Regulation of Src Homology 2-containing Tyrosine Phosphatase 1 during Activation of Human Neutrophils

ROLE OF PROTEIN KINASE C*

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The tyrosine phosphorylation of several proteins induced in neutrophils by soluble and particulate stimuli is thought to be crucial for initiating antimicrobial responses. Although activation of tyrosine kinases is thought to mediate this event, the role of tyrosine phosphatases in the initiation and modulation of neutrophil responses remains largely undefined. We investigated the role of Src homology 2-containing tyrosine phosphatase 1 (SHP-1; also known as protein tyrosine phosphatase 1C (PTP1C), hematopoietic cell phosphatase, PTP-N6, and SHPTP-1), a phosphatase expressed primarily in hemopoietic cells, in the activation of human neutrophils. SHP-1 mRNA and protein were detected in these cells, and the enzyme was found to be predominantly localized to the cytosol in unstimulated cells. Following stimulation with neutrophil agonists such as phorbol ester, chemotactic peptide, or opsonized zymosan, a fraction of the phosphatase redistributed to the cytoskeleton. Agonist treatment also induced significant decreases (30–60%) in SHP-1 activity, which correlated temporally with increases in the cellular phosphotyrosine content. Phosphorylation of SHP-1 on serine residues was associated with the inhibition of its enzymatic activity, suggesting a causal relationship. Accordingly, both the agonist-evoked phosphorylation of SHP-1 and the inhibition of its catalytic activity were blocked by treatment with bisindolylmaleimide I, a potent and specific inhibitor of protein kinase C (PKC) activity. Immnoprecipitated SHP-1 was found to be phosphorylated efficiently by purified PKC in vitro. Such phosphorylation also caused a decrease in the phosphatase activity of SHP-1. Together, these data suggest that inhibition of SHP-1 by PKC-mediated serine phosphorylation plays a role in facilitating the accumulation of tyrosine-phosphorylated proteins following neutrophil stimulation.

These findings provide a new link between the PKC and tyrosine phosphorylation branches of the signaling cascade that triggers antimicrobial responses in human neutrophils.

Polymorphonuclear (PMN)1 leukocytes, particularly neutrophils, destroy pathogenic microorganisms via a series of rapid and coordinated responses that include chemotaxis, phagocytosis, secretion of a variety of granules and vesicles, and production of reactive oxygen intermediates. These responses are mediated by the interaction of cell surface receptors with specific ligands found on microbial targets or in the inflammatory milieu. These receptor-ligand interactions, in turn, activate intracellular signal transduction cascades that couple the activating stimulus to physiological responses (1, 2).

One of the earliest biochemical events that follows receptor engagement is the accumulation of phosphate on tyrosine residues of cellular proteins. Increases in tyrosine phosphorylation can be initiated by a variety of soluble and particulate stimuli and temporally correlate with the appearance of cellular responses (3–5). The importance of tyrosine phosphorylation to neutrophil function is further underlined by the finding that inhibitors of protein tyrosine kinases block many neutrophil responses, including adherence (6), chemotaxis (7), phagocytosis (8), and production of reactive oxygen intermediates (9, 10).

Phosphotyrosine accumulation is regulated by the competing activities of protein tyrosine kinases and protein tyrosine phosphatases (PTPs). Increased activities of tyrosine kinases have been demonstrated in neutrophils treated with chemotactic peptides (11), cytokines (12, 13), and other ligands (14) and have been postulated to account for the increased tyrosine phosphorylation observed following stimulation with these agents. The subcellular localization of tyrosine kinases and their substrates may also play a role in regulating tyrosine phosphorylation. For example, neutrophils contain at least four types of secretory granules within their cytoplasm (15), some of which have been shown to contain tyrosine kinases. Following stimulation, granular fusion with the plasma membrane and/or

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1 The abbreviations used are: PMN, polymorphonuclear; BSA, bovine serum albumin; BIM, bisindolylmaleimide I; IMLP, formyl-Met-Leu-Phe; OPZ, opsonized zymosan; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; PTP, protein tyrosine phosphatase; SHP, Src homology 2-containing tyrosine phosphatase; TPA, 12-O-tetradecanoylphorbol-13-acetate; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid.
phagosome may allow associated kinases access to their substrates (16, 17).

