Regulation of Src Family Tyrosine Kinase Activities in Adherent Human Neutrophils

EVIDENCE THAT REACTIVE OXYGEN INTERMEDIATES PRODUCED BY ADHERENT NEUTROPHILS INCREASE THE ACTIVITY OF THE p58crfgr AND p53/56c lyn TYROSINE KINASES*

(Received for publication, April 12, 1996, and in revised form, June 19, 1996)

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Src family tyrosine kinases have been implicated in the adhesion-dependent activation of neutrophil functions (Yan, S. R., Fumagalli, L., and Berton, G. (1995) J. Inflamm. 45, 297–312; Lowell, C. A., Fumagalli, L., and Berton, G. (1996) J. Cell Biol. 133, 895–910). Because the activity of tyrosine kinases can be affected by oxidants, we investigated whether reactive oxygen intermediates (ROI) produced by adherent neutrophils regulate Src family kinase activities. Inhibition of ROI production by diphenylene iodonium, an inhibitor of NADPH oxidase, or degradation of H2O2 by exogenously added catalase inhibited the adhesion-stimulated activities of p58crfgr and p53/56c lyn. In addition, adhesion-stimulated p58crfgr and p53/56c lyn activities were greatly reduced in neutrophils from patients with chronic granulomatous disease (CGD) that are deficient in the production of ROI. Exogenously added H2O2 increased p58crfgr and p53/56c lyn activities in nonadherent neutrophils. Although ROI regulated the activities of p58crfgr and p53/56c lyn, they did not affect the redistribution of the two kinases to a Triton X-100-insoluble, cytoskeletal fraction that occurs in adherent neutrophils. Tyrosine phosphorylation of proteins in adherent, CGD neutrophils was only partially inhibited, suggesting that the full activation of p58crfgr and p53/56c lyn, which depends on endogenously produced ROI, does not represent an absolute requirement for protein tyrosine phosphorylation. The adhesion-stimulated activity of the tyrosine kinase p72hck was not affected by catalase in normal neutrophils, and it was comparable in normal and CGD neutrophils. These findings suggest that ROI endogenously produced by adherent neutrophils regulate Src family kinase activities selectively and establish the existence of a cross-talk between reorganization of the cytoskeleton, production of ROI, and Src family tyrosine kinase activities in signaling by adhesion.

In the presence of inflammatory mediators, neutrophils (PMN)1 interacting with appropriate adhesion molecules reorganize their cytoskeleton and spread over the adhesive surface, produce reactive oxygen intermediates (ROI), and release specific granule constituents (1–6). Activation of PMN functions by adhesion is mediated by integrins. In fact, PMN from patients affected by the leukocyte adhesion deficiency type I syndrome that do not express β2 integrins are defective in adhesion-dependent functions (3, 6, 7, 8). In addition, soluble anti-integrin antibodies (Abs) inhibit PMN functions stimulated by adhesion (5, 7–9). Finally, surface-bound anti-β2(10–13) or anti-β3 integrin (12, 13) Abs trigger both reorganization of the actin-based cytoskeleton and ROI production. These last findings strongly suggest that PMN integrins generate intracellular signals activating selective cell functions (“outside-in signaling”).

In the last few years, signal transduction by integrins has been the object of extensive investigations (reviewed in Ref. 14). These studies established that reorganization of the cytoskeleton and activation of tyrosine kinases play a central role in signal transduction by integrins (14). The actin-based cytoskeleton and tyrosine kinases are also essential for signaling by PMN integrins. In fact, cytochalasins (1, 4) and tyrosine kinase inhibitors (11, 15) block PMN spreading, as well as adhesion-dependent ROI production. In addition, inhibition of phosphotyrosine phosphatase activity by vanadyl hydroperoxide induces an increase in tyrosine phosphorylation and a rearrangement of the cytoskeleton in PMN (16). Interestingly, one of the proteins that is tyrosine phosphorylated in adherent PMN is the cytoskeletal protein Paxillin, whose tyrosine phosphorylation depends on expression of β2 integrins (17, 18).

Src family kinases likely play a central role in integrin signaling in PMN. In fact, the tyrosine kinase p58crfgr is activated upon PMN adhesion to fibrinogen, and this depends on expression of β2 integrins (19). In addition, modulation of PMN integrin adhesiveness by diveral cations also regulate the activity of both p58crfgr and p53/56c lyn (20). Studies with mice in which the fgr and the hck genes were inactivated (21) provided a formal proof that Src family kinases play an essential role in integrin-dependent PMN adhesion and ROI production (13). In fact, PMN from these mice do not spread over plasma, extra-cellular matrix proteins, or surface-bound anti-integrin Abs and do not produce ROI on these surfaces.

