A Regulatory Role for ADP-ribosylation Factor 6 (ARF6) in Activation of the Phagocyte NADPH Oxidase*

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In activated neutrophils NADPH oxidase is regulated through various signaling intermediates, including heterotrimeric G proteins, kinases, GTPases, and phospholipases. ADP-ribosylation factor (ARF) describes a family of GTPases associated with phospholipase D (PLD) activation. PLD is implicated in NADPH oxidase activation, although it is unclear whether activation of PLD by ARF is linked to receptor-mediated oxidase activation. We explored whether ARF participates in NADPH oxidase activation by formyl-methionine-leucine-phenylalanine (fMLP) and whether this involves PLD. Using multicolor forward angle light scattering analyses to measure superoxide production in differentiated neutrophil-like PLB-985 cells, we tested enhanced green fluorescent fusion proteins of wild-type ARF1 or ARF6, or their mutant counterparts. The ARF6(Q67L) mutant defective in GTP hydrolysis caused increased superoxide production, whereas the ARF6(T27N) mutant defective in GTP binding caused diminished responses to fMLP. The ARF1 mutants had no effect on fMLP responses, and none of the ARF proteins affected phorbol 12-myristate 13-acetate-elicited oxidase activity. PLD inhibitors 1-butanol and 2,3-diphosphoglycerate, or the ARF6(N48R) mutant assumed to be defective in PLD activation, blocked fMLP-elicited oxidase activity in transfected cells. The data suggest that ARF6 but not ARF1 modulates receptor-mediated NADPH oxidase activation in a PLD-dependent mechanism. Because PMA-elicited NADPH oxidase activation also appears to be PLD-dependent, but ARF-independent, ARF6 and protein kinase C may act through distinct pathways, both involving PLD.

The phagocyte NADPH oxidase is an important innate defense system against bacterial and fungal infections. Inherited deficiencies of this enzyme result in chronic granulomatous disease, which is characterized by enhanced susceptibility to microbial infection and dysregulated inflammatory responses. Although the components of this superoxide-generating system have been the subject of intensive investigation, the signaling mechanisms responsible for oxidase activation (the respiratory burst) are complex and not clearly defined. Many studies indicate that several GTPases of the Ras superfamily are involved at various levels of regulation of this inflammatory process. Rac was identified as a third cytosolic component required for activation of the NADPH oxidase, with Rac1 as the active component in guinea pig macrophages (3) and Rac2 in human neutrophils (4). In addition, Rap1A, which localizes to the plasma membrane and granule membranes in human neutrophils, was shown to associate with cytochrome b558 of the oxidase (5). Mutant Rap1A inhibits oxidase activity in transfected B cells (6), although its role in the system is not entirely clear.

The ADP-ribosylation factor (ARF) subfamily of Ras-related proteins consists of six mammalian GTPases (ARF1–ARF6), five of which are detected in man (ARF1, 3–6) (7). Originally identified as cofactors required for cholela toxin-catalyzed ADP-ribosylation of Gox (8), the ARFs have been shown to play critical roles in vesicular transport (9). Evidence supporting a role for ARF in granulocyte functions came from studies in neutrophils and HL-60 cells, where ARF1 and ARF3 were identified as cytosolic regulators of phospholipase D (PLD) (10, 11). However, these studies were conducted with cell-free reconstitution assays using recombinant ARF1 or cytosol from bovine brain, and therefore the identity of the endogenous ARF(s) participating in PLD activation and subsequent phagocyte functions is not known. PLD activity and its product phosphatidic acid have been implicated in a variety of responses by stimulated phagocytes, including secretion (12, 13), phagocytosis (14–16), and activation of NADPH oxidase (17). Several other studies suggest a role for ARF in receptor-dependent signaling in phagocytes (18, 19).

All human ARF mRNA species have been detected in HL-60 cells (20), and several of these ARF isoforms (ARF 1, 5, and 6) appear to activate rat brain PLD (21). The best characterized ARF protein, ARF1, is localized to the Golgi complex and is critical for vesicular transport along secretory pathways (9). Unlike ARF1, ARF6, which is the least conserved of the human ARF proteins, localizes at the cell periphery and cycles between the plasma membrane and endosomal compartments in a guanine nucleotide-dependent manner (22, 23). ARF6 was characterized as a regulator of membrane trafficking (22, 23) and remodeling of the plasma membrane and the underlying cytoskeleton (24–26). ARF6 has been linked functionally to PLD activation. In several mammalian cells ARF6(T27N) co-localizes with hPLD1a and hPLD1b (27), whereas in chromaffin cells ARF6 appears to activate PLD in vivo during exocytosis (28). Both ARF6 and PLD have been implicated in cells undergoing phagocytosis. ARF6 mutants defective in GTP binding (T27N)

