Endogenous Reactive Oxygen Intermediates Activate Tyrosine Kinases in Human Neutrophils*

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In response to invading microorganisms, neutrophils produce large amounts of superoxide and other reactive oxygen intermediates (ROI) by assembly and activation of a multicomponent enzyme complex, the NADPH oxidase. While fulfilling a microbicidal role, ROI have also been postulated to serve as signaling molecules, because activation of the NADPH oxidase was found to be associated with increased tyrosine phosphorylation (Fialkow, L., Chan, C. K., Grinstein, S., and Downey, G. P. (1993) J. Biol. Chem. 268, 17131-17137). The mechanism whereby ROI induces phosphoryrosine accumulation was investigated using electroporated neutrophils stimulated with guanosine 5'-O-3-thiotriphosphate in order to bypass membrane receptors. In vitro immune complex assays and immunoblotting were used to identify five tyrosine kinases present in human neutrophils. Of these, p56/59hck, p72syk, and p77hck were activated during production of ROI. Interestingly, the in vitro autoposphorylation activities of p53/p56hck and p59fgr were found to decline with ROI production. The mode of regulation of p56/59hck was explored in detail. Oxidizing agents were unable to activate p56/59hck in vitro and, once activated in situ, reducing agents failed to inactivate it, suggesting that the effects of ROI are indirect. Tyrosine phosphorylation of p56/59hck paralleled its activation, and dephosphorylation in vitro reversed the stimulation. We therefore conclude that tyrosine phosphorylation is central to the regulation of p56/59hck and likely also of p77hck which is similarly phosphorylated upon activation of the oxidase. Because ROI have been shown to reduce the activity of tyrosine phosphatases, we suggest that this inhibition allows constitutively active kinases to auto/transthosphorylate on stimulatory tyrosine residues, leading to an increase in their catalytic activity. Enhanced phosphoryrosine accumulation would then result from the combined effects of increased phosphorylation with decreased dephosphorylation.

Neutrophils play a central role in host protection against infection, killing pathogens by a series of rapid and highly regulated responses. These include chemotaxis, phagocytosis, secretion of anti-microbial agents, and generation of reactive oxygen intermediates (ROI)1 (reviewed by Sha’afi and Molski (1988)). Production of ROI is mediated by a multicomponent enzyme complex, the NADPH oxidase, present in the membrane of neutrophils and other leukocytes (Morel et al., 1991). Functional assembly of the oxidase facilitates the transfer of one electron from cytosolic NADPH to molecular oxygen, producing superoxide. Dismutation of superoxide in turn generates hydrogen peroxide, and both of these molecules can further generate other reactive oxygen intermediates, including hypochlorous acid, hydroxyl radical, and peroxynitrite (Halliwell and Gutteridge, 1990). Although the mechanisms whereby NADPH oxidase-derived ROI attack microbial targets are not completely understood, their importance in host defense is highlighted by a rare genetic disorder, chronic granulomatous disease. Patients afflicted with this disorder lack the ability to produce ROI and, as a result, suffer from chronic and recurring infections that can be lethal (Smith and Curnutte, 1991).

Although neutrophils are probably the most efficient source of superoxide, virtually all eukaryotic cells produce ROI, primarily as side products of electron transfer reactions in mitochondria and the endoplasmic reticulum (Halliwell and Gutteridge, 1985). In addition to their microbicidal role in phagocytosis, ROI have been suggested to act as signaling molecules in other cells (Schreck and Bauerele, 1991). In principle, ROI constitute good candidate signaling molecules because they are small, rapidly diffusible, and highly reactive. Moreover, both intra- and extracellular concentrations of ROI can be rapidly scavenged by several enzymes, including superoxide dismutase, catalase, and the glutathione peroxidase system, allowing tight control of ROI concentrations and rapid termination of signals. The notion that reactive, small inorganic molecules can function as intracellular signals is supported by the well-established role of nitric oxide in the regulation of vascular tone, neurotransmission, and cell-mediated immune responses (Nathan and Xie, 1994). By comparison, much less is known about the role of ROI in signaling, but suggested targets include the transcription factor NF-kB (Schreck et al., 1991), tyrosine phosphatases (Hecht and Zick, 1992, Fialkow et al., 1994), and phospholipase A2 (Zor et al., 1993).

