Isolation and Characterization of a Variant HL60 Cell Line Defective in the Activation of the NADPH Oxidase by Phorbol Myristate Acetate

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*J Immunol* 1998; 161:6885-6895; 
http://www.jimmunol.org/content/161/12/6885
Isolation and Characterization of a Variant HL60 Cell Line Defective in the Activation of the NADPH Oxidase by Phorbol Myristate Acetate

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Promyelocytic human leukemia HL60 cells can be differentiated into neutrophil-like cells that exhibit an NADPH oxidase activity through direct stimulation of protein kinase C (PKC) with PMA or through formyl peptide receptor activation. We have isolated a variant HL60 clone that exhibited a conditional PMA-induced oxidative response depending on the agent used for the differentiation. While cells differentiated with DMSO responded to either PMA or N-formyl peptide (N-formyl-Met-Leu-Phe-Lys or fMLFK), cells differentiated with dibutyryl-cAMP (Bt2cAMP) responded to fMLFK but very poorly to PMA. However, in Bt2cAMP-differentiated cells, the expression of the different PKC isoforms was similar to that observed in DMSO-differentiated cells. Moreover, PMA was able to induce a normal phosphorylation of the cytosolic factor p47phox and to fully activate extracellular signal-regulated kinases (Erk1/2). Interestingly, Bt2cAMP-differentiated cells exhibited a strong and sustained O2− production when costimulated with PMA and suboptimal concentrations of fMLFK which were, per se, ineffective. This sustained response was only slightly reduced by the conjunction of the mitogen-activated protein (MAP) kinase kinase (MEK) inhibitor PD98059 and wortmannin, a phosphatidylinositol-3 kinase (PI3K) inhibitor. Variant HL60 cells that were stably transfected with a constitutively active form of Rac1 were able, when differentiated with Bt2cAMP, to secrete oxidant following PMA stimulation. Altogether, the results suggest that, in addition to the phosphorylation of p47phox, the activation of NADPH oxidase requires the activation of a Rac protein through a pathway that diverges at a point upstream of MEK and that is independent of the activation of wortmannin sensitive PI3K. The Journal of Immunology, 1998, 161: 6885–6895.

Along with phagocytosis and degranulation, superoxide production is part of neutrophil responses to invading pathogens. These granulocytic functions are elicited by micromolar concentrations of chemotactic factors including N-formyl peptides, C5a and IL-8, which bind to specific G protein-coupled receptors. The activation of the enzyme responsible for superoxide production, NADPH oxidase, proceeds through a multistep assembly at the plasma membrane of several components including the two subunits of cytochrome b558 (p22phox and gp91phox), the small G proteins (Rac, Rap), and the cytosolic factors (p47phox, p67phox and p40phox) (1–3).

The signaling pathways linking chemoattractant receptors to NADPH oxidase activation are far from being elucidated. Stimulation by chemoattractants is accompanied by a rapid increase in Ca2+ and diacylglycerol and subsequently by the activation of protein kinase C (PKC) and the phosphorylation of several proteins including p47phox. A correlation has been established between agonist-induced phosphorylation of p47phox and the activation of the respiratory burst (4–6), but whether PKC is directly involved in this phosphorylation is still a matter of debate. The involvement of PKC in triggering the respiratory burst is supported by the ability of PMA to induce superoxide production, translocation, and phosphorylation of p47phox and by the ability of PKC to phosphorylate p47phox in vitro (7–12). It has also been shown that a recombinant p47phox that had been prephosphorylated by PKC could specifically activate NADPH oxidase in a cell-free system (13).

The Ras-related G protein Rac also contributes to the regulation of NADPH oxidase activity in phagocytic leukocytes (3, 14, 15). The different stages of action of Rac are not completely elucidated. Cell-free studies with neutrophil plasma membrane have shown that, in addition to p47phox and p67phox, the GTP-bound form of Rac is required to initiate the production of superoxide. Rac1 has been identified as the active component in guinea pig macrophages (16), whereas Rac2 was found to be predominant in human neutrophils (17–19). However, in a cell-free system either of them is able to trigger superoxide production (17, 20, 21). The decrease of Rac protein levels by antisense oligonucleotide strategies or the expression of a Rac-negative dominant result in a reduction of superoxide production, demonstrating the requirement for Rac in...
intact cell oxidase activation (22, 23). The addition of chemotactic tracts to neutrophils results in the exchange of GTP for GDP on Rac and in the translocation of this latter to the plasma membrane (24, 25). However, while the translocation of p67phox and p47phox kinetically parallels NADPH oxidase activity, the extent of translocation of Rac2 from the cytosol to the plasma membrane does not correlate with neutrophil NADPH oxidase activity, suggesting that the Rac proteins do not regulate NADPH oxidase activity stoichiometrically (26).

Several studies have demonstrated that the exposure of neutrophils to N-formyl peptides increases phosphatidylinositol-3 kinase (PI3K) activity (27, 28) and p24/44 mitogen-activated protein kinase (MAPK) (or Erk1/2) activities (29–31). The activation of these enzymes was timely correlated with the triggering of NADPH oxidase. Because PI3K inhibitors such as wortmannin (WT) and LY294002 completely abolished the superoxide secretion induced by N-formyl peptides but not that mediated by PMA (32–37), it has been suggested that PI3K is a key component, upstream of PKC, in the transduction pathway involved in chemotactant-mediated NADPH oxidase activation. However, the role of the p42/44 MAPKs is less clear. On the one hand, a functional link with NADPH oxidase activity is supported by the observation that, among the serine residues of p47phox that are phosphorylated upon stimulation, two are located within consensus sequences for proline-directed kinases (10) and correspond to the sites phosphorylated in response to priming described in neutrophils, where nonstimulatory L-formyl peptide (NH2-Y-E-S-T-K-R-K-L-A-S-A-V-OH) that was synthesized by BioCytex (Marseille, France). The anti-c-myc mAb (9E10) and competing c-myc peptide were purchased from Boehringer Mannheim. Cell culture medium, FCS, and geneticin (G418) were from Life Technologies (Grand Island, NY), except for phosphate-free RPMI, which was provided by ICN. (Orsay, France) [33P]Orthophosphoric acid was purchased from Isotopchim (Ganagobie, France). Redivue Pro-mix L-[35S] in vitro cell labeling mix was purchased from Amersham.