ROI and other oxidants have been shown to represent an important mechanism of regulation of protein tyrosine phosphorylation, and protein-tyrosine kinase and phosphatase activities. For example, H2O2, either alone or in combination with vanadate, enhances activities of receptor tyrosine kinases (see Refs. 22 and 23 and references quoted therein) and cytoplasmic tyrosine kinases such as p56fgr (24–28), p59c lyn (24, 25, 27), p72hck (29, 30), and ZAP-70 (31). In addition, H2O2 inhibits the protein-tyrosine phosphatase CD45 (24, 32). It is likely that regulation of tyrosine kinases and phosphatases is responsible

* This work was supported by grants from Italian Association for Cancer Research and the National Research Council of Italy (Strategic Project: Cytokines). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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† The abbreviations used are: PMN, polymorphonuclear neutrophil; DPI, diphenyleneiodonium chloride; CGD, chronic granulomatous disease; HBSS, Hank’s balanced salt solution; ROI, reactive oxygen intermediate(s); TNF, tumor necrosis factor-α; Ab, antibody.
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for the H\(_2\)O\(_2\)-induced phosphorylation of multiple cellular proteins, as well as specific substrates (33–35). In PMN, ROI produced by NADPH oxidase have been shown to regulate tyrosine phosphorylation of several proteins, including the mitogen-activated protein (MAP) kinase as well as tyrosine kinases (32, 36–38).

Previous studies established that there is a strict link between reorganization of the cytoskeleton, tyrosine kinase activities, and production of ROI in adherent PMN (see above). Although it was shown that Src family tyrosine kinases are essential to promote rearrangement of the cytoskeleton and ROI production in adherent PMN (13), we were interested to know whether ROI produced as a consequence of adhesion and spreading play any role in regulation of tyrosine kinase activities. In this report we provide evidence that ROI produced by adherent PMN enhance the activity of the Sry family tyrosine kinases p58\(^{c-fgr}\) and p53/56\(^{lyn}\).

EXPERIMENTAL PROCEDURES

Preparation of PMN—PMN were isolated from buffy coats of healthy volunteers or two patients affected by chronic granulomatous disease (CGD) by dextran sedimentation and centrifugation over Ficoll-Hypaque (Pharmacia Biotech Inc.) as described previously (10, 19). PMN from the two CGD patients did not produce superoxide anion and were used to be affected by the X-linked form of the disease by Western blot analysis of PMN lysates, which demonstrated a lack of expression of the gp91\(^{phox}\) and p22\(^{phox}\) components of NADPH oxidase (data not shown). Cells were resuspended in modified Hank's balanced salt solution (HBSS; 137 mM NaCl, 5.36 mM KCl, 0.34 mM NaHPO\(_4\), 0.44 mM KH\(_2\)PO\(_4\), 4.17 mM NaHCO\(_3\), 20 mM Hepes, 5.5 mM glucose), containing 5 mM EDTA and incubated at room temperature for 15 min (20, 39, 40). The cells were then washed and resuspended in HBSS containing no divalent cations, 1 mM MgCl\(_2\), 1 mM MgCl\(_2\) plus 1 mM CaCl\(_2\) or 0.2 mM MnCl\(_2\). PMN were incubated in fibrinogen-coated wells (10, 19, 20) in the presence or absence of 20 ng/ml TNF.

To inhibit NADPH oxidase, PMN were treated with 20 \(\mu\)M diphenylene iodonium chloride (DPI) (41) for 10 min at room temperature before plating. To degrade H\(_2\)O\(_2\), PMN were assayed in the presence of tyrosine kinase activities (data not shown). As shown in Fig. 2, the lyn- and p38\(^{c-fgr}\) and p53/56\(^{lyn}\) activities were analyzed by Western blotting exactly as described (19, 20). Tyrosine phosphorylated proteins were analyzed by Western blot analysis using the anti-phosphotyrosine Ab 4G10 (19).

RESULTS

TNF-stimulated PMN Adhesion to Fibrinogen and Generation of ROI Are Regulated by Divalent Cations—In previous studies, we demonstrated a strict correlation between PMN adhesion and activation of the Src family protein-tyrosine kinases p58\(^{c-fgr}\) and p53/56\(^{lyn}\) (20). The experimental approach we exploited to demonstrate this correlation was based on manipulation of the divalent cation composition of the assay medium (20). We then assayed adhesion-dependent ROI production by PMN suspended in media of different divalent cations composition (20, 39, 40). As shown in Fig. 1A, and in accord with our previous studies (20), TNF-stimulated PMN adhesion to fibrinogen occurred only in the presence of Mg\(^{2+}\) but not in the absence of divalent cations or in the presence of Ca\(^{2+}\) alone.