In addition to the effects of tyrosine kinases, decreases in the activity of tyrosine phosphatases may also lead to an increase in cellular tyrosine phosphorylation. In support of this notion, overall neutrophil phosphotyrosine phosphatase activity has been shown to decrease following stimulation with the chemotactrant fMLP or with phorbol esters, although the identities of the particular phosphatases responsible for this effect were not determined (18, 19). Similarly, the inhibition of tyrosine phosphatases with vanadate or its peroxides has been shown to potentiate fMLP-induced superoxide production in whole cells (20) and to activate a respiratory burst in electrophorated cells, providing further evidence that a reduction of phosphatase activity may lead to antimicrobial responses in PMN leukocytes (21, 22).

Although the spectrum of tyrosine phosphatases responsible for regulating neutrophil responses requires further definition, one PTP implicated in neutrophil signaling is CD45. A member of the transmembrane class of tyrosine phosphatases, CD45 has been found on secretory granules and the plasma membranes of neutrophils (23). Stimulation with a variety of agents has been shown to enhance the expression of CD45 on the plasma membrane (24), a phenomenon thought to contribute to the desensitization of neutrophils following further stimulation with fMLP (25). The intrinsic activity of CD45 is also modulated in neutrophils following production of reactive oxygen intermediates by the NADPH oxidase (26). The latter finding is thought to reflect the oxidation of critical cysteine residues within the catalytic domain of CD45 (which is conserved in all tyrosine phosphatases; see Ref. 27) and may represent a unique role for CD45 activity. In addition to oxidant-induced activation of mitogen-activated protein kinase (26), an enzyme thought to represent a substrate of CD45 (29).

In contrast to CD45, little is known about the role of soluble tyrosine phosphatases in neutrophil signal transduction. One such phosphatase, SHP-1 (also known as PTP1C, hematopoietic cell phosphatase, PTP-N6, and SHPTP-1), has recently been extensively studied as a potential regulator of the action of growth promoters in hematopoietic cells (reviewed in Ref. 30). SHP-1 has been shown to dephosphorylate and inactivate the erythropoietin (31, 32), stem cell factor (33), interleukin 3 (34), and growth promoters in hemopoietic cells (reviewed in Ref. 30). SHP-1 has been shown to dephosphorylate and inactivate the erythropoietin (31, 32), stem cell factor (33), interleukin 3 (34), and growth promoters in hemopoietic cells (reviewed in Ref. 30). SHP-1 has been shown to dephosphorylate and inactivate the erythropoietin (31, 32), stem cell factor (33), interleukin 3 (34), and growth promoters in hemopoietic cells (reviewed in Ref. 30). SHP-1 has been shown to dephosphorylate and inactivate the erythropoietin (31, 32), stem cell factor (33), interleukin 3 (34), and growth promoters in hemopoietic cells (reviewed in Ref. 30). SHP-1 has been shown to dephosphorylate and inactivate the erythropoietin (31, 32), stem cell factor (33), interleukin 3 (34), and growth promoters in hemopoietic cells (reviewed in Ref. 30). SHP-1 has been shown to dephosphorylate and inactivate the erythropoietin (31, 32), stem cell factor (33), interleukin 3 (34), and growth promoters in hemopoietic cells (reviewed in Ref. 30). SHP-1 has been shown to dephosphorylate and inactivate the erythropoietin (31, 32), stem cell factor (33), interleukin 3 (34), and growth promoters in hemopoietic cells (reviewed in Ref. 30). SHP-1 has been shown to dephosphorylate and inactivate the erythropoietin (31, 32), stem cell factor (33), interleukin 3 (34), and growth promoters in hemopoietic cells (reviewed in Ref. 30). SHP-1 has been shown to dephosphorylate and inactivate the erythropoietin (31, 32), stem cell factor (33), interleukin 3 (34), and growth promoters in hemopoietic cells (reviewed in Ref. 30). SHP-1 has been shown to dephosphorylate and inactivate the erythropoietin (31, 32), stem cell factor (33), interleukin 3 (34), and growth promoters in hemopoietic cells (reviewed in Ref. 30). SHP-1 has been shown to dephosphorylate and inactivate the erythropoietin (31, 32), stem cell factor (33), interleukin 3 (34), and growth promoters in hemopoietic cells (reviewed in Ref. 30). SHP-1 has been shown to dephosphorylate and inactivate the erythropoietin (31, 32), stem cell factor (33), interleukin 3 (34), and growth promoters in hemopoietic cells (reviewed in Ref. 30). SHP-1 has been shown to dephosphorylate and inactivate the erythropoietin (31, 32), stem cell factor (33), interleukin 3 (34), and growth promoters in hemopoietic cells (reviewed in Ref. 30). SHP-1 has been shown to dephosphorylate and inactivate the erythropoietin (31, 32), stem cell factor (33), interleukin 3 (34), and growth promoters in hemopoietic cells (reviewed in Ref. 30). SHP-1 has been shown to dephosphorylate and inactivate the erythropoietin (31, 32), stem cell factor (33), interleukin 3 (34), and growth promoters in hemopoietic cells (reviewed in Ref. 30). SHP-1 has been shown to dephosphorylate and inactivate the erythropoietin (31, 32), stem cell factor (33), interleukin 3 (34), and growth promoters in hemopoietic cells (reviewed in Ref. 30). SHP-1 has been shown to dephosphorylate and inactivate the erythropoietin (31, 32), stem cell factor (33), interleukin 3 (34), and growth promoters in hemopoietic cells (reviewed in Ref. 30). SHP-1 has been shown to dephosphorylate and inactivate the erythropoietin (31, 32), stem cell factor (33), interleukin 3 (34), and growth promoters in hemopoietic cells (reviewed in Ref. 30). SHP-1 has been shown to dephosphorylate and inactivate the erythropoietin (31, 32), stem cell factor (33), interleukin 3 (34), and growth promoters in hemopoietic cells (reviewed in Ref. 30). SHP-1 has been shown to dephosphorylate and inactivate the erythropoietin (31, 32), stem cell factor (33), interleukin 3 (34), and growth promoters in hemopoietic cells (reviewed in Ref. 30).
final protein concentration), sedimented rapidly, and resuspended (2.0 \times 10^8 cells/ml) in disruption buffer (see above). The cells were then disrupted by sonication, and nuclei and unbroken cells were removed by centrifugation at 14,000 \times g for 5 min. A high speed pellet, referred to hereafter as “membranes,” was isolated by centrifugation of the lysates at 100,000 \times g for 30 min. Washed three times with disruption buffer, and then dialyzed in Laemmli sample buffer. Remaining soluble (cytosolic) fractions were boiled in 2 × Laemmli sample buffer, and an identical number of cell equivalents (10^6) from both membrane and cytosolic fractions were subjected to SDS-PAGE and immunoblotting (see below).

