The Small GTP-binding Protein Rac Promotes the Dissociation of Gelsolin from Actin Filaments in Neutrophils

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Gelsolin is an actin filament-capping protein that has been shown to play a key role in cell migration. Here we have studied the involvement of phosphoinositide 3-kinase (PI 3-kinase) and GTP-binding proteins (G-proteins) in the regulation of gelsolin-actin interactions in neutrophils. Inhibition of PI 3-kinase activity in vitro by wortmannin did not affect the dissociation of actin-gelsolin (1:1) complexes induced by neutrophil stimulation with N-formyl-Met-Leu-Phe. Guanosine 5'-[γ-thio]triphosphate (GTPγS) indirectly promoted the dissociation of actin-gelsolin complexes in a cell-free system using neutrophil cytosol, and this effect was blocked by the GDP dissociation inhibitor for Rho (Rho-GDI). The GTPγS-loaded α2 and the β1γ2 subunits of heterotrimeric G-proteins (Gα2 and Gβ1γ2) also triggered actin-gelsolin dissociation in a Rho-GDI-sensitive manner. GTP-loaded activated Rac, but not activated Rho, induced the dissociation of cytosolic actin-gelsolin complexes. The guanine nucleotide exchange on Rac was increased by addition of GTPγS-loaded Gα2 or Gβ1γ2 to neutrophil cytosol. These findings suggest that activation of Rac by G-protein-coupled receptors in neutrophils triggers uncapping of actin filaments, independently of PI 3-kinase.

Gelsolin is a Ca2+- and polyphosphoinositide-regulated actin-binding protein involved in the signaling cascade activated by chemotactic agonists such as fMLP that controls actin polymerization in neutrophils (1, 2), a response thought to be essential for cell motility (3–5). In vitro and in the presence of Ca2+, gelsolin rapidly disassembles actin filaments by noncovalent severing and remains attached to their barbed ends (6, 7), a function that can be inhibited by the phosphoinositides PtdIns(4,5)P2 and PtdIns(4)P (2). Actin polymerization and actin-gelsolin dissociation are, however, maximal at a time (10–15 s after fMLP stimulation) when PtdIns(4,5)P2 levels drop (16, 17), implying that PtdIns(4,5)P2 synthesis does not correlate with these responses in vivo (18, 19).

PtdIns(3,4,5)P3 has thus been proposed to control actin-gelsolin dissociation (19–22) because, in contrast to PtdIns(4,5)P2, the cellular levels of this phospholipid rise upon fMLP stimulation, with a time course similar to both actin polymerization and actin-gelsolin dissociation. Moreover, PtdIns(3,4,5)P3 production is Ca2+-independent (19, 22). PtdIns(3,4,5)P3 is produced from PtdIns(4,5)P2 by the action of PI 3-kinase (17) and has been suggested to be a novel second messenger mediating cytoskeletal rearrangements and respiratory burst activation in response to chemoattractants (19–24).

There is now compelling evidence that cell surface receptor-activated actin rearrangements are controlled by small G-proteins of the Rho family in various motile cells. In fibroblasts, platelet-derived growth factor has been shown to control the formation of lamellipodia by activating Rac (25), whereas the assembly of stress fibers and focal contacts was mediated by Rho (26). In neutrophils, ADP-ribosylation of Rho by the C3 exoenzyme impaired chemotaxis (27), whereas in HL-60 cells inactivation of Rho by Clostridium limosum toxin inhibited basal but not fMLP-stimulated actin polymerization (28). Studies in permeabilized neutrophils showed that activation of G-proteins with GTPγS could trigger actin polymerization (29) by increasing the number of free barbed ends (30). The GTPγS-induced actin polymerization was not dependent on the presence of ATP, suggesting that this response is not dependent on protein or lipid phosphorylations (29). A recent report showed that GTPγS also induced actin polymerization in a cell-free system by activating Cdc42 and that this response did not correlate with PtdIns(4,5)P2 synthesis (31).

Here we assess the importance of PtdIns(3,4,5)P3 production in the fMLP-stimulated actin-gelsolin dissociation in neutrophils. We also provide evidence for the existence of a phosphoinositide-independent signaling pathway involving Rac that can trigger actin-gelsolin dissociation in neutrophil cytosol.

