β2 integrin-dependent phosphorylation of protein-tyrosine kinase Pyk2 stimulated by tumor necrosis factor α and fMLP in human neutrophils adherent to fibrinogen

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Abstract Tumor necrosis factor α and fMLP can activate a broad range of cellular functions in neutrophils adherent to biological surfaces. These functions are mediated by integrins and involve the activation of tyrosine kinases. Here, we report that Pyk2, a member of the focal adhesion kinase family, was present in human neutrophils and was rapidly phosphorylated and activated following tumor necrosis factor α and fMLP stimulation in an adhesion-dependent manner. Tyrosine phosphorylation of Pyk2 was attenuated by β_2 integrin blocking with specific antibodies. The tyrosine phosphorylation of Pyk2 was downstream of protein kinases Lyn, Syk and protein kinase C and cytoskeletal organization. The activation of Pyk2 may play a role in adhesion/cytoskeleton-associated neutrophils function.

Key words: Neutrophil; Protein-tyrosine kinase; Integrin; Cytoskeleton; Signal transduction; Focal adhesion

1. Introduction

The ligation of integrins on neutrophils (PMN) initiates a cascade of biochemical events that results in reorganization of the cytoskeleton, cell spreading over the adhesive surface and the release of reactive oxygen intermediates (ROI) and granule constituents [1]. Tyrosine phosphorylation of proteins plays a critical role in this signal transduction pathway [1–3]. Among the signal transduction molecules that are tyrosine-phosphorylated are paxillin [4,5], the focal adhesion kinase FAK [5–7], the mitogen activated protein kinase (MAPK) [8], the protein-tyrosine kinases (PTK) $p58^{c-fgr}$ [9], Lyn [10], Syk [11], phospholipase C-γ2 [12] and the proto-oncogene product Vav [13]. The phosphorylation of most of these molecules is commonly accompanied by the enhancement of their biological activities thereby activating a variety of cellular functions. A rapidly growing body of evidence has established that src family PTKs play a pivotal role in integrin signaling in human PMN. The activation of *src*-like kinases $p58^{c-fgr}$ and $p53/56^{lyn}$ in PMN is associated with integrin signaling [9,10]. Inhibition of this family of PTKs with a specific inhibitor PP1 blocked the integrin-dependent activation of PMN function by TNF [14]. More directly, PMN from $fgr^{-/-}hck^{-/-}$ double-mutant mice neither spread nor produce ROI on plasma and ECM proteins, the leukocyte integrin counter-receptor ICAM-1 and anti-integrin Ab (antibody)-coated surfaces [15]. In addition, $p72^{syk}$ is also activated by PMN integrin signals. Upon activation, $p72^{syk}$ forms multimolecular complexes with several tyrosine-phosphorylated proteins and tyrosine kinases $p58^{c-fgr}$ and $p53/56^{lyn}$ [11], indicating that $p72^{syk}$ may take part in integrin signaling in PMN.

In the current models of integrin signaling, another family of PTKs known as FAK occupies a more important position [3,16–19]. Integrin ligation induces the activation of FAK and their aggregation with integrins and cytoskeletal proteins at focal contacts. Thereafter, FAK transfers the integrin signals into diverse signaling pathways including those of PI-3 kinase, MAP kinase and cytoskeletal rearrangement [20,21]. FAK has been detected in the lysates of human PMN [5–7], but it does not appear to be activated by cell adhesion [5,7], suggesting that FAK has a less certain role in integrin signaling in PMN [19]. Recently, Pyk2 was identified as another member of the FAK family of PTKs [22–24]. Pyk2 (also called RAFTK for related adhesion focal tyrosine kinase, CAKβ for cell adhesion kinase β, CADTK and FAK2) is a cytoplasmic protein-tyrosine kinase and, like FAK, couples to several signaling pathways which regulate a variety of cellular functions [22,25,26]. Pyk2 is expressed in hematopoietic cells including primary bone marrow megakaryocytes [24,27,28], monocyte macrophages [29,30], platelets [31], mast cells [32] and T- [33–35] and B-cells [36]. The stimulation of many different cell surface receptors results in the tyrosine phosphorylation and activation of Pyk2 [22,24–26,37,38]. These stimuli include carbachol acting through nicotinic acetylcholine receptors, stress signals, membrane depolarization, cytokines and molecules that activate G-protein-coupled receptors. Recently, Pyk2 was found to be tyrosine-phosphorylated after integrin or immune receptor activation [27,33–36]. Pyk2 is also activated by addition of the calcium ionophore A23187 and PMA, suggesting that the activation of Pyk2 is downstream of the increase in intracellular calcium and the activation of protein kinase C (PKC) [22,28]. At the present time, no studies have reported on the presence or activation of Pyk2 in human PMN.

