Phosphoinositide 3-Kinase-dependent and -independent Activation of the Small GTPase Rac2 in Human Neutrophils*

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The small GTPase Rac participates in various cellular events such as cytoskeletal reorganization. It has remained, however, largely unknown about intracellular signaling pathways for Rac activation because of the lack of a simple and reliable assay to estimate the activation. Here we describe a novel method to detect the GTP-bound, active Rac in cells by pulling it down with the Rac-binding domain of the protein kinase PAK. Experiments using this method reveal that stimulation of human neutrophils with the G\textsubscript{i}-coupled receptor agonists N-formyl-methionyl-leucyl-phenylalanine (fMLP) and leukotriene B\textsubscript{4} (LTB\textsubscript{4}) leads to a rapid and transient increase in the GTP-bound state of Rac2, whereas phorbol myristate acetate (PMA) causes a slow but more sustained activation of Rac2. Pretreatment of cells with pertussis toxin results in defective activation of Rac2 in response to fMLP and LTB\textsubscript{4}, indicating that coupling of the receptors to G\textsubscript{i} plays a crucial role in the activation. Furthermore, the phosphoinositide 3-kinase (PI3K) inhibitors wortmannin and LY294002 block Rac2 activation elicited by the receptor agonists, but not that by PMA. Thus the G\textsubscript{i}-coupled receptors likely mediate Rac2 activation via PI3K, whereas PMA activates Rac2 in a PI3K-independent manner.

The small GTPases Rac1 and Rac2 are members of the Rho subfamily of the Ras-related GTP-binding proteins and serve as a molecular switch cycling between an active GTP-bound and an inactive GDP-bound state (1–3). In the active state, Rac interacts with a variety of effector proteins to elicit cellular responses, including cytoskeletal reorganization and gene activation (1–3). Growth factor or integrin-induced Rac activation in such responses is thought to require PtdIns(3,4,5)P\textsubscript{3}, \textsuperscript{1} that is generated from PtdIns(4,5)P\textsubscript{2} by PI3K (4), based on experiments using cells overexpressing constitutively active and dominant negative forms of Rac, and on those of microinjection of the mutant Rac proteins to cells (1–4). A major problem in this field has been the lack of the assay to evaluate activation of endogenous Rac.

In human neutrophils, Rac is considered to be involved in activation of the phagocyte NADPH oxidase. The oxidase, dormant in resting cells, is activated during phagocytosis of invading microorganisms to produce superoxide, a precursor of microbialicidal oxidants, thereby playing an important role in host defense (5–7). The activation, at a cell level, can be mimicked by soluble stimuli (5–8) including the chemotactic peptide fMLP and LTB\textsubscript{4}, which bind to their own G\textsubscript{i}-coupled receptors on the plasma membrane (8–10), and PMA, an activator of PKC (11). The NADPH oxidase is also activated with anionic amphiphiles such as arachidonic acid in a cell-free system reconstituted with five poly peptides; the membrane-bound catalytic core cytochrome \textsubscript{b}\textsubscript{558} comprising the two subunits gp91\textsubscript{phox} and p22\textsubscript{phox}, and the three cytosolic signaling proteins p47\textsubscript{phox}, p67\textsubscript{phox}, and Rac (5–7). In the system, solely the GTP-bound Rac, but not the GDP-bound protein, is able to induce superoxide production (12–14), probably via binding to p67\textsubscript{phox} (15–17). This implicates that Rac functions as a switch for the oxidase activation, although the oxidase activation also requires Rac-independent events such as stimulus-induced conformational change of p47\textsubscript{phox} that leads to its interaction with p22\textsubscript{phox} (18–21). In Epstein-Barr virus-transformed B lymphocytes or HL60 leukemic cells, introduction of Rac antisense oligonucleotides or expression of a dominant negative form of Rac2 is shown to partially inhibit superoxide production (22, 23), suggesting a role of Rac on the oxidase activation at a cell level. However, stimulus-dependent activation of Rac has not been demonstrated.