**Cell culture and differentiation**

Promyelocytic HL60 cells were cultured in RPMI 1640-glutMAX I medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% of heat inactivated FCS at 37°C in humidified atmosphere of 5% CO2/95% air. Cell differentiation was initiated either with 1.25% DMSO for 6 days when cell density was 0.5 × 10⁶ cells/ml or with 1 mM Br2cAMP for 3 days. In this latter case, optimal differentiation was obtained when cells were centrifuged and resuspended at a density of 10⁶ cells/ml in fresh complete RPMI before differentiation was initiated.

**Isolation of a variant HL60 clone**

Promyelocytic HL60 cells were diluted at a density of about 100 cells for 10 ml of conditioned RPMI filtered through a 0.2-μm sterile Milipore (Bedford, MA) filter. Before plating in tissue culture plates, the cell suspension was maintained at 37°C and supplemented with sterile agarose at the final concentration of 0.18%. After 2 wk, clones were picked with a Pasteur pipette, expanded in complete RPMI, and tested for the absence of PMA-induced superoxide production after differentiation with Br2cAMP.

**Superoxide production assay**

Cells were washed with PBS and resuspended at a concentration of 4 × 10⁷ cells/ml in PBS containing 0.5 mM CaCl₂, 1 mM MgCl₂, and 30 mM glucose (buffer A). Preincubation with various inhibitors was accomplished at 37°C for the indicated periods of time. Control cells without inhibitors were incubated in the same conditions. Fifty microliters of the cell suspension, kept at 4°C, were added to 1 ml of prewarmed PBS containing 200 μM of ferricytochrome C. Ferricytochrome C production was initiated by the addition of fMLF or PMA at the indicated concentrations. Ferricytochrome C reduction was continuously monitored at 550 nm.

**Cell lysis, subcellular fractionation, and Western blot analysis of PKC isozymes, rhoGDI, and Rac G proteins**

Cells were washed with PBS and resuspended at a concentration of 2 × 10⁶ cells/ml in buffer A. After stimulation with PMA (1 μg/ml) for 3 min, cells were harvested and resuspended into 50 mM Tris-HCl (pH 7.5) containing 2 mM EGTA, 50 mM 2-ME, 10 μg/ml leupeptin, 10 mM benzamidine, 5 μM pepstatin, 0.2 μg/ml aprotinin, and 1 mM PMSF. Cells were disrupted by sonication, and the resulting homogenate was centrifuged (800 × g for 5 min at 4°C) to remove unbroken cells and nuclei. The cytosolic fraction obtained by centrifugation at 100,000 × g for 1 h at 4°C was supplemented with Laemmli sample buffer containing 5 mM DTT. The remaining pellet was solubilized by sonication in Laemmli sample buffer containing 5 mM DTT (51). Samples were denatured by boiling for 5 min, subjected to electrophoresis on a 10% SDS-polyacrylamide gel, and electrotransferred to nitrocellulose. Nonspecific binding was blocked by incubation of the membrane with PBS containing 0.1% Tween-20 (PBS-T) and 3% BSA for 1 h at room temperature. The membrane was then incubated overnight at 4°C with appropriate dilutions (1:250 to 1:1000) of mAbs to the various PCK isozymes in PBS-T containing 3% BSA. After washing in PBS-T, the membrane was incubated with a 1:100 dilution of rabbit anti-mouse IgGs (Sigma) in PBS-T containing 1% BSA for 2 h. After washing of the membrane in PBS-T, detection of PCK isozymes was performed using 125I-labeled protein A and autoradiography on Fuji RX films (Fuji Medical Systeme, Clichy, France).

RhoGDI and Rac1 polyclonal Abs were used at the dilution 1:100. Bound RhoGDI IgGs were detected with 125I-labeled protein A whereas bound Rac1 IgGs were visualized with anti-rabbit IgGs peroxidase-linked Abs and ECL.
Immunoprecipitation of MAPKs

Differentially HL60 cells were resuspended in buffer A (10^7 cells/ml) and stimulated either with fMLFK (10 nM) or PMA alone (1 μg/ml) or with the combination of buffer A plus 10 nM fMLFK for a total time of 3 min. In the latter case, fMLFK was added 1 min after PMA. Stimulation was terminated by a brief centrifugation and lysis of the cell pellet at 4°C in 800 μl of lysis buffer containing 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EGTA, 1 mM sodium orthovanadate, and antiproteases (1 ml of a 1:10,000 dilution of AEBSF). Nuclei were pelleted at 10,000 × g and supernatants were incubated for 2 h at 4°C with 1 μg of a rabbit polyclonal Ab against Erk1 or Erk2. Eight milligrams of protein A-Sepharose in 100 μl PBS were added and incubation was pursued for 2 h. Protein A-Sepharose beads were washed twice with buffer B plus 0.2 mM sodium orthovandate, then twice with kinase buffer (20 mM Tris-HCl (pH 7.5), 20 mM MgCl2, 1 mM MnCl2, 1 mM DTT, 5 mM NaF, 200 μM sodium orthovandate). One-tenth of washed complexes were kept for Western blot analysis of immunoprecipitated MAPK. This was performed as described for PKC analysis with the following modifications. First Ab was used as the same as that used during the immunoprecipitation step (dilution 1/1000) and detection of MAPks was performed with peroxidase-conjugated protein A using the ECL Western blotting detection system.