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or GTP hydrolysis (Q67L) inhibit Fcy receptor (FcyR)-mediated phagocytosis in the RAW 264.7 macrophage cell line (29). Phagocytosis of complement-opsinized particles activates PLD in macrophages. Using *Mycobacterium tuberculosis*, Kusner and colleagues (14) correlated inhibition of phagocytosis with diminished PLD activity and demonstrated that phagocytosis could be restored by exogenous PLD (14). Finally, studies in neutrophils (15) and monocytic U937 cells (16) have demonstrated that stimulation of FcγR is tightly linked to PLD activation.

In light of the growing body of evidence linking ARF activation to receptor stimulation of phagocytes, and findings linking PLD activation to ARF, as well as to receptor-mediated oxidative responses, we explored the possible involvement of ARF1 and ARF6 in NADPH oxidase activation and whether this involves participation of PLD. For this purpose, we used the PLB-985 cell line induced to differentiate into a neutrophil-like phenotype following treatment with dibutyryl cAMP (Bt_cAMP). In previous work (30) we demonstrated that these cells are readily transfected while exhibiting phenotypic traits of differentiated phagocytes. Using this model, we explored possible roles of ARF1 and ARF6 through transfection of mutated forms produced as fusions with enhanced green fluorescent protein (EGFP), and demonstrated involvement of both ARF6 and PLD in formyl-methionine-leucine-phenylalanine (fMLP) receptor-mediated activation of the respiratory burst. In contrast, phorbol 12-myristate 13-acetate (PMA) activation, both apparently involving activation of PLD.

In this work, we present evidence that inhibition of PLD activation to ARF, as well as to receptor-mediated oxidase activity, was associated with either the electroporation or sedimentation protocols.

### EXPERIMENTAL PROCEDURES

**Cell Culture—**PLB-985 cells were grown in stationary suspension cultures in RPMI 1640 medium containing 10% bovine serum (HyClone Laboratories, Inc., Logan, UT), 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 12.5 units/ml nystatin at 37 °C in a humidified atmosphere of 5% CO₂. Cell number and viability were determined by trypan blue exclusion.

**Transfection of PLB-985 Cells—**To induce a granulocytic phenotype, PLB-985 cells were pretreated with 0.3 mM Bt_cAMP (Sigma) for 3 days. The cells were then transfected 24 h after Bt_cAMP induction and grown in RPMI 1640 medium containing 20% bovine serum in the presence of 0.3 mM Bt_cAMP for an additional 2 days. For transient transfections, PLB-985 cells (1 x 10⁶) cells washed in 0.3 ml of cold Ca²⁺- and Mg²⁺-free PBS were electroporated (99 μs, three pulses, 0.9 volt, using a BTX Electro square porator T 820) and 20 μg each of plasmid DNA. 48 h after electroporation (3 days postinduction), the living PLB-985 cells were obtained from Ficoll density gradients (30) and used for flow cytometric analysis. The ARF cDNAs (ARF6-WT, ARF6(Q67L), ARF6(T27N), ARF1-WT, ARF1(Q71L), ARF1(T31N)) were cloned into the vector pEGFP-N1 (CLONTECH) and fused to the Aequorea victoria fluorescence gene, which reacts with an extracellular epitope (33). The cells were then washed twice with PBS and incubated with goat anti-mouse IgG (H+L) Cy5-conjugated Fab’/f(ab’2), fragment (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) for 30 min at room temperature in the dark. After washing, the cells were analyzed on the flow cytometer, and gp91phox as detected by excitation at 590 nm and a 665/14 bandpass filter. Transfectants were detected independently by EGFP fluorescence, as described above. Histograms were constructed based on analysis of 0.5 x 10⁶ cells.

**Chemiluminescence Assay of NADPH Oxidase Activity—**Superoxide production by untransfected PLB-985 cells was assayed by chemiluminescence using a superoxide-specific, enhanced luminol-based substrate (DIOGENES, National Diagnostics), as described previously (34). The reaction was monitored for 15 min at 37 °C using a luminometer (Labsystem, Helsinki, Finland). Conditions for dose- and time-dependent inhibition of oxidation activity were explored with the PLD inhibitors 1-butanol and 2,3-diphosphoglycerate (2,3-DPG) (as well as 3-butanol, control) to determine the most effective treatment for inhibiting superoxide generation without reducing cell viability below 98%. Optimal concentrations were chosen at 0.5% 1-butanol, 0.5% 3-butanol, or 5 mM 2,3-DPG, which were added to the cells 10 min prior to activation.