Equal protein loading was confirmed by Comassie Blue staining.

Isolation of Triton X-100-insoluble (“Cytoskeleton-associated”) Proteins—Neutrophil suspensions were treated with or without stimuli, sedimented rapidly, and then rapidly resuspended in lysis buffer (150 mM NaCl, 2 mM EDTA, 1 mM NaVO_3, 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 50 mM Tris, pH 8.0) containing 1% Triton X-100. The samples were vortexed vigorously and left on ice for a minimum of 10 min to ensure complete lysis of cells. Insoluble (cytoskeleton-associated) proteins were isolated by centrifugation at 14,000 \times g for 5 min, washed three times with lysis buffer, and boiled in Laemmli sample buffer. Triton-soluble proteins were boiled with 2 × concentrated Laemmli sample buffer, and an identical number of cell equivalents (10^6) from both Triton-soluble and -insoluble fractions were subjected to SDS-PAGE and immunoblotting (see below).

SDS-PAGE and Immunoblotting—Proteins were separated by SDS-PAGE in 12% polyacrylamide gels and subsequently transferred to polyvinylidene difluoride membranes. Immunoblotting was carried out as described previously (45). Quantitation was performed by densitometry of exposed films using a Protein Databases (New York, NY) DNA 35 scanner with Discovery series 1D gel analysis software.

SHP-1 Immunoprecipitation and Phosphatase Assay—Neutrophil suspensions were treated with or without stimuli, sedimented rapidly, and resuspended in ice-cold lysis buffer containing 1% Triton X-100 (see above). Lysates were centrifuged at 14,000 \times g for 5 min and then precleared with 50 μl of Sepharose beads. Affinity-purified antibodies to SHP-1 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% BSA in lysis buffer, followed by incubation at 4 °C for 2 h. The immunoprecipitates were washed four to six times and then subjected to SDS-PAGE and immunoblotting or phosphatase assays. Immunoblotting confirmed that equal amounts of SHP-1 were immunoprecipitated from control and stimulated neutrophils.