Immune complex MAPK assay

MAPK assay was initiated by resuspending the immunoprecipitates in 40 μl of kinase buffer supplemented with 5 mM okadate acid, 10 mM p-nitrophenylphosphate, 50 μM ATP, 5 μCi [γ-32P]ATP and 10 μg MBP per assay. Phosphorylation was allowed for 30 min at 30°C and terminated by addition of 40 μl of 4-fold concentrated Laemmli buffer and boiling for 5 min. Phosphorylated MBP was analyzed under reducing conditions on a 15% SDS-polyacrylamide gel. Gels were fixed in 10% TCA and 20% methanol, dried, and visualized with a Molecular Dynamics PhosphorImager. Quantification of the phosphorylation levels was performed through PhosphorImager.

In vivo phosphorylation of p47phox

Differentially HL60 cells were washed twice with 175 mM NaCl, 2.5 mM KCl, and 10 mM HEPES (pH 7.5) and incubated for 3 h at 37°C in phosphate-free RPMI medium with 20 μM HEPES (pH 7.5), 20 mM glucose, and 1 μCi [γ-32P]orthophosphoric acid. Cells (2 × 10^7 cells/ml) were stimulated either with fMLFK (1 μM), PMA alone (1 μg/ml), or with the combination of PMA plus 10 nM fMLFK for a total time of 2 min. fMLFK being added 1 min after PMA. Stimulation was stopped by lysis in 500 μl RIPA buffer at 4°C (52) supplemented with antiphosphatases and antiproteases as described above. p47phox was immunoprecipitated as described above with a polyclonal rabbit Ab directed against the C-terminal end of p47phox. Protein A-Sepharose beads were washed sequentially with 50 mM Tris-HCl (pH 8) and 0.5 mM NaCl containing 1% Triton X-100 and 0.2% SDS, with the same buffer containing 1% Triton X-100 and 0.1% SDS and with the same buffer without detergent, then with PBS. Samples were re-suspended in 2-fold concentrated Laemmli buffer and loaded on a 10% SDS-polyacrylamide gel under reducing conditions. Gels were fixed in 10% TCA and 20% methanol, dried, and visualized with a Molecular Dynamics PhosphorImager.

p47phox phosphopeptide mapping

p47phox was phosphorylated and isolated from 2–4 × 10^7 cells/samples as described above. After separation on SDS-polyacrylamide gel under reducing conditions and electrophoresis on nitrocellulose, the phosphorylated p47phox band was excised after visualization by autoradiography. Two-dimensional peptideic maps were performed according to El Benna et al. (10). In brief, the excised band was soaked in 200 μl of 0.5% polyvinyl pyrrolidone 40 in 100 mM acetic acid for 30 min at 37°C. The nitrocellulose band was washed five times with water, three times with 1 ml of 50 mM NH4HCO3 (pH 8.0), and placed in 200 μl of the buffer later. Tryptic digestion was performed at 37°C for 3 h by adding 5 μl trypsin-TPCK every hour. Water (300 μl) was added and samples were freeze dried. The lyophilization step was repeated twice with 500 μl of water, once with 500 μl of electrophoresis buffer (formic acid/acetic acid/water, 1:3:36, v/v). Finally, the sample was solubilized in 6 μl electrophoresis buffer and loaded on 10 cm × 10 cm cellulose thin layer plates (Schleicher & Schüll, Cera-labo, Ecquevilly, France). Peptides were first separated by electrophoresis at 4°C for 35 min at 500 V with a LBK (Gaithersburg, MD) Multiphor apparatus. After drying, the second dimension was performed by chromatography in n-butanol/pyridine/acetic acid/water (75:50:15:60, v/v). Detection of 32P-labeled peptides was performed through PhosphorImager.

Expression of the constitutively active Rac1 clone in the variant HL60 clone

The pEF-myc-tagged Rac1V12 expression plasmid was constructed as followed. The pMT90 plasmid kindly provided by Dr. P. Chavrier (Marseille, France) was digested with NotI and BamHI. The resulting myc-tagged Rac1V12 containing fragment was cloned between the NotI and BamHI sites of the plasmid pMDNA3.1 (In Vitrogen, Groningen, The Netherlands). The resulting plasmid was digested with Pmel and the fragment containing the myc-tagged Rac1V12 sequence was cloned into the Smal site of a modified version of the pEF-neo plasmid kindly provided by Dr. M. C. Dinauer (Indianapolis, IN) (53).

Transfection of the variant HL60 clone was performed by electroporation with a Bio-Rad Gene Pulser apparatus, according to Tonnetti et al. (54) with slight modifications. In brief, 20 μg of supercoiled plasmid DNA in TE (10 mM Tris-HCl and 1 mM EDTA (pH 8.0)) were mixed with 10^7 cells in 0.5 ml of phosphate-buffered sucrose (272 mM sucrose and 7 mM Na3HPO4 (pH 7.4)). Cells were electroporated with a pulse of 250 V for 18–20 ms. Control cells were transfected in the same conditions with the pEF-neo plasmid. Following electroporation, cells were allowed to recover in 2 ml of RPMI culture medium for 48 h before selection with 0.6 mg of G418/ml of medium. G418-resistant transfected clones were obtained by limited dilution into 24-well microtiter plates.