Here we report that Pyk2 was present in human PMN. Stimulation of the cells with tumor necrosis factor α (TNFα) or the chemotactic peptide fMLP induced the tyrosine phosphorylation of Pyk2 which was mainly associated with a Triton X-100 insoluble cytoskeletal fraction in a β_2 integrin-dependent manner. By using different inhibitors, we also demonstrated that the phosphorylation of Pyk2 was a downstream event of the activation of $p53/56^{lyn}$, $p72^{syk}$, PKC and rearrangement of the cytoskeleton. These data suggest that Pyk2 may function in signaling events associated with PMN adhesion and cytoskeletal rearrangement.

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2. Materials and methods

2.1. Materials

Fibrinogen was obtained from Gibco BRL Products (Gaithersburg, MD, USA) and recombinant human TNFα from Pepro Tech (Rocky Hill, NJ, USA). Protein Assay (Bradford) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reagents were purchased from Bio-Rad Laboratories (Melville, NY, USA), Immoblot PVDF (polyvinylidene difluoride) transfer membranes from Millipore Corporation (Bedford, MA, USA), protease inhibitors from Boehringer Mannheim (Mannheim, Germany), the enhanced chemiluminescence (ECL) Western blotting detection reagents from Amersham Life Science (Arlington Heights, IL, USA) and protein A-bound Trisacryl beads from Pierce (Rockford, IL, USA). Abs used in this study were obtained from the following sources: monoclonal Ab (mAb) SZ21 (IgG1, anti-human CD61, β_3 integrin subunit) and mAb Lia1/2 (IgG1, anti-human CD29, β_1 integrin subunit) from Immunotech (Westbrook, ME, USA), mAbs L130 (IgG1, anti-human CD18, β_2 integrin subunit), G25.2 (IgG2a, anti-CD11a), D12 (IgG2a, anti-CD11b) and SHCL-3 (IgG2b, anti-CD11c) from Becton Dickinson (San Jose, CA, USA), mAb IB4 (IgG2b, anti-CD18) from Dr S.D. Wright (Merk Research Laboratories, Rathway, NJ, USA), goat anti-Pyk2 polyclonal Ab, horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG, anti-phosphotyrosine mAb PY20 and HRP-conjugated PY20 from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Inhibitors for PTKs (genistein), *src* kinases (PP1), *syk* kinase (piceatannol), PKC (bisindolylmaleimide I or Gö6850 and staurosporine), PI-3 kinase (wortmannin) or actin polymerization (cytochalasin B) were purchased from Calbiochem (La Jolla, CA, USA). All other reagents were purchased from Sigma BioScience (St. Louis, MO, USA).

2.2. PMN isolation

PMN were isolated from peripheral human venous blood of healthy volunteers using the method of dextran sedimentation and centrifugation over Ficoll-Paque as previously reported [14]. The cells were finally washed once in phosphate-buffered saline (PBS) and resuspended in KRPg (Krebs-Ringer phosphate buffer with glucose: 145 mM NaCl, 4.86 mM KCl, 1.22 mM $MgSO_4$, 5.7 mM Na_2HPO_4, 0.54 mM $CaCl_2$ and 5.5 mM glucose, pH 7.4) at a density of 10^7 cells/ml.

