A major function of Rac2 in neutrophils is the regulation of oxidant production important in bacterial killing. Rac and the related GTPase Cdc42 also regulate the dynamics of the actin cytoskeleton, necessary for leukocyte chemotaxis and phagocytosis of microorganisms. Although these GTPases appear to be critical downstream components of chemokine receptor signaling in human neutrophils, the pathways involved in direct control of Rac/Cdc42 activation remain to be determined. We describe an assay that measures the formation of Rac-GTP and Cdc42-GTP based on their specific binding to the p21-binding domain of p21-activated kinase 1. A p21-binding domain glutathione S-transferase fusion protein specifically binds Rac and Cdc42 in their GTP-bound forms both in vitro and in cell samples. Binding is selective for Rac and Cdc42 versus RhoA. Using this assay, we investigated Rac and Cdc42 activation in neutrophils and differentiated HL-60 cells. The chemotactant fMet-Leu-Phe and the phorbol ester phorbol myristate acetate stimulate formation of Rac-GTP and Cdc42-GTP with distinct time courses that parallel cell activation. We also show that the signaling pathways leading to Rac and Cdc42 activation in HL-60 cells involve G proteins sensitive to pertussis toxin, as well as tyrosine kinase and phosphatidylinositol 3-kinase activities.

Small GTPases of the Ras superfamily serve as key regulators in the control of intracellular signaling pathways. GTPases regulate molecular events by cycling from the inactive GDP-bound state to active GTP-bound forms. This GDP/GTP cycle is regulated by the interaction of the GTPases with guanine nucleotide exchange factors (GEFs), GDP dissociation inhibitors, and GTPase-activating proteins (GAPs), presumably under the control of signaling events initiated by cell-surface receptors (1). The activated GTPases interact with specific target proteins that serve as effectors to regulate downstream signaling cascades. The Rac GTPase subfamily, which consists of the closely related GTPases Rac, Cdc42, and RhoA, has been implicated in the regulation of diverse cellular functions, including actin cytoskeletal dynamics, oxidant generation, transformation, membrane trafficking, apoptosis, transcription, and cell cycle control (2–5).

Polymorphonuclear neutrophils are circulating cells that can be rapidly activated in response to inflammatory signals to adhere and migrate through the extracellular matrix to sites of infection and/or inflammation. At these sites, bacteria are phagocytosed and killed through the secretion of granules and oxidants. Many studies indicate that small GTPases are involved at various levels to regulate the cellular functions involved in the inflammatory process (1, 6). The first identified biological activity of Rac was regulation of oxidant production by the phagocyte NADPH oxidase (3, 7, 8). This function has been confirmed through studies utilizing cell-free systems (9, 10), as well as intact cells (11), and in a transgenic model (12). Rac2 appears to be a required NADPH oxidase component in human neutrophils, and there is evidence to implicate direct interactions with both p67phox (13) and the cytochrome b558 (14, 15) oxidase proteins. More recent work has established that Rho GTPases control leukocyte cytoskeletal dynamics as well. Cdc42 induces actin polymerization in neutrophil extracts (16), and Rho, Rac, and Cdc42 have been implicated in the migratory responses of leukocytes to chemotactic stimuli (17, 18) as well as in the phagocytic process (19, 20). Moreover, Rac, Rho, and Cdc42 are also involved in a variety of leukocyte signaling pathways, including activation of phospholipase D (21, 22), reviewed in Ref. 23. Whereas activation of the Rho GTPases is clearly critical for controlling the inflammatory responses of human leukocytes, the upstream signals and regulatory proteins controlling Rho family GTPase activation in these cells remain largely unknown.

