

β 2 Integrin-dependent Protein Tyrosine Phosphorylation and Activation of the *FGR* Protein Tyrosine Kinase in Human Neutrophils

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Abstract. Stimulation of adherent human neutrophils (PMN) with tumor necrosis factor (TNF) triggers protein tyrosine phosphorylation (Fuortes, M., W. W. Jin, and C. Nathan. 1993. *J. Cell Biol.* 120:777-784). We investigated the dependence of this response on β 2 integrins by using PMN isolated from a leukocyte adhesion deficiency (LAD) patient, which do not express β 2 integrins, and by plating PMN on surface bound anti- β 2 (CD18) antibodies. Protein tyrosine phosphorylation increased in PMN plated on fibrinogen and this phosphorylation was enhanced by TNF. Triggering of protein tyrosine phosphorylation did not occur in LAD PMN plated on fibrinogen either in the absence or the presence of TNF. Surface bound anti-CD18, but not isotype-matched anti-Class I major histocompatibility complex (MHC) antigens, antibodies triggered tyrosine phosphorylation in normal, but not in LAD PMN. As the major tyrosine phosphorylated proteins we found in our assay conditions migrated with an apparent molecular mass of 56–60 kD, we investigated whether β 2 integrins are impli-

cated in activation of members of the *src* family of intracellular protein-tyrosine kinases. We found that the *fgr* protein-tyrosine kinase (p58^{fgr}) activity, and its extent of phosphorylation in tyrosine, in PMN adherent to fibrinogen, was enhanced by TNF. Activation of p58^{fgr} in response to TNF was evident within 10 min of treatment and increased with times up to 30 min. Also other activators of β 2 integrins such as phorbol-12-myristate 13-acetate (PMA), and formyl methionyl-leucyl-phenylalanine (FMLP), induced activation of p58^{fgr} kinase activity. Activation of p58^{fgr} kinase activity, and phosphorylation in tyrosine, did not occur in PMN of a LAD patient in response to TNF. Soluble anti-CD18, but not anti-Class I MHC antigens, antibodies inhibited activation of p58^{fgr} kinase activity in PMN adherent to fibrinogen in response to TNF, PMA, and FMLP. These findings demonstrate that, in PMN, β 2 integrins are implicated in triggering of protein tyrosine phosphorylation, and establish a link between β 2 integrin-dependent adhesion and the protein tyrosine kinase *fgr* in cell signaling.

STUDIES performed in the last few years with cells of different lineage have established that, besides participating in cell adhesion, integrins may activate selective cell functions either directly or in synergy with other surface receptors (for review see Turner and Burridge, 1991; Hynes, 1992; Juliano and Haskill, 1993; Sastry and Horwitz, 1993). Also members of the β 2 integrin subfamily, which are referred to as leukocyte integrins due to their restricted expression in lymphocytes, monocytes, and granulocytes, have been shown to signal for activation of selective leukocyte functions. Retrospectively, the first evidence for the signaling capacity of a leukocyte integrin was obtained by Wright, Silverstein, and colleagues, who demonstrated that the complement receptor type 3 (CD11b/CD18) can be activated to signal for particle engulfment in monocytes and neutrophils (PMN)¹ (Wright and Silverstein, 1982; Wright

et al., 1983a; Wright and Meyer, 1986; for a review on activation of C3 receptors for phagocytosis see Wright and Griffin, 1985). Studies with lymphocytes showed that LFA-1 (CD11a/CD18) provides costimulatory signals for T cell activation (Wacholitz et al., 1989; Van Severter et al., 1990; Kuhlman et al., 1991), and enhances antigen presentation by B cell (Moy and Brian, 1992), and that gp150/95 (CD11c/CD18) is implicated in B cell proliferation (Postigo et al., 1991). LFA-1, and CR3 in monocytes, and LFA-1 in natural killer cells were also implicated in stimulation of cytokines expression (Couturier et al., 1990; Melero et al., 1993). Evidence has been also presented for an involvement of β 2 integrins in IgG-mediated phagocytosis, and LTB₄ generation by PMN (Gresham et al., 1991; Graham et al., 1993).

A clear example of leukocyte integrin-dependent signaling has been shown to be represented by the adhesion-dependent stimulation by tumor necrosis factor (TNF) of PMN respira-

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1. *Abbreviations used in this paper:* FMLP, formyl-methionyl-leucyl-phenylalanine; LAD, leukocyte adhesion deficiency; LFA-1, lymphocyte

function-associated antigen 1; LRI, leukocyte response integrin; MHC, major histocompatibility complex; PMN, neutrophils; TNF, tumor necrosis factor.

tory burst (Nathan, 1987). As originally described by Nathan, plating of PMN on extracellular matrix and serum proteins, or endothelial cells render them able to respond to TNF, or other cytokines (Nathan, 1989), with production of elevated amounts of toxic oxygen molecules; this occurs with a characteristic long lag time, proceeds up to 90–120 min, and is accompanied by spreading of the cell over the surface. The $\beta 2$ -integrin dependence of this phenomenon has been demonstrated by two sets of observation. Production of toxic oxygen molecules in response to TNF (Nathan et al., 1989), or FMLP (Shappel et al., 1990) by adherent PMN depends on expression of $\beta 2$ integrins as PMN of leukocyte adhesion deficiency (LAD) patient, which do not express $\beta 2$ integrins, do not respond to TNF in adherent conditions, and soluble anti- $\beta 2$ integrins antibodies inhibit this response. Interaction of both neutrophils, and eosinophils with surface-bound anti-leukocyte integrins antibodies, is accompanied by spreading and toxic oxygen molecules production (Berton et al., 1992; Laudanna et al., 1993b). Recent findings showed that also the $\beta 3$ integrin-related leukocyte response integrin (LRI) and its functionally associated integrin-associated protein (IAP) can signal for toxic oxygen molecules production in PMN (Zhou and Brown, 1993).

Intracellular messengers implicated in activation of selective PMN function by $\beta 2$ integrins have not been clearly identified. Leukocyte integrins have been reported to signal for an increase of cytosolic free-calcium (Pardi et al., 1989; Richter et al., 1990; Jaconi et al., 1991; Ng-Sikorski et al., 1991; Altieri et al., 1992), and, more recently, for activation of phospholipase D (Fällman et al., 1993). However, a causal relationship between cytosolic free-calcium increase, and/or phospholipase D-derived messengers and PMN spreading, and activation of a respiratory burst with production of toxic oxygen molecules has not been demonstrated. Since the early studies by Nathan (1987), the only clear requirement for the adhesion-dependent stimulation of PMN respiratory burst was recognized to be represented by spreading of the cell over the adhesive surface, as cytochalasins, elevation of cytosolic cAMP, and an inhibitor of myosin light chain kinase, wortmannin, inhibit both spreading and activation of a respiratory burst (Laudanna et al., 1990; Nathan and Sanchez, 1990; Laudanna et al., 1993a). Significantly, it was also demonstrated that protein tyrosine kinase inhibitors block both the $\beta 2$ -integrin dependent PMN spreading and respiratory burst (Fuortes et al., 1993; Laudanna et al., 1993a).

The understanding of the mechanisms of signal transduction by integrins has been recently highlighted by the demonstration of the existence of an integrin-dependent pathway of activation of protein tyrosine phosphorylation. Both members of the $\beta 1$ (Kornberg et al., 1991; Freedman et al., 1993), and $\beta 3$ (Ferrell and Martin, 1989; Golden et al., 1990; Shattil and Brugge, 1991) subfamilies of integrins trigger protein tyrosine phosphorylation. Significantly, a clear link has been established between reorganization of the cytoskeleton and this pathway of protein tyrosine phosphorylation (for review see Turner and Burridge, 1991; Burridge et al., 1992; Zachary and Rozengurt, 1992; Juliano and Haskill, 1993; Sastry and Horwitz, 1993).