The presence of the myc-tagged Rac1V12 mRNA in the G418-resistant clones was detected by a reverse transcriptase PCR assay of DNase-treated RNA. Poly(A)+ mRNA was isolated from 5 × 10^7 cells samples using the StrataSpin mRNA isolation system (Novagen, Madison, WI). First strand DNA synthesis was performed using random hexanucleotides as primers and Expand Reverse Transcriptase (Boehringer Mannheim). Fragments corresponding to myc-Rac1V12 and endogen Rac1 cDNAs were amplified by PCR with the following primers: 5’-CTCATACTCAGAAGAGGATCTGG-3’ (sense in the myc tag sequence), 5’-CTGCAATGTTATGTGATGATG-3’ (sense in Rac1 sequence), and 5’-AGACTGTCGTGGATCGTCTG-3’ (antisense in Rac1 sequence). The products were resolved in a 1% agarose gel and stained with ethidium bromide.

For detection of myc-tagged Rac1V12 protein, 5 × 10^7 HL60 cells transfected with either pEF-myc-Rac1V12 or pEF-neo plasmid were washed twice with 10 ml of methionine/cystine-deficient RPMI and cultured for 60 min in this culture medium. Cells at the density of 10^7 cells/ml were labeled by the addition of 3.75 μCi of RadioVivo Pro-mix L-[35S] in vitro cell labeling mix for 3 h. Cells were then washed once with 10 ml of complete RPMI and cultured for another hour in 10 ml of complete RPMI. Cell pellets were lysed in 1 ml of ice-cold RIPA buffer supplemented with protease inhibitors. Lysates were centrifuged for 5 min at 14,000 × g, and supernatants were subjected to immunoprecipitation with 10 μg of anti-c-myc mAb (9E10) coupled to protein-G Sepharose beads in the presence or absence of 40 μg/ml of competing c-myc peptide. After 16 h at 4°C, Sepharose beads were subjected to four washes with 1 ml of ice-cold RIPA buffer. Sepharose beads were treated with 80 μl of 2-fold Laemmli sample buffer with reducing agents for 5 min at 100°C, and loaded on a 14% SDS-polyacrylamide gel. After fixation and incubation in 1 M sodium salitycyle in 20% methanol, the gel was dried and exposed to x-ray film.

Results

Isolation and oxidase phenotype of the variant HL60 cell line

Wild-type HL60 cells from the American Type Culture Collection (Manassas, VA) can be differentiated to granulocyte-like cells with DMSO or Bt2CAMP. They similarly produce superoxide in response to PMA or N-formyl peptide (iMLFK) whatever the differentiation protocol used. While testing a new FCS, we selected a rapidly growing population of cells that proved to respond very poorly to PMA when differentiated with Bt2CAMP. A clone was further isolated on soft agar and characterized. When assayed for NADPH oxidase activity after differentiation with DMSO, the isolated variant HL60 cells exhibited a rapid and strong response to both N-formyl peptide and PMA (Fig. 1A, traces d and e). However, after differentiation with Bt2CAMP, the variant HL60 cells responded to a saturating concentration of N-formyl peptide (1 μM) but virtually failed to respond to PMA (1 μg/ml), even after a prolonged exposure (Fig. 1A, traces a and b, respectively). PMA was nevertheless able to induce a strong and sustained superoxide...
production in Bt2cAMP-differentiated cells when used in conjunction with a suboptimal concentration of fMLFK (10 nM) which failed to activate the NADPH oxidase (Fig. 1A, traces b and c). This sustained response was observed independently of the order of addition of the two agonists.

The possibility to evoke oxidative activity in Bt2cAMP-differentiated variant HL60 cells indicates that components of the NADPH oxidase complex were structurally functional. Moreover, the response elicited by the combination of the two activators was persistent over at least 10 min, while the response elicited by 1 μM N-formyl peptide alone was rapidly attenuated. To compare the ability of fMLFK to stimulate per se NADPH oxidase activity and its potency to rescue the PMA response, we quantified the amount of superoxide produced by Bt2cAMP-differentiated cells stimulated with varying concentrations of fMLFK in the absence or presence of PMA, respectively. When PMA was present, superoxide production was detectable with concentrations of fMLFK as low as 100–200 pM (Fig. 1B). A plateau was reached at ~10 nM and the concentration for a half-maximal restoration of the PMA response was about 3 nM. In the absence of PMA, superoxide production was detectable only for concentrations superior to 20 nM-50 nM fMLFK.

The rescue of PMA by N-formyl peptide is partially PTX-resistant

As expected, the NADPH oxidase stimulation in response to a saturating concentration of fMLFK alone was fully inhibited by pretreating cells for 5 h in the presence of 100 ng PTX/ml before cytochrome c reduction assay. A, Cytochrome c reduction kinetics upon stimulation with either 1 μM fMLFK or PMA plus 10 nM fMLFK. B, The total amount of superoxide produced after 6-min stimulation as in A was measured. For each set of agonists, the amount of superoxide produced by PTX-treated cells was expressed as the percentage of the amount obtained with control cells. Values represent the means ± SD of three independent experiments.

Phosphorylation of p47phox in the variant HL60 cell line

Phosphorylation of the cytosolic factor, p47phox, is thought to be a key step in PMA- and agonist-mediated activation of the NADPH oxidase. We therefore examined whether under PMA stimulation
p47phox was differentially phosphorylated in cells differentiated with DMSO or Bt2cAMP. After metabolic labeling of cells with [33P]orthophosphate and stimulation, p47phox was isolated by immunoprecipitation and submitted to SDS-PAGE. Consistent with the fact that about three times as much p47phox was expressed in cells differentiated with DMSO (Fig. 3A), the basal level of p47phox phosphorylation was about three times higher in cells differentiated with DMSO than in cells differentiated with Bt2cAMP (Fig. 3B, lanes 1 and 3). Stimulation with 10 nM fMLFK induced only a slight increase above this basal level of phosphorylation (data not shown). In DMSO-differentiated cells, PMA induced a 4-fold increase in the phosphorylation of p47phox while a 2-fold increase was observed in Bt2cAMP-differentiated cells (Fig. 3B, lanes 2 and 4, respectively). No further increase was noted when 10 nM fMLFK was subsequently added (Fig. 3B, lane 5).