In the same conditions of assays used for adhesion experiments, i.e. in 96-well plates and using 10 \(\mu\)M phorbol myristate acetate, ROI generation was strictly correlated with adhesion; in fact, it was almost undetectable if PMN were suspended in divalent cation-free or Ca\(^{2+}\)-containing medium (Fig. 1B). These findings were not due to alterations of NADPH oxidase activity in dependence of the divalent cation composition of the assay medium because the response to phorbol myristate acetate, an adhesion-independent stimulus of PMN O\(_2\) generation, was comparable in the absence of divalent cations or in the presence of Ca\(^{2+}\) or Mg\(^{2+}\) (see Fig. 1 legend). Because analysis of p58\(^{c-fgr}\) and p53/56\(^{lyn}\) activities required a higher number of cells (3 \(\times\) 10\(^6\)), which were plated in 24-well trays (see Ref. 20 and “Experimental Procedures”), we also analyzed O\(_2\) generation in these assay conditions (Fig. 1C). At high cell density, we could detect generation of O\(_2\) in response to TNF by PMN suspended in Ca\(^{2+}\) alone, but this was much higher in the presence of Mg\(^{2+}\) also in these assay conditions. Because the data reported in Fig. 1 showed that divalent cations can regulate, besides adhesion, also the adhesion-dependent production of ROI, we then addressed whether differences in ROI generation could account for different levels of activity of p58\(^{c-fgr}\) and p53/56\(^{lyn}\).

The Inhibitor of NADPH Oxidase Activity DPI Decreases the Adhesion-stimulated Activity of p58\(^{c-fgr}\) and p53/56\(^{lyn}\)—DPI is a strong inhibitor of NADPH oxidase (41), and we found that 20 \(\mu\)M DPI inhibited by more than 95% generation of ROI by adherent PMN in the conditions used to assay p58\(^{c-fgr}\) and p53/56\(^{lyn}\) activities (data not shown). As shown in Fig. 2, the Mg\(^{2+}\)-dependent, TNF-stimulated p58\(^{c-fgr}\) and p53/56\(^{lyn}\) activities were inhibited by DPI. In addition, DPI inhibited the activation of p58\(^{c-fgr}\) (Fig. 2) and p53/56\(^{lyn}\) (data not shown) accomplished by inducing PMN adhesion and spreading with Mn\(^{2+}\) in the absence of TNF (Fig. 2B). As illustrated in Fig. 2C for p53/56\(^{lyn}\), the inhibitory effects of DPI were evident also...
Fig. 1. ROI production by PMN incubated on fibrinogen in the presence of TNF correlate with adhesion. PMN were treated with EDTA and suspended in HBSS containing no divalent cation, 1 mM Ca\(^{2+}\), or 1 mM Mg\(^{2+}\) (see “Experimental Procedures” and Refs. 20, 39, and 40). A, PMN adhesion. PMN (0.15 \(\times\) 10\(^6\) cells in 200 \(\mu\)l) were incubated for 30 min in fibrinogen-coated, 96-well plates in the absence or the presence of 20 ng/ml TNF. At the end of incubation nonadherent cells were washed away and the percentage of adhesion was quantified as detailed in Ref. 20. B and C, \(O_2\) generation. In B, PMN were incubated as in A, whereas in C the assay was performed in 24-well plates with a cell density of 3 \(\times\) 10\(^6\) cells/400 \(\mu\)l. \(O_2\) was measured as described under “Experimental Procedures.” \(O_2\) generation in the same assay conditions used in B, but in response to phorbol myristate acetate (2 ng/ml) was (in mmol/30 min/0.15 \(\times\) 10\(^6\) PMN \(\pm\) S.D.): without cations 2.1 \(\pm\) 0.6, plus Ca\(^{2+}\), 2.7 \(\pm\) 0.6, plus Mg\(^{2+}\), 2.2 \(\pm\) 0.4. The mean results of six independent experiments \(\pm\) S.D. are reported.