Indirect studies with protein tyrosine kinase inhibitors (Laudanna et al., 1993a), and recent direct evidence (Fuortes et al., 1993) clearly indicated that the adhesion-dependent

stimulation of neutrophil spreading, and production of toxic oxygen molecules also implicate protein tyrosine phosphorylation. We provide in this paper a direct proof that $\beta 2$ integrin-dependent adhesion triggers protein tyrosine phosphorylation in neutrophils. Moreover, we show that $\beta 2$ integrins signaling involves activation of one member of the *src* family of intracellular protein tyrosine kinases, the p58^{src} protein.

Materials and Methods

PMN Preparation

PMN were isolated from buffy coats of healthy volunteers, or whole blood of a LAD patient, by dextran sedimentation, and centrifugation over Ficoll-Hypaque (Pharmacia LKB Biotechnology, Brussels, Belgium) as previously described (Berton et al., 1992). Cells were resuspended in Hank's balanced salt solution, containing 0.5 mM CaCl₂, and 5.5 mM D-glucose (HBSS). F.M. was a five month-old male child, hospitalized for recurrent infections, who was diagnosed as being affected by LAD by analysis of expression of $\beta 2$ integrins. This was performed independently in the Department of Pediatrics of the University of Brescia, and by ourselves. The patient lacked expression of CD18, CR3, and LFA-1 as revealed by FACS analysis of PMN, and mononuclear cell samples. Expression of L-selectin, and Class I-MHC antigens was comparable to control PMN.

Preparation of Fibrinogen and Antibody-coated Wells

0.3 or 0.7 ml of 250 μ g/ml fibrinogen (from human plasma; Sigma Chem. Co., St. Louis, MO) diluted in pyrogen-free water were dispensed in 2 cm² 24 well, or 10 cm² 6 well plates, respectively, and allowed to dry at 37°C. 24 well plates were coated with protein A from *Staphylococcus aureus* (Sigma), and then with the anti-CD18 mAb IB4 (Wright et al., 1983b), kindly donated by Dr. S. D. Wright, The Rockefeller University, New York, NY, or the anti-Class I MHC antigens B9.12.1 (Lemonnier et al., 1982), kindly donated by Dr. R. Accolla, Institute of Immunology and Infectious Diseases, University of Verona, Verona, Italy, exactly as described before (Berton et al., 1992).

Protein Tyrosine Phosphorylation

After isolation, PMN were treated for 10 min at room temperature with 5.4 mM diisopropyl fluorophosphate (DFP; Aldrich, Milwaukee, WI), washed with HBSS, and finally resuspended in HBSS at 6×10^6 /ml. 0.25 ml of the PMN suspension were dispensed in 24 well plates, and after incubation for 5 min at 37°C in air/5% CO₂, added with 0.05 ml of diluent (HBSS) or HBSS containing 120 ng/ml TNF, 120 ng/ml PMA, or 600 nM formyl-methionyl-leucyl-phenylalanine (FMLP). After incubation at 37°C in air/5% CO₂ for the times indicated in the results section, 0.1 ml of four-fold concentrated modified RIPA buffer were added. Composition of modified RIPA was as follows: 25 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium-deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM dithiothreitol (DTT), 0.1 mM sodium-orthovanadate, 0.01 mM phenylarsine oxide, 1 mM EDTA, 0.005 mg/ml pepstatin, 0.005 mg/ml leupeptin, 1 mM PMSF. After incubation at 4°C for 30 min, PMN lysates were aspirated from the wells with a 1-ml syringe connected to a 27G 3/4 0.4 \times 19 needle, and after several passages through the needle, clarified by centrifugation at 13,000 g for 10 min. Supernatants were assayed for protein concentration with the DC (Detergent-Compatible) Protein Assay of Bio-Rad Laboratories (Hercules, CA), using bovine serum albumin as standard. Equal amounts of PMN lysates (50–60 μ g protein per lane) were electrophoresed on 9, or 10% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes using a Bio-Rad Trans Blot apparatus, and the blot incubated for 3 h in TBS (170 mM NaCl, 50 mM Tris pH 7.5) containing 5% BSA, and 0.2% NP-40 (blocking solution). Immunoblots were probed overnight with the anti-phosphotyrosine mAb 4G10 (UBI, Lake Placid, NY) (1 μ g/ml) in blocking solution containing 3% instead of 5% BSA. After four washings for 10 min with TBS-0.2% NP-40, the blot was probed for 1–2 h with a 1:2,000 dilution, in blocking solution containing 3% BSA, of HRP-conjugated goat anti-mouse IgG (Amersham, Amersham, UK), and then washed again as described above. Immunoreactivity was detected using the Enhanced Chemiluminescence Western blot-

ting detection reagent (ECL, Amersham). In some experiments immunoblots were probed with 4G10 in the presence of 1 mM phosphotyrosine.

Analysis of p58^{Gr} Kinase Activity and Phosphorylation in Tyrosine

Antibodies against p58^{Gr}, were raised immunizing rabbits with synthetic peptides corresponding to the unique NH₂-terminal region of the protein. A peptide representing amino acids from position 16 to 30 of p58^{Gr} (KEDAGLEGDFRSYGA) was conjugated to ovalbumin with glutaraldehyde, and used as immunogen. Samples of sera were tested for reactivity with the peptide immobilized to plastic surfaces by ELISA. Anti-p58^{Gr} antibodies (Abs) did not react with synthetic peptides corresponding to position 8 to 24 of p59^{hck} (FLQVGGNTFSKTETSAS), and position 35 to 51 of p59^{lyn} (TPQHYPSTFGVTSIPNYN). p59^{lyn} peptide was kindly donated by Dr. S. Courtneidge, EMBL, Heidelberg, Germany. Abs were purified from sera by ammonium sulfate precipitation. Anti-p58^{Gr} Abs recognized a protein migrating at ~56–58 kD in Western blots, and immunoprecipitated from PMN lysates a protein displaying kinase activity towards the exogenous substrate enolase, and autophosphorylating kinase activity. When tested in immunocomplex kinase assay, the p58^{Gr} peptide used as immunogen, but not the p59^{hck}, and the p59^{lyn} peptides (all used at 50 µg/ml of PMN lysates), competed effectively with immunoprecipitation of p58^{Gr}. For analysis of p58^{Gr} kinase activity, PMN (15 × 10⁶ in 0.65 ml HBSS) were plated in 10 cm² 6 well plates, and, after addition of different stimuli, in 0.1 ml HBSS, incubated for different times, and then lysed by the addition of 0.25 ml of fourfold concentrated modified RIPA (see the above section on protein tyrosine phosphorylation). Samples of PMN lysates were adjusted to the same protein concentration with modified RIPA (250–300 µg in 400 µl) and incubated for 90–120 min with 2 µl of immobilized protein A to Trysacril (Pierce, Rockford, IL) which had been preadsorbed with 10 µg anti-p58^{Gr}, or control rabbit IgG. Immunocomplexes were collected by centrifugation in microfuge, washed twice with RIPA (25 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium-deoxycholate, 0.1% SDS), once with TBS, and once with kinase buffer (20 mM Hepes pH 7.4, 10 mM MnCl₂, 1 mM DTT). Immunocomplexes were resuspended in 20 µl kinase buffer containing 2 µCi ³²P-γ-ATP (Amersham, specific activity 5,000 Ci/mmole), and incubated at room T for 10–15 min. The reaction was stopped by the addition of 20 µl of twofold concentrated SDS sample buffer, and samples electrophoresed on a 10% SDS-polyacrylamide gel. Gels were stained, and destained, and after drying, exposed overnight at -70°C to X-Omat AR, or X-Omat MA film (Kodak, Rochester, NY). For analysis of phosphorylation of p58^{Gr} in tyrosine, this was immunoprecipitated from PMN lysates as above described. Proteins eluted from immunocomplexes were electrophoresed on 10% SDS-polyacrylamide gels and transferred to

nitrocellulose as described above. Immunoblots were probed with anti-phosphotyrosine Abs as described above. To detect the immunoprecipitated p58^{Gr}, blots, which had been probed with anti-phosphotyrosine Abs, were incubated first in denaturation buffer, and then in renaturation buffer exactly as described by Cooke et al. (1991) to remove anti-phosphotyrosine and second Ab, and then reprobbed with anti-p58^{Gr} Abs.