**Translocation and Immunoblot Analysis—**Bt_cAMP-differentiated PLB-985 cells were stimulated for 3 min with 1 μM fMLP or for 5 min with 100 ng/ml PMA at 37 °C in Hanks’ balanced saline solution with Ca²⁺ and Mg²⁺. Cell membranes were prepared following sonication by methodology described previously (30). 50 μg of membrane proteins were separated by electrophoresis on 12% polyacrylamide SDS gels and blotted to nitrocellulose. ARF1 and ARF6 were detected by using rabbit anti-ARF1 (provided by Dr. Richard A. Kahn, Emory University, Atlanta, GA (35)) and rabbit anti-ARF6 (provided by Dr. Julie G. Donaldson, NIH, Bethesda, MD (26)) according to standard protocols (30).

### RESULTS AND DISCUSSION

In previous studies we demonstrated the PLB-985 cell line to be a useful model system that is capable of developing a differentiated myeloid phenotype while being amenable to gene transfection protocols (30). Because of the relatively low transfection efficiency of these cells and the requirements for rapid transfection protocols, we developed a multicolor FACS analysis to identify and characterize small subpopulations of transiently transfected cells within mixed cell populations. Transfected cells were identified by detection of recombinant EGFP, expressed either alone or fused with various ARF proteins of interest. Following treatments with Bt_cAMP for 3 days to induce a neutrophil-like phenotype, the cells were analyzed for forward angle light scattering (FALS) and 90° side light scattering properties. Two distinct populations were observed based on distinct light scattering properties (Fig. 1A): low FALS population (10⁶ cells) and gate G1, and a high FALS population, indicated as gate G2 or G3 (depending on untransfected or transfected cell cultures, respectively). The high FALS population from transfected cell cultures (G3) exhibited higher 90° light scatter compared with control untransfected cells (G2), consistent with a greater granularity that was associated with either the electroporation or sedimentation proto-
cell surface-conjugated Ros-SE oxidase activity (F1/F0) is expressed as detection of the respiratory burst in differentiated PLB-985 cells using corrected for background counts observed in untransfected cells. was calculated from EGFP fluorescence cell counts observed above 30, infected (\( \frac{F1}{F0} \)) showed a significant elevation \((p \leq 0.03)\) in oxidase activity. The PLB-985 cells transiently transfected with WT forms of recombinant ARF1 or ARF6 produced as fusion proteins with EGFP (ARF1-WT, ARF6-WT) or with fusion proteins of two mutants of each ARF. ARF6(T27N) and ARF1(T31N) mutants represent putative dominant-negative mutants with reduced affinity for GTP, whereas the ARF6(Q67L) and ARF1(Q71L) mutants represent putative active forms with reduced GTPase activity. Earlier work has shown that the fusion of these proteins at their C terminus with EGFP does not interfere with GTP-dependent cycling between Golgi membrane and cytoplasmic compartments (ARF1) or signaling through PLD in response to receptor stimulation (ARF1, ARF6) in whole transfected cells (37, 38). We compared activation of NADPH oxidase in Bt2cAMP-differentiated PLB-985 cells, PLB-985 cells transfected with empty EGFP-N1 vector (control), and cells transfected with the various EGFP/ARF protein constructs in response to 1 \( \mu \text{M} \) fMLP or 100 ng/ml PMA. Fig. 2A presents results from a representative double fluorescence FACs analysis of transfected, fMLP-activated PLB-985 cells. Cells expressing the highest levels of recombinant EGFP/ARF proteins in R3 exhibited the most dramatic results (Figs. 2B and 3). Superoxide production observed in differentiated PLB-985 cells transfected with ARF1-WT, the two ARF1 mutants, or ARF6-WT was similar to activity observed in the differentiated, untransfected parental line or differentiated cells transfected with empty EGFP-N1 vector. In contrast, cells transfected with ARF6 mutants showed dramatic alterations in oxidase activity. The PLB-985 cells transiently transfected with ARF6(Q67L) showed a significant elevation \((p = 0.03)\) in oxidase activity, whereas PLB-985 cells transfected with ARF6(T27N) did not generate any detectable superoxide in response to fMLP. These effects were only observed when using fMLP as an agonist; transfected cells stimulated with PMA, a nonphysiological activator presumed to bypass early receptor-activated signaling intermediates, showed no effects on oxidative output with the expression of the same recombinant ARF fusion proteins (data not shown). Superoxide production in response to fMLP was similar in all cell populations that ex-
hibited low EGFP fluorescence (i.e. EGFP Fl < 30, R0) regardless of the ARF construct transfected (data not shown), indicating that all of the cultures analyzed had the same oxidative potential, and therefore the differences in fMLP-elicited oxidase activity observed were limited to cells expressing high levels of ARF6 mutants.