2.3. PMN incubation and lysate preparation

In some experiments, PMN were pretreated with 10 μg/ml of the Abs indicated in Section 3 for 45 min on ice with gentle mixing every 5 min. Plastic tissue culture 12 well plates were pre-coated with fibrinogen (250 μg/ml in PBS, 500 μl/well) at 4°C overnight followed by four washes in PBS. The cells were allowed to settle onto the pre-coated surfaces and to equilibrate to 37°C by incubating in the fibrinogen-coated plates (0.6 ml/well) or shaking in polypropylene round bottom tubes in a 37°C water bath for 10 min before stimulation. When necessary, genistein (50 μM), PP1 (5 μM), piceatannol (5 μM), cytochalasin B (CB, 5 μg/ml), wortmannin (10 nM), staurosporine (100 nM) or Gö6850 (50 nM) were added to the cells at the same time as they were dispensed into the wells. The cells were incubated in the absence or presence of TNFα (20 ng/ml) or fMLP (100 nM) for the times indicated in Section 3. The cells incubated in tubes were then transferred to Eppendorff tubes and spun down by microfuging for 10 s and the incubation media in the wells were removed by aspiration. Cytosolic proteins were removed from the pelleted cells and the cells adherent to the wells by treating with CSK buffer (25 mM PIPES, pH 6.9, 4 M glycerol, 1 mM EGTA, 0.2% Triton X-100, 5 μg/ml of pepstatin and leupeptin, 10 μM phenylarsine oxide, 5 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 100 μM sodium vanadate and 2.5 mM diisopropyl fluorophosphate) for 4 min at room temperature followed by centrifugation at $2000\times g$ for 4 min as previously described [39]. The resulting Triton X-100 insoluble cytoskeletal fractions were further solubilized by boiling in SDS-PAGE sample buffer. In some experiments, the cells were directly solubilized in RIPA buffer [10,11,39] or SDS-PAGE sample buffer.

2.4. Immunoprecipitation and Western blotting

Protein concentrations in the lysates, determined by the Bio-Rad Bradford Protein Assay method according to the manufacturer's instructions, were adjusted by appropriately diluting with SDS-PAGE sample buffer. Equal volumes (containing 150 μg of proteins) of each lysate were diluted eight times with Tris-buffered saline (TBS: 25 mM Tris-HCl, pH 7.5, 150 mM NaCl). Triton X-100 was added to a final concentration of 2% (v/v) and pre-cleared by rotating with 30 μl of a slurry of protein A-bound Trisacryl beads for 1 h at 4°C. The beads were then removed by microfuging for 2 min and the supernatants were precipitated with goat anti-Pyk2 Ab (2 μg/sample) or anti-PY mAb PY20 (5 μg/sample) immobilized on protein A-Trisacryl beads by rotating for 5 h at 4°C. After intensively washing with TBS/2% Triton X-100 supplemented with 1/8 volume of SDS-PAGE sample buffer, the precipitates were eluted by boiling in SDS-PAGE sample buffer and separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were detected by Western blotting using HRP-conjugated anti-PY mAb PY20 or goat anti-Pyk2 followed by HRP-conjugated rabbit anti-goat IgG and visualized by ECL reagents. Thereafter, the overlaid Ab was stripped with a stripping buffer as instructed by the manufacturer of ECL reagents and the membrane was re-blotted for the protein of Pyk2 using goat anti-Pyk2 Ab followed by an HRP-conjugated rabbit anti goat IgG and ECL reagents [14,39].