Previous studies have shown that phosphorylation of p47phox occurs on multiple serine residues under PMA stimulation (10). As the absence of phosphorylation of only one or two serine residues may be sufficient to impair NADPH oxidase activity, we further investigated p47phox phosphorylation using two-dimensional tryptic phosphopeptide mapping. The pattern of tryptic phosphopeptide map of p47phox immunoprecipitated from Bt2cAMP-differentiated variant cells after PMA stimulation (Fig. 3C) was identical to that obtained previously for p47phox isolated from human neutrophils (11). An identical pattern of phosphorylated peptides was observed whether cells were differentiated with DMSO or Bt2cAMP, albeit some spots appeared less intense when Bt2cAMP was used. However, the costimulation with 10 nM fMLFK, which restored a robust and sustained NADPH oxidase activity, did not modify the intensity of these spots. Thus, PMA-induced p47phox phosphorylation did not seem impaired in Bt2cAMP-differentiated cells. The failure of PMA to activate the NADPH oxidase might result from a deficiency in the activation of upstream regulatory enzymes. We therefore investigated the status of PKC, PI3K and MAPK in the variant HL60 cell line after differentiation with Bt2cAMP.

Expression of PKC isoforms in differentiated variant HL60 cells

Phorbol esters are direct activators of a number of PKCs. Upon cell activation with PMA, translocation of PKC to the cell membrane has been observed and is associated with the activation of the enzyme (56). To examine whether the failure of PMA to activate the NADPH oxidase in Bt2cAMP-differentiated cells could arise...
from a deficiency in the expression or function of one PKC isoform, we analyzed the distribution of the various isoforms in subcellular fractions of DMSO- and Bt2 cAMP-differentiated HL60 cells using specific Abs. In either type of differentiated cells, isoforms α, β, γ, δ, ε, θ, λ, and ζ were detected (Fig. 4). They corresponded to the isoforms previously detected in neutrophils (8, 12, 57) or in granulocyte-type differentiated HL60 cells (58, 59). PKC η, θ, and μ were not visible whether cells were differentiated with DMSO or Bt2 cAMP (data not shown). In nonstimulated cells, PKC α, β, and γ were detected predominantly in the cytosolic fraction, whereas PKC δ and ε were mainly recovered in the particulate membrane fraction. Distribution of PKC isoforms ζ, λ, and ζ appeared to be unchanged after PMA stimulation whichever conditions were used for the differentiation. The atypical isoforms λ, ζ, and ζ, which have been shown to be unaffected by phorbol esters (56), were not expected to be translocated. The distribution of the most easily detectable atypical isofrom (i.e., PKC ζ) was not modified even when cells were costimulated with PMA and a low concentration of N-formyl peptide. Altogether, the results indicate that there was no obvious abnormality in the PKC isoforms expression and distribution in Bt2 cAMP-differentiated cells.

Effects of WT and PD98059 on the NADPH oxidase and Erks activities in Bt2 cAMP-differentiated HL60

In neutrophils, the binding of chemoattractants to their cognate receptors results in activation of PI3K and of Erk1/2 MAPK. As these enzymes were proposed to participate in the induction of the oxidative burst, we examined in Bt2 cAMP-differentiated HL60 the effects of PI3K and MEK inhibitors, namely WT and PD98059, on superoxide production and on Erk2 phosphorylating activity elicited under the various conditions of stimulation.

As illustrated in Fig. 5, WT strongly inhibited the response to 1 μM fMLFK (hatched bars). The extent of inhibition obtained at 10 nM and 100 nM WT were of 50% and 80%, respectively. PD98059 at best reduced the same response by 30%. However, treatment of Bt2 cAMP-differentiated variant HL60 cells with 10 nM or 100 nM WT or with 50 μM or 100 μM PD98059 had only a slight inhibitory effect (inferior to 20%) on superoxide production when cells were costimulated with PMA plus 10 nM fMLFK (hatched bars). These results suggest that, under stimulation conditions, the activation of the NADPH oxidase complex elicited by substimulatory concentrations of chemoattractant proceeds through a pathway in which PI3K and MEK play no major role.

Both the Erk inhibitor and the PI3K inhibitor have been shown to alter MAPK activities in vivo (39–42, 44–46, 60, 61). Their respective effect in Bt2 cAMP-differentiated variant cells were thus checked on Erk2 activity as assayed by the ability of Erk2 immune complexes to phosphorylate the MBP in vitro (Fig. 6). Only a very weak basal activity was observed in control cells that have not been stimulated. Phosphorylation of MBP was markedly increased when Erk2 was immunoprecipitated from cells stimulated with 10 nM fMLFK (or 1 μM fMLFK; data not shown) or with PMA alone. The combination of the two agonists resulted in an even stronger Erk2 activity (Fig. 6, first track of last panel). Only partial inhibitions of stimulated Erk2 activity were obtained after a pretreatment of the cells with PD98059. Under stimulation with fMLFK, PMA, or the combination of both, Erk2 activity was reduced by 42 ± 15%, 68 ± 16%, or 27 ± 18% (n = 3), respectively. However, much stronger inhibitory effects were observed while combining PD98059 and WT (Fig. 6, last track in each panel). Fig. 5 shows that, in parallel, the NADPH oxidase response to 1 μM fMLFK was completely obliterated by cotreatment of the

**FIGURE 5.** Effects of inhibitors PD98059 and WT on superoxide production in Bt2 cAMP-differentiated variant HL60. PD98059 and WT, used separately or in combination, were added at the indicated concentration to the oxidase assay medium for 1 h or 10 min, respectively, before agonist stimulation. Bars represent the amount of superoxide produced for 6 min after stimulation with PMA plus 10 nM fMLFK (filled bars) or with 1 μM fMLFK (hatched bars). For each stimulatory condition, the values ± SD (n = 5) were expressed as percentage of control oxidase activity obtained in the absence of inhibitor.