To confirm the relationship between ARF6 expression and oxidative output in fMLP-activated cells, we compared superoxide production in cells analyzed within several regions based on EGFP fluorescence intensities. Fig. 3 compares oxidase activity between PLB-985 cells transfected with ARF6 protein constructs (T27N, or Q67L, or WT) and control EGFP-N1 vector. The results extend those obtained in Fig. 2B by showing that transfection with ARF6-WT and EGFP-N1 had no effect on oxidase function in all regions analyzed, while confirming that the alterations in oxidase activity by transfected ARF6(T27N) or ARF6(Q67L) correlated closely with the amount of these proteins produced. A similar analysis of PLB-985 cells transfected with ARF1 protein constructs showed no effect on oxidase activity (data not shown).

To address concerns of whether differences in oxidase activity could be explained simply by differences in differentiation or expression of essential oxidase components, we examined the cell surface expression of gp91phox in these cultures by FACS analysis using a monoclonal antibody directed against an extracellular epitope of this protein (33). Fig. 4 shows that all transfected cell populations detected in R3 expressed comparable amounts of cell surface gp91phox, as indicated by peak levels of secondary anti-mouse antibody detected. These results showed that the alterations in oxidase activity observed with ARF6(T27N) or ARF6(Q67L) expression were not caused by differences in gp91phox expression and confirmed that all transfected cultures differentiated to a comparable extent, consistent with findings that demonstrated comparable oxidative output in all populations exhibiting low EGFP fluorescence. These findings provide further support to the conclusion that ARF6, but not ARF1, has a specific signaling role in fMLP-mediated activation of the respiratory burst.

fMLP-mediated oxidase activation in neutrophils and HL-60 cells is inhibited by primary alcohols (39), suggesting that fMLP-stimulated oxidase activation through ARF6 is also PLD-dependent in PLB-985 cells. To test this hypothesis, we used inhibitors of PLD, 1-butanol (39) and 2,3-DPG (14, 40), substrates that inhibit formation of phosphatidic acid, and compared fMLP-mediated activation of NADPH oxidase in Bt_cAMP-differentiated PLB-985 cells transfected with ARF6-WT, ARF6(Q67L), or empty EGFP-N1 vector (Fig. 5). The PLD inhibitors 1-butanol (0.5%) and 2,3-DPG (5 mM) blocked superoxide production in PLB-985 cells transfected with empty EGFP-N1 or ARF6-WT. Furthermore, both inhibitors significantly blocked the enhanced superoxide production observed in ARF6(Q67L) transfected cells (2,3-DPG versus control, p = 0.007; 1-butanol versus control, p = 0.001), whereas 3-butanol (0.5%), which is not a substrate for PLD, had little effect on the oxidative response of these cells.

To explore further the notion that fMLP receptor-mediated activation of the respiratory burst involves ARF6 signaling

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**Fig. 2.** Comparison of the respiratory burst activity in PLB-985 cells transfected with various EGFP/ARF fusion proteins. A, representative double dot plot obtained from FACS analysis of respiratory burst activity of EGFP-transfected PLB-985 cells. R1–R3 represent areas of increasing EGFP fluorescence within the upper right (double positive) quadrant. B, fMLP-elicited (1 μM) NADPH oxidase activity deduced from oxidized Ros-SE fluorescence values (F1/F0) of transfected cell G3 populations that exhibited the highest levels of EGFP/ARF protein expression (R3). Data represent the means ± S.E. (n = 5–7). Differences between oxidase activity with either ARF6(Q67L) or ARF6(T27N) and control were statistically significant, p = 0.03 and p = 0.0011 respectively. PLB, untransfected, differentiated PLB-985 cells; EGFP-N1, differentiated PLB-985 cells transfected with empty EGFP-N1 vector; WT, wild-type. Various mutant EGFP/ARF vectors are described under “Experimental Procedures.”