Recent observations suggested a role for ROI in neutrophil signal transduction (Fialkow et al., 1993). Neutrophils stimulated to produce ROI were reported to undergo increased tyrosine phosphorylation of several proteins. Exogenous oxidants were able to mimic this response, whereas anti-oxidants could

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1 The abbreviations used are: ROI, reactive oxygen intermediate(s); GTPγS, guanosine 5'-O-(3-thiotriphosphate); NaV, sodium orthovanadate; NAC, N-acetylcysteine; PAG, polyacrylamide gel electrophoresis; MOPS, N-(morpholino)propanesulfonic acid.
block it. Several lines of evidence suggested that ROI generated by the NADPH oxidase were responsible for the effect, including the finding that the increased tyrosine phosphorylation failed to occur in neutrophils from patients with chronic granulomatous disease. As much as tyrosine phosphorylation is an important mediator in the regulation of anti-microbial responses (Berkow and Dodson, 1990; Grinstein and Furuya, 1991), ROI may play an important role in the control of auto-paracrine signaling at sites of inflammation.

The extent of tyrosine phosphorylation is determined by the activity of two competing enzyme families, tyrosine kinases and phosphatases. Earlier in vitro (Hecht and Zick, 1992) and in vivo (Zor et al., 1993) studies have suggested that ROI can inhibit the activity of certain tyrosine phosphatases by oxidation of a conserved cysteine residue within their catalytic domain. Although the inhibition of tyrosine phosphatases may account for the elevated tyrosine phosphorylation induced by ROI, increased activity of tyrosine kinases could conceivably contribute to the response. Indeed, tyrosine kinases have been reported to be activated in lymphocytes by oxidizing agents (Bauskin et al., 1991; Nakamura et al., 1993). For these reasons, we investigated whether endogenous ROI generated by the NADPH oxidase affected the activity of tyrosine kinases in human neutrophils.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ficoll 400, dextran T500, protein A-Sepharose, and Sepharose beads were purchased from Pharmacia. Medium RPMI, K-ATP, GTP-γS, NADPH, sodium orthovanadate (NaV), N-acetylcysteine (NAC), diamide, and dithiothreitol were from Sigma. Hydrogen peroxide was from Fisher. Bovine serum albumin and acrylamide were from Boehringer Mannheim. 2′-5′-PATP and MOPS were from ICN. Truncated T-cell phosphatase was the generous gift of Dr. C. Diltz (Department of Medicine, University of Washington, Seattle, WA). Diphosphoglycerate was synthesized in our laboratory as described previously (Collette et al., 1995).

**Antibodies**—Anti-sera to src family tyrosine kinases were generated as described (Li et al., 1992). Anti-sera were generated against syk (Kien et al., 1993), zap-70 (Tsyganov et al., 1994), and hck (Mahajan et al., 1995) as described. Monoclonal antibodies (4G-10 clone) to phosphotyrosine (free or conjugated to agarose beads) were from UBI. Goat anti-rabbit antibodies conjugated to horseradish peroxidase were from Jackson Immunotech and donkey anti-mouse antibodies conjugated to horseradish peroxidase were from Amersham Corp.

**Solutions**—Bicarbonate-free RPMI 1640 medium was buffered to pH 7.3 with 25 m M Na-HEPES. Permeabilization medium contained 140 mM KCl, 10 mM HEPES, 1 mM MgCl₂, 1 mM EGTA, 1 mM CaCl₂, 1 mM K-ATP, and 10 mM K-HEPES, pH 7.0. Phosphate-buffered saline (pH 7.4) was from Pierce. All medium was adjusted to 250 ± 5 mosm with the major salt.