**FIGURE 6.** Effects of inhibitors PD98059 and WT on MBP phosphorylating activity of Erk2 isolated from Bt2 cAMP-differentiated variant HL60 cells. Erk2 isoform was immunoprecipitated from lysates of Bt2 cAMP-differentiated cells that have been stimulated with PMA (1 μg/ml), fMLFK (10 nM), or both agonists for 3 min after incubation without inhibitor or with either 100 μM PD98059 for 1 h, 100 nM WT for 10 min, or a combination of both. The figure is representative of three independent experiments. Western blot analyses were systematically performed to check that the same amount of kinase had been immunoprecipitated (data not shown). The MBP phosphorylation assay was conducted as described in Materials and Methods. Assays were also performed with Erk1. Similar results were obtained although weaker activities were observed (data not shown).
cells with WT and PD98059, whereas the fMLFK-rescued PMA-dependent response was only slightly inhibited (20% inhibition). Thus, in cells simultaneously challenged with PMA and fMLFK, it was possible to reduce Erk2 activity by more than 80% while oxidant generation proved to be virtually unaltered. Although the association of the agonists resulted in a stronger Erk2 activity, the sustained superoxide production does not appear to be correlated with the activation of Erk2. This finding supports the former conclusion that the Erk proteins have little, if any, role in the chemotactant-dependent rescuing activity of NADPH oxidase in presence of PMA.

**Constitutively active Rac protein can restore PMA-dependent oxidative response in variant HL60 cells**

When we analyzed lysates of differentiated HL60 cells by immunoblotting, Rac1 was detected in the cytosolic fraction as a 23-kDa band whether cells were differentiated with DMSO or Bt2cAMP (Fig. 7). Rac1 protein was not immunodetected in the particulate fraction, and the small G protein Rac2 was detected neither in DMSO- nor in Bt2cAMP-differentiated cells with commercially available Abs (data not shown). As already observed for the expression of p47phox (see Fig. 3A), the level of expression of Rac1 and RhoGDI, a protein that associates with Rac in its GDP bound form, was lower in cells differentiated with Bt2cAMP than in cells differentiated with DMSO (Fig. 7). The fact that the NADPH oxidase complex is functional under chemotactant stimulation in both conditions of differentiation indicates that the low level of expression of these cytosolic components is not a limiting factor in the activation of the NADPH complex.

To examine whether the reduced PMA-mediated superoxide production in Bt2cAMP-differentiated variant HL60 cells could result from a default in the activation of Rac1, we stably expressed a constitutively active form of Rac1 (Rac1V12) by transfecting cells with the vector pEF-myc-Rac1V12. G418-resistant clones were tested for the expression of myc-Rac1V12 mRNA (lane 5) and a PCR product of 550 bp corresponding to myc-Rac1V12 mRNA (lane 7) were amplified from pEF-myc-Rac1V12-transfected cells. While the 410-bp product was always detected, the 550-bp PCR product was absent in sample derived from the control cells that were transfected with the empty vector pEF (lane 1). The myc-Rac1V12 protein was undetectable by immunoblotting of the cell lysates with an anti-myc Ab (data not shown). However, a 24- to 25-kDa species was immunoprecipitated from the cytosol of myc-Rac1V12-transfected cells after metabolic labeling with [35S]methionine (Fig. 8B). The identity of this band with myc-

**FIGURE 7.** Detection by immunoblotting of RhoGDI and Rac1 in differentiated variant HL60 cells. Variant HL60 cells were induced to granulocytes in the presence of DMSO or Bt2cAMP. The same number of differentiated cells (5 x 10^7 cells) were pelleted, and cytosolic fractions were prepared. Proteins were separated on a 14% polyacrylamide gel in the presence of SDS and processed for immunoblotting on nitrocellulose as indicated in Materials and Methods.

**FIGURE 8.** Effect of myc-Rac1V12 expression on NADPH oxidase activity in Bt2cAMP-differentiated variant HL60. A. Reverse transcriptase assay for the expression of myc-Rac1V12 mRNA in G418-resistant clones. Transfected cells were lysed and poly(A)^+ mRNA was isolated, treated with DNase and exposed to reverse transcriptase (RT +) or buffer (RT -). Specific primers were used for PCR amplification. The 410-bp PCR product in lanes 3 and 7 corresponds to endogenous Rac1. The 550-bp PCR product in lane 5 corresponds to myc-Rac1V12 expressed only in G418-resistant HL60 transfected cells. M denotes the m.w. markers. B. Immunodetection of myc-Rac1V12 protein. Cells transfected with pEF or pEF-myc-Rac1V12 were metabolically labeled with [35S]methionine as described in Materials and Methods. After lysis, protein samples were immunoprecipitated with anti-myc mAb (9E10) adsorbed to protein G Sepharose in the presence or absence of c-myc competing peptide. The immunoprecipitates were subjected to SDS-PAGE and autoradiography. Lanes corresponding to immunoprecipitates in the presence of c-myc peptide exhibit a higher radioactive background. C. Oxidase activity in variant HL60 cells expressing myc-Rac1V12. Control cells transfected with the empty vector pEF-neo (on the left) and cells expressing myc-Rac1V12 (on the right) were stimulated with 1 µg/ml PMA and cytochrome c reduction was continuously monitored. The figure is representative of three independent experiments with three independent clones.