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EXPERIMENTAL PROCEDURES

Materials—BAPTA-AM, Pluronic F-127, N-1-pyreneiodoacetamide, and fluorescein-phalloidin were from Molecular Probes; FMLP, PtdIns(4,5)P$_2$, PtdIns(4)P, and PtdIns were from Sigma; [α-32P]GTP (3000 Ci/mmol) was from Hartmann Analytic; wortmannin was a generous gift of T. G. Payne, Sandoz, Basel; 2C4 hybridoma producing anti-gelsolin monoclonal antibody was donated by L. A. Elvehjem, the lysate was centrifuged at 100 × g at 4 °C; the supernatant was centrifuged 20 min at 9000 × g at 4 °C, and the supernatant was centrifuged 30 min at 121,500 × g at 4 °C. The supernatant ("cytosol") was collected and stored at –80 °C until use. Ca$^{2+}$ Depletion of Neutrophils—For Ca$^{2+}$ depletion, neutrophils were resuspended in phosphate-buffered saline supplemented with 2 mM EGTA and incubated with 25 μM BAPTA-AM (in Me$_2$SO containing 20% (w/v) Pluronic F-127) for 30 min at room temperature. The cells were then pelleted by centrifugation and resuspended at the desired concentration in the respective assay buffer without Ca$^{2+}$ and containing 2 mM EGTA. The effectiveness of Ca$^{2+}$ depletion was checked by measuring changes in intracellular Ca$^{2+}$ levels in response to FMLP. For this purpose, cells were loaded with 5 μM Fura 2-AM and were stimulated with 100 nM FMLP. Two wavelength excitation was performed at 340 and 380 nm, and the intracellular [Ca$^{2+}$]$_i$ ([(Ca$^{2+}$)]) was calculated from the fluorescence intensity at 510 nm. GDP- or GTP-bound forms of the small G-proteins as GST fusion proteins and neutrophil gelsolin are due to an N-terminal extension of 25 amino acids on plasma gelsolin (44).

Quantification of Barbed End Nucleating Activity—Pyrene-labeled F-actin prepared as above was depolymerized and gel-filtered through a Sephadex G-150 column (37). Barbed end nucleating activity was analyzed (15). Neutrophils were resuspended at 1.5 × 10$^8$/ml in HBSSG$_2$ (14) and preincubated with Me$_2$SO (control) or wortmannin (dissolved in Me$_2$SO) for 10 min at 37 °C. 100 μl of cells were then added to an equal volume of HBSSG$_2$ containing Me$_2$SO (control) or 200 nM FMLP for 15 s at 37 °C. The stimulation was stopped by the addition of 200 μl of lysing buffer (14). After centrifugation, actin-gelsolin (1:1) complexes were immunoprecipitated from the supernatants using immobilized purified anti-gelsolin monoclonal antibody and washed as described (14). After SDS-PAGE and Coomassie Blue staining, the gels were scanned as above. The molar ratios of gelsolin and actin were calculated (14) assuming that the actin/gelsolin molar ratio is equal to the ratio of the measured absorbances of the 90-kDa (cytoplasmic gelsolin) and the 42-kDa (actin) protein bands on the gel multiplied by 2.14. The differences in molecular mass between plasma and neutrophil gelsolin are due to an N-terminal extension of 25 amino acids on plasma gelsolin (44).