Among the Rho GTPase targets identified in the neutrophil, the p21-activated kinases (PAK1 and -2) were initially found to be activated after fMet-Leu-Phe stimulation (24). Binding of Rac- or Cdc42-GTP leads to PAK autophosphorylation and activation of the ability to phosphorylate exogenous substrates on serine and/or threonine residues (25). Substrates for PAK in human neutrophils may include the p47phox and p67phox NADPH oxidase components (24, 26). Rac and Cdc42 activate PAK through binding to the p21-binding domain (PBD). This sequence, located in the N-terminal regulatory part of the protein, contains a highly conserved 14-amino acid CRIB domain (amino acids 74–88) found in many proteins interacting with Rac- or Cdc42-GTP (27). Whereas the minimal CRIB domain is sufficient for the binding of Rac and Cdc42, a larger sequence is required for high affinity interaction (amino acids 13198-IMM from the Scripps Research Institute. 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.
67–150) (28, 29) and effective activation by GTPases (30).

We have used the PBD domain of PAK1 as a probe to specifically isolate the active forms of Rac and Cdc42 from human neutrophil samples. Activation of Rac2 and Cdc42 by the chemoattractant fMet-Leu-Phe and the general stimulus phorbol myristate acetate (PMA) occurs with distinct time courses that parallel cellular activation by these agents. We have also investigated the signaling pathways involved in Rac2 and Cdc42 activation by chemoattractant, including the participation of heterotrimeric G proteins, tyrosine kinases, and PI 3-kinase.

The PAK PBD-based assay provides a simple and direct means to determine Rac and Cdc42 activation in cells.

**EXPERIMENTAL PROCEDURES**

**Biological Materials**—Human neutrophils of 90–95% purity were prepared from freshly drawn blood from healthy volunteers collected in acid-citrate/dextrose. Neutrophils were purified by dextran sedimentation, hypotonic lysis of erythrocytes, and centrifugation through Ficoll-Paque, as described (31). The cells were diisopropyl fluorophosphate-treated, washed with 0.9% NaCl, and finally resuspended in Krebs-Ringer Hepes buffer containing 5 mM glucose (KRHG) for experiments.

Human pro-myelocytic leukemic HL-60 cells stably transfected to express the DML receptor (32) were maintained in a selective RPMI 1640 medium containing 10% fetal bovine serum and 0.8 mg/ml bovine serum albumin, and the radioactivity bound to the beads was quantified by liquid scintillation counting.

**Intrinsic GTP Hydrolysis Activity**—Recombinant Rac1 or Cdc42 (50 ng) were preloaded with [γ-32P]GTP or [35S]GTPγS, and GTP hydrolysis was determined in the presence or absence of 4 μM of GST-PBD as described (35).

**RESULTS**

**Development of a PBD-based Assay of Rac/Cdc42 Activation**

**Specificity of the Interaction between Recombinant Rac and Cdc42**—The PAK protein exhibits a selective affinity for the GTP-bound form of Rac or Cdc42 (25). We first verified that this specificity for the active conformation of the GTPases is maintained in the isolated GST-PBD fusion protein. Purified GST-PBD was used as a probe in an affinity precipitation assay with different nucleotide-bound forms of recombinant Rac1. Fig. 1a shows that GST-PBD effectively interacts with the active GTPγS-bound form of the GTPase. There was little or no interaction with the inactive GDP-bound form. We verified that Rac1-GTPγS did not bind nonspecifically to GST beads or to glutathione-Sepharose beads alone. To confirm this result, we investigated the interaction of GST-PBD with overexpressed cytosolic GTPases. BHK cells overexpressing Rac2 wild type, the constitutively GTP-bound active form (Q61L), or T17N mutant with Rac1, Rac2, and Cdc42 but not Rho. The specificity of the interaction of GST-PBD with recombinant GTPases loaded with labeled nucleotides.