Fig. 2. DPI inhibits the adhesion-dependent activation of p58\(^{c-fgr}\) and p53/56\(^{lyn}\). PMN were treated with EDTA and suspended in HBSS containing different divalent cations (see “Experimental Procedures”). After incubation in fibrinogen-coated wells for 30 min, PMN were lysed with RIPA, and anti-p58\(^{c-fgr}\) (A and B) or anti-p53/56\(^{lyn}\) (C and D) immunoprecipitates were subjected to in vitro kinase assays. In the experiments reported in A and D, kinase activities were also assayed in the absence of divalent cations or in the presence of Ca\(^{2+}\) only (not shown), confirming previous findings (20) that TNF does not increase p58\(^{c-fgr}\) or p53/56\(^{lyn}\) activities in these assay conditions (see for example the first two lanes of B). In C, assays were performed in the presence of 2.5 \(\mu\)g of acid-denatured enolase. One representative experiment of three performed is reported.

Assaying the in vitro phosphorylation of an exogenous substrate, such enolase. The described effects of DPI were not due to an inhibitory effect on PMN adhesion. PMN adhesion in the presence of Mg\(^{2+}\) was (in percent; mean results of five independent experiments \(\pm\) S.D.): unstimulated (without DPI), 6.8 \(\pm\) 7.7; unstimulated (plus DPI), 4.6 \(\pm\) 2.0; TNF-stimulated (without DPI), 35.7 \(\pm\) 4.1; TNF-stimulated (plus DPI), 29.2 \(\pm\) 2.5. The inhibitory effects of DPI on p58\(^{c-fgr}\) and p53/56\(^{lyn}\) activities were not due to a direct effect on the two kinases. In fact, as shown in Fig. 3, DPI did not inhibit the two kinase activities either if added to PMN lysates or to the immunoprecipitated p58\(^{c-fgr}\) or p53/56\(^{lyn}\).

Catalase Inhibits the Adhesion-stimulated Activity of p58\(^{c-fgr}\) and p53/56\(^{lyn}\)—Studies with cells of different lineages established that the ROI species involved in activation of protein tyrosine phosphorylation is H\(_2\)O\(_2\) (see Introduction). To elucidate whether H\(_2\)O\(_2\) produced by adherent PMN played a role in modulation of p58\(^{c-fgr}\) and p53/56\(^{lyn}\) activities, we investigated the effect of the H\(_2\)O\(_2\)-degrading enzyme catalase (Fig. 4). As shown in Fig. 4, catalase blunted activation of p58\(^{c-fgr}\) and p53/56\(^{lyn}\) induced by Mg\(^{2+}\)-dependent, TNF-stimulated adhesion. This finding suggests that ROI produced at the plasma membrane level and released in the extracellular milieu are involved in regulation of p58\(^{c-fgr}\) and p53/56\(^{lyn}\) activities in adherent PMN. A critical role of H\(_2\)O\(_2\) in regulating p58\(^{c-fgr}\) and p53/56\(^{lyn}\) activities is also suggested by the finding that superoxide dismutase had no inhibitory effect (data not shown).

High Concentrations of H\(_2\)O\(_2\) Can Activate p58\(^{c-fgr}\) and p53/56\(^{lyn}\) Independently of Adhesion—Because H\(_2\)O\(_2\) can stimulate protein tyrosine phosphorylation (see Introduction), we then investigated the effect of H\(_2\)O\(_2\) on p58\(^{c-fgr}\) and p53/56\(^{lyn}\) activities in conditions in which PMN adhesion was hampered by incubating the cells in Ca\(^{2+}\) alone (see Fig. 1, and Ref. 20). As shown in Fig. 5, in the presence of Ca\(^{2+}\) alone, H\(_2\)O\(_2\) dose-dependently increased p58\(^{c-fgr}\) and p53/56\(^{lyn}\) activities in PMN incubated on fibrinogen in the absence of TNF. Interestingly, p58\(^{c-fgr}\) was more sensitive than p53/56\(^{lyn}\) to adhesion-independent activation by H\(_2\)O\(_2\). In fact, high concentrations of H\(_2\)O\(_2\) (5–10 mM) enhanced p58\(^{c-fgr}\), but not p53/56\(^{lyn}\), activity also in the absence of sodium azide (NaN\(_3\)), an inhibitor of the endogenous H\(_2\)O\(_2\)-degrading enzyme catalase. In the presence of NaN\(_3\), both kinases were activated by a concentration of 0.5–1 mM H\(_2\)O\(_2\). Comparable results were obtained with PMN suspended in divalent cation-free medium (data not shown). H\(_2\)O\(_2\) had no effect if added to PMN lysates or to immunoprecipitated p58\(^{c-fgr}\) or p53/56\(^{lyn}\) (data not shown). We conclude that H\(_2\)O\(_2\) does not act directly on the two kinases but its target is a molecule involved in regulation of p58\(^{c-fgr}\) and p53/56\(^{lyn}\) activities. As shown in Fig. 5, even in the presence of NaN\(_3\), the capability of H\(_2\)O\(_2\) to activate p58\(^{c-fgr}\) and p53/56\(^{lyn}\) in a fashion that was independent of adhesion and TNF stimulation, was
in 0.4 ml H2O2 produced in conditions of maximal stimulation, gen-coated wells, PMN were lysed with RIPA, and anti-p58c-fgr i.e. HBSS containing 1 mM Mg2+. We then analyzed p58c-fgr and p53/56 lyn activities in PMN from patients affected by CGD are deficient in spe-cific activities of p58c-fgr and p53/56 lyn but also causes a re-distribution of the two kinases to a cytoskeletal Triton X-100-insoluble fraction (20, 43). Interestingly, recent results demonstrated that p60c-src induces fibroblast spreading by a