Determination of Cellular Filamentous Actin—Filamentous actin was stained with fluorescein-labeled phallolidin according to Ref. 46. 47. Neutrophils were resuspended at 10$^8$/ml in HBSSG$_2$ and preincubated with wortmannin or Me$_2$SO (control) for 10 min at 37 °C. The cells were then stimulated with 100 nM FMLP or Me$_2$SO for 15 s, and 100 μl was added to an equal volume of HBSSG$_2$ containing Me$_2$SO (control) or 200 nM FMLP for 15 s at 37 °C. The stimulation was stopped by the addition of 200 μl of lysing buffer (14). After centrifugation, actin-gelsolin (1:1) complexes were immunoprecipitated and analyzed by SDS-PAGE. The fluorescence intensity at 407 nm between 100 and 200 s was used as a measure of barbed end nucleating activity. EGTA-resistant actin-gelsolin (1:1) complexes were immunoprecipitated using purified anti-gelsolin monoclonal antibody (clone 2C4) coupled to CNBr-activated Sepharose CL-4B. The immunoprecipitates were washed once with buffer A and analyzed by SDS-PAGE. After Coomassie Blue staining, the gels were scanned on a Bio-Rad GS-670 flatbed imaging densitometer. The molar ratios of gelsolin to actin were calculated as above, assuming that the actin/gelsolin molar ratio is equal to the ratio of the measured absorbances of the 90-kDa (plasma gelsolin) and 42-kDa (actin) protein bands multiplied by 2.21 (see Ref. 14).
Measurement of GDP-GTP Exchange on GST-Rac1 in Neutrophil Cytosol—Purified recombinant GST-Rac1 was preloaded with GDP and added (10 μM final concentration) to neutrophil cytosol containing 10 mM EGTA, 12.5 μM GTP, and 93.8 μM CaCl2 in [γ-32P]GTP in the presence of 2.5 μM recombinant GDP-S-loaded Gαo, HED pretreated with GTP-γ-S and MgCl2 (control), 2.5 μM recombinant Gβγ, or buffer D (control). The samples were incubated for 10 min at 37 °C and placed on ice, and glutathione-Sepharose CL-4B (40 μl of a 1:1 suspension in HEPES buffer) was added. After incubation for 30 min at 4 °C, the beads were collected by centrifugation and washed three times with Wash buffer (48). The samples were split in two. One-half was assayed for bound radioactivity by scintillation counting, and the other was analyzed for protein content by SDS-PAGE and Coomassie Blue staining. The ratio of bound radioactivity (cpm) to protein content (μg) was used as an indicator of the rate of exchange of GDP to GTP on GST-Rac1.

RESULTS

Modulation of Gelsolin/Actin Interactions by Phosphoinositides in Vitro—It has been shown in previous reports that increased phosphorylations of the inositol head group of phosphoinositides enhance their ability to dissociate actin-gelsolin complexes (PtdIns(4,5)P2 > PtdIns(4)P > PtdIns) (8-10). We have now evaluated the capacity of PtdIns(3,4,5)P3 to dissociate EGTA-resistant equimolar actin-gelsolin complexes and to inhibit the actin filament severing activity of gelsolin in vitro. When purified actin-gelsolin complexes were exposed to phosphoinositides prior to immunoprecipitation, PtdIns(3,4,5)P3 was found to be equally potent to PtdIns(4,5)P2 in dissociating actin from gelsolin (EC50 = 19 μM, Fig. 1A). The actin filament severing activity of gelsolin was measured by the relative rate of pyrene-labeled F-actin depolymerization. In these experiments, PtdIns(3,4,5)P3 was somewhat more efficient than PtdIns(4,5)P2 in inhibiting the gelsolin-mediated severing of actin filaments (EC50 of 4 μM versus 10 μM, Fig. 1B).

Effect of PI 3-Kinase Inhibition and Ca2+ Depletion on Agonist-induced Actin-Gelsolin Dissociation and Actin Polymerization in Neutrophils—While in resting neutrophils a large fraction of gelsolin is associated with actin, and exposure of the cells to FMLP leads to the rapid release of actin from gelsolin (14). This is paralleled by an increase in free barbed ends (14, 15) and a doubling of the cellular F-actin content (45, 49, 50). At a time point when these three transient responses reach their maximum, the effects of the PI 3-kinase inhibitor wortmannin and of Ca2+ depletion were studied. As shown in Fig. 2A, 100 nM FMLP caused a decrease of the amount of actin associated with gelsolin to 1/7 of the levels in unstimulated cells within 15 s, which is in agreement with previous reports (14).

Pretreatment of neutrophils with 0.1–10 μM wortmannin prior to stimulation did not significantly affect the FMLP-induced breakdown of actin-gelsolin complexes as compared with control cells (Fig. 2A). While wortmannin at 0.1 μM completely inhibits PI 3-kinase in a specific manner (24, 51), the substance also affects myosin light chain kinase activity at micromolar concentrations (52). Since the signal controlling actin-gelsolin dissociation has been proposed to be Ca2+-independent (14), we analyzed the levels of complexes in Ca2+-depleted cells. Ca2+ depletion resulted in a dramatic decrease in the basal levels of actin-gelsolin complexes (actin/gelsolin ratio = 0.07, Fig. 2A). Stimulation of Ca2+-depleted neutrophils lowered the actin/gelsolin ratio further from 0.07 to 0.02. The effect of wortmannin in Ca2+-depleted cells was pronounced, as it inhibited the agonist-mediated dissociation of actin and gelsolin completely at 1 μM.