**Fig. 3.** Correlation between mutant ARF6 protein production and the enhancement (Arf6(Q67L)) or inhibition (ARF6(T27N)) of respiratory burst activity in transfected PLB-985 cells stimulated with fMLP. Oxidase activities were compared within the three regions of dot plots illustrated in Fig. 2A. Data represent the means ± S.E. (n = 4).
through activation of PLD, we also examined mutant forms of ARF1 and ARF6 which are thought to be defective in their activation of PLD. A recent report (13) identified regions within the crystallographic structure of ARF1 important for hPLD1 activation. The substitution of asparagine to arginine at position 52 (N52R) completely abolished the ability of ARF1 to activate hPLD1 in vitro, while not affecting another ARF1-associated response, the recruitment of coatamer to membranes. Based on this observation, we mutated asparagine 52 to arginine (N52R) in ARF1/EGFP, as well as the corresponding site within ARF6 (N48R)/EGFP. As shown in Fig. 6, PLB-985 cells transfected with ARF1-WT or ARF1(N52R), as well as ARF6-WT, showed no effect on superoxide production, whereas cells transfected with ARF6(N48R) exhibited significant inhibition (p < 0.01) of superoxide production. Although the effects of this mutation on PLD activation were not examined directly, these findings provide additional support to the notion that ARF6 participates in receptor-mediated oxidase activation and suggest that ARF6 acts through activation of PLD, consistent with results obtained with the pharmacological agents shown in Fig. 5.

Phorbol esters are also effective stimuli of PLD activity in a wide range of intact cells (41). In neutrophils and HL-60 cells, most agonists that activate the respiratory burst also activate protein kinase C and PLD (39). To clarify further whether protein kinase C-dependent stimulation of the oxidase involves PLD in Bt2cAMP-differentiated PLB-985 cells, we tested the effect of the same PLD inhibitors on PMA-elicited superoxide production in these cells (Fig. 7). PLD inhibitors, 0.5% 1-butanol or 5 mM 2,3-DPG, caused significant inhibition (p < 0.005 and p < 0.001, respectively) of superoxide production in response to PMA, whereas the control compound 3-butanol (0.5%) had no effect on superoxide. These observations, together with the absence of any demonstrable effects of dominant negative mutants of ARF1 or ARF6 on PMA-stimulated oxidase activity, suggest that protein kinase C activation of the oxidase is PLD-dependent but ARF-independent.

As another correlate to ARF involvement in oxidase activation in differentiated PLB-985 cells, we examined membrane translocation of ARF1 and ARF6 following stimulation with either 1 μM fMLP or 100 ng/ml PMA. Fig. 8 shows that stimulation by either of these agonists caused enhanced membrane binding of both the ARF1 and ARF6 isoforms. These observations are consistent with previous reports demonstrating that both fMLP and PMA stimulate translocation of ARF to the plasma membrane in HL-60 cells and neutrophils, although these studies did not distinguish between the two isoforms (18, 19). Thus, PMA stimulates ARF1 and ARF6 activation and translocation to membranes in PLB-985 cells but has other direct or overriding effects on oxidase activation which appear to involve PLD but are insensitive to the effects of the dominant ARF1 or ARF6 isoforms.

The present study provides novel evidence for unique involvement of ARF6 in N-formyl peptide receptor-stimulated activation of NADPH oxidase because expression of the GTP-binding deficient
mutant (ARF6(T27N)) inhibited superoxide production, whereas the GTPase-deficient mutant (ARF6(N48R)) enhanced superoxide production. Furthermore, the effects of these ARF6 mutants on superoxide production were not observed when the corresponding ARF1 proteins were expressed, indicating that this response is specific for ARF6. Furthermore, the inhibitory effects of the ARF6(N48R) mutant, as well as those of the PLD inhibitors 1-butanol and 2,3-DPG, provide evidence that the GTPase-deficient mutant (ARF6(Q67L)) enhanced superoxide production, whereas the dominant-negative and positive effects on superoxide production were not observed when the corresponding ARF1 proteins were expressed. These observations, together with our findings, indicate that both ARF1 and ARF6 become activated in stimulated myeloid cells but that the two proteins are involved in different functions which may relate to their segregation into different cellular compartments (23).

In conclusion, we have developed a unique assay using molecular approaches to study the signaling cascade leading from N-formyl peptide receptor stimulation to NADPH oxidase activation in intact neutrophil-like cells. Using this assay we demonstrated that ARF6 has a direct role in NADPH oxidase regulation and suggest that this physiological function of ARF6 is mediated through PLD. ARF6 and protein kinase C appear to activate the oxidase in parallel pathways. PLD apparently participates in both pathways and is located downstream of either protein kinase C or ARF6. Future work should address the identity of the PLD isozyme that links ARF6 activation to the oxidase, as well as upstream signaling intermediates responsible for ARF6 activation.

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