In immunoprecipitation assays, immunoprecipitates were washed once with assay buffer (0.5 mM EGTA, 25 mM HEPES, pH 7.0) and then incubated with 200 μl of assay buffer containing 10 μM p-nitrophenyl phosphate at 37 °C for 4–16 h while shaking. Reactions were stopped by addition of 800 μl of 0.2 M NaOH, beads were sedimented by brief centrifugation, and phosphatase activity was assessed by measuring the absorbance at 400 nm of the supernatant.

\^\textsuperscript{32}P\textsuperscript{32}Orthophosphate Labeling and Phosphoamino Acid Analysis—Neutrophil suspensions (2.0 \times 10^8 cells/ml) were incubated for 3 h at 37 °C in Na\textsuperscript{+}-rich medium containing 0.5% BSA in the presence of \textsuperscript{32}P-labeled orthophosphate (2.0 μCi/ml). The cells were washed with Na\textsuperscript{+}-rich medium and treated with or without stimuli before lysis and immunoprecipitation of SHP-1, as above. Following washing of immunoprecipitates, the samples were then subjected to SDS-PAGE and blotted onto polyvinylidene difluoride membranes. Quantitation of \textit{in situ} phosphorylation was performed with a Molecular Dynamics PhosphorImager, using the ImageQuant software. Phosphoamino acid analysis was performed as described previously (46).

In Vitro Protein Kinase C Phosphorylation—Neutrophil suspensions were treated with or without 10\textsuperscript{-7} M TPA for 10 min, sedimented rapidly, and then resuspended in ice-cold lysis buffer. SHP-1 was immunoprecipitated as above, and immune complexes were washed with kinase assay buffer (1 mM β-mercaptoethanol, 1 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, and 20 mM MOPS, pH 7.0). The beads were then incubated for 30 min at 30 °C in kinase buffer in the presence of lipid activators (100 μM TPA and 100 μM Na\textsubscript{2}ATP, and 400 μC/ml [\textsuperscript{32}P\textsuperscript{32}P]ATP. Control experiments were performed in the absence of PKC or with immunoprecipitates obtained with nonimmune sera. When the phosphatase activity of SHP-1 was to be determined following PKC phosphorylation, treatments were performed in the absence of [\textsuperscript{32}P\textsuperscript{32}P]ATP, and 1 mM Na\textsubscript{2}ATP was present during the incubation of immunoprecipitates with PKC.

**RESULTS**

Presence of SHP-1 in Human Neutrophils—Although previous studies have demonstrated SHP-1 expression in a variety of hematopoietic cells and cell lines (47–49), the presence of SHP-1 in neutrophils has not been documented. To address this issue, we used reverse transcriptase-polymerase chain reaction. Reactions were performed using RNA purified from neutrophils and oligonucleotide primer pairs designed for amplification of the catalytic domain of the SHP-1 cDNA (40). As shown in Fig. 1A, results of this analysis revealed amplification of a 120-base pair mRNA fragment of SHP-1 from neutrophil RNA (lane 3). No products were seen in the absence of either reverse transcriptase or neutrophil RNA (Fig. 1A, lanes 1 and 2, respectively), ensuring the specificity of these reactions.

SHP-1 in Human Neutrophils

**Fig. 1. Identification of SHP-1 in human neutrophils.** A, reverse transcriptase-polymerase chain reaction was performed using primers designed to amplify a 120-base pair sequence of SHP-1 (lanes 1–3) or interleukin 1α (lane 4). Reactions were performed in the presence (+) or absence (−) of reverse transcriptase (RT), freshly isolated RNA from human neutrophils (PMN RNA) or RNA encoding interleukin 1α (pAW109RNA, Perkin-Elmer). Samples of the final reaction mixture were subjected to agarose gel electrophoresis and visualized by ethidium bromide staining. B, quantitation of SHP-1 in human neutrophils by immunoblotting. Glutathione S-transferase (50 ng), increasing amounts (in ng) of glutathione S-transferase-SHP-1, whole neutrophil lysate (PMN; 10^6 cell equivalents), and an SHP-1 immunoprecipitate (IP) were loaded onto SDS-PAGE gels and subjected to immunoblotting with affinity-purified antibodies to SHP-1. Also indicated in the SHP-1 immunoprecipitate is the immunoglobulin heavy chain (IgG). A and B are representative of at least three similar experiments. C, comparison of the mobility of SHP-1 in neutrophils and HL60 cells. Whole cell extracts (10^6 cell equivalents) of human neutrophils (PMN) and undifferentiated HL-60 cells were analyzed by immunoblotting with affinity-purified polyclonal antibodies to SHP-1. The position of the α splice variant of SHP-1 is indicated.
SHP-1 in Human Neutrophils

