Activation State-Dependent Interaction between Goi and p67phox

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The phagocyte NADPH oxidase consists of multiple protein subunits that interact with each other to form a functional superoxide-generating complex. Although the essential components for superoxide production have been well characterized, other proteins potentially involved in the regulation of NADPH oxidase activation remain to be identified. We report here that the Goi subunit of heterotrimeric G proteins is a novel binding partner for p67phox in transfected HEK293T cells and peripheral blood polymorphonuclear leukocytes. p67phox preferably interacts with inactive Goi. Expression of p67phox caused a dose-dependent decrease in intracellular cyclic AMP concentration, suggesting altered function of Goi. We identified a fragment of p67phox, consisting of the PB1 domain and the C-terminal SH3 domain, to be critical for the interaction with Goi. Because these domains are involved in the interaction with p47phox and p40phox, the relationship between the respective binding events was investigated. Wild-type Goi, but not its QL mutant, could promote the interaction between p67phox and p47phox. However, the interaction between p67phox and p40phox was not affected by either Goi form. These results provide the first evidence for an interaction between p67phox and an alpha subunit of heterotrimeric G proteins, suggesting a potential role for Goi in the regulation or activation of NADPH oxidase.

Professional phagocytes play a critical role in the innate immune response to pathogens. The detection of microbial products such as fMet-Leu-Phe (fMLF) by resting neutrophils is an essential activating event that results in a spectrum of activities aimed to eliminate the causes of infection. In particular, neutrophils have the ability to generate toxic oxygen intermediates via activation of the NADPH oxidase (2), a tightly regulated multiprotein enzyme complex (32). Numerous studies have established that the active oxidase is composed of at least five essential subunits: membrane-associated gp91phox and p22phox, which form the redox core flavocytochrome b558 of the enzyme, and the cytosolic factors p67phox, p47phox, and p40phox (23, 29, 42, 43). Membrane translocation of the cytosolic subunits together with the active monomeric G protein Rac1/2 is a crucial step for assembly of a fully functional enzyme (1, 17).

p67phox is a multidomain protein implicated in essential NADPH oxidase protein-protein interactions. Its activation domain binds to the catalytic core of gp91phox and activates the electron transfer process (7, 27). The stretch of tetrapricopeptide repeat (TPR) motifs in its amino terminus is responsible for recruitment of active Rac (19). The phox and bem1 (PB1) domain binds p40phox (12), and the C-terminal Src-homology 3 (SH3) sequence is necessary for association with p47phox (9). Therefore, p67phox appears as a central coordinator for NADPH oxidase assembly.

Chemoattractants such as fMLF stimulate G protein-coupled receptors, leading ultimately to O2− generation. The majority of signals arising from the chemoattractant receptors are pertussis toxin sensitive and therefore mediated by Gi proteins (5, 46). Moreover, it is the Gβγ dimer that regulates a variety of effectors, such as phosphatidylinositol 3-kinase, phospholipase C-β, and most likely specific Rac guanine nucleotide exchange factors, leading to oxidase activation. The Goi subunit, however, has not been implicated in the assembly or regulation of the NADPH oxidase.

Recent studies have led to the identification of several novel binding partners for Go proteins besides the conventional Gβγ, downstream effectors, and specific G protein-coupled receptors. With a few exceptions, these proteins fall mainly into two defined groups: the regulators of G protein signaling (RGS) and the Goi/o-Loco (GoLoco)-containing proteins. RGS proteins attenuate G protein signaling by accelerating the intrinsic GTPase activity in Go (8, 36). The GoLoco interaction motif is found in a variety of proteins, such as activators of G protein signaling (AGS), Leu-Gly-Asn repeat-enriched protein (LGN), Pcp2, Rap1GAP, and others (20, 25). GoLoco proteins interact with GDP-bound Goi and act as guanine nucleotide dissociation inhibitors while impeding binding of Gβγ (44). Of particular interest is the R12 class of RGS, comprising RGS10, 12, and 14, which possess both the characteristic RGS box and GoLoco domains in their sequences (16).

In the present study, we investigated whether p67phox could interact with the alpha subunits of heterotrimeric G proteins, because p67phox was previously shown to bind the small GTPase Rac (19, 22). We show that p67phox directly interacts with GDP-bound Goi in both transfected cells and human neutrophils. The binding site for Goi was localized to the C-terminal SH3 domain on p67phox. Overexpression of p67phox in our system dose dependently decreased basal levels of cyclic AMP (cAMP), a readout for activation of Goi signaling and also a negative regulator of O2− production. Furthermore, the association of p47phox with p67phox was affected by the Goi activation state, suggesting that Goi not only is a binding partner of...
p67phox but also may participate in the regulation of NADPH oxidase activation.