Activation-specific probes for small GTPases have recently been constructed, which allows determination of the activity of endogenous Ras and Rap1 (24–26) and hemagglutinin-tagged Rac1 and Cdc42 (27) without radioactive in vivo labeling. Using a similar procedure, we have developed here a novel assay to estimate activation of endogenous Rac in cells, by pulling down the GTP-bound, active Rac with the Rac-binding domain (RBD) of the protein kinase PAK2 expressed as a GST fusion. This assay is based on the finding that PAK2-RBD binds to the GTP-bound Rac with a high affinity, whereas the affinity for the GDP-bound protein is undetectably low (28). Using this method, we show here that Rac2, the predominant isoform in human neutrophils (29), is rapidly and transiently converted to the GTP-bound, active state, in response to the G\textsubscript{i}-coupled receptor agonists fMLP and LTB\textsubscript{4}. The activation appears to require PI3K that is known to be stimulated by the G\textsubscript{i} signaling in neutrophils (8, 30–33). On the other hand, PMA induces a slow but more sustained activation of Rac2, which is dependent on PKC but independent of PI3K.

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\textsuperscript{1} The abbreviations used are: PtdIns(3,4,5)P\textsubscript{3}, phosphatidylinositol 3,4,5-trisphosphate; PtdIns(4,5)P\textsubscript{2}, phosphatidylinositol 4,5-bisphosphate; PI3K, phosphoinositide 3-kinase; fMLP, N-formyl-methionyl-leucyl-phenylalanine; LTB\textsubscript{4}, leukotriene B\textsubscript{4}; G\textsubscript{i}, the G\textsubscript{i} class of heterotrimeric GTP-binding protein; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; GST, glutathione S-transferase; RBD, Rac-binding domain; PTX, pertussis toxin; GST-558 comprising the two subunits gp91\textsubscript{phox} and p22\textsubscript{phox}; PAK2, protein kinase PAK2 expressed as a GST fusion.
Chemicals—fMLP and wortmannin were purchased from Wako Chemical (Osaka, Japan), LTβ, from Cayman Chemical (Ann Arbor, MI), PMA and pertussis toxin (PTX) from Research Biochemicals International (Natick, MA), and LY294002 and GF109203X from Biomol Research Laboratories (Plymouth Meeting, PA). Other chemicals used were of the highest purity commercially available.

Preparation of the Rac-binding Domain of Human PAK2 as a GST Fusion Protein—We isolated a DNA fragment encoding the Rac-binding domain of human PAK2 (PAK2-RBD; amino acids 66–147) from total RNA of the neuroblastoma cell line SH-SYSY by reverse transcriptase-polymerase chain reaction, according to the protocol of the manufacturer (Perkin Elmer). The polymerase chain reaction product was subcloned into pGEX-2T (Amersham Pharmacia Biotech). The cDNAs for human Rac1 and Rac2 subcloned into pGEX-2T were prepared as described previously (17). The identities of all the constructs were verified by DNA sequencing. The GST fusion proteins were prepared as described above, followed by cleavage with thrombin, according to the protocol of the manufacturer (Amersham Pharmacia Biotech).

Specific Detection of the GTP-bound Rac by GST-PAK2-RBD—Recombinant Rac1 or Rac2 was incubated for 30 min at 30 °C in 90 μl of a nucleotide-exchange buffer (137 mM NaCl, 2.7 mM KCl, 2 mM EDTA, 4.3 mM NaHPO4, and 1.4 mM KHPO4, pH 7.0) in the presence of 11 mM GTP-γS, GTP, GDP, ATP, CTP, or UTP. The exchange reaction was terminated by the addition of 10 μl of 100 mM MgCl2. The nucleotide-loaded proteins were incubated at 4 °C with GST-PAK2-RBD and glutathione-Sepharose-4B beads in buffer A (20 mM Hepes, pH 7.4, 142.5 mM NaCl, 1% Nonidet P-40, 10% glycerol, 4 mM EGTA, 4 mM EDTA). After washing the beads three times with buffer A, the samples were subjected to 12% SDS-PAGE and stained with Coomassie Brilliant Blue.

Assay for Rac2 Activation in Human Neutrophils—Human neutrophils were isolated from fresh venous blood of healthy volunteers by dextran sedimentation, hypotonic lysis, and the Conray/Ficoll method (10, 18). More than 98% of the cells were neutrophils in the preparation. After washing the beads three times with buffer A, the samples were subjected to 12% SDS-PAGE and stained with Coomassie Brilliant Blue.