Fig. 2. Subcellular localization of SHP-1 in human neutrophils. A, unstimulated neutrophils were fixed in suspension with paraformaldehyde, allowed to adhere to poly-L-lysine-coated coverslips, permeabilized with a buffer containing 0.1% Triton X-100, and blocked with 5% donkey serum. Fixed cells were then stained with affinity-purified antibodies to SHP-1 (left) or an affinity-purified nonimmune serum (right), and detection was performed by incubation with fluorescein isothiocyanate-conjugated donkey anti-rabbit antibodies. Samples were analyzed using a Zeiss laser confocal microscope. Bars, 5 μm. B, subcellular fractionation of human neutrophils was performed by nitrogen cavitation followed by Percoll gradient centrifugation (see “Experimental Procedures”). Fractions displaying the maximal activity of marker protein for primary (1°), secondary (2°), and tertiary (3°) granules, the combined secretory vesicle and plasma membrane fraction (sv/pm), and the cytosol (cyto) were selected, and an equal amount of protein (25 μg) from each fraction was analyzed by immunoblotting with affinity-purified antibodies to SHP-1. For reference, 25 μg of a whole neutrophil lysate (PMN) was also loaded. C, association of SHP-1 with the Triton-insoluble fraction following stimulation. Neutrophil suspensions were treated without (Control) or with phorbol ester (TPA) for 10 min or with chemotactic peptide (FMLP) for 2 min and then rapidly sedimented and resuspended in a lysis buffer containing 1% Triton X-100. After vortexing and incubation for 10 min on ice, soluble and insoluble proteins were isolated by centrifugation of lysates at 14,000 g for 5 min. An equal number of cell equivalents (106) from the Triton-soluble (TS) and -insoluble (TI) fractions were subjected to SDS-PAGE and immunoblotting with affinity-purified antibodies to SHP-1. The downward curve of the SHP-1 immunoreactive band in the Triton-soluble fractions was due to the presence of 0.1% BSA in the lysis buffer. D, immunoblots from three experiments like those in C were analyzed by densitometry, and the amount of SHP-1 isolated in the Triton-insoluble fraction is shown as a percentage of the total (soluble + insoluble) immunoreactive protein (mean ± S.E. (bars) of three experiments). *p < 0.05.

S-transferase fusion protein encompassing residues 1–296 of the phosphatase (Fig. 1B). A single immunoreactive band of approximately 65 kDa is apparent in neutrophil cell lysates, and the same band was identifiable in immunoprecipitates obtained with the SHP-1 antibody. This corresponds to the reported molecular mass of one of the splice variants of SHP-1 (40, 50). To quantify the level of SHP-1 expression, increasing amounts of the fusion protein were loaded into SDS-PAGE gels along with neutrophil lysates and subjected to immunoblotting. By interpolation of the absorbance of these bands, we determined that neutrophils contain 12 ± 5 ng (n = 3) of SHP-1/106 cells or approximately 530 nM (based on a volume of 350 fL/cell), determined using a Coulter Channelizer.

Two variants of SHP-1 have been identified, generated by the alternative splicing of 39 amino acids within the C-terminal SH2 domain of SHP-1 (38). To determine which splice variant is expressed in human neutrophils, whole cell extracts were analyzed by immunoblotting with affinity-purified antibodies to SHP-1 and compared with HL-60 cells, a promyelocytic cell line found earlier to express only the higher molecular weight α splice variant of SHP-1 (50). As shown in Fig. 1C, the SHP-1 immunoreactive band of neutrophils co-migrated precisely with that seen in HL-60 lysates. Similar results were obtained using polyclonal and monoclonal antibodies to SHP-1. We conclude that neutrophils express only the α splice variant of SHP-1.