Rac1V12 was ascertained by the fact that it was not recovered in lysate of control cells and when competing c-myc peptide was added during the immunoprecipitation. The absence of detection of myc-Rac1V12 protein by direct immunoblotting with anti-myc Abs in clones expressing myc-Rac1V12 mRNA most probably results, as mentioned by others (62), from the fact that high expression levels of the constitutively active form of Rac may cause lethality of the cells.
Clones expressing myc–Rac1V12 were differentiated with Bt2cAMP and tested for oxidase activity under stimulation by PMA. As shown in Fig. 8C, these cells were able to produce superoxide in response to PMA to a similar extent as DMSO-differentiated variant cells. This appeared specifically due to Rac1V12 expression as the clones that were transfected with the empty vector failed to produce superoxide under PMA stimulation when differentiated with Bt2cAMP. This finding suggests that the failure of PMA to activate the NADPH oxidase in the variant HL60 cells is consecutive to a defect in Rac activation.

**Discussion**

P47phox phosphorylation is not sufficient to induce oxidase activity

We have isolated a variant HL60 cell line that is poorly responsive to PMA with respect to the activation of the NADPH oxidase after differentiation into neutrophil-like cells with Bt2cAMP. However, a normal PMA-mediated superoxide production is observed when cells are differentiated with DMSO. Although PMA is inefficient to trigger NADPH oxidase activation in Bt2cAMP-differentiated cells, it is nevertheless able to induce the phosphorylation of p47phox. Judging from the two-dimensional tryptic phosphopeptide analysis of p47phox immunoprecipitated from both Bt2cAMP- and DMSO-differentiated cells, the inability of PMA to induce superoxide production is unlikely to result from a defect in the phosphorylation of only one or a few serine phosphorylation sites of p47phox.

Thus, our results suggest that the phosphorylation of p47phox is not sufficient to trigger the activation of the NADPH oxidase. This result is consistent with the notion that the levels of p47phox phosphorylation and the rates of superoxide production are not correlated. Based on the observation that p47phox is phosphorylated in cells treated with the protein phosphatase inhibitor calyculin A without any stimulation of NADPH oxidase, it has been suggested that the phosphorylation of discrete residues may negatively regulate the NADPH oxidase (63). Several other studies have underscored that a marked reduction in the phosphorylation of p47phox is not always accompanied by an inhibition of formyl peptide-induced oxidase activity (7, 64). However, in a cell-free system, arguments have been provided for the necessity of p47phox to be phosphorylated to serve as a switch in the first step of oxidase activation (13). Our results do not allow us to conclude on the exact role of the phosphorylation of p47phox in triggering the production of superoxide, but they point out to the requirement of a second signal independent of p47phox phosphorylation.

Absence of PMA-mediated superoxide production in Bt2cAMP-differentiated variant HL60 cells does not result from a defective activation of PKC or MAPK

As phorbol esters are direct activators of a number of PKC isoforms, the inability of PMA to activate oxidase in Bt2cAMP-differentiated variant HL60 cells may be due to a defective expression or a functional alteration of PKC. It has been proposed that the PKC-sensitive β isoforms could be involved in the phosphorylation of p47phox (8). The lack of any obvious difference between DMSO- and Bt2cAMP-differentiated cells in the expression and subcellular localization of the immunonanalyzed PKC isoforms indicates that the poor PMA-induced superoxide production in Bt2cAMP-differentiated cells does not result from a defective expression of any PKC isoform. One cannot conclude on the functionality of the isoforms known not to be translocated upon PMA stimulation (ζ, λ, and ι). However, since PMA is able to activate Erk1/2 in Bt2cAMP-differentiated cells and based on recent studies indicating that PKC ζ is critically involved in the activation of MEK/Erk1/2 pathway (65–67), it is tempting to speculate that the phenotype of the variant HL60 cells does not result from a deficiency in PKC ζ.

Chemoattractants stimulate a second pathway that bypasses PI3K and MEK and is involved in rescuing the activation of NADPH oxidase by PMA

A major observation in this study is that despite the failure of PMA alone to trigger a production of superoxide anions in Bt2cAMP-differentiated cells, its association with nonstimulatory doses of chemoattractant restored a full and sustained NADPH oxidase response. The use of specific inhibitors gave us some hints in deciphering the signaling pathway elicited by substimulatory concentrations of chemoattractants. Interestingly, the oxidative response elicited by the addition of a substimulatory concentration of fMLFK with PMA was not inhibited by either WT, PD98059, or the combination of both inhibitors. Moreover, under costimulation conditions, Erk2 activity was dramatically repressed by the combination of WT and PD98059. These observations suggest that low concentrations of chemoattractant activate a pathway bypassing PI3K, MEK, and the MAPK Erk1/2.