MATERIALS AND METHODS

Antibodies. Monoclonal antibody to c-Myc was purchased from Covance (Berkley, CA) and that to FLAG from Sigma-Aldrich (St. Louis, MO). The anti-p67phox monoclonal antibody was from BD Transduction Laboratories (Lexington, KY). Antibodies specific to Goi and Go3 were from Santa Cruz Biotechnology (Santa Cruz, CA), as were the anti-p47phox polyclonal and the anti-β-actin monoclonal antibodies. The anti-Go2 serum was produced in a rabbit (with amino acids 213 to 354 as an antigen), as was the anti-p67phox serum (raised against the purified glutathione S-transferase [GST]–p67phox fusion protein). The anti-p47phox polyclonal antibody was acquired from Upstate (Lake Placid, NY). Monoclonal antibodies specific to Gαi2, Gαi3, and p40phox were from Sigma (St. Louis, MO) and the anti-p47phox polyclonal and the anti-gp91phox monoclonal antibody were from BD Transduction Laboratories (Lexington, KY). Antibodies specific to Goi and Go3 were produced in a rabbit (with amino acids 213 to 354 as an antigen), as was the anti-p67phox serum (raised against the purified glutathione S-transferase [GST]–p67phox fusion protein). The anti-p47phox polyclonal antibody was acquired from Upstate (Lake Placid, NY). Monoclonal antibodies specific to Gαi2, Gαi3, and p40phox were from Sigma (St. Louis, MO) and the anti-p47phox polyclonal and the anti-gp91phox monoclonal antibody were from BD Transduction Laboratories (Lexington, KY). Antibodies specific to Goi and Go3 were produced in a rabbit (with amino acids 213 to 354 as an antigen), as was the anti-p67phox serum (raised against the purified glutathione S-transferase [GST]–p67phox fusion protein). The anti-p47phox polyclonal antibody was acquired from Upstate (Lake Placid, NY). Monoclonal antibodies specific to Gαi2, Gαi3, and p40phox were from Sigma (St. Louis, MO) and the anti-p47phox polyclonal and the anti-gp91phox monoclonal antibody were from BD Transduction Laboratories (Lexington, KY). Antibodies specific to Goi and Go3 were produced in a rabbit (with amino acids 213 to 354 as an antigen), as was the anti-p67phox serum (raised against the purified glutathione S-transferase [GST]–p67phox fusion protein). The anti-p47phox polyclonal antibody was acquired from Upstate (Lake Placid, NY). Monoclonal antibodies specific to Gαi2, Gαi3, and p40phox were from Sigma (St. Louis, MO) and the anti-p47phox polyclonal and the anti-gp91phox monoclonal antibody were from BD Transduction Laboratories (Lexington, KY). Antibodies specific to Goi and Go3 were produced in a rabbit (with amino acids 213 to 354 as an antigen), as was the anti-p67phox serum (raised against the purified glutathione S-transferase [GST]–p67phox fusion protein). The anti-p47phox polyclonal antibody was acquired from Upstate (Lake Placid, NY). Monoclonal antibodies specific to Gαi2, Gαi3, and p40phox were from Sigma (St. Louis, MO) and the anti-p47phox polyclonal and the anti-gp91phox monoclonal antibody were from BD Transduction Laboratories (Lexington, KY). Antibodies specific to Goi and Go3 were produced in a rabbit (with amino acids 213 to 354 as an antigen), as was the anti-p67phox serum (raised against the purified glutathione S-transferase [GST]–p67phox fusion protein). The anti-p47phox polyclonal antibody was acquired from Upstate (Lake Placid, NY). Monoclonal antibodies specific to Gαi2, Gαi3, and p40phox were from Sigma (St. Louis, MO) and the anti-p47phox polyclonal and the anti-gp91phox monoclonal antibody were from BD Transduction Laboratories (Lexington, KY). Antibodies specific to Goi and Go3 were produced in a rabbit (with amino acids 213 to 354 as an antigen), as was the anti-p67phox serum (raised against the purified glutathione S-transferase [GST]–p67phox fusion protein). The anti-p47phox polyclonal antibody was acquired from Upstate (Lake Placid, NY). Monoclonal antibodies specific to Gαi2, Gαi3, and p40phox were from Sigma (St. Louis, MO) and the anti-p47phox polyclonal and the anti-gp91phox monoclonal antibody were from BD Transduction Laboratories (Lexington, KY).