Localization of SHP-1 in Human Neutrophils: Cytoskeletal Association following Stimulation—Immunofluorescence staining of untreated neutrophils fixed in suspension revealed a diffuse, predominantly cytosolic localization of SHP-1 (Fig. 2A, left panel). In these cells, weak nuclear staining and a variable degree of punctation in the cytosol was also noted, whereas no staining was seen in fixed cells stained with an affinity-purified nonimmune serum (Fig. 2A, right panel). To confirm that SHP-1 was predominantly cytosolic in resting cells, subcellular fractions obtained by Percoll gradient centrifugation were analyzed by immunoblotting (see “Experimental Procedures”). As shown in Fig. 2B, the results of this analysis also revealed SHP-1 to be almost exclusively located in the cytosolic fraction. Minute amounts of the phosphatase were occasionally found in the combined secretory vesicle and plasma membrane fraction, which is known to trap cytosolic components on rescaling of vesicles. No SHP-1 was detected in fractions containing primary, secondary, or tertiary granules.

To determine whether SHP-1 translocates between cellular compartments on neutrophil stimulation, SHP-1 localization was examined by separating the high speed pellet and supernatant (i.e. whole membrane and cytosolic fractions) from cells stimulated with the following agents: a chemotactic peptide (fMLP), which activates a GTP-binding protein-coupled receptor, a phorbol ester (TPA), which directly activates PKC, and opsonized zymosan (OPZ), a particulate stimulus that signals via Fcγ receptor and complement receptor 3. Immunoblotting of these fractions revealed minimal amounts of SHP-1 to be associated with membranes (high speed pellet) in unstimulated cells, and this amount was not substantially altered following stimulation (data not shown). These findings were confirmed by analysis of subcellular fractions isolated by Percoll gradient centrifugation from cells treated with or without phorbol ester (not shown).

Although SHP-1 could not be found to associate with membranes during stimulation, a fraction of this phosphatase was found to associate with the Triton X-100-insoluble residue of...
activated cells. The Triton-insoluble material, thought to represent mostly cytoskeletal components, bound little SHP-1 before activation (<10%), but significant amounts were associated following stimulation with fMLP, TPA (Fig. 2C), or OPZ (not shown). Quantitation by densitometry revealed that stimulation by TPA induced a 2–3-fold increase in cytoskeletal-associated SHP-1 (Fig. 2D). A more modest increase in the cytoskeletal association of SHP-1 was noted following stimulation with OPZ and fMLP (Fig. 2D), although this change was not statistically significant. These findings suggest that SHP-1 may play a role in the cytoskeletal remodeling that occurs during neutrophil activation by dephosphorylating cytoskeletal-associated proteins.

Inhibition of SHP-1 Activity—Agonist-induced activation of neutrophils is known to be associated with increased cellular tyrosine phosphorylation (see the Introduction). This phenomenon is illustrated in the experiment in Fig. 3A, in which whole-cell lysates from control and stimulated cells were immunoblotted with anti-phosphotyrosine antibody. This effect can be mimicked by treatment with oxidizing agents such as diamide, which have been shown to inhibit the activity of tyrosine phosphatases (51) and in vivo (52). We have previously demonstrated that activation of the NADPH oxidase, an endogenous source of reactive oxygen species in neutrophils, can lead to inhibition of CD45 and concomitant increased tyrosine phosphorylation (26, 53). We therefore tested whether inhibition of SHP-1 was similarly mediated by oxidation. However, under conditions that greatly inhibit CD45, SHP-1 was minimally affected (not shown), implying that these phosphatases are differentially regulated.

Phosphorylation has been shown to alter the activity of several phosphatases, including SHP-1 (54–56). We therefore examined the phosphorylation state of this phosphatase in resting and activated neutrophils. As shown in Fig. 4A, immunoprecipitation of SHP-1 from [32P]orthophosphate-labeled cells revealed a low but detectable amount of phosphorylation in untreated cells. By contrast, phosphorylation was markedly increased by treatment of cells with the agonists.

Inhibition of SHP-1 activity by agents that induce tyrosine phosphorylation. A, neutrophil suspensions were treated without (Control) or with the indicated agents for 10 min, except fMLP stimulation, which was for 2 min. Following treatment, cells were resuspended in boiling Laemmli sample buffer and subjected to SDS-PAGE and immunoblotting with a monoclonal antibody to phosphotyrosine. B, neutrophil suspensions were treated as in A with the indicated stimulus, and the activity of SHP-1 was determined in vitro following immunoprecipitation (see “Experimental Procedures”). Results (mean ± S.E. (bars) of three experiments) are presented as the percentage of maximal activity, which was always found in untreated cells. Immunoblots of these immunoprecipitates confirmed that equivalent amounts of SHP-1 were immunoprecipitated following stimulation with each agent (inset, SHP-1 is indicated with an arrowhead). C, neutrophil suspensions were treated with $10^{-7}$ M TPA for the indicated time (min), and the activity was then determined in vitro as in B. Means ± S.E. (bars) of three experiments are shown. D, neutrophil suspensions were treated with $10^{-7}$ M TPA for the indicated time (min) and subjected to anti-phosphotyrosine immunoblotting as in A.