Results

TNF Triggers Protein Tyrosine Phosphorylation in Adherent PMN

PMN were plated in fibrinogen-coated wells in the presence or the absence of TNF, and incubated up to 60 min at 37°C before lysis with RIPA buffer (see Materials and Methods), and analysis of tyrosine-phosphorylated proteins by antiphosphotyrosine immunoblots. PMN plated on fibrinogen in the absence of TNF remained mainly rounded, and slightly adhered to the bottom of the well; PMN plated in the presence of TNF underwent spreading and firm adhesion to fibrinogen-coated plates. Spreading was evident already after 10 min from plating, and, as judged by the morphology of the cells, was maximal at 30 min, and maintained up to 60 min.

In some experiments, which are illustrated by the antiphosphotyrosine immunoblot reported in Fig. 1 A, tyrosine phosphorylation of several proteins increased with time of incubation on fibrinogen, and up to 30 min, even in the absence of TNF. TNF increased tyrosine phosphorylation at all time points tested. In most of several experiments performed, the maximal increase of protein tyrosine phosphorylation induced by TNF reproducibly occurred after 30 min, and only in a few of these after 60 min from plating. Among proteins whose phosphorylation increased substantially, and reproducibly as a consequence of TNF treatment, we found proteins migrating with an apparent molecular weight of 50, 68, 71, 86, and 115 kD. However, the most consistent increase in tyrosine phosphorylation was found in three proteins migrating at a

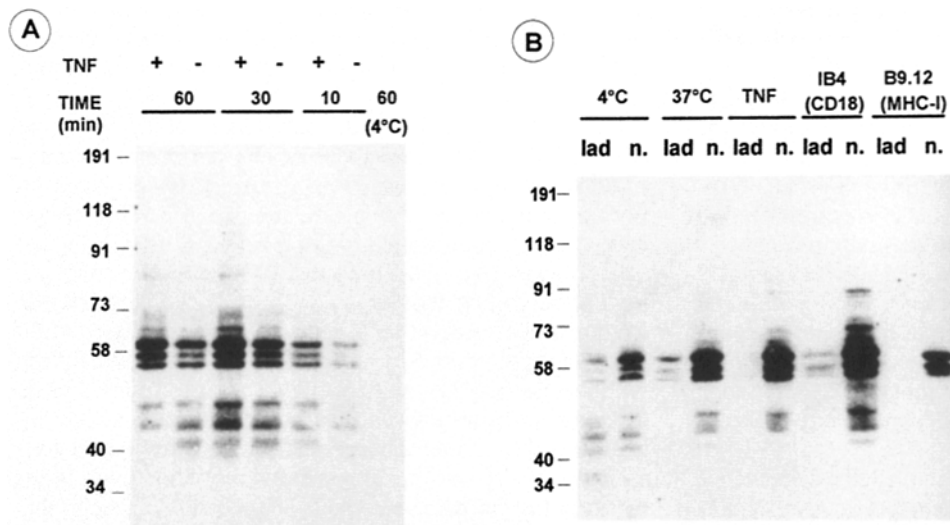


Figure 1. TNF-stimulated protein tyrosine phosphorylation in PMN adherent to fibrinogen is dependent on $\beta 2$ integrins. (A) PMN were kept in ice (4°C), or plated in fibrinogen-coated wells for the times indicated in the absence, or the presence of 20 ng/ml TNF. Proteins were extracted with RIPA buffer as described in Materials and Methods, and after electrophoresis in SDS-polyacrylamide gels, transferred to nitrocellulose and probed with the anti-phosphotyrosine mAb 4G10. In experiments in which incubation with 4G10 was done in the presence of 1 mM phosphotyrosine no reactivity was detected (not shown). Numbers at the left of the figure show migration of molecular

weight markers. (B) PMN isolated from a LAD patient (LAD) or a healthy donor (n.) were kept in ice (4°C), or plated for 30 min in fibrinogen-coated wells in the absence (37°C), or the presence (TNF) of 20 ng/ml TNF. PMN were also plated for 30 min in wells in which the anti-CD18 mAb IB4, or the anti-MHC Class I Ag mAb B9.12 were immobilized through the Fc fragment to Protein A (see Materials and Methods, and Berton et al., 1992). Proteins were extracted and analyzed as described above and in Materials and Methods.

molecular weight of ~56–60 kD. The effect of TNF on tyrosine phosphorylation in PMN plated on fibrinogen was reproduced with equal results in a total of 12 experiments. The only variations we found in the course of these studies regarded the basal protein tyrosine phosphorylation detected in PMN which, after isolation, were kept in ice before lysis, and that detected in PMN plated on fibrinogen in the absence of TNF. In the experiment reported in Fig. 1 *A*, PMN kept in ice, and then lysed in RIPA (4°C) did not show any reactivity to antiphosphotyrosine Abs. However, in some experiments, we found a basal tyrosine phosphorylation of the three major bands migrating at ~56–60 kD (See Figs. 1 *B* and 6 *A*). Also protein tyrosine phosphorylation in PMN plated on fibrinogen in the absence of TNF showed some variation. This increased with time (Fig. 1) in some experiments, but did not change, or even slightly decrease (see for example Figs. 4 and 6, *A*) in most of the experiments. Variations in the extent of the basal phosphorylation might be explained taking into account that PMN purification include steps in the course of which cells are maintained at room temperature for 30–45 min which might permit cell–cell interaction. We could not clarify the reasons of the variability in the extent of protein tyrosine phosphorylation in PMN plated on fibrinogen in the absence of TNF; however, in the course of this, and previous studies (Laudanna, et al., 1990; Laudanna et al., 1993), we noted that also adhesion of PMN to fibrinogen displays some variations as some cell preparations adhered more tightly to fibrinogen. The presence of “activating” factors which PMN were exposed to during blood collection, or cell purification might explain this finding, as well as stimulation of protein tyrosine phosphorylation in the absence of TNF. Despite this outlined variability, TNF reproducibly and consistently increased protein tyrosine phosphorylation in PMN plated on fibrinogen.

Triggering by TNF of Protein Tyrosine Phosphorylation in Adherent PMN Is Dependent on $\beta 2$ Integrin Expression

Previous studies showed that stimulation of the respiratory burst of PMN plated on different adhesive surfaces by TNF depends on expression of $\beta 2$ integrins (Nathan et al., 1989; Nathan and Sanchez, 1990; Shappel et al., 1990), or can be mimicked by plating PMN on immobilized anti-integrin Abs (Berton et al., 1992; Zhou and Brown, 1993). We therefore sought to investigate whether also triggering of protein tyrosine phosphorylation depended on $\beta 2$ integrin expression. We addressed this issue by using two different approaches.

The first was based on the use of PMN isolated from a LAD patient which did not express $\beta 2$ integrins (see Materials and Methods). As shown in Fig. 1 *B*, antiphosphotyrosine immunoblots of lysates of LAD PMN plated on fibrinogen for 30 min either in the absence or the presence of TNF, revealed the presence of almost undetectable antiphosphotyrosine reactive proteins.