Culture and transient transfection of mammalian cells. The human embryonic kidney epithelial cell line 293T (HEK293T) was maintained in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT). Cells were transfected using LipofectAMINE 2000 reagent (Life Technologies) according to the manufacturer's instructions. For superoxide generation experiments, the monkey kidney epithelial transgenic COS-phox cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% FBS and in the presence of 0.2 mg/ml hygromycin (Sigma), 0.8 mg/ml neomycin sulfate (Invitrogen), and 1 μg/ml puromycin (Calbiochem). Cells were transiently transfected using LipofectAMINE 2000 reagent (Life Technologies) according to the manufacturer's protocol.

Immunoprecipitation and Western blotting. Twenty-four hours after transfection, the cells were lysed in buffer containing 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM MgCl2, 1 mM EDTA, 1% Triton X-100, 1 mM diethiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor cocktail set I (Calbiochem). For immunoprecipitation studies, the cleared lysates were incubated overnight at 4°C with either the MYC-specific monoclonal antibody (10 μg/ml, anti-Gαi2 [1:250], or anti-p47phox [1 μg/ml]) as indicated. Proteins A/G PLUS-agarose (Santa Cruz Biotechnology) was added to the samples, and samples were incubated for 1.5 h at 4°C. The beads were washed and resuspended in 50 μl of 5× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and boiled for 5 min to release bound proteins. The resolved samples were detected by Western blotting. When working with human neutrophils (107 cells/sample), the same fractionating protocol was followed with minor modifications. Briefly, lysis was achieved in the presence of 2× protease inhibitor cocktail set I and 100 μM E-64 (Sigma). Lysis, immunoprecipitation, and washing were performed in the presence of 10 μM GDP or 10 μM GDP, 30 μM AlCl3, and 10 mM NaF (AMF). Electrophoresis of the proteins on a 10% SDS-polyacrylamide gel was followed with transfer to a nitrocellulose membrane (Schleicher & Schuell). The blots were blocked with 5% nonfat dry milk in Tris-buffered saline–Tween buffer (20 mM Tris-HCl, pH 7.4, 1% Tween 20, washed, and incubated with primary antibodies overnight at 4°C. Anti-rabbit (Bio-Rad) or antimoouse (Calbiochem) peroxidase-conjugated secondary antibodies were added to the membranes at a 1:3,000 dilution for 1 h at room temperature. The bands on the blots were visualized by chemiluminescence (Pierce).

Preparation of human neutrophils. Blood from healthy donors was collected following a procedure approved by the Institutional Review Board at the University of Illinois at Chicago by using ACD buffer (1.365% citric acid, 2.5% sodium citrate, and 2% dextrose). Erthrocytes were removed by sedimentation with Hespan (6% hetastarch; Abbott Laboratories). Polymorphonuclear leukocytes were further fractionated by centrifugation at 450 × g for 1 h at 12°C on a discontinuous Percoll (Amersham Pharmacia Biotech) gradient (74% and 55%). In a routine preparation, approximately 97% of the cells were neutrophils and the viability was about 98%, as determined by Trypan blue exclusion.

Expression and purification of recombinant GST fusion proteins in Escherichia coli and pulldown assay. The pGEX-2T and pGEX-p67phox constructs, encoding GST and full-length GST-p67phox fusion proteins, respectively, were introduced into the E. coli strain DH10B. Protein expression was induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 2 h at 30°C for GST-p67phox and p67phox-MYC GST. The bacterial pellet was resuspended in 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mg/ml lysozyme, 1× protease inhibitor cocktail set II (Calbiochem), and 2 mM diethiothreitol and sonicated. The lysate was complemented with 1% Triton X-100 and shaken for 1 h at 4°C. After centrifugation at 12,000 × g for 10 min at 4°C, the supernatant was snap-frozen for storage in 10% glycerol.

RESULTS

The interaction between Goi2 and p67phox is direct and depends on the Go activation state. To evaluate the potential interaction of p67phox and Goi, an expression construct was created to produce C-terminal MYC-tagged p67phox (p67phox-MYC). Moreover, activity of recombinant p67phox-MYC was verified in a whole cell-based reconstitution assay, which required exogenous p67phox for FMLP- and PMA-induced O2 generation (11). The p67phox-MYC DNA construct was transiently cotransfected with vectors encoding each of the three isoforms of Goi into HEK293T cells, which do not express any of the NADPH oxidase components except for Rac1. Both inactive wild-type Goi (Goiwt) and the GTPase-deficient (constitutively active) Goi mutants (Q204L for Goi1 and Goi3 and Q205L for Goi2) were examined. Twenty-four hours after transfection, p67phox-MYC was immunoprecipitated from cell lysates, and the precipitates were analyzed by immunoblotting. Surprisingly, Goi1wt, Goi2wt, and Goi3wt were all detected in the immunoprecipitates (Fig. 1). Additionally, we also observed a modest interaction between p67phox and the endogenous Goi (vector, mock-transfected cells). Interestingly, the interaction between p67phox and the active GoiQL was significantly reduced to levels as low as or even below those of the vector controls (Fig. 1A, lanes 3 versus lanes 1). The specificity of this interaction was validated by the observation that neither the GDP- nor the GTP-bound forms of Goi could associate with p67phox (data not shown).

