to reorganize their cytoskeleton and undergo a large-scale respiratory burst (8). This work demonstrates that most tyrosine phosphoproteins in TNF-stimulated PMN are confined to focal adhesions, that the focal adhesion protein paxillin is present in human PMN, and that the tyrosine phosphorylation of paxillin is induced by a cytokine and microbial products, provided that the cells can adhere, utilize β2 integrins, and reorganize ac-
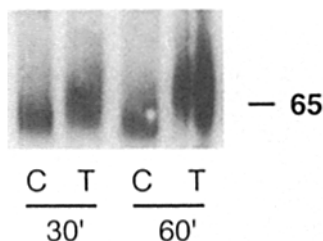

Figure 7. Paxillin undergoes a posttranslational modification different from tyrosine phosphorylation. PMN were plated on FBS-coated dishes and stimulated with TNF (T) (250 ng/ml) or left untreated (control, C) for the indicated times. Cell lysates (350 μg) were immunoprecipitated with anti-phosphotyrosine polyclonal Ab. The supernatants were immunoprecipitated again with anti-paxillin mAb. The phosphotyrosine-immunodepleted anti-paxillin immunoprecipitates were subjected to nonreducing SDS-PAGE and the separated proteins transferred to nitrocellulose and probed with anti-paxillin followed by ECL detection. Molecular mass marker is indicated in kD.
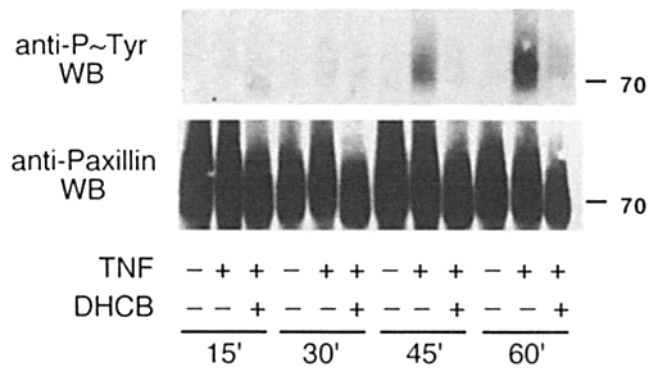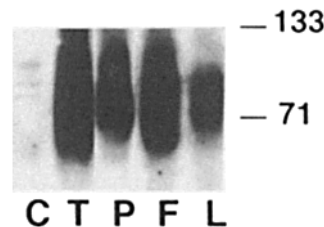

Figure 9. Effect of different stimuli on tyrosine phosphorylation of paxillin. PMN were plated on FBS-coated plates (A) and stimulated with TNF (T; 250 ng/ml), PMA (P; 100 ng/ml), fMLP (F; 1 μM), opsonized bacteria (L; 6 × 10⁶/ ml), or left untreated (C) for 60 min. Cell lysates (125 μg) were immunoprecipitated with anti-paxillin mAb. Proteins were separated by nonreducing SDS-PAGE, transferred to nitrocellulose, and probed with HRP-conjugated anti-phosphotyrosine mAb followed by ECL detection. Molecular mass markers are indicated in kD.

tin. This is one of the few specific identifications of a protein to undergo tyrosine phosphorylation in response to any agonist in PMN or in response to TNF in any cell.

These studies also identify p125^{FAK} in human PMN. Due to the common localization of p125^{FAK} and paxillin in focal adhesions (28, 31), their concomitant phosphorylation (5, 25, 39), and the ability of immunoprecipitated p125^{FAK} to tyrosine phosphorylate purified paxillin in vitro (33), it has been suggested that tyrosine phosphorylation of paxillin may result from the activation of p125^{FAK} (39). However, in the present work, p125^{FAK} did not change its tyrosine phosphorylation state in response to stimulation with TNF. Since tyrosine phosphorylation of p125^{FAK} is generally considered one means of activation, this finding directs attention to the probable existence in neutrophils of a non-p125^{FAK} pathway for integrin- and cytokine-dependent tyrosine phosphorylation of a focal adhesion-associated protein. Consistent with this, Greenberg et al. (12) observed tyrosine phosphorylation of paxillin after Fc-receptor mediated phagocytosis by macrophages in the absence of p125^{FAK} tyrosine phosphorylation. They suggested that p72^{syk}, a tyrosine kinase present also in PMN (2), might be responsible. Similarly, Huang et al. (13) observed tyrosine phosphorylation of a protein of 68 kD in platelets whose integrins were ligated under conditions that did not stimulate tyrosine phosphorylation of p125^{FAK}. Alternatively, it is possible in our studies that constitutively active p125^{FAK} did phosphorylate paxillin, but only after TNF acted to bring the two proteins together, or that p125^{FAK} was activated by a mechanism other than tyrosine phosphorylation. Recently, Berton et al. (3) reported that the tyrosine kinase p58^{fgr} is activated in a $\beta2$ integrin-dependent manner in adherent neutrophils, and that its phosphorylation and activation are enhanced by TNF, making p58^{fgr} another candidate to tyrosine phosphorylate paxillin.