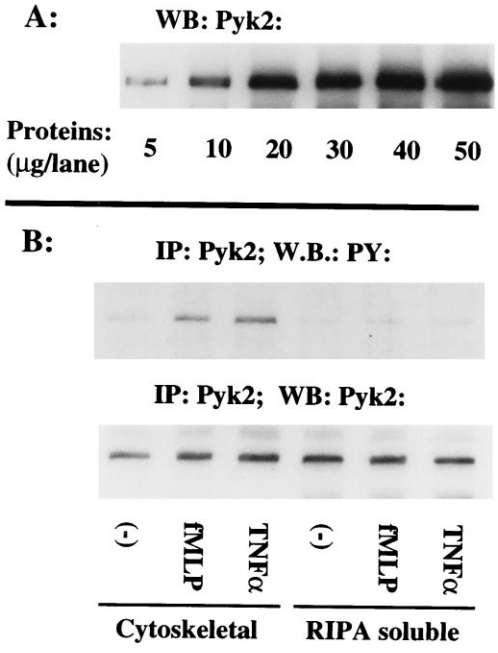

Fig. 1. Pyk2 is expressed in human PMN and its phosphorylated form is mainly associated with a Triton X-100 insoluble cytoskeletal fraction. (A) Human PMN were lysed with SDS-PAGE sample buffer immediately after isolation and the indicated amounts of protein were loaded and separated by SDS-PAGE. The protein was detected by Western blotting (WB) using a goat anti-Pyk2 Ab followed by an HRP-conjugated rabbit anti-goat IgG. The bound Abs were visualized by using ECL reagents as describe in Section 2. (B) Fibrinogen adherent PMN were stimulated with TNF or fMLP for 5 min. At the end of each incubation, the cells were either directly solubilized in RIPA buffer followed by centrifugation at $12\,000\times g$ for 15 min to provide a RIPA soluble fraction (the right three lanes) or treated with a Triton X-100 containing buffer (CSK) followed by centrifugation at $2000\times g$ for 4 min to provide a Triton X-100 insoluble pellet as the cytoskeletal fraction (the left three lanes) which were further solubilized in boiling SDS-PAGE sample buffer. The lysates were diluted eight times and immunoprecipitated (IP) using the anti-Pyk2 Ab immobilized on protein A-Trisacryl beads. The precipitates were analyzed by Western blotting (WB) using HRP-conjugated anti-phosphotyrosine mAb PY20 (upper panel). The membranes were stripped and re-blotted for Pyk2 protein (lower panel) as described in A. This figure represents one of four independent experiments with similar results.

3. Results

3.1. Pyk2 is expressed by human PMN and TNF or fMLP induces its tyrosine phosphorylation when associated with a cytoskeletal fraction

It has long been noted that cellular proteins with molecular weights of –115–120 kDa are phosphorylated on tyrosine residues following β_2 integrin activation in human PMN [9,10,14,40,41]. Since Pyk2 is a –115 kDa molecule which is involved in integrin signaling in megakaryocytes, platelets and T- and B-cells [27,35,36], we examined whether Pyk2 was expressed and tyrosine-phosphorylated in human PMN. By immunoblotting, the Pyk2 PTK was easily detectable in human PMN even when as low as 5 μg of lysate proteins were loaded on a single lane (Fig. 1A), suggesting that human PMN express large amounts of this enzyme. However, when assays were carried out to assess the tyrosine phosphorylation of this kinase in PMN stimulated without or with TNF or fMLP and lysed with RIPA buffer, no significant enhancement of tyrosine phosphorylation was detected (the right three lanes in the upper panel of Fig. 1B). Since RIPA buffer can extract most of the cytosolic proteins but cannot solubilize those tightly associated with the cytoskeleton, while Pyk2 is found associated with cytoskeletal proteins in several cell lines [28,42,43], we therefore sought to examine the relationship of this enzyme with the cytoskeleton in human PMN. When the cellular proteins of PMN were separated into cytosolic and cytoskeletal fractions using a Triton X-100 containing buffer CSK according to the method reported earlier by the author [39], an amount of Pyk2 was found associated with the cytoskeletal fraction (data not shown). We then immunoprecipitated the kinase from the SDS-PAGE sample buffer lysates of the cytoskeletal fraction and blotted for tyrosine phosphorylation of the molecules. As shown in the left three lanes in the upper panel of Fig. 1B, an enhanced tyrosine phosphorylation of Pyk2 associated with the cytoskeletal fraction was detected upon TNF or fMLP stimulation. When the membrane was stripped and re-blotted for protein, comparable amounts of Pyk2 protein were detected in each sample (lower panel of Fig. 1B, as well as in the lower panels of other figures), demonstrating that the difference in tyrosine phosphorylation detected by anti-phosphotyrosine (PY) blotting was not due to differences in the amount of protein in each precipitate.