The activity of SHP-1 was examined after fMLP treatment for 15 s–20 min. A statistically significant decrease in activity was not observed within this period.
residues. Anti-phosphotyrosine immunoblotting of SHP-1 immunoprecipitates confirmed that neither TPA nor any of the other agonists studied were capable of inducing tyrosine phosphorylation of this phosphatase (not shown).

Role of PKC—Activation of PKC is known to occur following stimulation by either fMLP or opsonized zymosan. As both of these agents, as well as the direct PKC activator TPA, induced phosphorylation of SHP-1, we next examined the possibility that phosphorylation of this phosphatase is mediated by PKC. As demonstrated in Fig. 6A, pretreatment of [32P]orthophosphate-labeled neutrophils with 2 μM BIM, a potent and specific inhibitor of PKC (57), inhibited both TPA- and OPZ-induced phosphorylation of SHP-1. However, SHP-1 phosphorylation was not entirely abrogated by BIM treatment, suggesting that PKC was not fully inhibited or that other kinases also contribute to SHP-1 phosphorylation. By contrast, prior treatment with BIM fully prevented the agonist-induced decrease in SHP-1 activity (Fig. 5B). Thus, it appears that PKC plays a significant role in regulating SHP-1, apparently through a phosphorylation-dependent mechanism.

Finally, we wished to determine whether PKC was responsible for direct phosphorylation and inhibition of SHP-1 or was instead acting upstream of the regulatory kinase(s). To this end, immunoprecipitates of SHP-1 were prepared from resting or TPA-treated neutrophils, washed extensively, and subjected to in vitro phosphorylation using PKC purified from rat brain. As shown in Fig. 6A, immunoprecipitated SHP-1 was readily phosphorylated by PKC (lane 2). Moreover, stimulation of cells with TPA prior to immunoprecipitation decreased the amount of in vitro PKC-mediated SHP-1 phosphorylation (Fig. 6A, lane 3), presumably due to the incorporation in situ of nonradioactive phosphate into sites on SHP-1 that are substrates of PKC. Treatment of these immunoprecipitates with [γ-32P]ATP alone, in the absence of PKC, did not result in phosphorylation of SHP-1 (Fig. 6A, lane 1), confirming that phosphorylation of SHP-1 in the in vitro assay is not mediated by co-precipitating kinases. Similarly, no PKC-induced phosphorylation was evident in experiments performed with immunoprecipitates of nonimmune serum (Fig. 6A, lane 4).

We next determined the effect of in vitro phosphorylation on the activity of SHP-1. Immunoprecipitates of the phosphatase were subjected to phosphorylation by purified PKC, as in Fig. 6A, and assayed for activity. In vitro phosphorylation by PKC was found to inhibit the activity of SHP-1 to 54 ± 3.9% (three experiments with duplicate determinations) of the control level. This inhibition of phosphatase activity was comparable with that induced by TPA pretreatment of intact cells prior to immunoprecipitation of SHP-1. Together, these results suggest that direct phosphorylation of SHP-1 on serine residues by PKC mediates, at least in part, the inhibition of SHP-1 phosphatase activity following neutrophil stimulation.

DISCUSSION

Little is known about the role of tyrosine phosphatases in regulating neutrophil antimicrobial responses. In this report, we established that SHP-1 is expressed in human neutrophils and that the concentration of SHP-1 in these cells is approxi-

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3 Although mitogen-activated protein kinase has been proposed to phosphorylate and inhibit the activity of the related tyrosine phosphatase SHP-2 (65), we observed no effect on the phosphorylation of SHP-1 following TPA treatment in the presence of 50 μM PD98059, a potent and specific inhibitor of mitogen-activated protein kinase kinase.
In the current study, tyrosine phosphorylation of SHP-1 was not observed with any of the agonists used. Nevertheless, tyrosine phosphorylation of SHP-1 has been observed in other cell types (61, 62) and, contrary to our findings of serine phosphorylation-mediated inhibition, is thought to increase the activity of this phosphatase (54, 55). The apparent lack of phosphotyrosine-mediated regulation of SHP-1 in human neutrophils may reflect the absence of specific tyrosine kinases responsible for the related tyrosine phosphatase SHP-2 (58).