**Cell Isolation and Permeabilization**—Neutrophils were isolated from fresh heparinized blood of healthy human volunteers by dextran sedimentation, followed by Ficoll-Hypaque gradient centrifugation. Contaminating red cells were removed by NH₄Cl lysis. Neutrophils were counted using a Model ZM Coulter Counter, resuspended in HEPES-buffered RPMI medium at 10⁷ cells/ml, and maintained in this medium at room temperature until use. To minimize proteinosis following extraction of cells in detergent, the cells (10⁶/ml) were pretreated with 5 mM diisopropyl fluorophosphate (Calbiochem) for 30 min at room temperature. For electroporation, 1.5 × 10⁷ cells were sedimented and resuspended in 1 ml of ice-cold permeabilization medium. This suspension was transferred to a Bio-Rad Pulser cuvette and permeabilized with two discharges as described previously (Grinstein and Furuya, 1988). The cells were rapidly sedimented and resuspended in fresh, ice-cold permeabilization medium between discharges. Electroporated cells were used immediately. Where indicated, cells were preincubated for 2 min at 37°C in the presence of 2 mM diaphosphoglycerate or 2 mM NAC prior to stimulation.

**SDS-PAGE and Immunoblotting**—SDS-PAGE and immunoblotting were performed essentially as described (Brumell and Grinstein, 1994). Briefly, samples were subjected to electrophoresis in 12% acrylamide gels and blotted onto polyvinylidene fluoride membranes (Millipore). Neutrophil whole cell lysates and immunoprecipitates were blotted with monoclonal anti-phosphotyrosine antibodies (1:5000 dilution) or with anti-sera to the specified tyrosine kinase (1:2000 dilution). Donkey anti-mouse and goat anti-rabbit secondary antibodies coupled to horseradish peroxidase were used at a 1:5000 dilution. Detection was made using the enhanced chemiluminescence system from Amersham Corp. Quantitation of radiograms was performed by densitometry using a Protein Databases Inc. (Huntington Station, NY) model DNA 35 scanner and the Discovery series software.

Immunoprecipitation and Immunocomplex Kinase Assay—Electroporated cells (1.5 × 10⁶/ml) were solubilized in ice-cold lysis buffer, which contained 1% Nonidet P-40, 150 mM NaCl, 2 mM EGTA, 1 mM NaV, 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 µM pepstatin A, and 50 mM Tris-HCl, pH 8.0. Lysates were centrifuged at 100,000 × g for 5 min to remove unbroken cells and insoluble debris and then prewarmed with 50 µl of Sepharose beads. Antibodies to the indicated tyrosine kinase or to phosphotyrosine were incubated with these lysates for 2 h at 4°C while rotating end over end. Immunocomplexes were precipitated by addition of 100 µl of a 50% slurry of protein A-Sepharose beads, previously blocked with 10% bovine serum albumin in lysis buffer, followed by incubation at 4°C for 2 h. The addition of beads was unnecessary for phosphotyrosine antibodies, which were covalently attached to Sepharose. The immunoprecipitates were washed 4–6 times and then resuspended in 1 ml of lysis buffer. Equal aliquots of this suspension were used for immunoblotting and for analysis of in vitro kinase activity.

The kinase activity of immunocomplexes was determined essentially as described (Burkhardt and Bolen, 1993). In brief, immunoprecipitates were washed with 1 ml of kinase buffer (5 mM MnCl₂, 20 mM MOPS, pH 7.0), and autophosphorylating activity was assayed by incubation with 25 µl of kinase buffer containing 12.5 µCi of [γ-32P]ATP and 1 µM K-ATP. Where specified, 1 µg of rabbit muscle enolase was included as an exogenous substrate. Samples were incubated at 25°C in an Eppendorf Thermomixer, and reactions were stopped by the addition of boiling 2× concentrated Laemmli sample buffer. The samples were subjected to SDS-PAGE, and the gels were stained with Coomassie Blue and dried in gel wrap (Biodesign Inc.). Dried gels were used for direct quantitation of radioactivity with a Molecular Dynamics PhosphoImager using Imagequant software or were subjected to radiography with an intensifying screen, Seay (U. Colorado). To study the effect of oxidizing agents on the tyrosine kinase activity of hck, immunoprecipitates of this kinase were isolated from untreated, electroporated cells. After washing, immune complexes were treated for 30 min with 1 mM diamide or 1 mM hydrogen peroxide at 30°C while shaking in a Thermomixer. As above, identical aliquots were used in parallel for immunoblotting and in vitro kinase assay. To study the effect of reducing agents on hck, immune complexes from GTP-γS-stimulated cells were treated with 20 mM NAC or 1 mM dithiothreitol for 30 min at 37°C and processed as above.