3.2. Pyk2 phosphorylation induced by TNF or fMLP is mediated by β_2 integrin-dependent adhesion

The fact that tyrosine-phosphorylated Pyk2 was detected when associated with the cytoskeletal fraction of human PMN led us to anticipate that the activation of this enzyme might be tightly related to cell adhesion [1]. We then compared the phosphorylation of Pyk2 in cells adherent to fibrinogen and in suspension. Both TNF and fMLP induced Pyk2 tyrosine phosphorylation in adherent cells. Pyk2 phosphorylation was clearly evident after 5 min and gradually dephosphorylated thereafter (Fig. 2). The phosphorylation of Pyk2 was detectable even when the cells were incubated for as long as 60 min on fibrinogen (data not shown). In contrast, when cells were incubated in suspension by shaking in tubes, neither TNF nor fMLP triggered a detectable enhancement of tyrosine phosphorylation of the kinase (Fig. 2). These results were confirmed by two different experimental methods: immunoprecipitating with the anti-Pyk2 Ab followed by Western blotting using anti-PY mAb PY20 (Fig. 2A upper panel) or immunoprecipitating with PY20 followed by Western blotting using the anti-Pyk2 Ab (Fig. 2B). Both methods of detection demonstrated similar results (Fig. 2). In both cases, similar amounts of Pyk2 protein were assessed for tyrosine phosphorylation from adherent and non-adherent cells (Fig. 2A, lower panel).

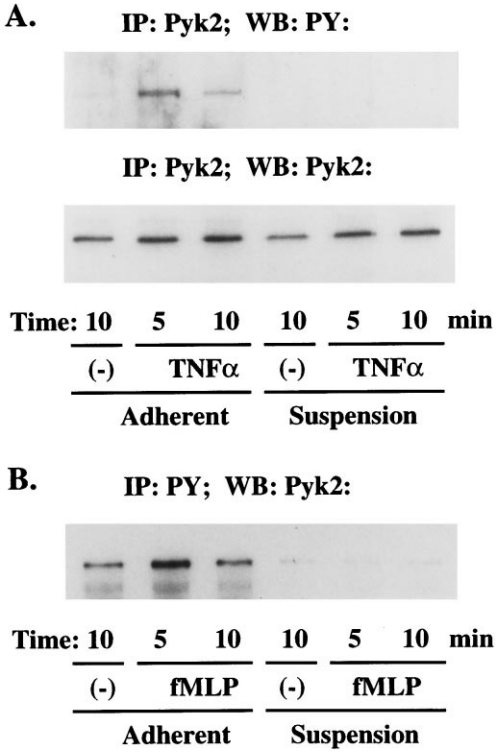

Fig. 2. Stimulation of Pyk2 phosphorylation by TNF and fMLP in human PMN is adhesion-dependent. PMN were incubated in fibrinogen-coated wells (adherent) or shaking tubes (suspension) in the absence or presence of TNF or fMLP for the indicated time and cytoskeletal fractions were prepared from these cells as described in the legend to Fig. 1B. The immunoprecipitation (IP) was performed using Abs anti-Pyk2 (A) or anti-phosphotyrosine (PY) (B) and the Western blotting (WB) was done using the method described in the legend to Fig. 1B for A and that described in Fig. 1A for B. This figure represents one of four experiments with similar results.

Because fibrinogen is a well known ligand for β_2 integrins of PMN [1], the involvement of integrins in the induction of Pyk2 phosphorylation following TNF or fMLP stimulation was evaluated using specific Abs to block selected integrin activation. PMN were pre-treated with anti-integrin Abs prior to plating on fibrinogen-coated wells. As shown in Fig. 3A, the anti-β_2 chain integrin Ab IB4 and to a lesser extent another anti-β_2 chain integrin Ab L130 (Fig. 3B), but not the anti-β_3 chain integrin Ab SZ21 or control mouse IgG, attenuated the tyrosine phosphorylation of Pyk2 induced by TNF or fMLP in PMN adherent to fibrinogen. Blocking of the β_1 chain integrin with a mAb Lia1/2 (Immunotech, Westbrook, ME, USA) showed no inhibitory effect on Pyk2 phosphorylation in fibrinogen adherent cells (data not shown). Similar to our previous observations of the activation of *src*-like kinases by TNF in fibrinogen adherent PMN [10], the α-chains of β_2 integrins were not involved in the TNF- or fMLP-stimulated phosphorylation of Pyk2 in PMN adherent to fibrinogen since