Since the resting and activated NADPH oxidase states are tightly regulated by complex protein-protein interactions (35), we examined whether the binding between p67phox and Goi could occur in the presence of the other NADPH components...
in an environment where the enzyme is fully functional. We isolated neutrophils from human peripheral blood, lysed them, and immunoprecipitated endogenously expressed Gαi2 in the absence or presence of AMF, which mimics the transition state of GTP hydrolysis. As expected from the previous observations, an interaction between p67phox and Gαi2 was detectable in nontreated cell lysates and was markedly diminished in the presence of AMF (Fig. 1B, lane 2 versus lane 1). Since fMLF stimulates neutrophil O2− production via activation of the Gαi proteins, we treated isolated neutrophils with 1 μM fMLF before lysis and immunoprecipitation with an anti-Gαi2 serum. In this case too, the interaction between the endogenous p67phox and Gαi2 was markedly decreased compared to that observed in unstimulated neutrophils (Fig. 1B, lane 3 versus lane 1). Thus, the binding of p67phox with Gαi2 is dependent on the G protein activation state and is observed in resting neutrophils.

To further investigate whether this interaction was direct, we performed an in vitro binding assay between purified, bacterially expressed GST-p67phox and Sf9-expressed Gαi1 (Fig. 2). The GST-p67phox fusion protein coupled to glutathione-Sepharose beads, but not the GST control protein, specifically bound Gαi1 in the presence of GDP but not when the Gαi was preloaded with either AMF or GTPγS. Thus, these data demonstrate a direct interaction between p67phox and Gαi1 and again show that the interaction is modulated by the Gαi activation state.
Expression of \( p67^{\text{phox}} \) partially inhibits cAMP formation. Activation of the Goi family of G proteins is responsible for the inhibition of adenylyl cyclase and subsequent reduction in the basal and inducible levels of cAMP. To gain an insight into the inhibition of adenylyl cyclase and subsequent reduction in cAMP level (data not shown). HEK293T cells transiently transfected to express increasing amounts of untagged \( p67^{\text{phox}} \), cAMP production was dose dependently inhibited (Fig. 3A), with a 50% reduction at the highest DNA concentration used for transfection. As a control, similar amounts of a vector encoding AGS3 did not influence the cAMP level (data not shown).

Heterotrimeric G proteins are composed of the guanine nucleotide-binding Go subunit and the G\( \beta \gamma \) dimer, which are regarded as one functional unit. G proteins are inactive in the GDP-bound state. Since \( p67^{\text{phox}} \) preferentially binds to inactive Goi, we expected that it might compete with G\( \beta \gamma \) for association with GDP-bound Goi. Surprisingly, in transiently transfected HEK293T cells, increasing amounts of \( p67^{\text{phox}} \), MYC did not disrupt the interaction between G\( \beta \)1\( \gamma 2 \) and Goi (Fig. 3B). These observations suggest that \( p67^{\text{phox}} \) most likely binds to Goi on a site distinct from the G\( \beta \gamma \) contact surface. Indeed, overexpression of \( p67^{\text{phox}} \) showed no effect on the basal phosphorylation of Akt, a characterized readout for Goi activation (data not shown). Finally, we verified that the ability of G\( \beta \gamma \) to coimmunoprecipitate \( p67^{\text{phox}} \) was mediated by the inactive Goi (Fig. 3C). Indeed, FLAG-tagged G\( \beta 1 \) was found to bind to \( p67^{\text{phox}} \) only when Goi2 was overexpressed in the cells. Endogenous Goi appeared to be insufficient to mediate the association between FLAG-G\( \beta 1 \) and \( p67^{\text{phox}} \), MYC, most likely because it already forms heterotrimers with endogenous G\( \beta \gamma \) proteins, which cannot be detected with the anti-FLAG monoclonal antibody (MAb). Based on these data, we concluded that the association between G\( \beta \gamma \) and \( p67^{\text{phox}} \) is indirect and mediated through the inactive, GDP-bound Goi.