To study the role of tyrosine phosphorylation in hck activation, immunoprecipitates obtained from GTP-γS-treated cells were incubated at 37°C for 30 min with or without 2 µg/ml of T-cell phosphatase. Lysates of the beads were subjected to kinase assays, and for immunoblotting with anti-phosphotyrosine antibodies to confirm the effectiveness of dephosphorylation by T-cell phosphatase.

**RESULTS**

**NADPH Oxidase-derived ROI Induce Tyrosine Phosphorylation**—The effect of ROI on tyrosine phosphorylation was studied in electroporated, activated cells treated with GTP-γS. This approach was chosen for two reasons. First, direct stimulation of GTP-binding proteins bypasses cell surface receptors, circumventing possible direct effects of the latter on the kinases and obviating receptor down-regulation, which can greatly reduce the magnitude and duration of the respiratory burst (reviewed by Klotz and J esaitis (1994)). Using GTP-γS, activation of the oxidase is sustained, resembling the physiological stimulation elicited by phagocytic stimuli (Grinstein and Furuya, 1991). Second, equilibration of the permeabilized cells with EGTA-containing buffers precludes changes in cytosolic calcium concentration, which might alter tyrosine phosphorylation (Berkow and Dodson, 1990).

As shown in Fig. 1A, the addition of GTP-γS and NADPH to
permeabilized cells induced the accumulation of phosphotyrosine on a number of proteins, as determined by immunoblotting (cf. lanes 1 and 4). Treatment of the electroporated cells with GTP·S and NADPH alone was found to have little effect (lanes 2 and 3). The stimulatory effect of GTP·S or NADPH was moderated by the presence of active tyrosine phosphatases. This is indicated by the pronounced enhancement in phosphotyrosine accumulation noted when vanadate, a phosphatase inhibitor, was included during stimulation (Fig. 1A). For this reason, 10 μM sodium orthovanadate was included routinely in subsequent assays to minimize dephosphorylation, thereby magnifying the responses. At the concentration used, vanadate itself had negligible effects on tyrosine phosphorylation (see lanes 1 and 2 in Fig. 1C), consistent with earlier findings (Bourgoin and Grinstein, 1992). Moreover, whereas vanadate increased the extent of phosphotyrosine accumulation, the phosphorylated substrates and the time course of phosphorylation were similar in the presence and the absence of the phosphatase inhibitor. As illustrated in Fig. 1B, phosphotyrosine accumulation induced by GTP·S stimulation was rapid (evident after 1 min) and time-dependent, with a maximal response seen after 10 min.

FIG. 1. Effect of GTP·S on tyrosine phosphorylation. A, NADPH dependence and potentiation by sodium vanadate. Electroporated neutrophils were incubated at 37°C without (−) or with (+) the following agents for 5 min: 10 μM GTP·S, 2 mM NADPH, and 10 μM NaV, as indicated. Cells were then rapidly sedimented, boiled in sample buffer, and subjected to SDS-PAGE. Analysis was performed by immunoblotting with a monoclonal antibody to phosphotyrosine. B, time course of phosphotyrosine accumulation. Electroporated neutrophils were treated without (−) or with (+) 10 μM GTP·S, 2 mM NADPH, and 10 μM NaV for the indicated time (min) and processed as in A. C, dependence of tyrosine phosphorylation on NADPH oxidase-derived ROI. Electroporated neutrophils were treated without (−) or with (+) 10 μM GTP·S and/or 2 mM NADPH for 5 min at 37°C. Where specified, the cells were treated with 2 mM diphenylene iodonium (DPI) or 2 mM NAC for 2 min at 37°C prior to GTP·S stimulation. The presence of 10 μM NaV during treatment is indicated. The results shown are representative of three separate experiments.