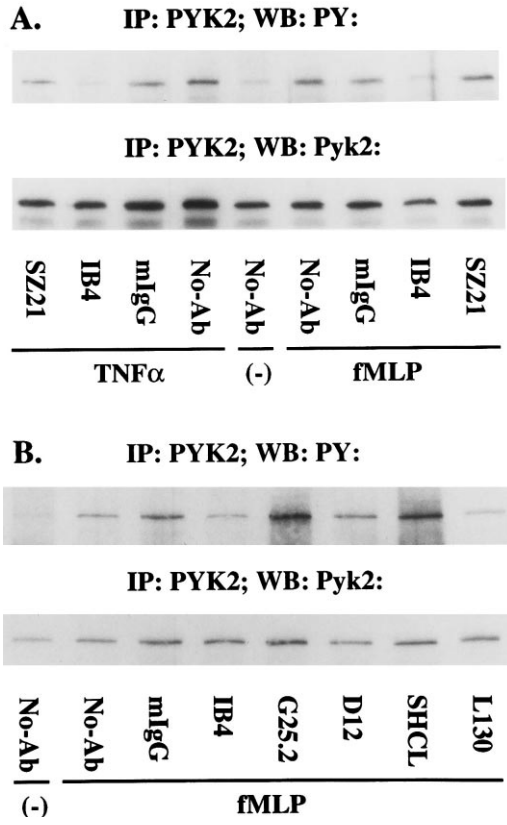

Fig. 3. Stimulation of tyrosine phosphorylation of Pyk2 by TNF and fMLP in human PMN adherent to fibrinogen is β_2 integrin-dependent. PMN were pre-incubated with 10 μg/ml of either normal mouse IgG (mIgG), IB4 (anti-CD18, the β_2 integrin β-chain), SZ21 (anti-CD61, the β_3 integrin β-chain), G25.2 (anti-CD11a), D12 (anti-CD11b), SHCL-3 (SHCL, anti-CD11c) or L130 (anti-CD18) for 45 min on ice before they were incubated in fibrinogen-coated wells. After being stimulated without or with TNF or fMLP for 5 min, the cytoskeletal fractions of the cells were prepared and Pyk2 was immunoprecipitated (IP) and analyzed by Western blotting (WB) as described in the legend to Fig. 1B. This figure represents one of three experiments with similar results.

pre-blocking of CD11a, CD11b or CD11c with mAbs G25.2, D12 and SHCL-3, respectively, failed to inhibit the phosphorylation of the kinase induced by fMLP (Fig. 3B) or TNF (data not shown). These data suggest that the activation of Pyk2 by TNF and fMLP in human PMN is a β_2 integrin-dependent event.

3.3. *Phosphorylation of Pyk2 is a downstream event of the activation of p53/56^{lyn}, p72^{syk}, PKC and cytoskeletal rearrangement*

As addressed above, integrin signaling can activate PTKs, PI-3 kinase and cytoskeletal rearrangement [3]. In addition, the phosphorylation of Pyk2 appears to be associated with β_2 integrin activation. Therefore, we sought to further characterize the signaling pathway which links β_2 integrin activation to Pyk2 phosphorylation using some specific inhibitors. When PMN were treated with the tyrosine kinase inhibitor genistein (50 μM) for 10 min before the addition of stimuli, the enhanced tyrosine phosphorylation of Pyk2 induced by TNF or fMLP was mostly attenuated (Fig. 4A and data not shown). It was not certain if this was due to the direct effect of genistein on this kinase or due to the inhibition of other PTKs, especially those of the *src* family, since genistein inhibits mainly *src*-like kinases. We then introduced a highly specific inhibitor (PP1) of *src* family PTKs which we have previously demonstrated to be highly specific for p53/56^{lyn} in human PMN [14]. The inhibition of p53/56^{lyn} with PP1 gave rise to a similar decrease in tyrosine phosphorylation of Pyk2 induced by TNF or fMLP (Fig. 4A) to that observed in genistein-treated cells, indicating that Pyk2 may be downstream of p53/56/^{lyn}. Nevertheless, it should be noted that neither genistein nor PP1 could completely abolish the phosphorylation of Pyk2 (Fig. 4A), suggesting that there may be signaling pathway(s) other than *src* family kinases which can lead to the activation of Pyk2. Actually, the activation of p72^{syk} and the rearrangement of the cytoskeleton may play a more important role in TNF or fMLP-induced phosphorylation of Pyk2. In PMN treated with piceatannol (5 μM), a specific inhibitor for p72^{syk} [7,14], or cytochalasin B (5 μg/ml), which blocks actin-based cytoskeleton polymerization, the phosphorylation of Pyk2 was almost completely abolished (Fig. 4A and data not shown). As with other cell lines [22,28,30,31,44], the activity of PKC is involved in the activation of Pyk2 in human PMN since treatment of the cells with PKC inhibitors Gö6850 or staurosporine caused a profound inhibition of Pyk2 phosphorylation stimulated by TNF or fMLP (Fig. 4B and data