The second approach we used to establish the dependence on $\beta 2$ integrins of the increase of protein tyrosine phosphorylation in adherent PMN was to plate PMN on immobilized anti- $\beta 2$ chain Abs. We (Berton et al., 1992), and other investigators (Zhou and Brown, 1993), demonstrated that plating of PMN on surface bound anti-integrin Abs triggers PMN functions without the requirement of costimulation by TNF. When

normal PMN were plated on anti- $\beta 2$ chain Abs a strong increase of phosphorylation of several proteins was detected (Fig. 1 *B*). Proteins whose tyrosine phosphorylation was increased by anti- $\beta 2$ chain Abs migrated at the same molecular weight of proteins whose tyrosine phosphorylation was increased by TNF in PMN adherent to fibrinogen. Significantly, protein tyrosine phosphorylation in PMN plated on an isotype matched Ab, reacting with Class I MHC antigens, was comparable to that detected in PMN maintained in ice before lysis. Consistent with the finding that increase in protein tyrosine phosphorylation did not occur in LAD PMN plated on fibrinogen either in the absence or the presence of TNF, also plating of LAD PMN on anti- $\beta 2$ chain Abs did not cause any response. The lack of any effect of the isotype matched anti-Class I MHC antigens Ab, and the evidence that LAD PMN did not respond to anti- $\beta 2$ chain Abs, clearly excludes that protein tyrosine phosphorylation triggered by the anti- $\beta 2$ chain Ab in normal PMN could also depend on the interaction with the Fc portion of the Ab.

TNF Enhances Kinase Activity and Tyrosine Phosphorylation of p58^{src} in Adherent PMN

The results so far described demonstrate that the TNF-stimulated protein tyrosine phosphorylation in PMN adherent to fibrinogen is dependent on ligation of fibrinogen by $\beta 2$ integrins. Since we reproducibly found that the major proteins which are tyrosine phosphorylated in this experimental system migrate with a molecular weight of ~56–60 kD, we started to address whether they might be represented by members of the *src* family of intracellular protein tyrosine kinases. As the bands stained by antiphosphotyrosine Abs could well represent different proteins migrating at the same molecular weight as also suggested by their strong reactivity with antiphosphotyrosine Abs, we addressed the issue of a possible $\beta 2$ integrin-dependent activation of members of the *src* family by a direct approach based on immune precipitation of p58^{src}, a member of the *src* family which is expressed in myelomonocytic cells (Ley et al., 1989; Notario et al., 1989; Inoue et al., 1990; Willman et al., 1991), and analysis of its enzymatic activity by *in vitro* kinase assays, and its extent of tyrosine phosphorylation by antiphosphotyrosine immunoblots.

Fig. 2 shows results of *in vitro* kinase assays (*A*), and antiphosphotyrosine immunoblots (*B*) performed on anti-p58^{src} immunoprecipitates from lysates of PMN plated on fibrinogen in the presence or the absence of TNF. TNF enhanced p58^{src} autophosphorylating activity in PMN plated on fibrinogen (*A*); in TNF-treated PMN a higher p58^{src} autophosphorylating activity was evident already after 10 min from plating, maximal at 30 min, and then decreased at 60 min. As reported in *B*, a close correlation existed between the increase in p58^{src} autophosphorylating activity, and an enhanced tyrosine phosphorylation of the protein. An enhanced p58^{src} autophosphorylating kinase activity as a consequence of TNF treatment was found reproducibly in 14 independent experiments in which also the time course of this enhancement displayed a high reproducibility being maximal, in most of the experiment, at 30 min, or, in only two experiments, at 60 min of treatment. Fig. 3 shows results of two other experiments which illustrate the reproducibility of the enhancement of p58^{src} autophosphorylating activity, and

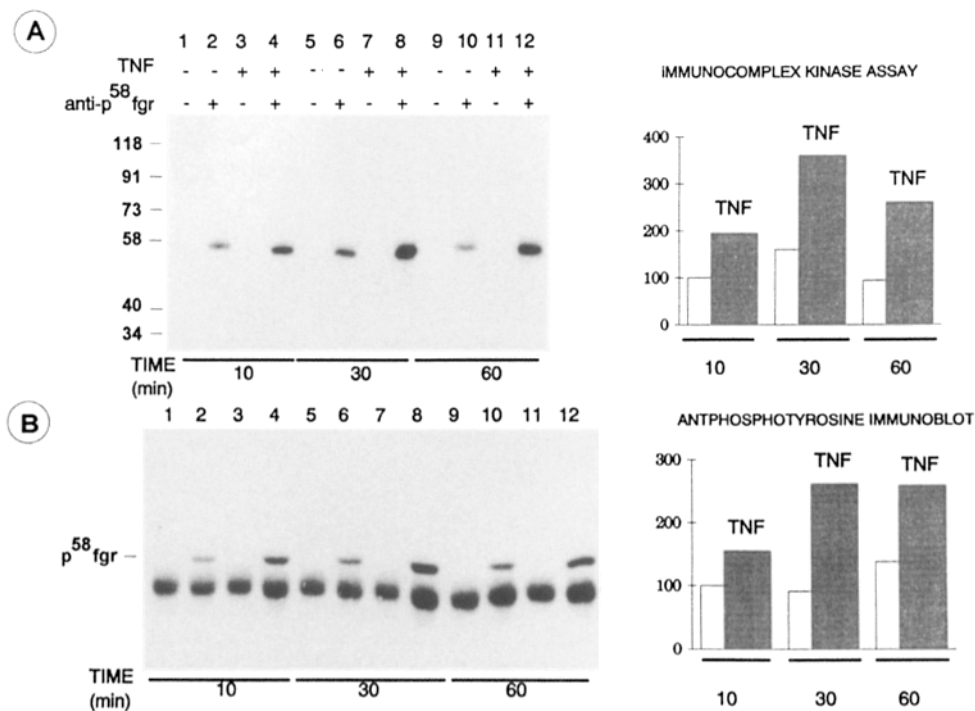


Figure 2. TNF enhances autophosphorylating kinase activity, and tyrosine phosphorylation of p58^{fgr} in PMN adherent to fibrinogen. PMN were plated in fibrinogen-coated wells for 10 (lanes 1-4), 30 (lanes 5-8), or 60 (lanes 9-12) min in the absence, or the presence of 20 ng/ml TNF. PMN lysates were immunoprecipitated with control rabbit IgG (-; odd numbers), or anti-p58^{fgr} Abs (+; even numbers). Aliquots from the same lysates were immunoprecipitated with control rabbit IgG (-; odd numbers), or anti-p58^{fgr} Abs (+; even numbers). Panels at the right of the figure show intensity of the band revealed by autoradiography, and quantified by densitometry. The signal detected in lysates of PMN plated for 10 min in the absence of TNF was given an arbitrary value of 100 and the other values calculated according to the formula: actual density/density at 10 min in the absence of TNF × 100.

intensity of the band revealed by autoradiography, and quantified by densitometry. The signal detected in lysates of PMN plated for 10 min in the absence of TNF was given an arbitrary value of 100 and the other values calculated according to the formula: actual density/density at 10 min in the absence of TNF × 100.

tyrosine phosphorylation by TNF (see also Figs. 5, 6, and 7). Enhancement of p58^{fgr} activity by TNF was also found in assays in which phosphorylation of the exogenous substrate enolase was analyzed (not shown). As illustrated in Fig. 3, enhanced p58^{fgr} activity and its extent of phosphorylation in tyrosine did not depend on variation of the amount of immunoprecipitated protein; in fact, probing of the same immunoprecipitates with anti-p58^{fgr} Abs showed that comparable amounts of the protein were immunoprecipitated.

As shown in Figs. 2 and 3, and also illustrated in Fig. 6, activation of p58^{fgr} kinase activity by TNF was also accompanied by an enhanced reactivity of the protein with antiphosphotyrosine Abs. To strengthen the evidence that TNF enhances tyrosine phosphorylation of p58^{fgr}, we performed experiments with the tyrosine kinase inhibitors genistein. In PMN treated with 5 μg/ml genistein the reactivity of the immunoprecipitated p58^{fgr} with antiphosphotyrosine Abs did not change as a consequence of treatment with TNF; immunocomplex kinase assays performed in parallel also showed that treatment with genistein blocked the enhancement of the p58^{fgr} autophosphorylating kinase activity by TNF (not shown).