The involvement of Erk1/2 in the activation of NADPH oxidase by saturating concentrations of formyl peptide is still a matter of debate. These MAPKs have been proposed as possible effectors of NADPH oxidase activation, mainly because the activation of both NADPH oxidase and Erk1/2 is inhibited by WT (39, 61) or by PD98059 (39–42). However, the dose-dependent effects of these inhibitors are dissimilar regarding Erks activity or NADPH oxidase activity (44–46) and Erk1/2 activity could be dissociated from the oxidative burst in both human neutrophils and differentiated HL60 cells (43). In this study, the lack of a strong inhibitory effect of PD98059 on the production of superoxide could result from a partial inhibition of Erk2 activity when cells were stimulated by the chemoattractant. Our observation that the MEK activation inhibitor (PD98059) modestly repressed Erk2 activation by formyl peptide while having a stronger effect on the activation by phorbol ester suggests that the activation of Erk2 by formyl peptide is partially independent from MEK. Another possibility is that MEK is differentially inhibited by PD98059 depending on whether MEK is activated by formyl peptide or PMA. It has been recently shown that MEK can be differentially activated (via a Raf-dependent or a Raf-independent pathway) according to the PKC isotype that is activated (67).

Taken together, our results suggest that chemoattractant-mediated activation of NADPH oxidase involves two pathways. The first pathway, which requires high concentrations of chemoattractant, flows through PI3K, whereas the second pathway would diverge at a point upstream of WT-sensitive PI3K and MEK. This second pathway can be activated by low concentrations of chemoattractant but is not sufficient, alone, to elicitoxidant secretion. It is likely that the two pathways actually act in concert when NADPH oxidase is activated by a saturating concentration of chemoattractant (see Fig. 8). In the variant HL60 cells differentiated with Bt2cAMP, PMA appears to place the complex in a primed state awaiting further stimulus before the NADPH oxidase response is elicited. The aforementioned PI3K and MEK-independent pathway provides the switch to turn on the oxidase complex. The extremely low concentrations of chemoattractant that are required to turn on this “oxidase switch” suggest that the second pathway is similar to that involved in the chemotaxis of phagocytic leukocytes. Noticeably, some authors using the MEK/Erk inhibitor PD98059 failed to inhibit granulocytes functions typically induced
by low doses of chemoattractant, i.e., chemotaxis and actin polymerization (42, 46, 68). Although they affected chemotaxis, PI3K inhibitors did not prevent the increase in total F actin in agonist-stimulated cells (33, 37, 69).

Role of Rac in the complementation of the PMA defect in Bt2 cAMP-differentiated variant HL60 cells

Rac regulates the activity of the NADPH oxidase in phagocytic leukocytes by mechanisms that are not fully understood (3, 15). In variant HL60 cells, the restoration of the PMA-dependent respiratory burst following expression of the constitutively active myc-Rac1V12 further underlines a role of Rac in oxidase activation in intact cells. The contribution of Rac as part of the oxidase enzymatic complex has been fairly demonstrated by the requirement of the Rac1/2 protein in oxidant-generating cell-free systems (16, 17, 20) and by the agonist-induced translocation of Rac to the plasma membrane (24, 25). In addition to its role in switching on oxidant formation at the level of the plasma membrane, Rac also appeared to be connected with upstream signaling mechanisms. It has been previously reported that the p21-activated kinase (PAK) and its upstream regulator Rac regulate the activity of p38 SAPK (70–72). PAK also proved to phosphorylate p47phox in vitro in a Rac-GTP-dependent manner (73). The involvement of p38 SAPK in the signal pathway leading to superoxide production has been recently underscored in several studies (38, 41, 47–50). Therefore, the question arises as to whether the restoration of the respiratory burst with the active mutant Rac1V12 takes place at the level of the oxidase complex or in upstream signaling. However, in Bt2 cAMP-differentiated variant cells, endogenous Rac is supposedly upregulated based upon the fact that the enzymatic complex is functional under chemoattractant stimulation. The weak ability of PMA-challenged Bt2 cAMP-differentiated cells to secrete oxidant is much probably due to an inefficient signaling upstream of Rac. Although the phenotype is not expressed in DMSO-differentiated cells, one cannot exclude that the defect does not exist since it could be compensated by the higher level of expression of the NADPH cytosolic factors, p47phox and Rac1. Because PMA-induced oxidase could be restored either by addition of low doses of chemoattractant or by expression of myc-Rac1V12, we suggest that Rac belongs to the second aforementioned, PI3K- and MEK-independent pathway. As suggested above, this second pathway may be part of the pathway involved in chemotaxis and F-actin assembly for Rac is also known to regulate cytoskeleton rearrangements (74). Interestingly, Arcaro et al. (75) recently reported that the chemoattractant-dependent activation of Rac in neutrophils triggered the uncapping of actin filaments, independently of PI3K.

In summary, PMA and low concentrations of chemoattractant activate two distinct, restricted pathways that complement each other to fulfill the requirements for NADPH oxidase activation. As illustrated in Fig. 9, at high concentrations of chemoattractant the array of activated effector enzymes overlaps with that activated by PMA alone, leading to NADPH oxidase activation. The observation that a PMA-dependent activation is restored in cells transfected with myc-Rac1V12 cDNA suggests that, in Bt2 cAMP-differentiated cells, a cross-talk between a PMA-activated PKC pathway and components upstream the activation of Rac is impaired. The reason of this defect is unknown but may result from a lower expression of regulatory components involved in PMA-mediated Rac activation in cells differentiated with Bt2 cAMP. It is tempting to speculate that low concentrations of chemoattractant can restore PMA-mediated NADPH activation by stimulating a WT- and PD98059-resistant signaling pathway in which Rac plays a pivotal role. The possibility to stably transfect the cells and the sensitivity of the superoxide production assay (especially when using low concentrations of chemoattractant combined with PMA) make this variant HL60 cell line an interesting tool to decipher the hierarchy of signaling events upstream and downstream of Rac.

Aknowledgments

We thank Dr M. Dinauer (Indianapolis, IN) for kindly providing the pEF-neo plasmid and Dr P. Chavrier (Marseille, France) for kindly providing the pMT90 plasmid.

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