**Selectivity of the Interaction**—It has previously been shown that the PAK1 binds Rac and Cdc42 but not Rho (24, 25). To determine if the GST-PBD domain behaves with the same selectivity, cytosolic fractions of neutrophils, known to express Rac1, Rac2, Cdc42, and RhoA, were loaded with GTPγS or GDP. The affinity precipitation assay with GST-PBD was performed, and the presence of each GTPase was assessed with the detection method using Kodak X-Omat AR film, as determined with Ref. 36.
totally prevent, GTP hydrolysis by Cdc42 (Fig. 2c). To confirm that we were measuring GTP hydrolysis and not nucleotide dissociation, the same experiment was performed with Cdc42 loaded with the poorly hydrolyzable nucleotide [35S]GTPyS. We observed that the amount of [35S]GTPyS remaining bound to the Cdc42 only decreased very slowly, indicating that appreciable dissociation was not taking place under the conditions of the binding assay. The lack of complete inhibition of GTP hydrolysis by the PBD suggests that, in order to avoid loss of GTP-bound GTPase due to hydrolysis, the GST-PBD incubation period should be kept short. This is balanced, however, by the time needed for association of the GTP-GTPase with the PBD protein; 30-min to 1-h incubations appear to give optimal results.

**Rac and Cdc42 Activation in fMLP-stimulated Leukocytes**

Neutrophils were stimulated with 1 μM fMLP or 100 ng/ml PMA, and activation of Rac and Cdc42 was investigated using the GST-PBD binding assay. Stimulation by fMLP led to a rapid and transient activation of both Rac2 and Cdc42. Analysis at various times after stimulation with fMLP showed that activation of Rac2 and Cdc42 peaked between 30 s to 1 min, followed by a decrease in levels of active GTPase (Fig. 3a). Stimulation with PMA also induced Rac2 and Cdc42 activation, but the formation of GTP-GTPase was slower, reaching the maximal level of activation at 5 min after stimulation. The relative amount of activated GTPase formed with each stimulus was compared with the total amount of activable GTPases present in the cell lysates, as determined by preloading the total GTPase in the sample with GTPyS (leftmost panels in Fig. 3, a and b). We estimated that ~2 (fMLP) to ~5% (PMA) of Rac2 and ~5 (fMLP) to 10% (PMA) of Cdc42 are activated out of the total available cellular GTPase pool.

Previous studies from our laboratory had used the stimulus-dependent translocation of Rac2 to the plasma membrane as a measure of GTPase activation in human neutrophils (34). Translocation of Rac2 in response to either fMLP or PMA occurs with an identical time course as does Rac2 activation determined with the PBD assay; in both cases this correlates well with activation of NADPH oxidase activity (not shown here, but see Ref. 34). Additionally, the amounts of Rac2 shown to translocate upon stimulation with fMLP or PMA were similar to the fraction that we could directly measure as being activated, suggesting that the assay was accurately measuring the level of GTP-Rac2 formed. Interestingly, however, whereas the formation of Rac2-GTP measured here was transient, peaking by 1 or 5 min, respectively, with fMLP and PMA, the translocated Rac2 remains membrane-associated for a much longer period (34). This suggests that once membrane-bound, the Rac2 may be stabilized in a protected complex with other NADPH oxidase components.

We observed a similar time course of GTPase activation in the promyelocytic cell line HL-60 differentiated into neutrophil-like cells (Fig. 3b). The time course of activation by fMLP and the relative fraction of active Rac2 and Cdc42 formed were essentially the same as in peripheral human neutrophils (Fig. 3a), suggesting that similar mechanisms of activation for Rac and Cdc2 may exist in this differentiated promyelocytic cell line. In contrast to fMLP, stimulation with PMA in HL-60 cells appeared to somewhat slower in stimulating the increase in active Rac and Cdc42, although peak activation was still observed by 5 min. Again, this correlated well with the kinetics of NADPH oxidase activation (not shown).

Analysis of GTPase activation in neutrophils and HL-60 cells at early times was difficult due to the rapidity of the response and because of the well-known propensity of the leukocytes to
become partially activated by contact with test tube surfaces. This surface-induced activation seemed to cause increases in the basal (unstimulated) levels of active Rac and Cdc42. Stimulation in response to fMLP at early times was therefore carefully analyzed by averaging the early time point data obtained from several separate experiments with different leukocyte preparations (Fig. 3c). Activation was clearly evident by 30 s and remained the same or slightly increased by 1 min.