In conclusion, our findings suggest that SHP-1 plays an important role in regulating the balance of protein tyrosine phosphorylation in neutrophils. Although the substrates for SHP-1 in these cells have not been defined, our data indicate a role for this enzyme in dephosphorylating both cytosolic and cytoskeleton-associated proteins. In addition, the finding that PKC acts to inhibit SHP-1 activity following neutrophil stimulation provides a new link between the serine-threonine and tyrosine phosphorylation branches of the signaling cascade that triggers antimicrobial responses in human neutrophils.

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**FIG. 6.** In vitro phosphorylation and inhibition of SHP-1 by PKC. A, neutrophil suspensions were treated without (−; lanes 1, 2, and 4) or with (+; lane 3) 10−7 M TPA prior to immunoprecipitation of SHP-1. After washing extensively, SHP-1 immunoprecipitates were subjected to in vitro phosphorylation in the absence (−; lane 1) or presence (+; lanes 2–4) of purified PKC with added TPA, phosphatidylinositol serine, and [32P]ATP. Control experiments were performed using immunoprecipitates obtained with an affinity-purified nonimmune serum or with beads alone displayed phosphatase activities that were consistently less than 25% of the activity of SHP-1 immunoprecipitates from untreated cells. Data are means ± S.E. (bars) of three experiments, each with duplicate determinations.

mately 530 nM. In contrast to other hemopoietic cell types, which can express alternatively spliced forms (66 and 62 kDa) of SHP-1 (40, 50), only the a splice variant of SHP-1 was found to be expressed in human neutrophils. Although the molecular basis for this observation is unclear, the preferential expression of one splice variant may be of functional relevance, as has been suggested for the related tyrosine phosphatase SHP-2 (58).

In unstimulated cells, SHP-1 was found to be predominantly in the cytosol, although minimal amounts were found to be associated with the nucleus, the plasma membrane, and the cytoskeleton. Stimulation with TPA and, to a lesser extent, with OPZ and FMLP induced an increase in the amount of SHP-1 associated with the cytoskeleton. Cytoskeletal association of SHP-1 has been demonstrated in platelets stimulated with thrombin, and this translocation was postulated to mediate dephosphorylation of cytoskeletal-associated substrates (59). By analogy, we suggest that the functional effects of SHP-1 on neutrophil function reflect its action on both cytoskeletal and cytosolic targets.

Agents that induce tyrosine phosphorylation of neutrophil proteins, such as OPZ and TPA, were found to inhibit the activity of SHP-1. Furthermore, the time course of SHP-1 inhibition following stimulation paralleled that of cellular tyrosine phosphate accumulation. These results suggest that inhibition of SHP-1 contributes to phosphotyrosine accumulation and may play a role in the regulation of antimicrobial responses. Stimuli that inhibited the activity of SHP-1 were also found to induce its phosphorylation on serine residues. TPA-induced serine phosphorylation of SHP-1 had been reported in HL-60 cells, but inhibition of catalytic activity was not described (60). The increased SHP-1 expression induced by TPA in these cells may have masked the inhibitory effect of phosphorylation on enzyme activity (50, 60). PKC-dependent phosphorylation of SHP-1 has been demonstrated in human thymocytes, and this translocation was shown to inhibit its activity (56). That phosphorylation by PKC is responsible for the functional inhibition of SHP-1 in human neutrophils is suggested by the experiments using BIM. This PKC antagonist diminished phosphorylation of the phosphatase while precluding the inhibition of its catalytic activity. It is noteworthy that concentrations of BIM that inhibited phosphorylation incompletely resulted in almost complete reversal of the functional inhibition. This can be interpreted to mean that phosphorylation at multiple sites is required for inhibition of catalytic activity. This interpretation would also account for the observation that, although inducing phosphorylation of SHP-1, FMLP failed to significantly inhibit its activity. In accord with this notion, phosphorylation of SHP-1 on multiple sites has been described in stimulated platelets (55).