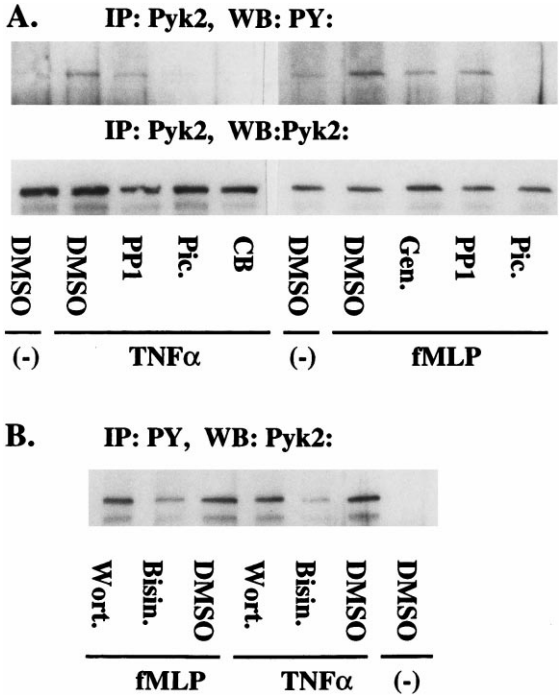

Fig. 4. *Src* and *Syk* tyrosine kinases and PKC as well as actin polymerization are involved in the induction of Pyk2 phosphorylation by TNF and fMLP in human PMN adherent to fibrinogen. PMN were pretreated with the solvent (DMSO) or inhibitors for PTKs (genistein or Gen.), src kinases (PP1), syk kinase (piceatannol or Pic.), PKC (bisindolylmaleimide I or Bisin.), PI-3 kinase (wortmannin or Wort.) or actin polymerization (cytochalasin B or CB) for 10 min at 37°C in fibrinogen-coated wells before stimulating without or with TNF or fMLP for 5 min. The SDS-PAGE sample buffer lysates of the cytoskeletal fractions were immunoprecipitated (IP) using anti-Pyk2 (A) or anti-PY (B) Abs and analyzed by Western blotting (WB) using HRP-PY20 (A) or anti-Pyk2 (B) as described in the legends to Fig. 1 and Fig. 2. This figure represents one of three experiments with similar results.

not shown). Direct activation of PKC by phorbol 12-myristate 13-acetate (PMA) triggered a more intense tyrosine phosphorylation of Pyk2 in comparison to stimulation by TNF or fMLP (data not shown). The PI-3 kinase signaling pathway, although associated with Pyk2 in THP1 cells [29], is not necessary for Pyk2 activation in PMN. As shown in Fig. 4B, inhibition of PI-3 kinase with wortmannin affected neither TNF nor fMLP-induced tyrosine phosphorylation of Pyk2 in human PMN adherent to fibrinogen.

4. Discussion

These studies indicated that Pyk2 was present in human PMN and was tyrosine-phosphorylated after cell stimulation with TNFα or fMLP. The induction of Pyk2 phosphorylation by these stimuli in PMN was dependent on cell adhesion to fibrinogen and was mediated by β_2 integrins. Activation of *src* family kinases ($p53/56^{lyn}$), *syk* family kinases ($p72^{syk}$), PKC and cytoskeletal rearrangement, all important downstream events of integrin signaling, were important in the phosphorylation of Pyk2 in PMN. More notably, the phosphorylated Pyk2 was mainly found associated with a Triton X-100 insoluble cytoskeletal fraction. These results strongly suggest that the tyrosine phosphorylation and activation of Pyk2 in PMN may play a role in the regulation of adhesion/cytoskeleton-associated PMN functions.