not evident at <0.5 mm. In the conditions of assays we rou-tinely used to assay adhesion-dependent stimulation of p58c-fgr and p53/56 lyn activities (this study, Figs. 2, 3, and 4, and Ref. 20), i.e. in 24-well trays and with a cell density of 3 × 106 PMN in 0.4 ml H2O2 produced in conditions of minimal stimulation, i.e. in the presence of Mg2+, either alone or together with Ca2+, and with optimal stimulatory doses of TNF (20 ng/ml), was <10 µM. This strongly suggests that much higher doses of H2O2 are required to activate p58c-fgr and p53/56 lyn when adhesion and the cytoskeleton reorganization underlying PMN spreading are prevented.

Adhesion-dependent Activation of p58c-fgr and p53/56 lyn Is Defective in PMN from Chronic Granulomatous Disease Patients—PMN of patients affected by CGD are deficient in specifc components of the NADPH oxidase and are therefore unable to produce ROI (44). We then analyzed p58c-fgr and p53/56 lyn activities in PMN from CGD patients. As reported in Fig. 6A, PMN from CGD patients did not show any impairment of basal or TNF-stimulated adhesion to fibrinogen. In PMN from two of these patients, p58c-fgr activity was higher in conditions that permitted adhesion (compare activity in the presence of Mg2+ with that in the absence of divalent cations); however, TNF treatment did not increase this activity (Fig. 6B). In PMN from one of these patients we found that p53/56 lyn was slightly activated in response to TNF (Fig. 6C). We also analyzed p58c-fgr and p53/56 lyn activities in CGD PMN after different times of incubation on fibrinogen. As shown in Fig. 7, in normal PMN p58c-fgr activity increased in response to TNF already after 7.5 min of incubation, and it was further enhanced with time up to 30 min. At short times of incubation (7.5 min), p58c-fgr activity in CGD PMN was almost comparable with that of normal PMN, and it was enhanced by TNF treatment. However, a consistent difference between normal and CGD PMN was found in the basal and TNF-stimulated p58c-fgr activity increasing the time of incubation. Analysis of p53/56 lyn in PMN of the same CGD patient showed that TNF caused an increase of the kinase activity, which was, however, lower than that detected in normal PMN (Fig. 7). Importantly, the addition of H2O2 to adherent CGD PMN enhanced p53/56 lyn activity (Fig. 3) and p58c-fgr activity in CGD PMN was almost comparable with that of normal PMN, and it was enhanced by TNF treatment. Although the results obtained with PMN from CGD patients displayed some small variability (see for example the 30-min time point of Figs. 6 and 7), they definitively confirm that ROI are required for an optimal activation of p58c-fgr and p53/56 lyn. In addition, the results reported in Fig. 7 suggest that the early activation of at least p58c-fgr is not altered in CGD PMN. At these early time points and in accord with previous studies (1, 4), we did not detect any production of ROI by normal PMN. These findings suggest that ROI are required to boost the activity of p58c-fgr and p53/56 lyn.

ROI Are Not Required to Redistribute p58c-fgr and p53/56 lyn to a Triton X-100-insoluble Cytoskeletal Fraction—TNF-stimulated β2 integrin-dependent adhesion not only affects enzymatic activities of p58c-fgr and p53/56 lyn but also causes a re-distribution of the two kinases to a cytoskeletal Triton X-100-insoluble fraction (20, 43). Interestingly, recent results demonstrated that p60c-src induces fibroblast spreading by a
kinase-independent mechanism and that this correlates with p60c-src association with a cytoskeletal fraction (45). As reported in Fig. 8, in both normal and CGD PMN, TNF-stimulated adhesion was accompanied by a redistribution of p58c-fgr to a Triton X-100-insoluble fraction. Also p53/56lyn redistribution was independent of ROI production as it occurred, albeit to a lower extent than in normal PMN, also in adherent CGD PMN (Fig. 8). That p58c-fgr and p53/56lyn redistributions to a cytoskeletal fraction did not require or only partially required ROI production, respectively, was confirmed in experiments with normal PMN. In fact, DPI and catalase did not affect redistribution of p58c-fgr and only partially inhibited redistribution of p53/56lyn in normal PMN (data not shown). We conclude that ROI regulate activities of p58c-fgr and p53/56lyn but not the redistribution of the two kinases to a cytoskeletal fraction.