**Determination of Goi binding site on \( p67^{\text{phox}} \).** \( p67^{\text{phox}} \) is a multidomain protein (Fig. 4A). The N-terminal TPR domain (amino acids 3 to 154) is composed of four TPR motifs and directly binds Rac-GTP (19, 22). This segment (amino acids 1 to 199) was also demonstrated to mediate direct interaction with gp91\( ^{\text{phox}} \) (7). A short activation domain (amino acids 199 to 210) immediately follows the TPR domain (27), and the C-terminal segment contains two SH3 modules flanking a PB1 domain. The PB1 and C-terminal SH3 domains are involved in the direct interactions with \( p40^{\text{phox}} \) and \( p47^{\text{phox}} \), respectively (9, 12). In an attempt to localize the region of \( p67^{\text{phox}} \) required for Goi binding, a series of truncated MYC-tagged \( p67^{\text{phox}} \) mutants was generated (Fig. 4A), verified by sequencing, and separately expressed into HEK293T cells together with Goi2wt (Fig. 4B). In immunoprecipitation and immunoblotting experiments, the \( p67^{\text{phox}} \) C-terminal fragment (amino acids 213 to 526) precipitated Goi2 as effectively as did full-length \( p67^{\text{phox}} \), whereas the N-terminal segment (amino acids 1 to 213) did not retain binding to the Go protein (Fig. 4B, lanes 2 and 3 versus lane 1). Furthermore, the fragment covering residues 340 to 526 was shown to be sufficient for interaction with Goi2, whereas the sequence extending from amino acids 303 to 455 did not bind Goi2. Therefore, at first view the C-terminal SH3 was the most likely candidate for the Goi binding site, and two additional \( p67^{\text{phox}} \) mutants that both contained \( p47^{\text{phox}} \) and \( p40^{\text{phox}} \) mutants was generated (Fig. 4A), verified by sequencing, and separately expressed into HEK293T cells together with Goi2wt (Fig. 4B). In immunoprecipitation and immunoblotting experiments, the \( p67^{\text{phox}} \) C-terminal fragment (amino acids 213 to 526) precipitated Goi2 as effectively as did full-length \( p67^{\text{phox}} \), whereas the N-terminal segment (amino acids 1 to 213) did not retain binding to the Go protein (Fig. 4B, lanes 2 and 3 versus lane 1). Furthermore, the fragment covering residues 340 to 526 was shown to be sufficient for interaction with Goi2, whereas the sequence extending from amino acids 303 to 455 did not bind Goi2. Therefore, at first view the C-terminal SH3 was the most likely candidate for the Goi binding site, and two additional \( p67^{\text{phox}} \) mutants that both contained \( p47^{\text{phox}} \) and \( p40^{\text{phox}} \) seemed to confirm this conclusion. However, the C-terminal SH3 and its flanking regions (residues 429 to 526) failed to coimmunoprecipitate Goi2. Therefore, the C-terminal SH3 in \( p67^{\text{phox}} \) could be necessary but not sufficient for recruitment of Goi, and perhaps both PB1 and the second SH3 in \( p67^{\text{phox}} \) contribute to the binding interaction with Goi.

**The interaction between \( p47^{\text{phox}} \) and \( p67^{\text{phox}} \) but not between \( p40^{\text{phox}} \) and \( p67^{\text{phox}} \) is dependent on the Goi activation state.** Since the C-terminal SH3 domain of \( p67^{\text{phox}} \) can interact directly with \( p47^{\text{phox}} \) and its PB1 domain associates with the
phox and Cdc (PC) domain of p40phox (9, 12), we investigated whether G~i2~ overexpression could influence these binding interactions. p67phox and p47phox were coexpressed in HEK293T cells together with G~i2~ (Fig. 5A). Interestingly, the wild type but not the GTPase-deficient mutant of G~i2~ increased the interaction between p67phox and p47phox (Fig. 5A, top panel, lanes 4 and 5 versus lane 3). However, in the presence of p47phox the association between p67phox and G~i2~ was