Activation of p58^{fgr} in Adherent PMN by Phorbol Myristate Acetate and Formyl-methionyl-leucyl-phenylalanine

Activation of β2 integrin avidity for different ligands has been demonstrated to occur in response to different agonists, including PMA and chemotactic peptides (see Arnaout, 1990; Springer, 1990). We therefore addressed whether activation of p58^{fgr} in adherent PMN was also triggered by

other agonists able to enhance β2 integrin-dependent adhesion. Treatment of PMN plated in fibrinogen-coated wells with PMA, or FMLP triggered spreading of the cells over the fibrinogen surface (not shown). As illustrated in Fig. 4, PMA and FMLP increased protein tyrosine phosphorylation with a time-course which was different from that found with TNF (see also Fig. 1). In fact, after 10 min, protein tyrosine phosphorylation was comparable in TNF-treated and unstimulated cells, but PMA and FMLP increased this to the extent found in PMN treated for 30 min with TNF. We then investigated in more detail the time course of activation of p58^{fgr} kinase activity by PMA, and FMLP. As shown in Fig. 5, a clear increase of p58^{fgr} kinase activity was found already after 5 min of treatment with PMA, or FMLP. Consistently with the results reported in Figs. 2 and 3, p58^{fgr} kinase activity did not change in TNF-treated PMN up to 10 min but increased at 30 min.

Activation of p58^{fgr} Kinase Activity by TNF Is Dependent on Expression of β2 Integrins

To demonstrate that activation of p58^{fgr} kinase activity in adherent PMN by TNF depended on β2 integrins, we performed studies with PMN of a LAD patient. As shown in A of Fig. 6, we reproduced with another sample of PMN from the same donor the finding reported in Fig. 1 B that TNF does not enhance protein tyrosine phosphorylation in LAD PMN. In the experiment reported in Fig. 6 A, we detected a basal tyrosine phosphorylation in both normal and LAD PMN kept in ice before lysis. This basal tyrosine phosphorylation decreased slightly after incubation of the cells on fibrinogen for 30 min at 37°C. Treatment with TNF stimu-

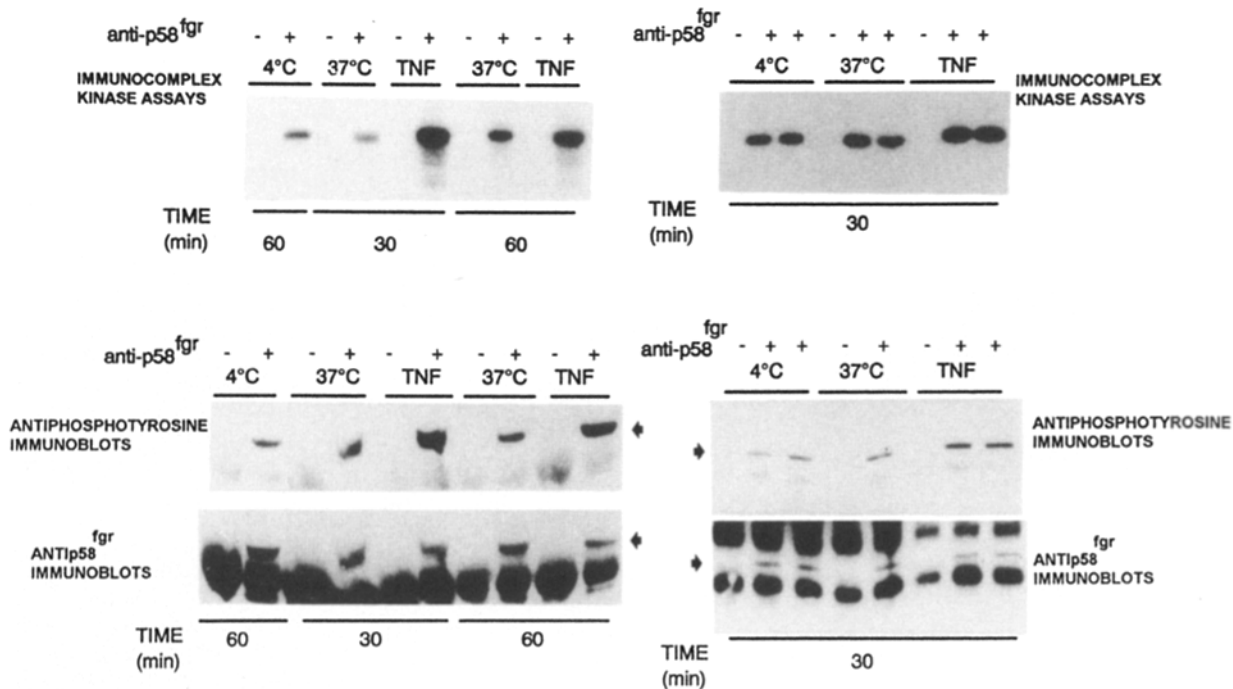


Figure 3. TNF enhances autophosphorylating kinase activity, and tyrosine phosphorylation of p58^{fgr} in PMN adherent to fibrinogen. PMN were kept in ice (4°C), or plated in fibrinogen-coated wells for 30, or 60 min, as indicated, in the absence (37°C), or the presence (TNF) of 20 ng/ml TNF. PMN lysates were immunoprecipitated with control rabbit IgG (-), or anti-p58^{fgr} Abs (+). Aliquots from the same lysates were immunoprecipitated and analyzed in parallel for p58^{fgr} autophosphorylating kinase activity, or p58^{fgr} tyrosine phosphorylation. After analysis of phosphorylation in tyrosine, blots were stripped of the Abs (see Materials and Methods) and reprobed with anti-p58^{fgr} Abs. Results of two independent experiments are reported. Where indicated, immunoprecipitates and assays were run in duplicates.

lated protein tyrosine phosphorylation in normal, but not in LAD PMN.

As shown in *B* of Fig. 6, immunocomplex kinase assays showed that p58^{fgr} in adherent LAD PMN is not activated by TNF. p58^{fgr} specific kinase activity assayed in immunoprecipitates from lysates of normal, or LAD PMN which had been kept in ice before lysis, was comparable. As also illustrated in Fig. 5 (compare p58^{fgr} kinase activity of cells kept at 4°C, or maintained at 37°C for 30 min in fibrinogen-coated wells), in some experiments, including that reported in Fig. 6, p58^{fgr} kinase activity decreased upon incubation of normal PMN on fibrinogen for 30 min. While TNF increased p58^{fgr} kinase activity in normal PMN either in comparison to cells kept in ice before lysis, or incubated on fibrinogen in the absence of TNF, no increase of p58^{fgr} kinase activity was detected in LAD PMN as a consequence of TNF treatment. As shown in *C* of Fig. 6, also the TNF-induced increase in tyrosine phosphorylation of p58^{fgr} did not occur in LAD PMN. To perform the experiments reported in Fig. 6, we had to omit analysis of p58^{fgr} phosphorylation in PMN kept in ice before lysis due to the limitation in the available number of cells. However, while TNF clearly increased tyrosine phosphorylation of p58^{fgr} in normal PMN plated on fibrinogen for 30 min, it had no effect on LAD PMN.

