Activation of the Small GTPase Rap1 in Human Neutrophils

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The small GTPase Rap1 is highly expressed in human neutrophils, but its function is largely unknown. Using the Rap1-binding domain of RapGDS (RapGDS-RBD) as an activation-specific probe for Rap1, we have investigated the regulation of Rap1 activity in primary human neutrophils. We found that a variety of stimuli involved in neutrophil activation, including f-Met-Leu-Phe (fMLP), platelet-activating factor (PAF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and IgG-coated particles, induce a rapid and transient Rap1 activation. In addition, we found that Rap1 is normally activated in neutrophils from chronic granulomatous disease patients that lack cytochrome b588 or p47phox and have a defective NADPH oxidase system. From these results we conclude that in neutrophils Rap1 is activated independently of respiratory burst induction. Finally, we found that Rap1 is activated by both the Ca2+ ionophore ionomycin and the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA), indicating that phospholipase C (PLC) activation leading to elevated levels of intracellular free Ca2+ and diacylglycerol (DAG) can mediate Rap1 activation. However, inhibition of PLC and Ca2+ depletion only marginally affected fMLP-induced Rap1 activation, suggesting that additional pathways may control Rap1 activation.

Neutrophils play an important role in the host defense to microbial pathogens. Stimulation of these cells induces multiple responses, including cell adhesion, migration, secretion, phagocytosis, and the generation of reactive oxygen species. Deregulated activation of neutrophils is implicated in the pathogenesis of a variety of inflammatory diseases leading to tissue damage. Therefore, neutrophil function is under tight control. A diverse array of receptors are expressed on the surface of neutrophils, allowing regulation by a wide range of agonists. Tyrosine kinase-linked receptors (eg, granulocyte-macrophage colony-stimulating factor [GM-CSF] receptors), serpine receptors (eg, N-formylmethionyl-leucyl-phenylalanine [fMLP] and platelet-activating factor [PAF] receptors), and receptors that are stimulated by immune complexes such as the Fc receptors, activate distinct and overlapping signaling pathways regulating neutrophil responses (and references therein).

Several small GTPases have been implicated in controlling neutrophil function. In particular, Rac1 was shown to play an important role in the formation of the NADPH oxidase complex, thereby controlling the respiratory burst. In addition, receptor-dependent activation of Ras was recently demonstrated. Another small GTPase suggested to play a role in neutrophil function is the Ras-like small GTPase Rap1. Rap1 has an effector domain virtually identical to Ras, and it has been shown that ectopic expression of Rap1, under certain conditions, antagonizes Ras signaling. Of the two known isozymes of Rap1, Rap1A is highly expressed in human neutrophils, and its function is largely unknown, because Rap1 is not necessary to normally activated in neutrophils from chronic granulomatous disease patients that lack cytochrome b588 or p47phox and have a defective NADPH oxidase system. From these results we conclude that in neutrophils Rap1 is activated independently of respiratory burst induction. Finally, we found that Rap1 is activated by both the Ca2+ ionophore ionomycin and the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA), indicating that phospholipase C (PLC) activation leading to elevated levels of intracellular free Ca2+ and diacylglycerol (DAG) can mediate Rap1 activation. However, inhibition of PLC and Ca2+ depletion only marginally affected fMLP-induced Rap1 activation, suggesting that additional pathways may control Rap1 activation.

MATERIALS AND METHODS

Isolation of human neutrophils. Blood was obtained from healthy volunteers from the Red Cross Blood Bank (Utrecht, The Netherlands). Mixed granulocytes were isolated from the buffy-coat of 500 mL 0.4% (wt/vol) tri-sodium citrate (pH 7.4) -treated blood as previously described. Mononuclear cells were removed by centrifugation over isotonic Percoll (1.078 g/mL) from Pharmacia (Uppsala, Sweden). After lysis of the erythrocytes in isotonic NH4Cl solution, neutrophils were washed and resuspended in incubation buffer (20 mM HEPES, 132 mM NaCl, 6 mM N-acetate, 1 mM MgSO4, 1.2 mM KH2PO4, 5 mM glucose, 1 mM MgCl2) containing 0.5% human serum albumin (HSA; Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). Neutrophils were incubated for 30 minutes at 37°C before stimulation. Neutrophils isolated in this manner were in the resting state. Neutrophils for the experiments described in Fig 4 were isolated as described. In all experiments, a concentration of 107 cells/mL was used for stimulation.