The observation that depletion of intracellular calcium pools with BAPTA strongly lowered the basal levels of complexes is probably due to the fact that there is a turnover of gelsolin molecules at the barbed ends of actin filaments in the cell and that the reassociation of gelsolin to actin is blocked in the absence of calcium (11, 12) (as well as the severing activity of gelsolin that could also produce actin oligomers with capped barbed ends). This decrease in basal levels of complexes is probably not due to increased cellular levels of PtdIns(4,5)P2 or PtdIns(4)P (possibly caused by inhibition of Ca2+-dependent phospholipase C), as in 32P2-labeled neutrophils the basal levels of [(32)P]PtdIns(4,5)P2 and [(32)P]PtdIns(4)P were not significantly affected by Ca2+ depletion (data not shown). Ca2+-
depletion totally abolished the fMLP-stimulated breakdown of $[^{32}P]PtdIns(4,5)P_2$, but not the production of $[^{32}P]PtdIns(3,4,5)P_3$, as previously reported (22).

We tested various pharmacological agents for their ability to inhibit the fMLP-mediated breakdown of actin-gelsolin complexes in neutrophils. Inhibitors of protein kinases like staurosporine, genistein, and erbstatin did not inhibit the response, which was also insensitive to preincubation with 0.5% ethanol (an inhibitor of fMLP-induced phosphatidic acid production), but required metabolic energy, as it was inhibited by incubating the cells with 2-deoxyglucose and antimycin A. Stimulation of heterotrimeric G-proteins with AlF$_4^-$ (50 $\mu$m, 5 min) could mimic the effect of fMLP on the complexes in the intact cell (data not shown).

When neutrophils were stimulated with 100 nM fMLP for 15 s and subsequently treated with detergent for a barbed end nucleating activity assay, the stimulation elevated the relative rate of actin polymerization by a factor of 2 (Fig. 2B), in accordance with previous reports (15). Wortmannin showed no significant effect at 100 nM, although doses higher than 1 $\mu$m produced a 50% inhibition of the fMLP-induced rise in nucleating activity (Fig. 2B). The same amounts of inhibitor produced no significant effect on the barbed end nucleating activity in resting cells (data not shown). Ca$^{2+}$-depletion per se induced an increase in free barbed ends (Fig. 2B). The stimulation of Ca$^{2+}$-depleted cells with 100 nM fMLP caused a further (1.7-fold) increase in barbed end nucleating activity, which is 80% of the response in normal cells (see also Ref. 15). The effect of wortmannin in Ca$^{2+}$-depleted cells was similar to normal cells.

The basal levels of cellular F-actin content were lowered by wortmannin (1 $\mu$m) pretreatment (by 23%) or Ca$^{2+}$-depletion (by 13%). On the other hand, both treatments left the fMLP-mediated actin polymerization intact (Fig. 2C), as also reported under different conditions previously (45–47).

**Effect of GTP$_{\gamma}$S on Cytosolic Actin-Gelsolin Complexes—**To study the regulation of actin-gelsolin dissociation by G-proteins, we developed a cell-free system using cytosolic fractions of neutrophils obtained by nitrogen cavitation. The cytosolic preparations displayed levels of EGTA-resistant actin-gelsolin (1:1) complexes ranging from 0.5 to 1 (mol/mol), thus reflecting the levels of complexes in the soluble fraction of Triton X-100 extracts of resting neutrophils.