FIG. 3. Dose-dependent effect of p67phox on G~i~ activity and interaction with G~βγ~. (A) Accumulated cAMP levels were measured in vector-versus p67phox-transfected HEK293T cells. Increasing levels of p67phox expression and equivalent amounts of endogenous G~i2~ in each sample were determined by immunoblotting with the respective antisera. Values for cAMP concentration were expressed as a percentage relative to the basal level (vector), which was assigned a value of 100%. The histograms show the means ± standard errors of the means of triplicate experiments (n = 3). Student’s t test was used to compare the significance of the decrease to the basal level (*, P < 0.01; **, P < 0.001). (B) FLAG-tagged Gβ1 and Gγ2 were coexpressed alone (Vector) and either with G~i2wt~ or in combination with G~i2wt~ and two different concentrations of p67phox-MYC as indicated. Cell homogenates were subjected to immunoprecipitation (IP) with an anti-FLAG MAb. The integrity of G~i/Gβγ~ interaction was verified by blotting the immunoprecipitates with an anti-G~i2~ serum (top panel). The homogenates were analyzed for protein expression (two bottom panels), and total FLAG-G~β1~ in the immunoprecipitates serves as a loading control (middle panel). (C) HEK293T cells were transiently transfected to express a C-terminal MYC-tagged p67phox either alone (Vector) or with Gβ1 and Gγ2 in the absence or presence of exogenous wild-type G~i2~, which directly interacts with G~βγ~. The cell homogenates were subjected to immunoprecipitation using an anti-MYC MAb and analyzed by Western blotting (IB). Blots shown were reproduced in three independent experiments.
barely detectable (Fig. 5A, second panel from top, compare lanes 4 and 5 to lanes 1 and 2). This finding suggests that p47phox competes with Gαi for binding to p67phox.

Similar experiments were conducted in cells expressing p67phox, Gαi2, and p40phox (Fig. 5B). The interaction between p67phox and p40phox was not affected by the expression of either Gαi2wt or Gαi2QL (Fig. 5B, top panel, lanes 3, 4, and 5). Surprisingly, p67phox, which preferentially binds to inactive
Gai in the absence of p40phox, switched its affinity for the active form of the Gai protein when p40phox was present (Fig. 5B, second panel from top, lanes 1 and 2 compared to lanes 4 and 5). The decreased but still apparent coimmunoprecipitation between p67phox and inactive Gai in the presence of p40phox suggests that p40phox might only partially displace Gai from p67phox.

Finally, all four binding partners were coexpressed together (Fig. 5C). Interestingly, in the presence of both p47phox and p40phox, the characteristic difference in p67phox binding to Gai2wt versus Gai2QL remained (Fig. 5C, top panel, lanes 4 and 5 versus lanes 2 and 3). Taken together, these data confirm the delicate dynamics in protein-protein interactions involved in the formation and regulation of p67phox-containing complexes.

The phox components p67phox and p47phox associate with Gai in one large complex. In HEK293T cells and in the absence of p40phox, p47phox was found to completely block the binding of Gai2 to p67phox (Fig. 5A). However, when the three phox proteins and Gai2 were coexpressed, as a closer mimic of human neutrophils, both p47phox and Gai2 coimmunoprecipitated with MYC-tagged p67phox (Fig. 5C). Therefore, we tested whether p67phox, p47phox, and Gai2 would associate in the same large complex or bind differently to form a variety of smaller ones. Human neutrophils were purified from peripheral blood and either unstimulated or stimulated with 1 μM fMLF before lysis and immunoprecipitation with antibodies against p47phox and Gai2 (Fig. 6). Both Gai2 and p67phox were detected in the anti-p47phox precipitates in either resting or fMLF-stimulated cells. Similarly, both p47phox and p67phox coimmunoprecipitated with Gai2. Interestingly, the interaction between p47phox and p67phox increased upon fMLF stimulation, with Gai2 concomitantly dissociating from the complex (Fig. 6, “IP: p47phox” set of panels). As expected, p67phox interaction with Gai2 markedly diminished after fMLF treatment and so did the association between p47phox and Gai2 (Fig. 6, “IP: Gai2” set of panels). p67phox most likely bridges p47phox and Gai2, causing them to coimmunoprecipitate. Indeed, we have not observed any interaction between Gai2 and p47phox in the absence of p67phox in HEK293T cells (data not shown).
shown). Taken together, these results support the presence of a multiprotein complex containing p47phox, Goi, and p67phox.

Functional implications of Goi interaction with p67phox on NADPH oxidase activity. We took advantage of the COS-phox system, a whole-cell-based reconstitution assay manipulated to generate fMLF-induced O$_2^-$ (11, 31). The transgenic cells stably expressing gpg91phox, p22phox, p67phox, and p47phox were transiently transfected with DNA coding for the formyl peptide receptor (FPR), as well as protein kinase C (PKC) using specific antibodies against each component as indicated. The expression levels in neutrophils of the three proteins were detected by probing the homogenates with the respective antibodies (panels on the right side). Data are representative of similar results obtained in three independent experiments.