Neutrophil stimulation. One milliliter of neutrophil suspension was stimulated with one of the following stimuli: fMLP (1 µmol/L), PAF (1 µmol/L), TPA (100 ng/mL), thapsigargin (100 µmol/L) (all from Sigma, St Louis, MO), GM-CSF (0.1 µmol/L; Genzyme, Boston, MA), and

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ionomycin (100 nmol/L; Calbiochem, La Jolla, CA). The concentrations used are known to prime neutrophils or activate the respiratory burst.\(^1,5,17\) In some experiments, cells were preincubated as described in the legends of the figures with one of the following inhibitors: IBMX, prostaglandin E\(_2\) (PGE\(_2\)), wortmannin, staurosporine (all from Sigma), GFI09203X, U73122, or LY294002 (all from Biomol, Plymouth, PA). At different time points, 0.5 mL 3\(\times\) RIPA (1\(\times\) RIPA: 150 mmol/L NaCl, 10 mmol/L Tris-HCl [pH 7.4], 1% NP-40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate [SDS], 2 mmol/L phenylmethylsulfonyl fluoride [PMSF], 2 mmol/L benzamidine, 4 mmol/L aprotinin, 4 mmol/L leupeptin, and 4 mM trypsin inhibitor) was added.

For stimulation with IgG-coated particles,\(^14\) 500 µL of 0.8-µm latex beads (Difco, Augsburg, Germany) was washed with phosphate-buffered saline (PBS) and suspended in PBS (pH 8.5). One hundred microliters of human IgG (150 mg/mL; Lister Institute, Hertfordshire, UK) was added and incubated for 30 minutes at 37°C. Beads were washed twice with incubation buffer, resuspended in 500 µL incubation buffer, and added to 10\(^6\) cells in 500 µL incubation buffer. Samples were stirred continuously and 200-µL aliquots were lysed at indicated time points by adding 200 µL 2× RIPA.

Rap1 activation assay. Rap1 activation was determined essentially as described.\(^12\) Cell lysates were put on ice for 8 minutes and clarified by centrifugation at 14,000 rpm in an Eppendorf centrifuge for 8 minutes at 4°C. Per sample, 14 µg His-RalGDS-RBD or glutathione S-transferase (GST)-RalGDS-RBD was precoupled for 1 hour to 15 µL of 75% Ni-NTA (nickel-nitrilotriacetic acid agarose; Qiagen, Hilden, Germany) (His-RalGDS-RBD) or 40 µL of 10% glutathione beads (GST-RalGDS-RBD). After coupling, beads were washed 4 times with RIPA, added to the cell lysate, and incubated for 30 minutes at 4°C. Samples were washed 3 times in RIPA and bound proteins were eluted in 15 µL of Laemmli sample buffer. The samples were put on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF; NEN, Boston, MA). Rap1 was detected with a monoclonal antibody against Rap1. The amount of Rap1 GTP was isolated at different time points. Cells were lysed and Rap1 GTP was isolated with the 97 amino acid RalGDS-RBD. Rap1 was detected by Western blotting.\(^12\) Stimulation with 1 µmol/L fMLP-induced a rapid increase in Rap1 activity with biphasic kinetics (Fig 1). An initial activation peak was observed by 10 seconds, which decreased around 30 seconds. Activity peaked again at 5 minutes, followed by a slow decline toward basal levels observed in resting neutrophils. The extent of activation varied somewhat between different donors (compare Figs 1, 3, and 4B for variation in Rap1 activation after fMLP-stimulation), but the kinetics of Rap1 activation remained essentially constant in all experiments. Approximately 1% of Rap1 present in the total lysate of stimulated cells was found to be bound to RalGDS-RBD (Fig 1). This was concluded after comparing the amount of Rap1 present in total lysate and that of Rap1 in the GTP bound state on Western blot. Stimulation with 1 µmol/L PAF also resulted in a rapid activation of Rap1, which peaked around 10 seconds, followed by a slow decline towards basal level. In contrast to fMLP, no second activation peak was observed. fMLP and PAF both act via serpentine receptors. To investi-
gate whether Rap1 could be activated via receptor-associated tyrosine kinases, we stimulated neutrophils with GM-CSF (0.1 nmol/L). This stimulation resulted in a delayed and weaker activation of Rap1, compared with fMLP- or PAF-induced Rap1 activation, reaching its maximum around 5 to 10 minutes.

Various studies have implicated Rap1 functioning in the production of oxygen radicals. Therefore, we analyzed Rap1 activation by stimuli known to induce a respiratory burst in resting neutrophils. Incubation with IgG-coated particles resulted in a slow but steady increase of Rap1 activation detectable after 30 seconds, which reached its maximal activity after 4 minutes (Fig 2). A slow increase of Rap1 activation was also observed after treating resting neutrophils with TPA (100 ng/mL). In this case, Rap1 activation was detectable after 1 minute and reached a maximum after 5 minutes of TPA treatment.