RESULTS

The Rac GTPases in the GTP-bound State, but Not in the GDP-bound State, Bind to GST-PAK2-RBD—To test the validity of the RBD of human PAK2 (amino acids 66 to 147) as a tool to identify the active GTP-binding state of Rac, we expressed and purified the domain as a GST fusion protein (GST-PAK2-RBD).

Recombinant Rac1 and Rac2 preloaded with GTP-γS were precipitated using glutathione-Sepharose-4B beads coupled to the fusion protein, washed three times, and analyzed by SDS-PAGE (Fig. 1). Under the conditions, the binding of the active Rac1/2 to GST-PAK2-RBD appeared stable because further washing did not affect the recovery of the GTPases (data not shown). The same results were obtained when GTP was loaded instead of GTP-γS (data not shown). On the other hand, the beads coupled to GST-PAK2-RBD did not retain the wild-type Rac1 and Rac2 in GDP-bound states (Fig. 1). When Rac2 was loaded with ATP, CTP, or UTP, no interaction with the GST fusion protein was observed (data not shown). In addition, neither GTP- nor GDP-bound Rac interacted with the beads coupled to GST alone (data not shown). Thus PAK2-RBD specifically recognizes the GTP-bound, active state of Rac to form a stable complex and thereby can be used as an activation-specific probe for Rac.

Rac2 Is Activated in Human Neutrophils Stimulated with fMLP, LTB4, and PMA—After human neutrophils were stimulated with the G i-coupled receptor agonist fMLP and then lysed, Rac was precipitated with GST-PAK2-RBD bound to glutathione-Sepharose beads and identified by Western blot with a polyclonal antibody against Rac2. This antibody is specific to Rac2 and does not recognize other members of the Rho family GTPases such as Rac1, Cdc42, or RhoA (data not shown). As shown in Fig. 2A, stimulation with fMLP led to a rapid and transient increase in the amount of Rac2 that bound to PAK2-RBD: the increase occurred within 30 s and reached its maximum level at 1 min. The amount of Rac2 detected were dependent on the concentration of fMLP (Fig. 2B). Increases do not seem to be because of changes in Rac protein levels because the same amount of Rac2 was detected during the stimulation in the whole cell lysates by Western blot analysis (data not shown). At the maximal level, about 10–12% of total Rac2 could be precipitated with GST-PAK2-RBD, when estimated by Western blot using various amounts of the whole cell lysates (Fig. 2C). In contrast with the GST fusion protein, GST alone bound to the beads failed to precipitate Rac2 in the fMLP-stimulated cells (data not shown). When an antibody specific to Rac1 was used instead of the anti-Rac2 antibody, no specific bands on Western blot were observed (data not shown). This is consistent with Rac2 being largely predominant (>96%) in human neutrophils (29). Since PAK2-RBD associates exclusively with the GTP-bound state of Rac2, with no detectable affinity for the GDP-bound protein (Fig. 1), we conclude that fMLP induces a rapid and transient conversion of Rac2 to the GTP-bound active state. LTβ, another G i-coupled receptor agonist, also caused a rapid and transient activation of Rac2 in a dose-dependent manner (Fig. 2, A and B). The maximal amount of the GTP-bound Rac2 formed by LTβ (about 10% of total Rac2) was slightly less than that in response to fMLP (Fig. 2C).
FIG. 2. Rac2 activation in human neutrophils stimulated with fMLP, LTB₄, or PMA. A, human neutrophils were stimulated for the indicated time with fMLP (1 μM), LTB₄ (1 μM), or PMA (1 μg/ml). B, neutrophils were stimulated for 1 min with fMLP or LTB₄, or for 10 min with PMA at indicated concentrations. After the incubation, the cells were lysed, and the GDP-bound, active Rac2 was precipitated with GST-PAK2-RBD bound to glutathione-Sepharose-4B beads. Rac2 was identified by Western blot with an anti-Rac2 antibody. For details, see "Experimental Procedures." C, neutrophils were stimulated for 1 min with fMLP (1 μM) or LTB₄ (1 μM), or for 10 min with PMA (1 μg/ml). After the incubation, the cells (2.8 × 10⁷ cells) were lysed, and the GDP-bound, active Rac2 was precipitated with GST-PAK2-RBD bound to glutathione-Sepharose-4B beads. The precipitates (2.8 × 10⁷ cell equivalents) or the whole cell lysates (derived from the indicated cell numbers of human neutrophils) were subjected to 12% SDS-PAGE and analyzed by Western blot.