The effect of GTP·S on tyrosine phosphorylation was entirely dependent on the presence of NADPH. As shown in Fig. 1C (as well as in Fig. 1A), treatment of electroporated cells with GTP·S had little effect when the nucleotide was omitted (cf. lanes 3 and 4). This finding suggests that generation of superoxide by the NADPH oxidase is required for the increase in tyrosine phosphorylation following stimulation with GTP·S. In support of this hypothesis, we found that NAC, a powerful anti-oxidant that has been shown to scavenge ROI and increase cytosolic levels of reduced glutathione (Halliwell and Gutteridge, 1985), effectively attenuated the tyrosine phosphorylation produced by GTP·S in the presence of NADPH. Moreover, diphenylene iodonium, an inhibitor of the flavoprotein component of the NADPH oxidase (Ellis et al., 1988), had a comparable effect (lane 6). These findings are in agreement with those of Fialkow et al. (1993) and indicate that NADPH oxidase-derived ROI promote tyrosine phosphorylation in neutrophils.

Tyrosine Kinases Present in Neutrophils—As an initial step in the study of the mechanism of action of ROI, we determined which of the known tyrosine kinases are present and active in GTP·S-stimulated neutrophils. Electroporated cells were activated with the nucleotide and immediately solubilized for immunoprecipitation with one of a battery of antibodies to tyrosine kinases. The immune complexes were used for in vitro kinase assays and then subjected to SDS-PAGE and autoradiography. Of the 11 antibodies tested, 5 were found to immunoprecipitate active kinases detectable by autophosphorylating activity, suggesting by the close correspondence of the phosphorylated bands to the known molecular weight of the kinase immunoprecipitated (Fig. 2A). The active kinases included three src family members; lyn (53 and 56 kDa), hck (56 and 59 kDa), and fgr (59 kDa). Also included were syk (72 kDa) and btk (77 kDa). In contrast, no significant activity was measurable in src, fyn, yes, blk, lck, and zap-70 immunoprecipitates or when rabbit nonimmune serum (Fig. 2A, cont.) was used. The presence of lyn, hck, fgr, syk, and btk in neutrophils has been reported previously (Yamashita et al., 1987; Ziegler et al., 1987; Gutkind and Robbins, 1989; Asahi et al., 1993; Yamada et al., 1993).

In good agreement with the kinase assays of Fig. 2A, the presence of lyn, hck, fgr, syk, and btk in neutrophils was confirmed by immunoblotting whole cell lysates with the same antisera used for precipitation (Fig. 2B). Both the full-length (72 kDa) syk protein as well as its ~40-kDa degradation product were observed upon immunoblotting (Fig. 2B, closed arrowheads). A band of ~65 kDa was also recognized by the syk anti-serum. It is not presently clear whether this polypeptide is related to syk or is merely a fortuitously cross-reacting protein. It is noteworthy, however, that a band of similar mobility was often found to be phosphorylated in syk immune complex assays (Fig. 3A), suggesting that the 65-kDa polypeptide co-immunoprecipitates and can be phosphorylated by syk.

Modulation of Tyrosine Kinase Activity by ROI—The effect of ROI on neutrophil kinase activity was studied next. To this end, immunoprecipitates were prepared from control and

2 Hereafter, "GTP·S stimulation" refers to treatment of electroporated neutrophils with 10 μM GTP·S in the presence of 2 mM NADPH and 10 μM NaV at 37°C.

3 We observed a phosphoprotein of about 60–62 kDa in lyn immunoprecipitates. Lyn has been extensively studied and only two isoforms have been identified, suggesting that the third band may be a separate protein. Several kinases have been shown to co-precipitate with lyn in other systems, including syk (Sidorenko et al., 1995), btk (Cheng et al., 1994), and a cell cycle regulatory protein, p34cdc2 (Yuan et al., 1995).
mechanism of tyrosine kinase activation by endogenous ROI was studied in detail for hck. This kinase was chosen because it is virtually quiescent in unstimulated cells yet is the most active in immunoprecipitates from activated cells, providing an optimal signal to noise ratio. We first considered the possibility that activation of hck by ROI resulted from direct oxidation of critical residues on the kinase. To test this notion, hck immunoprecipitates obtained from unstimulated cells were treated in vitro with two strong oxidizing agents, diamide and H₂O₂. Comparable concentrations of these oxidants have been shown to promote phosphotyrosine accumulation when added to intact cells (Fialkow et al., 1994). Neither diamide nor H₂O₂, however, was capable of activating isolated hck in immune complexes (Fig. 4A). Conversely, we found that reducing agents could not reverse the activation of hck immunoprecipitated from lysates of GTPγS-treated cells. As shown in the rightmost lanes of Fig. 4A, when added directly to the immunoprecipitate neither NAC nor dithiothreitol diminished the activation of hck. These findings contrast the preventive effect of NAC seen when added to permeabilized cells during the respiratory burst, described in Fig. 1B.