Activation of primed neutrophils by fMLP also induces the respiratory burst (Fig 3A). To investigate whether Rap1 activation is modified under these conditions, we primed neutrophils with 0.1 nmol/L GM-CSF followed by stimulation with 1 µmol/L fMLP. As shown in Fig 3B, fMLP induced Rap1 activation to a similar extent both in resting and in primed neutrophils. In general, the kinetics of activation were slightly different, with the first activation peak delayed and the absence of a second peak in the case of primed cells. As a consequence, usually only a single activation peak was observed around 30 seconds, which decreased after 1 minute. Similar results were obtained after priming neutrophils with PAF (data not shown).

Multiple signaling pathways direct fMLP-induced Rap1 activation. Although Rap1 was activated by all agents used, the kinetics of Rap1 activation differed, suggesting that multiple signaling pathways might regulate Rap1 activation. To determine the mechanisms by which Rap1 GTPase is regulated, we investigated fMLP-induced Rap1 activation, because some of
the signaling pathways used by fMLP in resting neutrophils are defined. fMLP activates a serpentine receptor that is coupled to various heterotrimeric G-proteins (Bokoch7 and references therein). After stimulation, phospholipase C β (PLCβ) is activated, resulting in diacylglycerol (DAG)-mediated activation of protein kinase C (PKC) and inositol 3,4,5 triphosphate (IP3)-mediated Ca2+ mobilization.7,21 In addition, phosphatidylinositol-3-kinase (PI-3K) is activated, which may be responsible for the activation of protein kinase B (PKB) and of Rac GTPases.21-23

A possible signaling pathway which may mediate Rap1 activation involves changes in intracellular free Ca2+ concentration ([Ca2+]i). We first investigated whether Ca2+ was able to induce Rap1 activation, because in human platelets α-thrombin induced Rap1 activation is Ca2+-dependent.12 Resting neutrophils were incubated with ionomycin and thapsigargin to mimic Ca2+ influx and Ca2+ release from internal stores. As shown in Fig 4A, ionomycin induced a rapid activation of Rap1 to a level similar to fMLP, whereas induction of Rap1 by thapsigargin, although detectable, was clearly lower than that induced by fMLP. Because these experiments indicated that Ca2+ influx may be sufficient to induce Rap1 activation in neutrophils, we next investigated whether an increase in [Ca2+]i was essential for Rap1 activation. We therefore depleted neutrophils of Ca2+ by pretreatment with 1 mmol/L EGTA, 1.5 µmol/L indo-1/AM, or 100 nmol/L thapsigargin. Under these conditions, [Ca2+]i decreased to less than 10 nmol/L and no increase in [Ca2+]i, levels was observed after fMLP stimulation (Fig 4B). Ca2+ depletion did not affect the rapid fMLP-induced Rap1 activation, but we did observe a reduction in Rap1 activation at the second activation peak as compared with the control fMLP treatment. Similar results were obtained after inhibition of Ca2+ influx by blocking Ca2+ channels with La3+ (data not shown).

From these results we concluded that elevated [Ca2+]i is sufficient but not essential to induce Rap1 activation in human neutrophils.

A role for PKC in Rap1 activation was suggested, because TPA induced Rap1 activation in neutrophils (see Fig 2). Therefore, we examined whether inhibitors of PKC could abolish fMLP-induced Rap1 activation. However, the broad-specificity PKC inhibitors staurosporine and GF109203X (bis-indoxylmaleimide) did not inhibit fMLP-induced Rap1 activa-

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**Fig 4.** Ca2+ dependency of Rap1 activation. (A) Neutrophils were incubated with ionomycin (100 nmol/L) or thapsigargin (100 nmol/L) for the indicated time points. Rap1 GTP was detected as in the legend to Fig 1. (B) Ca2+-depleted neutrophils were stimulated with 1 µmol/L fMLP. As a control, non-Ca2+-depleted cells were stimulated with 1 µmol/L fMLP. Ca2+-depleted and untreated neutrophils of the same donor were taken to measure [Ca2+] after 1 µmol/L fMLP stimulation. Representative examples of at least three independent experiments are shown.
tion, indicating that the fMLP-induced Rap1 activation is independent of PKC (Fig 5A). Furthermore, most of the TPA-induced Rap1 activation was insensitive to staurosporine, whereas, at the concentrations used, staurosporine effectively abolished TPA-induced respiratory burst (Fig 5B). Thus, Rap1 can be activated directly by TPA and does not require PKC.