Because in PMN of CGD patients we found that adhesion-dependent activation of p58c-fgr and p53/56lyn was substantially lower than in normal PMN but not totally inhibited and that redistribution of the two kinases to a cytoskeletal fraction was not affected, we were interested to know which are the consequences of a total lack of ROI production on protein tyrosine phosphorylation and analyzed tyrosine phosphorylated proteins in the Triton-soluble and insoluble fraction of normal and CGD PMN (Fig. 9). As we previously reported (43), tyrosine phosphorylated proteins were mainly recovered in the Triton-insoluble fraction, and TNF-stimulated adhesion caused an increase of the tyrosine phosphorylation of several proteins. Not surprisingly, the extent of tyrosine phosphorylation was lower in CGD than in normal PMN. However, TNF-stimulated adhesion increased tyrosine phosphorylation of several proteins present in the Triton-insoluble fraction also in CGD PMN, i.e. in the total absence of ROI production. This finding sug-

**Fig. 6.** CGD PMN adhere normally to fibrinogen, but adhesion-dependent activation of p58c-fgr and p53/56lyn is defective. A, adhesion assays of normal and CGD PMN that had been treated with EDTA and suspended in HBSS containing 1 mM Mg2+. Adhesion was assayed after 30 min of incubation in fibrinogen-coated wells. B and C, p58c-fgr and p53/56lyn kinase activities in normal and CGD PMN. In B, EDTA-treated PMN were suspended in HBSS containing either 1 mM Mg2+ or no divalent cation. In C, EDTA-treated PMN were suspended in 1 mM Mg2+. After 30 min of incubation, PMN were lysed with RIPA, and anti-p58c-fgr or anti-p53/56lyn immunoprecipitates were subjected to in vitro kinase assays.

**Fig. 7.** Adhesion-dependent activation of p58c-fgr and p53/56lyn is defective in CGD PMN. PMN from one CGD patient and one healthy subject were treated with EDTA and suspended in 1 mM Mg2+. After incubation in fibrinogen-coated wells for the times indicated, PMN were lysed with RIPA, and anti-p58c-fgr or anti-p53/56lyn immunoprecipitates were subjected to in vitro kinase assays. In the experiment reported in the bottom right panel, normal or CGD PMN were incubated for 30 min on fibrinogen before lysis with RIPA buffer. CGD PMN were treated or not with 5 mM H2O2 in the presence of 2 mM NaN3.

**Fig. 8.** Adhesion-dependent redistribution of p58c-fgr and p53/56lyn to a Triton X-100-insoluble fraction is unaffected in CGD PMN. Normal and CGD PMN were assayed in the same conditions described in the legend to Fig. 6. PMN were incubated in fibrinogen-coated wells for 30 min and then lysed in a buffer containing Triton X-100 (see “Experimental Procedures” and Refs. 20 and 43). Both the Triton-soluble and insoluble fractions were solubilized in SDS-sample buffer and p58c-fgr and p53/56lyn analyzed by Western blotting. One representative experiment of two performed is reported.
suggests that the low activity of p58<sub>fgr</sub> and p53/56<sub>lyn</sub> we detected in CGD PMN (see Figs. 6 and 7) is sufficient to trigger protein tyrosine phosphorylation or that other tyrosine kinases, whose activity is totally independent of ROI production, are activated as a consequence of TNF-stimulated adhesion.