DISCUSSION

In phagocytic cells, functional assembly of the NADPH oxidase in the phagosome and plasma membrane is a rapid and complex process and is essential for host defense against pathogens (32). Chemoattractant-induced O$_2^-$ generation is mediated through Gi$\beta$$\gamma$ originating from activation of heterotrimeric Gi proteins (5). However, it is unclear whether the Goi proteins also play a role in NADPH oxidase activation through a different mechanism. A major finding of this study is the identification of GDP-bound Goi as a direct p67phox binding partner. To our knowledge, this is the first report that suggests a potential role for Goi in oxidase enzyme assembly and raises the possibility that Goi may be a distinct player in NADPH oxidase regulation.

Using coimmunoprecipitation and in vitro binding analysis, we observed an interaction between the inactive, GDP-bound Goi and the cystolic factor p67phox. This binding exists not only in transiently transfected cells (Fig. 1A) but also, and more importantly, in human neutrophils (Fig. 1B). The interaction significantly decreases upon activation of Goi, suggesting that p67phox can recognize the conformational changes associated with Goi activation. Since p67phox does not appear to compete with Gi$\beta$ for association with GDP-bound Goi (Fig. 3B), the binding site for p67phox on Goi must be different from the Goi/Gi$\beta$ interface. Furthermore, there is no observable difference in Goi2 and p67phox coimmunoprecipitation between untreated and pertussis toxin-treated (500 ng/ml, 4 h) cells (data not shown), which irreversibly ADP-ribosylates a specific C-terminal cysteine residue on the Goi subunit that leads to uncoupling from the receptor. Thus, the C-terminal sequence of Goi does not appear to be involved in the interaction with p67phox.

The possible relationship between Goi and the three cytosolic factors of NADPH oxidase is depicted in Fig. 8. Analysis of the deletion mutants of p67phox revealed that the C-terminal SH3 motif of p67phox (Fig. 8A), previously characterized as a direct binding site for p47phox (9), also contributes to the recruitment of Goi (Fig. 4). Indeed, p47phox can fully compete inactive Goi off of p67phox (Fig. 5A). However, in neutrophils p67phox clearly binds better with inactive Goi (Fig. 1B), even in the presence of endogenous p40phox and p47phox. Moreover, Goi2, p47phox, and p67phox were found to associate in the same complex (Fig. 6). Therefore, we tested whether coexpression of p67phox, p47phox, and p40phox in HEK293T cells, together with Goi2, would mimic what we observed in neutrophils (Fig. 5C). Indeed, the absence of p67phox for inactive Goi2 is maintained in the presence of both p47phox and p40phox. In addition, the binding of p47phox and Goi2 to p67phox is no longer mutually exclusive in the presence of p47phox. Coimmunoprecipitation assays performed in neutrophils have confirmed the presence of a multisubunit complex containing p47phox, p67phox, and Goi2 (Fig. 6). However, it is possible that only subpopulations of p67phox and Goi are associated at a given time, and it is also likely that a variety of multimers coexists in the cell. Additionally, evidence suggesting that p67phox can homodimerize may support the observation that p47phox and Goi are both present in the same large complex with p67phox and most likely have overlapping binding sites on p67phox. Although the intriguing prospect that p67phox can homodimerize has been approached in only a few biochemical studies drawing contradictory conclusions (10, 21), we have observed homodimerization of p67phox in transfected HEK293T cells (data not shown). Taken together, these findings suggest the possibility that the p47phox- and Goi-containing complex may comprise two or more copies of p67phox.

It is presently unclear how the binding of Goi to p67phox facilitates or influences assembly of the enzyme complex. One possibility is that this association promotes colocalization of the relevant interacting proteins in specific subcellular compartments, most likely in the cytosol or in the cytoskeletal fraction. Indeed, members of the large TPR-containing proteins, such as AGS3 (14, 30) and TPR1 (24), are emerging as
novel adaptors and scaffolds for G protein signaling. A possible explanation for the increased interaction between p67 phox and p47phox in the presence of the GDP-bound G/H9251 is that this G protein, through its association with p67 phox, positions p67phox in close proximity to p47 phox, thereby facilitating p47 phox-p67phox interaction (Fig. 8B). These interactions most likely occur within the cell, as both p67 phox and p47phox are cytosolic factors, and their association precedes membrane translocation of the complex. Moreover, several groups have confirmed the presence of two pools of Goi2, which are located in the plasma membrane and the cytosol fractions of unstimulated neutrophils (4, 15, 37, 38). Studies have also shown Goi binding to F-actin and tubulin in cytoplasmic structures and at the plasma membrane (34). The cytoskeleton provides a dynamic network between cellular structures and a docking surface for various signaling proteins, including Goi, in response to cell activation (13). Consistent with these observations, p67phox and p47phox are principally recovered in the cytoskeletal fraction of unstimulated and stimulated neutrophils (26, 41, 45). Thus, reorganized cytoskeleton may provide a scaffold for activation of the NADPH oxidase components in response to cell stimulation (33). Taken together with these previous observations, our findings support the hypothesis that the association between Goi and p67phox occurs in the cytosol of resting cells to favor the interaction between p67phox and p47phox, presumably involving the cytoskeleton. Upon stimulation, the readily mobilizable, preformed cytosolic phox complex can then translocate to the membrane for full assembly and activation of NADPH oxidase (18). Exactly how fMLF stimulation triggers activation of an intracellularly localized Goi has not been investigated. The availability of Goi as well as its activation state may also be influenced by multiple factors and regulated by fMLF. Indeed, Sarndahl et al. (39) showed activation and dissociation of cytoskeleton-bound Goi upon fMLF stimulation.