Because both elevated levels of \([Ca^{2+}]_i\) and TPA-treatment (which mimics the formation of DAG) activated Rap1, PLCβ may mediate fMLP-induced Rap1 activation. We investigated whether the PLCβ-inhibitor U73122 could inhibit fMLP-induced Rap1. However, no inhibition of fMLP-induced Rap1 activation was observed (Fig 5C). From these results we concluded that another, distinct signaling pathway regulating Rap1 activation is activated by fMLP or that a U73122-insensitive PLC isoform is responsible for the fMLP-induced Rap1 activation. As expected, both inhibition of PKC and Ca2+ depletion did not abolish fMLP-induced Rap1 activation either (Fig 5C).

To investigate whether Rap1 activation is mediated by PI-3K, we treated resting neutrophils with the PI-3K inhibitors wortmannin and LY294002. Both inhibitors failed to inhibit fMLP-induced Rap1 activation under conditions that did abolish respiratory burst (Fig 5D and data not shown).

Rap1 activation is not inhibited by PGE2 and is independent of oxidase assembly and function. In platelets, Rap1 activation by α-thrombin is completely inhibited by prostacyclin, a platelet antagonist that elevates the levels of cAMP. PGE2 is an antagonist of neutrophils and also elevates the levels of cAMP. In addition, PGE2 was reported to induce Rap1 phosphorylation via cAMP-dependent protein kinase A (PKA), resulting in the dissociation of Rap1 from cytochrome b558 in vitro. We therefore examined if PGE2 could antagonize fMLP-dependent Rap1 activation in neutrophils. However, cotreatment of neutrophils with PGE2 and the phosphodiesterase inhibitor IBMX (which further elevates cAMP levels) did not affect fMLP-induced Rap1 activation (Fig 6). The observation that a similar treatment did inhibit fMLP-induced respiratory burst in GM-CSF–primed neutrophils indicated that cAMP does not interfere with the activation of Rap1 GTPase.

That Rap1 activation was not dependent on Rap1 association with cytochrome b558 or the formation of the oxidase complex was further confirmed by analysis of Rap1 activation in patients

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**Fig 5.** fMLP- and TPA-induced Rap1 activation is independent of PKC. (A) fMLP-induced Rap1 activation is not inhibited by PKC inhibitors. Neutrophils were preincubated for 5 minutes with 200 nmol/L staurosporine or 10 minutes with 5 μmol/L GF109203X. Neutrophils were then stimulated with 1 μmol/L fMLP for 10 seconds and 5 minutes. As a control, untreated neutrophils of the same donor were used. (B) TPA-induced Rap1 activity is not inhibited by a PKC inhibitor. Neutrophils were stimulated with 100 ng/mL TPA for 5 and 10 minutes after preincubation for 5 minutes with 200 nmol/L staurosporine or buffer. Oxygen consumption was measured with a Clark oxygen electrode to measure the functionality of staurosporine treatment. Representative examples of at least three independent experiments are shown. (C) Inhibition of PLCβ does not influence Rap1 activity. Neutrophils were preincubated with 1 μmol/L U73122 (PLCβ inhibitor) for 3 minutes and subsequently stimulated with 1 μmol/L fMLP. (D) Rap1 activity is not inhibited by PI-3 kinase inhibitors. Neutrophils were preincubated with the PI-3 kinase inhibitors LY294002 (10 μmol/L) or wortmannin (300 nmol/L) for 5 minutes. Samples were taken after preincubation and 10 seconds and 5 minutes after 1 μmol/L fMLP stimulation. As a control, neutrophils of the same donor without preincubation were used. Representative examples of at least three independent experiments are shown.
with chronic granulomatous disease (CGD), which lack a functional oxidase complex. As shown in Fig 7, Rap1 is still activated by both fMLP and TPA in neutrophils isolated from CGD patients, lacking either cytochrome b558 (p91phox) or p47phox.

**DISCUSSION**

In this report we have investigated the signaling events in human neutrophils that resulted in the activation of Rap1, ie, an increase in levels of Rap1GTP. We have used an assay that uses the Rap1 binding domain of a putative Rap1 effector (RalGDS-RBD) to specifically precipitate Rap1GTP. We observed that a variety of stimuli can induce Rap1 activation, but the extent and kinetics of activation varied. A very rapid activation of Rap1 was observed after fMLP and PAF stimulation, whereas slower activation was observed after stimulation with TPA, incubation with IgG-coated particles, or GM-CSF.