Human neutrophils, in response to fMLP, produce superoxide, the production which is catalyzed by the phagocyte NADPH oxidase that is activated upon cell stimulation (31–34). LTB₄ also triggers superoxide production but to a lesser extent (10). The kinetics of the production by these agonists, i.e. rapid onset and short duration of 1–2 min (10, 32, 34), bear a resemblance to those of Rac2 activation (Fig. 2A), which suggests that the agonist-induced conversion of Rac2 to the active state is linked to the NADPH oxidase activation. We next tested whether PMA, a potent inducer of superoxide production (34), affects states of Rac2 in neutrophils or not. As shown in Fig. 2B, PMA induced Rac2 activation in a dose-dependent manner. The activation occurred slowly but in a more sustained manner: it was observed as early as 1–2 min and reached a maximum after 5–10 min, followed by a decrease at 20 min (Fig. 2A). The time course also resembles that of PMA-activated Rac2-GTP production (Fig. 3C).

Effects of Pertussis Toxin on Rac2 Activation in Human Neutrophils—It is well documented that both fMLP and LTB₄ receptors on human neutrophils are coupled to the Gᵢ class of heterotrimeric G-proteins (8, 9). The finding that both agonists cause activation of Rac2 in neutrophils (Fig. 2) suggests a role for Gᵢ. To clarify the involvement of Gᵢ in Rac2 activation, we tested the effect of PTX, which catalyzes ADP-ribosylation of Gᵢα to uncouple Gᵢ from the receptors (8). The toxin treatment of human neutrophils resulted in a complete loss of the fMLP-induced superoxide production, while it did not affect the PMA-induced one (data not shown). In the PTX-treated cells, neither fMLP nor LTB₄ was capable of activating Rac2 (Fig. 3), indicating that coupling of the receptors to Gᵢ is required for the Rac2 activation. On the other hand, the treatment did not affect PMA-triggered activation of Rac2 (Fig. 3).

FIG. 3. Effects of pertussis toxin on Rac2 activation in human neutrophils. Human neutrophils were preincubated for 2 h at 37 °C with or without PTX (8 μg/ml) and then stimulated for 1 min with fMLP (1 μM), LTB₄ (1 μM), or for 10 min with PMA (1 μg/ml). The GTP-bound, active Rac2 was detected as described in the legend to Fig. 2.

DISCUSSION

In the present study, we have developed a novel method to directly detect the GTP-bound active Rac that is converted from the GDP-bound state in intact cells. Using this method, we show that the Gᵢ-coupled receptor agonists fMLP and LTB₄ elicit a rapid and transient activation of Rac2 in human neutrophils and that the activation is mediated via not only Gᵢ but also PI3K. On the other hand, PMA causes a slow but more sustained activation of Rac2 in a PI3K-independent manner. Recent studies have suggested that PI3K is located upstream
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In addition to the PI3K-dependent mechanism, GEFs for Rac can be regulated by posttranslational modifications such as phosphorylation by a protein kinase. It has been reported that tyrosine-phosphorylated Vav, but not the unphosphorylated protein, enhances GDP/GTP exchange on Rac (44). In this context, it should be noted that PMA, a direct activator of PKC, increases the GTP-bound, active state of Rac2, independently of PI3K. Phosphorylation by PKC might regulate GEF(s) to activate Rac2 in neutrophils.

Activation of Rac in stimulated neutrophils has been postulated to occur because the GDP-bound Rac, but not the GTP-bound protein, can activate the phagocyte NADPH oxidase in vitro (12–14). However, stimulus-dependent activation of Rac has not been demonstrated. This study, using the novel method, shows that three stimulants for the NADPH oxidase activation in vivo, FMLP, LTB4, and PMA, are all capable of activating Rac2 in human neutrophils. The kinetics of Rac2 activation by the stimulants correspond well with those of superoxide production. Although it is well documented that PI3K inhibitors block superoxide production triggered by FMLP but not that by PMA (31–33), it has remained elusive how PI3K functions in the oxidase activation. As described above, PI3K is required for activation of Rac2 in the FMLP signaling, whereas PMA activates Rac2 in a PI3K-independent manner. Thus the present findings support the idea that Rac2 serves as a switch for the NADPH oxidase activation.

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