In addition to p67phox and p47phox, neutrophils contain p40phox, a cytosolic factor copurified with p67phox (40). The exact function of p40phox in promoting or inhibiting neutrophil NADPH oxidase assembly remains debatable. Interestingly, the addition of p40phox to our system causes a change in pref-
erence of p67phox from the inactive to the active form of G/G/H i (Fig. 5B). However, in the presence of p47phox, this change is reverted (Fig. 5C). We speculate that under these conditions, p67phox, p47phox, and p40phox cooperate in a temporal and spatial manner to favor formation of the p67phox/GDP-bound G/G/H i. The mechanism underlying this event is currently unknown and is a subject of our ongoing research. Based on data obtained from the in vitro binding assay with GTPS-loaded G/G/H i (Fig. 2), the coimmunoprecipitation between p67phox and the active form of G/G/H i in the presence of p40phox is most likely indirect and may require an unidentified factor (Fig. 8C). Thus, the prospect of an additional cofactor(s) raises the order of complexity in the sequence of interactions that takes place in NADPH oxidase assembly.

The interaction between p67phox and G/G/H i may functionally impact NADPH oxidase assembly and activation. In the intact COS-phox cell-based assay, overexpression of G/G/H i2 promoted not only fMLF- but also PMA-stimulated O2− generation (Fig. 7). This observation supports the possibility that G/G/H i interaction with p67phox facilitates the formation of the p67phox-p40phox cytosolic complex, which favors NADPH oxidase activation. Stimulation of the G/G/H i-coupled FPR by fMLF not only activates G/G/H i but also makes G/G/H i available for its downstream effectors. Along with these events, the GTP-bound G/G/H i can dissociate from p67phox (Fig. 6). Therefore, in activated cells, the increased binding between p40phox and p67phox and enhanced NADPH oxidase activity can be attributed to a combination of factors, including signals arising from G/G/H i. To our surprise, exogenous expression of p67phox dose dependently reduced cAMP basal levels in HEK293T cells, appearing as a positive regulator of the G/G i signaling pathway (Fig. 3A). The cAMP-dependent protein kinase A is a characterized inhibitor of O2− production in inflammatory cells (3, 28). Although it is still unclear whether p67phox has an effect on cAMP in neu-

FIG. 8. Model for the participation of G/G i in interactions between NADPH oxidase components. (A) Linear representations of p47phox, p67phox, and p40phox. The C-terminal SH3 domain of p67phox interacts with the proline-rich (PR) region of p47phox, and the PB1 domain binds to the phox and cdc motif (PC) of p40phox. AD, activation domain; SH3, Src-homology 3 domain; PB1, phox and bem1 domain; PX, phox domain. (B) A potential role of G/G i in targeting p67phox to specific subcellular compartments. Inactive G/G i, tethered to either the membrane or the cytoskeleton, may position p67phox in close proximity to p47phox and promote their interaction. The association between the two phox proteins then competes G/G i off of p67phox. (C) The recruitment of p40phox to p67phox is not significantly influenced by G/G i. p40phox may only partially displace G/G i from p67phox, allowing the formation of various complexes which may or may not include G/G i. Based on our experimental results, we hypothesize that upon activation of the G protein an additional partner may be involved in the association between the active G/G i and p67phox.
trophils, it is conceivable that a camp of levels in neutrophils may contribute to priming for O$_2^-$ generation. Given the ability of Gai-coupled receptors, such as the interleukin-8 receptor, to potentiate NADPH oxidase activation, the direct and activation state-dependent interaction between p67phox and Gai may represent another regulatory mechanism for NADPH oxidase activation.

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REFERENCES