**Signaling Pathways Involved in Rac2 and Cdc42 Activation in HL-60 Cells**

The fMLP receptor is known to couple to activation of neutrophil functional responses via a pertussis toxin-sensitive heterotrimeric G protein (37). Treatment of HL-60 cells with pertussis toxin effectively inhibited Rac2 activation (Fig. 4). The pathway leading to Rac/Cdc42 activation thus requires the initial coupling of the fMLP receptor to G, and places the activation of these small GTPases downstream of the heterotrimeric G protein.

The involvement of soluble tyrosine kinases in fMLP receptor signaling has been suggested by the ability of tyrosine kinase inhibitors to block fMLP-mediated cell activation, including the fMLP-induced membrane translocation of Rac2 (38). It has also been shown that fMLP receptor stimulation causes activation of the src-related kinase, Lyn, through an association with the Shc adaptor protein (39). We observed that treatment of HL-60 cells with 100 μM genistein prior to fMLP stimulation blocks Rac2 and Cdc42 activation (Fig. 5). These data directly indicate the involvement of tyrosine kinase activity in the pathway leading to Rac2 and Cdc42 activation.

PI 3-kinase has been shown to be involved in an upstream signaling pathway required for Rac activation in fibroblasts (40). PI 3-kinase activity is also required for chemoattractant receptor signaling, as the specific PI 3-kinase inhibitors wortmannin and LY294002 are able to decrease superoxide production and many other neutrophil functions (41–44). The majority of the phosphatidylinositol 1,4,5-trisphosphate formed in human neutrophils requires a tyrosine kinase-linked pathway involving the classical forms of PI 3-kinase (45), although a G protein subunit-regulated enzyme may also be involved (46). In order to determine if PI 3-kinase activity was involved for fMLP-induced Rac2 and Cdc42 activation in human neutrophils, we treated cells with 5–30 nM of wortmannin or 5–20 μM of LY294002 prior to stimulation with fMLP. Substantial, but not complete, inhibition of Rac or Cdc42 activation was observed with both inhibitors (Fig. 5). In each experiment,
NADPH oxidase activity was measured and was found to be totally inhibited at the inhibitor concentrations utilized. Thus, both PI 3-kinase-regulated and independent mechanisms for Rac and Cdc42 activation may exist in human neutrophils.

**DISCUSSION**

Although roles for Rac and Cdc42 in human leukocyte function have been demonstrated, the ability of inflammatory mediators to stimulate the formation of Rac-GTP or Cdc42-GTP has not been previously established. By using a specific assay based on the GTPase-binding domain of PAK, we demonstrate the formation of GTP-Rac2 and GTP-Cdc42 in human neutrophils stimulated with the chemoattractant fMLP or the phorbol ester PMA. Activation of both Rac2 and Cdc42 in response to fMLP is rapid, peaking at 0.5 to 1 min. This time course corresponds well to the activation of the Rac- and Cdc42 effectors PAK1 and -2 in these cells (24), as well as to activation of the NADPH oxidase (34). The receptor-induced activation of Rac2 also correlates with the translocation of Rac2 to the plasma membrane, as previously reported (34). This translocation has been shown to require the formation of Rac2-GTP in vitro (47). An interesting difference observed was that while Rac2 activation was transient, the Rac2 protein itself appears to remain membrane-associated for longer periods. It is possible that we are only measuring that fraction of activated GTPase that remains accessible to the GST-PBD in our assay and that the membrane-associated Rac2 enters into a higher affinity complex with NADPH oxidase and/or other effectors. The formation of such a protected complex was previously