Tyrosine kinase substrates in PMN treated with inflammatory polypeptides fall into three classes. Those which are rapidly phosphorylated and dephosphorylated may participate in early stages of signal transduction. The only member of this class identified to date has been MAP kinase (11). Tyrosine phosphorylation of the second class of substrates (pp150, pp115, pp75, pp65) is slow in onset and prolonged in duration (8). These appear likely to be involved in the reorganization of the cytoskeleton in adherent PMN, in that: (a) they colocalize with vinculin in focal adhesions (Fig. 1); (b) inhibition of their phosphorylation aborts cell spreading (8); (c) the kinetics of their phosphorylation matches that of cell spreading (8, 23, 24); and (d) an intact cytoskeleton protects them from premature dephosphorylation (8). On the other hand, as monitored by anti-phosphotyrosine immunoblot, most of the slowly phosphorylated proteins do not require actin polymerization to undergo tyrosine phosphorylation (8). Paxillin falls into a third class in TNF-treated PMN, not previously described, in that actin polymerization was essential for its tyrosine phosphorylation to reach a detectable level. Similarly, cytochalasin D blocked bombesin- and PDGF-stimulated tyrosine phosphorylation of paxillin (and p125^{FAK}) in Swiss 3T3 cells (25, 38, 39). In the latter cells, PDGF stimulated paxillin tyrosine phosphorylation up to a concentration of PDGF at which it began to cause actin cytoskeleton disassembly. At higher concentrations, PDGF was no longer effective (25). Therefore it appears that in PMN as in 3T3 cells, an intact cytoskeleton is necessary ei-

ther to permit activation of the tyrosine kinase responsible for paxillin phosphorylation or to bring paxillin and the kinase into apposition. In phagocytizing macrophages, on the other hand, cytochalasin D did not affect paxillin phosphorylation confined to the phagocytic cup (12).

Paxillin tyrosine phosphorylation was undetectable in a PMN from a patient with LAD. These cells lacked expression of all three leukocyte integrins—CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1, CR3), and CD11c/CD18 (p150,95)— that share the β_2 chain (CD18). The same phenotype was conferred on normal PMN by incubation with mAb anti-CD18. The availability of these integrins is a requirement for several TNF-induced PMN responses, including hydrogen peroxide production (24), changes in intracellular cAMP (21), degranulation (26), and activation of unidentified kinase(s) (8).

A posttranslational modification(s) also occurred in TNF-treated PMN involving those paxillin molecules which escaped tyrosine phosphorylation. Preliminary experiments (Fuortes, M., manuscript in preparation) indicate that this represented phosphorylation on serine. Different phosphorylation states of paxillin may regulate its association with different subsets of proteins.

The role of paxillin in any cell remains to be established. The present work suggests that tyrosine phosphorylation of paxillin by a kinase which is probably distinct from p125^{FAK} may help regulate the dramatic changes in cell shape and secretory behavior with which PMN respond to a major inflammatory mediator, TNF, and to microbes and their products. Paxillin is well positioned to play a key role in these processes, given its its localization in focal adhesions and its ability to interact with tyrosine kinases like p125^{FAK} (31), src (10), Csk (27), and lyn (19), "adaptor" proteins like crk (4), and structural proteins like vinculin (31). Indeed, paxillin now appears to be a point of convergence within focal adhesions for the actions of cytokines and microbial products (this study), neuropeptides (39), oncogenes (20), and integrins (5).

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