When neutrophil cytosol was exposed to GTP$_{\gamma}$S, the actin/gelsolin ratio in subsequently obtained anti-gelsolin immunoprecipitates was significantly reduced to less than 60% (Fig. 3A). The maximal effect of GTP$_{\gamma}$S occurred already at 1 $\mu$m. Increasing concentrations of GTP$_{\gamma}$S up to 100 $\mu$m did not further affect the level of complexes in the cytosol (Fig. 3B). 100 $\mu$m GDP did not dissociate the complexes (data not shown). ATP-$\gamma$S up to 100 $\mu$m was less effective than GTP$_{\gamma}$S in dissociating actin from gelsolin, indicating that GTP is not utilized by a diphosphonucleotide kinase to generate ATP and that the latter is not involved in the dissociation process (Fig. 3, A and B). When actin-gelsolin complexes were preformed from purified actin and gelsolin analyzed by SDS-PAGE and densitometry. Mean of three experiments $\pm$ S.E. B, isolated untreated (filled boxes), Pluronic F-127-treated, or Ca$^{2+}$-depleted neutrophils (open boxes) were preincubated with Me$_2$SO (control) or wortmannin at indicated concentration prior to stimulation with 100 nM fMLP for 15 s. The cells were then lysed, and barbed end nucleating activity was determined as described under “Experimental Procedures.” Mean of three experiments $\pm$ S.E. C, untreated (filled boxes) or Ca$^{2+}$-depleted (open boxes) neutrophils were preincubated with Me$_2$SO (control) or wortmannin 1 $\mu$m, prior to stimulation with 100 nM fMLP for 15 s. The cells were then fixed, permeabilized, and stained for F-actin content using fluorescein-phalloidin. The cellular F-actin content is expressed as % of the untreated control. Mean of three different experiments $\pm$ S.E.
plexes prepared from purified gelsolin and purified ADP- or ATP-actin complexes (purified from neutrophil cytosol by immunoprecipitation) could not dissociate the 1:1 complex (Fig. 3A). This indicates that GTPγS does not dissociate cytosolic actin-gelsolin complexes directly but that it activates additional factors in the cytosol that mediate its effect on the complexes. These observations are confirmed by the finding that actin-gelsolin (1:1) complexes purified from neutrophil cytosol by immunoprecipitation could not be dissociated directly by 100 µM GTPγS and ATPγS (data not shown).

It has been shown previously that Rho-GDI forms tight complexes with members of the Rho family of small G-proteins and that the complex is inactive in various processes (53, 54). When neutrophil cytosol was supplemented with 2.8 µM recombinant Rho-GDI, the protein reversed the action of subsequently added GTPγS but did not affect the level of actin-gelsolin complexes in untreated cytosol (Fig. 4A). Incubation of the cytosol with recombinant C3 transferase, ribosylating specifically Rho proteins (55–57), did not inhibit the GTPγS-mediated dissociation of actin and gelsolin, whereas 1 µM wortmannin did not inhibit the response either (data not shown).

The Action of GTP-binding Proteins on Cytosolic Actin-Gelsolin Complexes—Myristoylated recombinant GαS at 2.5 µM bound to GTPγS (but not to GDP) induced a significant decrease of about 40% in the levels of cytosolic actin-gelsolin (1:1) complexes, as compared with the control treated with HED buffer containing GTPγS and MgCl2 (Fig. 4B). The presence of GTPγS in the HED buffer used to preload the recombinant GαS (10 µM final concentration) induced by itself a significant decrease in the levels of the cytosolic complexes (Fig. 4B). The effect of recombinant GαS was indirect, since it was not observed on purified actin-gelsolin complexes. Recombinant GαS at 2.5 µM also induced a significant decrease of about 50% in cytosolic actin-gelsolin (1:1) complexes, when the cytosol was supplemented with 0.1 µM GTPγS (Fig. 4B). The effect of GTPγS-loaded GαS and of Gβγ on cytosolic complexes could be blocked by Rho-GDI (Fig. 4C), suggesting that heterotrimeric G-proteins can activate proteins of the Rho family in neutrophil cytosol, which in turn trigger actin-gelsolin dissociation. This is confirmed by the observation that the dissociation of actin-gelsolin in neutrophil cytosol could be induced by the addition of GTP-loaded, constitutively activated Val-12 Rac1 at 5 µM (Fig. 5A). Its dissociating potential was specific as GDP-loaded Val-12 Rac1, inactive GTP-Val-12 Ala-35 Rac1 or GTP-Val-14 RhoA did not cause dissociation of cytosolic actin-gelsolin complexes at the same concentration (Fig. 5A). The effect of GTP-loaded Val-12 Rac1 occurred in a concentration-dependent fashion with a 50% dissociation at around 10 µM (Fig. 5B). Preformed complexes of purified actin and gelsolin, however, could not be dissociated even in the presence of 25 µM recombinant GTPγS-loaded Val-12 Rac1 (data not shown), which suggests that a downstream target of Rac, but not the small G-protein itself, interacts with the actin-gelsolin complex. The decrease in cytosolic actin-gelsolin complexes induced by GTPγS-loaded Val-12 Rac1 was not inhibited by C3 transferase, confirming that Rho is not involved in the process (data not shown).