The inability of oxidants and reducing agents to affect hck autophosphorylation was confirmed by immunoblotting the immunoprecipitates with anti-phosphotyrosine antibodies (Fig. 1B). The kinase was found to be tyrosine-phosphorylated only after stimulation of the cells with GTPγS, and the phosphotyrosine content was unaffected by oxidizing and reducing agents. Together, these results suggest that ROI do not directly activate hck in GTPγS-stimulated neutrophils.

![Image 1](https://example.com/image1.png)

**Fig. 2.** Identification of tyrosine kinases present in neutrophils. A, immune complex kinase assays were performed in vitro using immunoprecipitates of the tyrosine kinases indicated, prepared from lysates of electroporated neutrophils treated with 10 μM GTPγS, 2 mM NADPH, and 10 μM NaV for 2 min. Kinase reactions were stopped, and the material was subjected to SDS-PAGE and autoradiography of the dried gel. The assay was also performed using a rabbit nonimmune serum (cont). B, whole neutrophil lysates were immunoblotted with antisera to the tyrosine kinases indicated. The closed arrowheads point to the tyrosine kinase. The open arrowhead indicates an unidentified protein of ~65 kDa that cross-reacts with the syk antibody.

![Image 2](https://example.com/image2.png)

**Fig. 3.** Modulation of tyrosine kinase activity by ROI. A, immune complex kinase assays were performed in vitro using immunoprecipitates of the tyrosine kinases indicated, prepared from lysates of electroporated neutrophils treated without (−) or with (+) 10 μM GTPγS, 2 mM NADPH, and 10 μM NaV for 1 min. B, immunoprecipitates of hck were prepared from lysates of electroporated neutrophils treated without or with 10 μM GTPγS, 2 mM NADPH, and 10 μM NaV for the indicated time (min) and subjected to immune complex kinase assays in the presence of enolase. The kinase reactions were stopped, and the samples were subjected to SDS-PAGE followed by autoradiography of the dried gels. A representative experiment is shown in the inset. Bands that correspond to autophosphorylation (closed arrow) and enolase phosphorylation (open arrow) were quantified with a PhosphorImager, and the results are presented as the percentage of maximal response in the main panel. C, in vitro autophosphorylation and enolase phosphorylation activities of lyn were determined as in B for hck. The data in B and C are the means ± S.E. of three experiments.

Role of Direct Oxidation in hck Activation by ROI—The GTPγS-stimulated cells using antisera to the kinases identified earlier in Fig. 2. As shown in Fig. 3A, the in vitro activity of hck, syk, and btk was noticeably increased following stimulation with GTPγS. The stimulation of hck was investigated in more detail in Fig. 3B, where immune complexes obtained at various times after the addition of GTPγS were assayed in the presence of the exogenous substrate enolase. Both the autophosphorylation of hck (closed arrowhead in inset to Fig. 3B) and its ability to phosphorylate enolase (open arrowhead) followed a biphasic course, peaking between 1–5 min and declining thereafter. A similar increase in the ability of syk and btk to phosphorylate enolase was also observed (data not shown). It should be noted that qualitatively similar responses of hck and lyn were seen when NaV was omitted during stimulation of electroporated cells (data not shown).