Our finding that several stimuli can activate Rap1 may indicate that multiple pathways are involved in the activation of Rap1. Thus far, a study of the activation of Rap1 has only been performed in human platelets and T lymphocytes. In these cells, Rap1 activation after α-thrombin stimulation is mediated by elevated [Ca^{2+}], which is necessary and sufficient. In neutrophils, it appears that, in addition to elevated [Ca^{2+}], other pathways lead to Rap1 activation. Indeed, both ionomycin and, to a lesser extent, thapsigargin induce Rap1 activation, but Ca^{2+} depletion did not abolish fMLP-induced Rap1 activation as well.

Because TPA efficiently induced Rap1 activation, DAG is a strong candidate to mediate Rap1 activation. PKC, a well-known target for DAG, is not involved in Rap1 activation, because both the fMLP-induced Rap1 activation and the TPA-induced Rap1 activation were not abolished after treatment with the broad PKC inhibitor staurosporine. This implies that a different DAG target is involved in the regulation of Rap1.

Surprisingly, affecting the elevation of [Ca^{2+}] and DAG formation by inhibition of PLCβ did not abolish fMLP-induced Rap1 activation. This could mean that U73122 is not sufficient to block an increase in [Ca^{2+}] and/or the formation of DAG. Indeed, PLC-independent pathways for Ca^{2+} influx and DAG formation have been described. Alternatively, a third, still-unknown pathway is involved in the regulation of Rap1.

It is unlikely that fMLP-induced Rap1 activation is mediated by PI-3K, because the PI-3K inhibitors wortmannin and LY294002 also failed to abolish Rap1 activation either.

It was shown previously that Rap1 is present in a complex with cytochrome b558 of the NADPH oxidase and cotranslocates with cytochrome b558 from the specific granules to the plasma or phagosome membrane after stimulation. Our results show that association of Rap1 with cytochrome b558 or an assembled oxidase complex is not necessary for Rap1 activation. This conclusion was based on our observation that Rap1 is normally activated in neutrophils from CGD patients that lack either p91phox or p47phox. Furthermore, PGE2, which was reported to induce Rap1 phosphorylation and dissociation from cytochrome b558, did not abolish Rap1 activation.

Our finding that Rap1 is activated after stimulation of both receptor associated-tyrosine kinases (GM-CSF) and serpentine
receptors (fMLP and PAF) indicates that Rap1 functions in a pathway common to both receptor types. Interestingly, only GM-CSF but not fMLP induces phosphorylation of Cbl.31 This protein has been implicated in the activation of Rap1 through the recruitment of C3G, an exchange factor for Rap1, to Cbl via the adaptor protein Crk or Crk-L.32,33 However, if indeed Cbl mediates Rap1 activation, pathways inducing Rap1 activation after fMLP stimulation would be independent of Cbl phosphorylation.

Although the function of Rap1 is unknown, it has been suggested that Rap1 activation is required for respiratory burst.10,11 Our results show that Rap1 activation in vivo is not sufficient to induce a respiratory burst. Perhaps Rap1 activation is only involved in one of the events regulating the multistep process of generating a respiratory burst. Rap1 activity has also been found in human platelets.12 Both neutrophils and platelets are highly specialized, and Rap1 is possibly involved in a specific function common to both cell types. The reported association of Rap1 to cytochrome b_{558} in human neutrophils may indicate that Rap1 is involved in the regulation of complex formation. In platelets, a close relative of Rap1, Rap2, has been found in a complex with the major platelet integrin αIIbβ₃,44 suggesting a similar role for Rap2 in platelets. Furthermore, in both cell types, Rap1 is at least partly localized on vesicular structures and is translocated to the plasma membrane upon activation.39 Interestingly, the Rap homologue in the budding yeast Saccharomyces cerevisiae, Rsr1 (Bud1) is involved in the selection of the site where a new bud will form.36 Perhaps Rap1 regulates the formation and/or translocation of protein complexes by specifying the fusion of different cellular compartments, such as the fusion of secretory vesicles with the plasma membrane. It is suggested that Rap1 is involved in regulated insulin secretion.37

To decipher the function of Rap1 it will be essential to know which effector protein binds to Rap1GTP. Interestingly, the effector domain of Rap1 is virtually identical to the effector domain of Ras,38,40 and it has been shown that Rap1 also binds to Ras effector proteins, including Ral guanine nucleotide exchange factors (RalGEFs) and Raf kinases.41,42 In neutrophils and platelets, Rap1 may activate one of the Raf kinase signaling cascades,43,44 or Rap1 may activate one of the RalGEFs resulting in the activation of the small GTPase Ras.45 This protein is involved in the activation of phospholipase D,46 which, together with members of the Arf family of small GTPases, may control translocation and fusion of granules.37

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