Activation of p58^{fgr} Kinase Activity by TNF, PMA, and FMLP Is Prevented by Soluble anti-CD18 Abs

To strengthen the conclusion inferred from experiments with

PMN of a LAD patient that $\beta 2$ integrins are implicated in activation of p58^{fgr}, we exploited previous observations that soluble anti-integrins Abs inhibit integrin-dependent activation of adherent PMN (Nathan et al., 1989; Shappel et al., 1990; Zhou and Brown, 1993). PMN were incubated for 30 min in ice with the anti-CD18 mAb IB4, or the isotype matched, anti-Class I MHC antigens B9.12, and then plated on fibrinogen with or without TNF, PMA, or FMLP. As shown in Fig. 7 (*A*), the anti-CD18 mAb IB4 (Wright et al., 1983b) totally blocked the activation of p58^{fgr} autophosphorylating kinase activity by TNF, and FMLP, and inhibited considerably the effect of PMA, an action which was accompanied by inhibition of PMN adhesion and spreading (not shown). It has been reported that, besides inhibiting $\beta 2$ integrins-dependent adhesion, the IB4 mAb increases cAMP levels in neutrophils (Gresham et al., 1991; Zhou and Brown, 1993). To exclude that the effects of IB4 were due to an elevation of cAMP, we tested the capability of HA1004, a protein kinase A inhibitor which was previously used for this purpose (Gresham et al., 1991; Zhou and Brown, 1993), to interfere with the inhibition by IB4 of p58^{fgr} activation. As illustrated in Fig. 7 (*B*), HA1004 had a slightly enhancing effect on p58^{fgr} activity of PMN which had been preincubated with the control mAb B9.12, and then plated on fibrinogen either in the absence or the presence of PMA. This is in line with previous evidence that increase of cAMP inhibits $\beta 2$ integrin-dependent spreading and activation of PMN functions (Nathan and Sanchez, 1990; Laudanna et al., 1990). Inhibition of p58^{fgr} activation by IB4 was how-

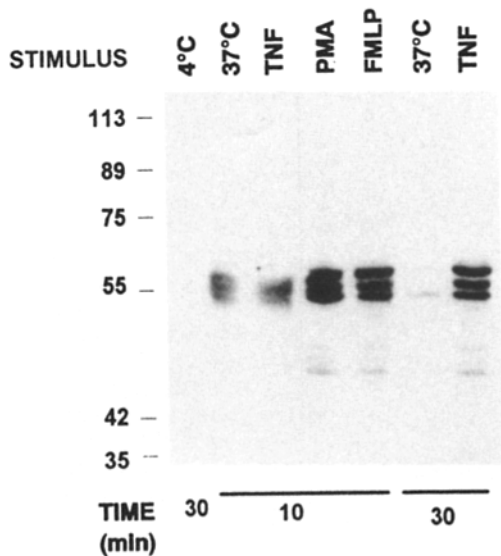


Figure 4. PMA and FMLP trigger protein tyrosine phosphorylation in PMN plated on fibrinogen. PMN were kept in ice (4°C), or plated in fibrinogen-coated wells for the times indicated in the absence (37°C), or the presence of 20 ng/ml PMA, 100 nM FMLP, or 20 ng/ml TNF. Proteins were extracted with RIPA buffer as described in Materials and Methods, and after electrophoresis in SDS-polyacrilamide gels, transferred to nitrocellulose and probed with the antiphosphotyrosine mAb 4G10.

ever unaffected by HA1004 in PMN adherent to fibrinogen and stimulated with PMA (Fig. 7 B), or TNF (not shown). These findings suggest that the capability of IB4 to interfere with p58^{tr} activation depended on binding to CD18, and inhibition of PMN adhesion and spreading to fibrinogen.

Discussion

The present studies demonstrate that, in PMN, $\beta 2$ integrins are involved in signaling for protein tyrosine phosphorylation, and that activation of p58^{tr} is likely implicated in this event. A possible role of $\beta 2$ integrins in signaling for protein tyrosine phosphorylation was suggested by studies showing that TNF triggers protein tyrosine phosphorylation in adherent PMN (Fuortes et al., 1993). In fact, as outlined in the Introduction section, activation by TNF of PMN functions when they adhere to different adhesive surfaces was demonstrated to depend on $\beta 2$ integrins. Moreover, it was shown that inhibitors of protein tyrosine kinases prevent the $\beta 2$ integrin-dependent stimulation of PMN functions by TNF (Fuortes et al., 1993; Laudanna et al., 1993a). Our demonstration that TNF does not trigger protein tyrosine phosphorylation in LAD PMN, which are defective in expression of $\beta 2$ integrins, provides a formal proof that this response to TNF is mediated by $\beta 2$ integrins. Furthermore, we found that surface bound anti- $\beta 2$ antibodies triggered directly a pattern of protein tyrosine phosphorylation comparable to that induced by TNF.

A growing body of evidence, obtained with cells of different lineage, points for a close link between triggering by integrins of protein tyrosine phosphorylation and reorganization of the cytoskeleton (for review see Turner and Burridge, 1991; Burridge et al., 1992; Zachary and Rozen-

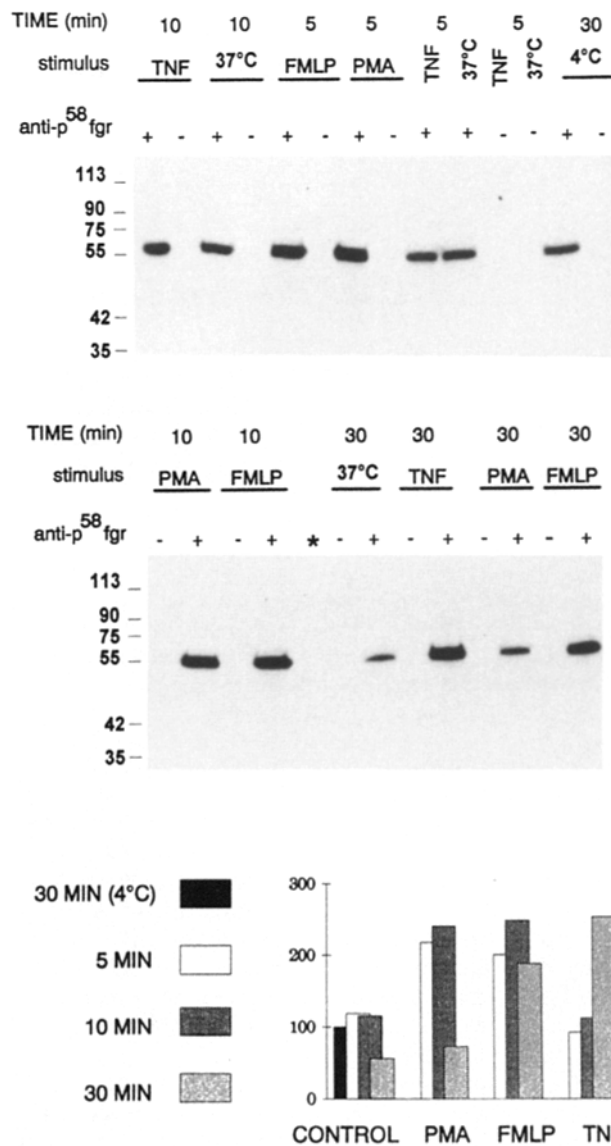


Figure 5. PMA and FMLP enhance p58^{tr} autophosphorylating kinase activity in PMN plated on fibrinogen. PMN were kept in ice (4°C), or plated in fibrinogen-coated wells for the times indicated in the absence (37°C), or the presence of 20 ng/ml PMA, 100 nM FMLP, or 20 ng/ml TNF. PMN lysates were immunoprecipitated with control rabbit IgG (-), or anti p58^{tr} Abs (+) and analyzed for autophosphorylating kinase activity. The bottom panel shows intensity of the bands revealed by autoradiography, quantified by densitometry and expressed in arbitrary units as described in Fig. 2 legend, but giving a value of 100 to the activity detected in lysates of PMN kept in ice. The lane marked with an asterisk was loaded with molecular weight markers.