Although production of ROI stimulated some tyrosine kinases, others were seemingly inhibited. The autophosphorylating abilities of lyn and fgr were diminished (73 ± 14% and 48 ± 16% of control activity, respectively; n = 3) following 1 min of GTPγS stimulation (Fig. 3A). As for hck, the detailed time course of the effects of ROI on lyn activity was analyzed with enolase as substrate (see Fig. 3C). Interestingly, quantitation of the auto- and enolase-phosphorylating activities of lyn immune complexes revealed a discrepancy. Phosphorylation of the exogenous substrate was markedly increased, whereas autophosphorylation decreased. These findings suggest that non-radioactive phosphate is incorporated into lyn in the cells, prior to immunoprecipitation, precluding subsequent incorporation of radiolabel into these sites. The reduced autophosphorylation is therefore an inaccurate indication of the enzymatic activity of lyn, which is at least transiently stimulated by GTPγS.
Role of Tyrosine Phosphorylation in the Regulation of Kinase Activity—Phosphorylation on tyrosine residues has been shown to be an important determinant of the activity of several tyrosine kinases, including those identified in neutrophils. Because tyrosine phosphorylation of hck was detectable when this enzyme was precipitated from stimulated cells (Fig. 4 B), we considered the possibility that ROI activate kinases in neutrophils indirectly by mediating their phosphorylation on tyrosine residues. As an initial approach to test this hypothesis, we tested the kinase activity of immunocomplexes obtained from control and GTP\(\gamma\)S-stimulated cells using anti-phosphotyrosine antibodies. The data in Fig. 5 demonstrate that phosphorylation was greater in precipitates from stimulated cells, suggesting that the relevant kinase activity may be associated with tyrosine phosphorylated proteins. A number of prominent bands displayed increased phosphorylation \textit{in vitro} (arrowheads in Fig. 5) with molecular masses of approximately 48, 54, 62, 68, 75, and 118 kDa.

The size of some of the phosphoproteins in phosphotyrosine immunoprecipitates correspond to that of the active kinases detailed in Fig. 2. To establish more directly whether the active kinases are tyrosine phosphorylated, immunoprecipitates of lyn, hck, fgr, syk, and btk were prepared from control and GTP\(\gamma\)S-treated cells and probed by immunoblotting with anti-phosphotyrosine antibodies. As illustrated in Fig. 6A, endogenous generation of ROI is accompanied by tyrosine phosphorylation of all the kinases studied (indicated with closed arrowheads), with the notable exception of btk, which remained unaffected. The figure also shows that both the intact form of syk as well as its 40-kDa proteolytic fragment (open arrowhead) was phosphorylated on tyrosine.

The correlation between the occurrence of tyrosine phosphorylation and the activation of the tyrosine kinases is further stressed by the similarity of the time courses of both events. In Fig. 6B, the degree of tyrosine phosphorylation was quantified in immunoprecipitates from cells stimulated for varying peri-
ROI-mediated Tyrosine Kinase Activation

In this report, we analyzed the mechanism leading to increased phosphotyrosine accumulation following ROI production in neutrophils. In electroporated cells treated with GTPγS, we detected an elevated activity of several kinases, measured in vitro. The activation of these kinases was rapid and correlated with the increase in tyrosine phosphorylation observed under these conditions.

Kinases of three separate families were found to be activated by ROI, as determined by autophosphorylation and phosphorylation of an exogenous substrate, enolase. hck, a member of the src family of tyrosine kinases and highly expressed in granulocytes and macrophages (Ziegler et al., 1987), displayed little activity in untreated cells but was rapidly stimulated following the addition of GTPγS. syk, which belongs to a separate family of kinases, also displayed increased activity following ROI production. In contrast, the closely related ZAP-70 tyrosine kinase, thought to be important in B- and T-cell receptor signaling (Sefton and Taddie, 1994) was not detectable in active neutrophils using our immune complex kinase assay. btk, a member of the tcr family of tyrosine kinases, is expressed in cells of myeloid and lymphoid lineage (Yamada et al., 1993) and was also activated by ROI. To our knowledge, activation of btk in neutrophils had not been reported previously.