**ROI Do Not Affect Adhesion-dependent Activation of the Protein-tyrosine Kinase p72<sub>syk</sub>**—Studies with platelets showed that integrin engagement activates both Src family tyrosine kinases and the tyrosine kinase p72<sub>syk</sub> (reviewed in Ref. 46). More recently, p72<sub>syk</sub> was shown to be involved in integrin signaling in monocytic cells (47). Because we found that integrin-dependent adhesion of PMN activates p72<sub>syk</sub><sup>3</sup>, we addressed whether ROI modulated the activity of p72<sub>syk</sub>. As shown in Fig. 10, TNF-stimulated PMN adhesion caused an early increase of p72<sub>syk</sub> autophosphorylating kinase activity. However, concentrations of catalase that strongly inhibited p58<sub>fgr</sub> and p53/56<sub>lyn</sub> activities (see Fig. 4) had no effect on p72<sub>syk</sub> activity. In addition activation of p72<sub>syk</sub> was not affected in PMN from one CGD patient. We conclude that ROI produced by adherent PMN are able to modulate the activity of p58<sub>fgr</sub> and p53/56<sub>lyn</sub> selectively, and adhesion-dependent activation of the p72<sub>syk</sub> tyrosine kinase is independent of ROI production.

**DISCUSSION**

The findings described in this paper implicate ROI as positive regulators of the activity of the protein-tyrosine kinases p58<sub>fgr</sub> and p53/56<sub>lyn</sub> in adherent neutrophils. This implication is substantiated by the findings that DPI, an inhibitor of NADPH oxidase, and catalase, an enzyme degrading H<sub>2</sub>O<sub>2</sub>, inhibited adhesion-dependent activation of p58<sub>fgr</sub> and p53/56<sub>lyn</sub>. In addition, adhesion activated p58<sub>fgr</sub> and p53/56<sub>lyn</sub> to a much lower extent in PMN from CGD patients, which are unable to produce ROI. However, ROI had no relevant effect on the redistribution of p58<sub>fgr</sub> and p53/56<sub>lyn</sub> to a cytoskeletal fraction, an event that we showed to be strictly adhesion-dependent (20, 43). It is likely that the capability of ROI produced by adherent PMN to increase the activity of p58<sub>fgr</sub> and p53/56<sub>lyn</sub> requires that the two kinases are redistributed to a cytoskeletal fraction (see below). The ability of ROI to increase p58<sub>fgr</sub> and p53/56<sub>lyn</sub> in adherent PMN displayed some selectivity. In fact, the p72<sub>syk</sub> kinase activity, which is enhanced by adhesion,<sup>3</sup> was not affected by ROI. That adhesion-dependent tyrosine phosphorylation of proteins was only partially inhibited in CGD PMN suggests that the low ROI-independent kinase activity of p58<sub>fgr</sub> and p53/56<sub>lyn</sub> that we detected in adherent CGD PMN or the activity of other kinases, such p72<sub>syk</sub>, are responsible for tyrosine phosphorylation of endogenous substrates.

Evidence has been recently accumulated that tyrosine kinases, and in particular Src family tyrosine kinases, play an essential role in a cascade of signals originating from PMN integrins and underlying the adhesion-dependent activation of ROI production (see Introduction). The findings described in this paper are apparently in contrast with the evidence that Src family tyrosine kinase activities are required both for adhesion and adhesion-dependent ROI production. In fact, they show that the activity of two Src family members, i.e. p58<sub>fgr</sub> and p53/56<sub>lyn</sub>, is largely dependent on ROI produced by the adherent PMN. Because production of ROI by adherent PMN requires the formation of an actin-based cytoskeleton (1, 4, 43), our findings would suggest that full activation of at least p58<sub>fgr</sub> and p53/56<sub>lyn</sub> is downstream reorganization of the cytoskeleton and ROI production. Therefore, one of the questions arising is whether the role of Src family kinases in promoting the formation of an actin-based cytoskeleton is partially independent on their kinase activity. Interestingly, the capability of p60<sup>src</sup> to promote fibroblast spreading was shown to be independent of its kinase activity and to correlate with its redistribution to focal adhesions (45). Hence, Src family kinases might also induce PMN spreading by a kinase-independent mechanism.

Interestingly, we found that redistribution of p58<sub>fgr</sub> and p53/56<sub>lyn</sub> to a Triton X-100 cytoskeletal fraction (20, 43) is unaffected or only partially affected by ROI (Fig. 8). However, tyrosine kinase inhibitors blunt the formation of an actin-based cytoskeleton and PMN spreading (11, 15), thus suggesting that tyrosine kinases enzymatic activity is important to promote reorganization of the cytoskeleton. Because we found that both in normal (Figs. 2 and 3) and CGD (Figs. 6 and 7) PMN adhesion induced a slight increase of p58<sub>fgr</sub> and p53/56<sub>lyn</sub> independently of ROI production, it is therefore possible that this ROI-independent Src family kinases activity may be sufficient to trigger the cascade of events leading to the formation of an actin-based cytoskeleton. In addition, it is of interest that in adherent PMN, activation of p72<sub>syk</sub> is totally independent of ROI production (Fig. 10). ROI-independent activities of p58<sub>fgr</sub> and p53/56<sub>lyn</sub> or p72<sub>syk</sub> activity might contribute to tyrosine phosphorylation of endogenous substrates, which, in fact was only partially inhibited in adherent CGD PMN (Fig. 10).