gurt, 1992; Juliano and Haskill, 1993; Sastry and Horwitz, 1993). Spreading of neutrophils triggered by TNF (Fuortes et al., 1993), or anti-integrins antibodies (Laudanna et al., 1993a) was shown to be inhibited by protein tyrosine kinase inhibitors. Also triggering of a selective PMN function such as production of toxic oxygen molecules in adherent conditions was shown to be strictly dependent on spreading and reorganization of the cytoskeleton. In fact, inhibition of spreading by protein tyrosine kinase inhibitors, cytochala-

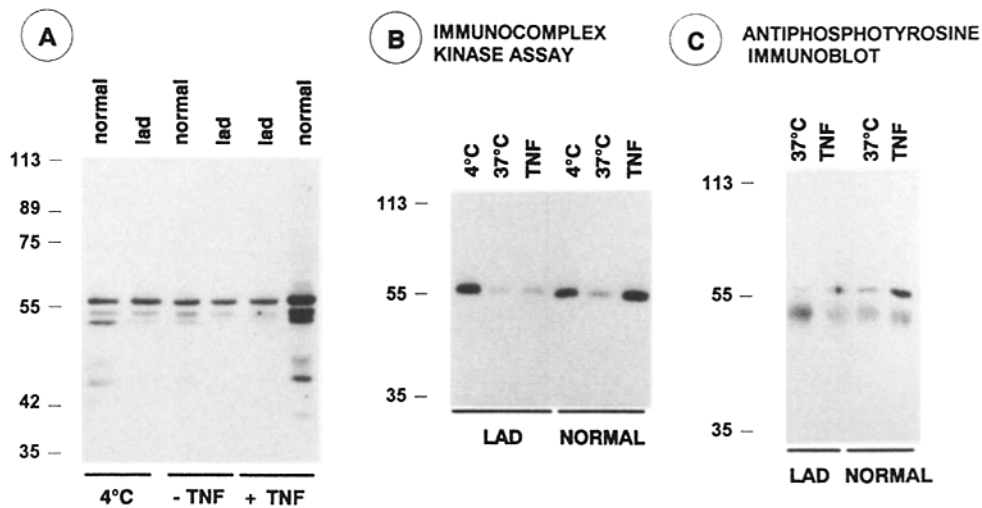


Figure 6. TNF does not enhance autophosphorylating kinase activity, and tyrosine phosphorylation of p58^{lck} in PMN of a LAD patient. (A) PMN from a healthy donor (*normal*), or a LAD patient (*lad*) were kept in ice (4°C), or plated for 30 min at 37°C in fibrinogen-coated wells in the absence or the presence of 20 ng/ml TNF. Protein tyrosine phosphorylation was analyzed as described in Fig. 1 legend and Materials and Methods. The results shown were obtained with PMN isolated from a blood sample different from that used for the experiment reported in Fig. 1. (B)

Lysates of PMN used for the analysis reported in panel A were immunoprecipitated, and analyzed for p58^{lck} autophosphorylating kinase activity. 4°C refers to PMN kept in ice before lysis; 37°C and TNF refer to PMN plated for 30 min in fibrinogen-coated wells in the absence or the presence of 20 ng/ml TNF, respectively. Similar results were obtained in two other experiments performed with PMN obtained from different blood samples of the LAD patient. (C) Lysates of PMN plated in fibrinogen-coated wells for 30 min at 37°C in the absence (37°C), or the presence of 20 ng/ml TNF, and used for the analysis reported in panel A and B, were immunoprecipitated with anti-p58^{lck} Abs and analyzed for reactivity with the antiphosphotyrosine mAb 4G10.

sins, the myosin light chain kinase inhibitor wortmannin, and elevation of intracellular cAMP also blocks the $\beta 2$ integrin-dependent activation of PMN respiratory burst (Laudanna et al., 1990; Nathan and Sanchez, 1990; Laudanna et al., 1993a). As outlined before (Nathan et al., 1993), these findings permit to envisage that stimulation of adherent PMN requires triggering of tyrosine phosphorylation and reorganization of the cytoskeleton; components of the NADPH oxidase, the system responsible for generation of toxic oxygen molecules (Malech, 1993) are then assembled in sites of polymerized cytoskeleton.

The present studies provide the first evidence that $\beta 2$ integrins can be implicated in activation of one member of the *src* family of intracellular tyrosine kinases, i.e., p58^{lck}. Members of this family have been clearly implicated in $\beta 3$ integrins signaling in platelets (Shattil and Brugge, 1991; Clark and Brugge, 1993). p58^{lck} is expressed in myelomonocytic cells, and the extent of its expression correlates with myelomonocytic cell differentiation (Ley et al., 1989; Notario et al., 1989; Inoue et al., 1990; Willman et al., 1991). The evidence that in PMN a chemotactic agonist induces translocation of p58^{lck} from an intracellular compartment to the plasma membrane (Gutkind and Robbins, 1989), and p58^{lck} is associated to type II receptors for the Fc of IgG (Hamada et al., 1993) already pointed for an important role of this protein in signal transduction. We showed that agonists of $\beta 2$ integrin activation such as TNF, PMA, and FMLP (Arnaout, 1990; Springer, 1990) enhance the kinase activity of p58^{lck}. We could demonstrate the $\beta 2$ integrin dependence of this event by showing that activation of p58^{lck} does not occur in PMN of a LAD patient, and that a soluble anti- $\beta 2$ chain mAb, inhibited activation of p58^{lck} by three unrelated stimuli. In PMN adherent to fibrinogen TNF (Figs. 2, 3, and 6), as well as PMA and FMLP (unpublished observation) caused also an enhanced phosphorylation of p58^{lck} in tyrosine. As kinase activity of members of the *src*

family can be regulated by dephosphorylation of critical phosphotyrosine residues at the COOH-terminal, or tyrosine phosphorylation of tyrosine residues within the kinase domain (Cooper and Howell, 1993), either activation of a tyrosine phosphatase acting on the COOH-terminal phosphotyrosine, or of a tyrosine kinase causing hyperphosphorylation of the tyrosine within the kinase domain, can explain our findings. In the former case, the enhanced tyrosine phosphorylation of p58^{lck} we found would be due to activation of p58^{lck} itself and its autophosphorylation. Although the use of the protein kinase inhibitor genistein allowed us to strengthen the dependence on a tyrosine kinase activity of p58^{lck} phosphorylation we detected with antiphosphotyrosine immunoblots, its action can be exerted either on a tyrosine kinase phosphorylating p58^{lck}, or the p58^{lck} kinase activity itself. The mechanisms of p58^{lck} activation by $\beta 2$ integrins in PMN appear to be more complex than those identified with members of the *src* family which are associated with the cytoplasmic tail of transmembrane molecules (Klausner and Samelson, 1991). We did not find any kinase activity associated to the $\beta 2$ integrin chain, or distinct α chains performing *in vitro* kinase assays on immunoprecipitates from Triton-X 100, or NP-40 PMN lysates (unpublished observation).