Although ROI production led to the activation of some tyrosine kinases, it appeared to have an opposite effect on the activity of others when estimated from autophosphorylation in immune complex kinase assays. Thus, lyn and fgr displayed high activities in untreated, electroporated neutrophils, which decreased following GTPγS stimulation. However, at least in the case of lyn, the apparent decrease in activity likely reflected occupancy of substrate sites by nonradioactive phosphate, which may have occurred in situ, prior to immunoprecipitation. Indeed, the ability of the enzyme to phosphorylate exogenous substrates was increased following stimulation of the respiratory burst. Therefore, caution must be exercised when equating the autophosphorylating and catalytic activities of tyrosine kinases.

None of the other tyrosine kinases tested were found to be activated following generation of ROI. These included yes, which is reported to be present in neutrophils, where it can be stimulated by granulocyte macrophage colony-stimulating factor (Corey et al., 1993). Clearly, although 11 different antisera were used, our survey was incomplete, because other tyrosine kinases are likely to exist in neutrophils.

The mechanism underlying the activation of the kinases by ROI was explored in some detail using hck as a prototype. Although ROI production in situ appeared to activate hck, oxidizing agents could not mimic this effect when applied to hck immunoprecipitates in vitro. Moreover, reducing agents failed to reverse the activation of hck isolated from GTPγS-treated cells. We conclude that hck activity is not regulated directly by ROI but rather by some other post-translational modification. Though not tested directly, we suggest by extension that activation of the other kinases is similarly indirect.

Because tyrosine phosphorylation of lyn, hck, fgr, and syk was found to occur upon stimulation by GTPγS, this post-translational modification was considered as a possible mechanism of regulation. This notion was evaluated using T-cell phosphatase to dephosphorylate activated hck. This procedure was found to eliminate the activity of the kinase, suggesting that tyrosine phosphorylation mediates the effect of ROI on hck activation.

Tyrosine kinase activity of src family members is thought to be suppressed by phosphorylation of a C-terminal residue, conserved among family members (Cooper, 1988; Liu et al., 1993; Cooper and Howell, 1993). Dephosphorylation of this residue has been shown to increase the activity of src family kinases (Cooper and King, 1987), in apparent conflict with our findings with hck, where complete dephosphorylation of the enzyme led to its inactivation. However, recent evidence has questioned this simple model of regulation. This includes the finding that a T-cell line lacking CD45 (the phosphatase that activates the src family member lck) was found to have higher lck activity even though its inhibitory C-terminal tyrosine residue was hyperphosphorylated (Burns et al., 1994). Although dephosphorylation of the C terminus may be important for the derepression of src family members, a number of unique tyrosine resi-
dyes have been reported to be phosphorylated upon activation. These include the so-called autophosphorylation site within the kinase domain (Smart et al., 1981; Patchinsky et al., 1982) and sites within the N-terminal domain of some src-family members (Souda et al., 1993). Evidence exists that phosphorylation of these residues is essential for kinase activity, possibly by stabilizing the active kinase (Veillette and Fournel, 1990; Mustelin, 1994). Recent evidence has also implied serine phosphorylation in the regulation of src-family members (Winkler et al., 1993; Watts et al., 1993). Whereas the regulation of src-family kinases remains incompletely understood, our findings imply that tyrosine phosphorylation is necessary to maintain the activity of hck following activation by endogenous ROI.

The steps that follow ROI generation and lead to kinase phosphorylation are unknown, but some insight is provided by recent reports that (a) critical conserved cysteine residues exist in the catalytic domain of many tyrosine kinases (Fisher et al., 1991) and (b) that both exogenous (Zor et al., 1993; Fialkow et al., 1994) as well as endogenous oxidants4 can inactivate tyrosine phosphatases, likely by targeting their critical sulphydryl moieties. In view of these considerations, the following scenario can be envisaged. Under basal conditions, the accumulation of tyrosine phosphoproteins and the autophosphorylation and stabilization of tyrosine kinases, some of which are constitutively active, are prevented by theOffsettingaction of tyrosine phosphatases. This delicate balance can be disrupted when ROI diminish the rate of dephosphorylation by one or more tyrosine phosphatases. Indeed, in neutrophils, the reaction with sulphydryl side chains in the catalytic domain of CD45 has been shown to be susceptible to inactivation by ROI or other cell products present in the inflammatory milieu, including lymphocytes and macrophages.

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