The effect of GTPγS in the cytosol was not caused by an increase in the polyphosphoinositide concentration of the cytosol, since we failed to detect the corresponding amount of PtdIns(3,4,5)P3, PtdIns(4,5)P2, or PtdIns(4)P that would have accounted for the decrease in the levels of complexes in the GTPγS-treated cytosol (data not shown). GTPγS did not induce an incorporation of 32P into phospholipids when [γ-32P]ATP was included in the cytosol (data not shown).

The effects of GTPγS-loaded GαS, Gβγ, and GTPγS-loaded Val-12 Rac1 on cytosolic actin-gelsolin (1:1) complexes were not significantly affected by the presence of neomycin (10 µM) or wortmannin (1 µM) in the cytosol (data not shown), excluding the involvement of phosphoinositides such as PtdIns(3,4,5)P3 or PtdIns(4,5)P2. Furthermore, a gelsolin-derived peptide that has been shown to bind specifically to PtdIns(4,5)P2 (32) did not inhibit the response to GTP-bound Val-12 Rac1 (Fig. 5C) and to GTPγS-loaded GαS and Gβγ (data not shown), at a concentra-
Rac-stimulated Dissociation of Actin-Gelsolin Complexes

The results presented in this article show that gelsolin is not specifically regulated by PtdIns(3,4,5)P₃ in vitro, since PtdIns(3,4,5)P₃ was able to dissociate actin-gelsolin complexes with a potency similar to PtdIns(4,5)P₂. Considering that, upon neutrophil stimulation with fMLP, the maximal concentration of PtdIns(3,4,5)P₃ does not exceed 10% of the concentration of PtdIns(4,5)P₂ (17), it can be speculated that PtdIns(4,5)P₂ as compared with PtdIns(3,4,5)P₃ production is probably not the signal triggering actin-gelsolin dissociation, assuming a uniform distribution of the polyphosphoinositides in the inner leaflet of the cell membrane. The observed lack of selectivity of the severing activity of gelsolin in vitro for PtdIns(3,4,5)P₃ as compared with PtdIns(4,5)P₂ supports this idea. The fMLP-stimulated breakdown of actin-gelsolin (1:1) complexes in neutrophils and the increase in barbed end nucleating activity were not significantly affected by wortmannin at a concentration of 0.1 μM, which shows that activation of PI 3-kinase is not responsible for these responses and supports the conclusion of the in vitro studies.

Since Ca²⁺ depletion caused a strong decrease in the basal levels of actin-gelsolin complexes and also increased the basal barbed end nucleating activity of neutrophil lysates, it has to be concluded that increasing the number of free barbed ends in the cell is not sufficient to induce actin polymerization, since the F-actin levels in Ca²⁺-depleted cells were not increased in comparison to normal cells. A possible explanation for this is from six different experiments and are plotted as % of buffer-treated controls. * indicates a significant difference as compared with control (Student’s t test, p < 0.05).

**DISCUSSION**

The results presented in this article show that gelsolin is not specifically regulated by PtdIns(3,4,5)P₃ in vitro, since PtdIns(3,4,5)P₃ was able to dissociate actin-gelsolin complexes with a potency similar to PtdIns(4,5)P₂. Considering that, upon neutrophil stimulation with fMLP, the maximal concentration of PtdIns(3,4,5)P₃ does not exceed 10% of the concentration of PtdIns(4,5)P₂ (17), it can be speculated that PtdIns(4,5)P₂ production is probably not the signal triggering actin-gelsolin dissociation, assuming a uniform distribution of the polyphosphoinositides in the inner leaflet of the cell membrane. The observed lack of selectivity of the severing activity of gelsolin in vitro for PtdIns(3,4,5)P₃ as compared with PtdIns(4,5)P₂ supports this idea. The fMLP-stimulated breakdown of actin-gelsolin (1:1) complexes in neutrophils and the increase in barbed end nucleating activity were not significantly affected by wortmannin at a concentration of 0.1 μM, which shows that activation of PI 3-kinase is not responsible for these responses and supports the conclusion of the in vitro studies.