**FIG. 3.** Rac2 and Cdc42 activation in human neutrophil and HL-60 cells after fMLP or PMA stimulation. a, time course of Rac2 and Cdc42 activation in human neutrophils. Neutrophils (2 × 10⁷ cells/ml) were stimulated with 1 μM fMLP or 100 ng/ml PMA in KRHG/Ca²⁺ buffer at 37 °C. At appropriate times, activation was stopped by addition of ice-cold 2× lysis buffer. The resulting cell lysate was clarified and used for the affinity precipitation assay for 1 h at 4 °C in the presence of 8 μg of GST-PBD. Proteins bound to GST-PBD were separated on SDS-PAGE, transferred to nitrocellulose membrane, and blotted for Rac2 or Cdc42, followed by ECL detection. Representative results of eight (fMLP) and two (PMA) independent experiments are shown. Results quantified by densitometry are shown below each panel. The small inset at the left of each experiment shows the total signal detected using cytosol pre-exchanged with either GTPγS or GDP, as described under “Experimental Procedures.” b, time course of Rac2 and Cdc42 activation in HL-60 cells. Granulocyte-like MeSO-differentiated HL-60 (2 × 10⁷ cells/ml) were stimulated with 1 μM fMLP or 100 ng/ml PMA and treated as described above for neutrophils. Representative result of seven (fMLP) or two (PMA) independent experiments are shown. c, quantification of GTPase activation at early times. In order to evaluate the reproducibility of our determinations in different experiments using distinct neutrophil preparations, we quantified the PBD immunoblots by densitometry. The values obtained for Rac2 and Cdc42 activation at early times in human neutrophils and HL-60 cells were averaged after normalization of the 30-s values to an arbitrary value of 100. The results shown are the mean ± S.E. of n = 3–4 separate experiments using distinct donors (neutrophils).

**FIG. 4.** Inhibition of Rac2 activation by pertussis toxin. MeSO-differentiated HL-60 cells were cultured 24 h in the presence (+) or the absence (−) of 20 ng/ml pertussis toxin (PTX). Cells were then washed, resuspended in KRHG/Ca²⁺ buffer (2 × 10⁷ cells/ml), and stimulated for 30 s with 1 μM fMLP. Cells were lysed by addition of ice-cold 2× lysis buffer, and the lysate was used for the affinity precipitation assay in the presence of 8 μg of GST-PBD, as indicated under “Experimental Procedures.” Proteins bound to GST-PBD were separated on SDS-PAGE, transferred to nitrocellulose membrane, and blotted for Rac2, followed by alkaline phosphatase detection. Control lysates were loaded with GTPγS or GDP, as indicated. Figure is a representative result of four independent experiments.
suggested by the inability of addition of a Rac-GAP to inhibit oxidase activity once the membrane-associated complex was assembled (48). Alternatively, the Rac2 may remain membrane-associated even after conversion to the inactive GDP-form, suggesting cycling to the cytosol (via GDP dissociation inhibitors) may lag behind inactivation of the GTPase.

Quantitative comparison of the amount of Rac2 or Cdc42 which becomes PBD-associated versus the total level of GTPase present which can be loaded with GTP indicates only a fraction of the total becomes active in response to fMLP or PMA. Again, this conclusion is consistent with the previous observation that only a (similar) fraction of Rac2 translocates in response to this receptor. The activation was also sensitive to the tyrosine kinase inhibitor genistein and, partially, to PI 3-kinase inhibitors, indicating the need for tyrosine kinase activity and PI 3-kinase activity upstream of the putative GEF(s). Although this could reflect the activity of multiple GEFs regulated by each pathway independently, it is of interest that both tyrosine phosphorylation and phosphatidylinositol 4,5-triphosphate binding are necessary components for activation of the Vav family. In conclusion, we have developed and characterized a specific assay for the formation of GTP-Rac and GTP-Cdc42 in cells. By using this assay, we demonstrate the activation of Rac2 and, for the first time, Cdc42 in human neutrophils in response to chemoattractant receptor activation and phorbol ester stimulation.

The similarities in the activation kinetics of both GTPases and their similar pharmacologic inhibition profile suggest that their activation may be catalyzed by the same GEF. In addition, because we show Cdc42 becomes activated rapidly, with kinetics correlating with those of leukocyte functional responses, Cdc42 can potentially be involved in regulation of some of these responses. Identification of the GEF(s) responsible for chemoattractant receptor signaling to Rac and Cdc42 remains an important goal for future investigations.

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