**Fig. 9. Tyrosine phosphorylation of cytoskeleton-associated proteins is only partially affected in CGD PMN.** Conditions of incubation and assay were as described in the legend to Fig. 8. Tyrosine phosphorylated proteins were detected by Western blot analysis.

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<sup>3</sup>S. R. Yan, M. Huang, and G. Berton, manuscript in preparation.
concentrations of H$_2$O$_2$ at least 50–100-fold higher than those actually produced by PMN are required to increase p58$^{c-fgr}$ and p53/56$^{lyn}$ kinase activity (see "Results").

H$_2$O$_2$ was shown to induce tyrosine phosphorylation of multiple cellular proteins in different cell types, and its effects were attributed to either a capability to inhibit protein-tyrosine phosphatases or to activate tyrosine kinases (see Refs. 22–37).

An inhibitory effect of H$_2$O$_2$ on the phosphatase activity of CD45 has been reported (24, 32). We do not know the role of CD45 in our experimental system. CD45 regulates Src family kinase activity in lymphocytic cells through dephosphorylation of the regulatory tyrosine present at the C terminus of these kinases (48–50). Therefore, it is unlikely that inhibition of CD45 could be responsible for activation of p58$^{c-fgr}$ and p53/56$^{lyn}$ in our experimental system. Src family kinases can be also regulated by tyrosine phosphorylation of the tyrosine present within their kinase domain (49, 50). Interestingly, it was recently demonstrated that H$_2$O$_2$ regulates p56$fgr$ kinase activity by inducing the tyrosine phosphorylation of the so-called autophosphorylation site, an effect that was suggested to be mediated by a yet unidentified protein kinase distinct from p56$hck$ (28). We found that H$_2$O$_2$ activated p58$^{c-fgr}$ and p53/56$^{lyn}$ only in intact cells, thus suggesting that its target is a molecule regulating the activity of the two kinases. Whichever is the mechanism of action of H$_2$O$_2$ in increasing p58$^{c-fgr}$ and p53/56$^{lyn}$ activities in adherent PMN, this may be relevant for regulation of specific cell functions upon adhesion to endothelial cells or extracellular matrix proteins. For example, PMN transcribe cytokine genes and release cytokines (51), and tyrosine kinases regulate gene transcription (50, 52).

The findings described in this and previous papers (13, 19, 20) can be explained by the model proposed in Fig. 11. This model, which proposes possible mechanisms of signaling by PMN integrins, is also based on recent findings on signaling by $\beta_1$ integrins in other cell types (53–55).

According to this model, activation of integrin adhesiveness ("inside-out signaling") by receptor agonists, such as TNF, activate PMN integrin adhesive activities (inside-out signaling). Cytoskeletal proteins, i.e. $\alpha$-actinin and filamin (56, 57), together with tyrosine kinases promote the formation of an actin-based cytoskeleton. Fgr and Hck play an essential role in integrin signaling in PMN (13). The role of Src family kinases may be in part independent on their tyrosine kinase activity (see the text for major details). TNF and/or tyrosine kinases can promote a partial assembly of NADPH oxidase (thick arrows; Refs. 4, 11, and 43). H$_2$O$_2$ produced by NADPH oxidase causes a full activation of p58$^{c-fgr}$ and p53/56$^{lyn}$ and the potential recruitment of other signaling molecules. Integrin-dependent adhesion activates p72$^{tyk}$ independently of ROI formation. Regulation of p58$^{c-fgr}$ and p53/56$^{lyn}$ activities by H$_2$O$_2$ requires redistribution of the two kinases to the cytoskeleton. This finding suggest the existence of a possible link between activation of Src family kinases by oxidants and integrins. See text for major details.

The finding presented in this paper demonstrate that endo-
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...genously produced ROI participate in amplifying signals originating from PMN integrins, thus adding further complexity to the mechanisms of signaling by adhesion. It is tempting to speculate that ROI, which trigger gene transcription in different cell types, may act by cooperating with integrin-dependent signals, i.e. modulating the activity of tyrosine kinases distributed to sites of integrin clustering. Our findings encourage studies along this line.

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J. Biol. Chem. 1996, 271:23464-23471.
doi: 10.1074/jbc.271.38.23464

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