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the fact that actin monomers are sequestered in the resting cell (58), so that they cannot add to the freed barbed ends. This hypothesis is supported by experiments in other cell types (59) and suggests that fMLP induces actin polymerization in neutrophils both by increasing the number of free barbed ends and by monomer desequestration (2).

The neutrophil cytosol appears to contain a GTP \(_{S}\)-stimulable factor that can trigger the dissociation of endogenous actin-gelsolin complexes. Considering the evidence that the response of the cytosol to GTP \(_{S}\) is blocked by Rho-GDI, it must be assumed that a member of the Rho family of small G-proteins is involved in this response. As the effect of GTP \(_{S}\) on actin-gelsolin complexes was mimicked by GTP-loaded Val-12 Rac1 and not Val-14 RhoA, it can be proposed that cytosolic Rac is the GTP \(_{S}\)-stimulable factor responsible for actin-gelsolin dissociation. The insensitivity of this response to C3 transferase confirms that Rho is not involved in the pathway leading to actin-gelsolin dissociation.

\(G_{\alpha 2}\) and \(G_{\beta 1 \gamma 2}\) were both found to be able to trigger actin-gelsolin dissociation, which is in agreement with previous studies reporting effector activation both by \(G_{\alpha}\) and \(G_{\beta \gamma}\) subunits (reviewed in Ref. 60). Since the \(G_{\alpha 2}\)- and \(G_{\beta 1 \gamma 2}\)-stimulated dissociation of cytosolic actin-gelsolin was Rho-GDI-sensitive, heterotrimeric G-proteins may be able to trigger Rac activation in the neutrophil cytosol, a model supported by other studies (61, 62). However, the GTP \(_{S}\)-stimulated dissociation of the cytosolic actin-gelsolin complexes is probably due to a direct and GTP. C, neutrophil cytosol was supplemented with a PI(4,5)P\(_2\)-binding peptide (50 \(\mu\)M final concentration) or buffer (control). GTP-loaded Val-12 Rac1 (4.8 \(\mu\)M) or buffer supplemented with GTP (control) was then added, and actin-gelsolin complexes were analyzed as in A. Values are plotted as % of the buffer-treated control (100%) \(\pm\) S.E., \(n = 4\). * indicates a significant difference as compared with control (Student’s t test \(p < 0.05\)).
activation of endogenous Rac proteins, since we detected only a very small amount of G\(_{\alpha_S}\) in neutrophil cytosol, representing only a few percent of the protein present in membrane preparations (data not shown). This cytosolic pool of G\(_{\alpha_S}\) does not represent an amount of protein equivalent to the amount of recombinant G\(_{\alpha_S}\) that has to be added to the cytosol to trigger dissociation of actin-gelsolin complexes. It has been shown that Rac is complexed with Rho-GDI in neutrophil cytosol (63) and that GTP\(_S\) does not dissociate the Rac-Rho-GDI complex directly (62). It must thus be proposed that a fraction of the Rac present in the cytosolic fraction used in our studies is not bound to Rho-GDI and is activated by the addition of GTP\(_S\). This hypothesis is consistent with the observation that preincubation of the cytosol with Rho-GDI inhibits the effect of subsequently added GTP\(_S\). Dissociation of Rac from Rho-GDI may occur during the preparation of the cytosolic fraction and exposure of the Rac-Rho-GDI complex to anionic phospholipids (64), during the cavitation of the cellular membranes. Our results demonstrate, however, that PI 3-kinase is not responsible for the fMLP-stimulated dissociation of actin-gelsolin complexes, implying that an anionic phospholipid different from PtdIns(3,4,5)\(_P\) is responsible for the Rac-Rho-GDI dissociation in this signaling pathway in vivo.