Protein tyrosine phosphorylation, and activation of p58^{lck} by $\beta 2$ integrins in PMN appears to occur in times longer than protein tyrosine phosphorylation found in platelets upon binding of fibrinogen to the $\alpha_{IIb}\beta 3$ integrin (see Huang et al., 1993). The shortest time point at which we could detect increase of protein tyrosine phosphorylation, and p58^{lck} activation was of 5 min, but both events increased up to 30 min. Although in our assay conditions we could not quantify the extent of PMN spreading, this also increased with time at least up to 30 min. It could therefore be that triggering of protein tyrosine phosphorylation and p58^{lck} activation is due to clustering of tyrosine kinases and their substrate in

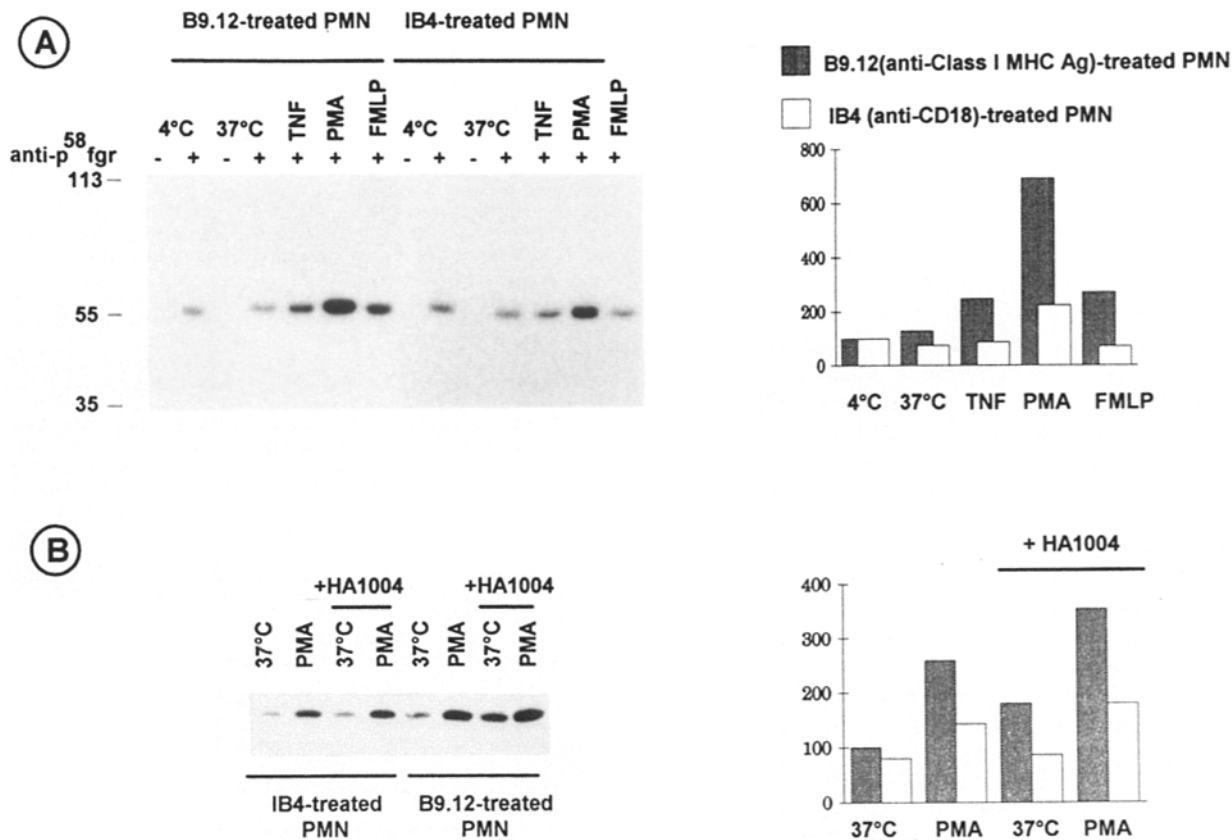


Figure 7. A soluble anti-CD18 mAb (IB4) inhibits enhancement of p58^{gr} autophosphorylating kinase activity triggered by TNF, PMA, and FMLP in PMN adherent to fibrinogen. (A) PMN were kept in ice for 30 min in the presence of 30 μ g/ml of IB4 (anti-CD18) or B9.12 (anti-Class I MHC antigens), and then lysed in RIPA (4°C), or plated for 30 min at 37°C in fibrinogen-coated wells in the absence (37°C), or the presence of 20 ng/ml TNF, 20 ng/ml PMA, or 100 nM FMLP. Kinase activity was analyzed by immunocomplex kinase assays as described in Materials and Methods in control rabbit IgG (-), or anti-p58^{gr} (+) immunoprecipitates. One of two experiments is reported. The panel at the right shows intensity of the bands revealed by autoradiography, and quantified by densitometry. The signal detected in lysates of PMN kept in ice (4°C) was given an arbitrary value of 100 and the other values calculated as described in Fig. 2 legend. (B) PMN were kept in ice for 30 min in the presence of 30 μ g/ml of IB4 or B9.12, and then incubated at 37°C in fibrinogen-coated wells in the absence, or the presence of 5 μ M HA1004. After 5 min, 20 ng/ml PMA (PMA), or diluent (37°C) were added, and the incubation prolonged for an additional 30 min. p58^{gr} autophosphorylating kinase activity was analyzed by immunocomplex kinase assays. The panel at the right shows intensity of the bands revealed by autoradiography, and quantified by densitometry. The signal detected in lysates of B9.12-treated PMN incubated on fibrinogen in the absence of HA1004 was given an arbitrary value of 100 and the other values calculated as described in Fig. 2 legend. One of two experiments is reported.

sites of cytoskeletal polymerization. Significantly, the β 2 integrin chain has been recently shown to be associated with α -actinin in PMN (Pavalko and LaRoche, 1993). Whether polymerization of the cytoskeleton is upstream or downstream to activation of tyrosine kinases in this model of β 2 integrin-dependent signaling needs to be addressed by further investigations. As neutrophil adhesion can be mediated by mechanisms independent of β 2 integrins, for example by the leukocyte response integrin (LRI) (Gresham et al., 1992; Zhou and Brown, 1993), it might be that also β 2 integrin-independent mechanisms of neutrophil adhesion and spreading trigger for protein tyrosine phosphorylation.

The demonstration of an essential role for β 2 integrin to signal for protein tyrosine phosphorylation in PMN plated on fibrinogen and challenged with TNF, strengthen previous evidence that PMN display a β 2 integrin-dependent signaling system which is likely involved in regulation of important cell functions. A possible explanation of our finding is that

the response of PMN adherent to fibrinogen to TNF originates from activation of avidity of integrins for the ligand: upon ligation of fibrinogen, β 2 integrins signal for protein tyrosine phosphorylation. This mechanism of "outside-in" signaling has been previously described to occur in cells of different lineage upon ligation of integrins (Arnaout, 1990; Springer, 1990; Hynes, 1992). This, and our previous findings which showed that surface bound anti-integrin antibodies trigger directly PMN functions without the requirement for costimulation by agonists of other receptors (Berton et al., 1992), permit to envisage a β 2 integrin-dependent pathway of signaling in PMN which displays several analogies with that which has been characterized in details in platelets (see Shattil and Brugge, 1991). Significantly, it was recently shown that ligands for the phagocyte integrin, which is referred to as LRI, act as agonist of PMN functions without the requirement of costimulation by other agonists (Zhou and Brown, 1993). Furthermore, some responses of PMN to

stimuli recognized by distinct receptors have been shown to be $\beta 2$ integrin-dependent (Gresham et al., 1991; Graham et al., 1993).

Our findings do not however exclude that, in vivo, the $\beta 2$ integrin-dependent stimulation of PMN by TNF and other agonists reflects a true costimulatory event which requires ligation of both $\beta 2$ integrins and agonists receptors.

The $\beta 2$ integrin-dependent stimulation of PMN functions finds a possible significance in almost all the physiopathological events this cell has been implicated. For example, hydrogen peroxide released by PMN upon interaction with ICAM-1 of endothelial, and other cells has been implicated in the phenomenon of PMN transmigration into tissues (Shappell et al., 1990; Entman et al., 1992). Interaction of LFA-1 with ICAM-3, which is expressed by PMN (de Fourgerolles and Springer, 1992) could trigger functions responsible for generation of toxic products when PMN aggregates accumulate in the micro circulation. Finally, a role for $\beta 2$ integrin-dependent adhesion has been clearly established in different models of PMN-dependent tissue injury (Argenbright and Barton, 1992; Mulligan et al., 1993a; Mulligan et al., 1993b; Burch et al., 1993). Identification of the cascade of signals generated by $\beta 2$ integrins in PMN will provide important clues also for new therapeutical strategies aimed to interfere with $\beta 2$ integrins signaling to control inflammation and tissue injury.

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