TNF and fMLP bind through distinct receptors and therefore, their signaling pathways should be different. Actually, they activate PMN in very distinct ways. In vitro, the optimum activation of PMN function by TNF can only be achieved when the cells are allowed to adhere to certain surfaces [1]. When the cells are incubated in suspension, TNF mainly acts to prime the cells [45,46]. In contrast, fMLP can activate PMN when adherent and in suspension [1]. In vivo, while fMLP induces PMN to migrate (chemotaxis), TNF immobilizes PMN by activating cell adhesion [47,48]. When we tried to assess the activation of Pyk2 in human PMN by these two activators, we found unexpectedly that these contradictory pathways of PMN activation induced Pyk2 phosphorylation in a very similar manner: similar in time-course, in cytoskeletal association and in β_2 integrin-dependence as well as in the sensitivity to inhibitors for PTKs, *src* family kinases, $p72^{syk}$, PKC and cytoskeleton polymerization. It appears that there must be a mechanism which is shared by the two stimuli that leads to the activation of Pyk2.

Pyk2 belongs to the FAK family and, like its homolog FAK, localizes to sites of cell to cell contact and to focal adhesion-like structures [23,27]. At these sites, some structural and signaling molecules form focal adhesion complexes that contain cytoskeletal proteins such as talin, vinculin, α-actinin, filamin, FAK and other phosphoproteins [3,27,49]. Although PMN do not form classical focal adhesion complexes, integrin activation results in tyrosine phosphorylation of several proteins and the formation of focal adhesion-like structures [50]. Here, we observed that the stimulated tyrosine phosphorylation of Pyk2 was dependent on the adhesion of PMN to fibrinogen (Fig. 2). Therefore, cell adhesion may serve as a key step in the activation of Pyk2 in human PMN. It should be noted that both TNF and fMLP are powerful activators of PMN adhesion [1]. Their effects on the stimulation of Pyk2 phosphorylation may be achieved through their activation of PMN adhesion even though in other cell systems, stimulation by G-protein-coupled receptors (such as fMLP) results in the tyrosine phosphorylation of Pyk2 [22,24,26,28].

Adhesion of human PMN to fibrinogen has been demonstrated to be mediated by β_2 integrins [1] and therefore, β_2 integrin signaling may account for Pyk2 phosphorylation. It has been shown that the aggregation of β_1 integrins in B-cells and β_3 integrins in T-cells results in tyrosine phosphorylation of Pyk2 [35,36]. We herein provide evidence that the phosphorylation of Pyk2 in human PMN adherent to fibrinogen in response to stimulation by TNF or fMLP is a function of β_2 integrin ligation since it is attenuated by β_2 integrin blocking (Fig. 3). After appropriate activation, integrins can generate intracellular signals which rapidly induce the activation of several signaling pathways including the activation of PTKs (especially those of the *src* family and *syk* family), of PKC and of actin polymerization [1,3]. Hence, it is not surprising to note that, as with the results obtained from other cell lines [22,28,30–32,34,44,51,52], the activities of *src*-like kinases (especially $p53/56^{lyn}$), *syk* kinase $p72^{syk}$, PKC and cytoskeletal rearrangement are all involved in the induction of Pyk2 phosphorylation, in human PMN.

The biological function of Pyk2 in distinct cell lines is still unclear. This enzyme has been reported to interact with signaling molecules such as paxillin [42,43], Grb2 [22,33] and $p130^{cas}$ [36]. Although *src* family kinases such as *Fyn*, *Lck* and *c-Src* associate by their SH2 domains with Pyk2 [26,33], we could not detect association of Pyk2 with the *src*-like kinases $p53/56^{lyn}$, $p58^{c-fgr}$ and $p59^{hck}$ during immunoprecipitation experiments (data not shown). Pyk2 tyrosine phosphorylates the potassium channel and suppresses channel currents [22] and also acts as an upstream regulator for stress signal activation of c-Jun N-terminal kinase [25]. As many of these pathways are activated in stimulated PMN [2], tyrosine phosphorylation of Pyk2 may play an important role in signal transduction and cell function.

In summary, these experiments indicate that stimulation of PMN with TNF and fMLP induces the tyrosine phosphorylation of Pyk2 which is dependent on cell adhesion. The phosphorylated Pyk2 is mainly associated with a Triton X-100 insoluble cytoskeletal fraction. The activation of $p53/56^{lyn}$, $p72^{syk}$ and PKC by β_2 integrin signaling appears to be involved in the phosphorylation of Pyk2 in human PMN adherent to fibrinogen. Pyk2 may therefore play a role in the regulation of adhesion/cytoskeleton-associated PMN function.

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