The evidence that both GTP\(_S\)-loaded G\(_{\alpha_S}\) and G\(_{\beta \gamma}\) were able to significantly increase the exchange rate of GDP to GTP on GST-Rac1 in neutrophil cytosol suggests the existence of a cytosolic nucleotide exchange factor for Rac that can be activated by heterotrimeric G-proteins. The identity of the exchange factor is not known at present, but several guanine nucleotide exchange factors specific for Rac have been described and are possibly involved in this pathway (reviewed in Ref. 65). PI 3-kinase has been reported to be involved in the activation of Rac downstream of growth factor receptors (66), which is in contrast with the results presented here, since wortmannin did not inhibit the heterotrimeric G-protein-induced activation of nucleotide exchange on GST-Rac1 and dissociation of actin-gelsolin complexes. A possible explanation for this discrepancy is that serpentine receptors, like the neutrophil fMLP receptor, activate an alternative signaling cascade controlling Rac activation, which involves a G-protein-activated exchange factor for Rac. This hypothesis is consistent with the observation that bombesin-stimulated membrane ruffling, which requires Rac, is not blocked by wortmannin in Swiss 3T3 cells (67).

The cytosolic component downstream of Rac mediating its effect on actin-gelsolin complexes is unknown. Two targets of Rac1 have been described in neutrophils (68) as follows: p67\(_{\text{phox}}\) and a 68-kDa kinase analogous to p65 PAK (69). We tested both purified recombinant p67\(_{\text{phox}}\) and an activated version of p65 PAK, which failed to induce cytosolic actin-gelsolin dissociation in the assay described here. These two proteins are probably not the only downstream targets of Rac, since it has been shown that Rac interacts with numerous proteins, some of which contain a consensus Rac-binding motif (CRIB) (70). POR-1, a Rac-binding protein that is involved in membrane ruffling (71), and IQGAP, which binds Rac and is localized to membrane ruffles (72), are two potential mediators of the Rac-stimulated dissociation of actin-gelsolin dissociation described in this report.

In platelets, activated Rac induced an increase in barbed end nucleating activity, which was explained by an increase in the cellular levels of PtdIns(4,5)\(_P\), since this response was blocked by a PtdIns(4,5)\(_P\)-binding peptide derived from gelsolin (32). When tested in our assay, the peptide did not inhibit the actin-gelsolin dissociation induced by GTP-loaded Val-12 Rac1, GTP\(_S\)-loaded G\(_{\alpha_S}\), and G\(_{\beta \gamma}\), which is in agreement with the finding that these responses are insensitive to neomycin treatment. It must thus be concluded that the G-protein-activated decrease in cytosolic actin-gelsolin complexes we observe is not dependent on phosphoinositides. The increase in barbed end nucleating activity induced by Rac in platelets (32) may be due to the dissociation of a capping protein distinct from gelsolin. Alternatively, it can be argued that the Rac-induced actin polymerization observed in permeabilized platelets (32) involves the membrane-associated cytoskeleton, which is absent from the cytosolic extracts used in the present study. Actin-gelsolin dissociation could thus be regulated differentially, depending on the cellular compartmentalization, membrane-associated complexes being a target for polyphosphoinositides, whereas the complexes found in the cytosol may be regulated by protein-protein interactions and/or protein phosphorylation.

GTP\(_S\) and Cdc42 were shown to induce actin polymerization in neutrophil lysates, independently of PtdIns(4,5)\(_P\) synthesis (31). By using a similar experimental approach, we show that Rac can trigger the dissociation of gelsolin from the barred ends of cytosolic actin oligomers, a response that is not mediated by PtdIns(3,4,5)\(_P\) or PtdIns(4,5)\(_P\). The discrepancy between the inability of Rac to induce actin polymerization (31) and its effect on actin-gelsolin complexes can be explained by the fact that dissociation of the complexes may not be sufficient to trigger actin polymerization in the cytosol, which is supported by the results presented above on intact, calcium-depleted neutrophils. Assuming that Cdc42 acts upstream of Rac in controlling cytoskeletal rearrangements (73, 74), it can be speculated that Cdc42 increases barbed end availability via Rac and actin monomer desequstration by a Rac-independent pathway. Since the ability of Cdc42 to induce actin polymerization was attributed to an interaction with PAK1 (31), an activated version of which (p65 PAK) was inactive in the actin-gelsolin dissociation assay, an attractive hypothesis would be that Cdc42-mediated activation of PAK1 controls actin monomer availability.

The involvement of cascades of G-proteins in the control of agonist-induced actin rearrangements in fibroblasts (25, 26, 73, 74), mast cells (61), and neutrophils (27–31) is now well established. We provide evidence that activation of Rac by G-protein-coupled receptors in neutrophils controls the release of gelsolin from the barred ends of actin filaments, independently of PI 3-kinase.

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