Roles for βII-Protein Kinase C and RACK1 in Positive and Negative Signaling for Superoxide Anion Generation in Differentiated HL60 Cells*

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β-Protein kinase (PKC) is essential for ligand-initiated assembly of the NADPH oxidase for generation of superoxide anion (O\textsubscript{2}\textsuperscript{-}). Neutrophils and neutrophilic HL60 cells contain both β and βII-PKC, isotypes that are derived by alternate splicing. βII-PKC-positive and βI-PKC null HL60 cells generated equivalent amounts of O\textsubscript{2}\textsuperscript{-} in response to fMet-Leu-Phe and phorbol myristate acetate. However, antisense depletion of βI-PKC from βI-PKC null cells inhibited ligand-initiated O\textsubscript{2}\textsuperscript{-} generation. fMet-Leu-Phe triggered association of a cytosolic NADPH oxidase component, p47\textsuperscript{phox}, with βII-PKC but not with RACK1, a binding protein for βII-PKC. Thus, RACK1 was not a component of the signaling complex for NADPH oxidase assembly. Inhibition of β-PKC/RACK1 association by an inhibitory peptide or by antisense depletion of RACK1 enhanced O\textsubscript{2}\textsuperscript{-} generation. Therefore, βII-PKC but not βI-PKC is essential for activation of O\textsubscript{2}\textsuperscript{-} generation and plays a positive role in signaling for NADPH oxidase activation in association with p47\textsuperscript{phox}. In contrast, RACK1 is involved in negative signaling for O\textsubscript{2}\textsuperscript{-} generation. RACK1 binds to βII-PKC but not with the p47\textsuperscript{phox}-βII-PKC complex. RACK1 may divert βII-PKC to other signaling pathways requiring β-PKC for signal transduction. Alternatively, RACK1 may sequester βII-PKC to down-regulate O\textsubscript{2}\textsuperscript{-} generation.

Ligand-initiated activation of superoxide anion (O\textsubscript{2}\textsuperscript{-}) generation by phagocytic cells such as neutrophils and neutrophilic-differentiated HL60 cells (dHL60 cells), involves translocation of cytosolic components p47\textsuperscript{phox} and p67\textsuperscript{phox} to the membrane and interaction with membrane-associated cytochrome b\textsubscript{558} (1–2). Protein kinase C (PKC), a phospholipid-dependent family of serine/threonine kinases, acts in the signal transduction pathway for O\textsubscript{2}\textsuperscript{-} generation and is critical for assembly of an active NADPH oxidase (3–7). Phosphorylation of p47\textsuperscript{phox} allows a conformational change, translocation of the p47\textsuperscript{phox} to the membrane, and assembly of an active NADPH oxidase (7). p47\textsuperscript{phox} contains multiple phosphorylation sites including classical PKC substrate sites; p47\textsuperscript{phox} is phosphorylated by β-PKC in vitro and is phosphorylated in ligand-activated phagocytic cells (1–3, 8).

PKC is a family of structurally related isotypes, with differing cofactor requirements but similar substrate specificity (9–12). Classical PKC isotypes α-PKC, β-PKC, and γ-PKC are phosphatidylserine (PS)-, diglyceride (DG)-, and Ca\textsuperscript{2+}-dependent; novel PKC isotypes δ-PKC, ε-PKC, and θ- and η-PKC also require PS and DG but are Ca\textsuperscript{2+}-independent. The atypical PKC isotypes, ζ-PKC, and λ-PKC, require PS but are DG- and Ca\textsuperscript{2+}-independent (9–12). PKC isotypes differ in their tissue distribution and localization within the cell, suggesting that each isotype plays a specific role in specific signal transduction pathways. dHL60 cells and neutrophils possess multiple PKC isotypes including α-PKC, βI-PKC, βII-PKC, δ-PKC, and ζ-PKC (8, 13–15).

Depletion of β-PKC by antisense pretreatment was previously shown to inhibit phosphorylation of p47\textsuperscript{phox}, translocation of p47\textsuperscript{phox} to the membrane, and generation of O\textsubscript{2}\textsuperscript{-} in response to cell activation by ligands such as fMet-Leu-Phe or to the PKC activator phorbol myristate acetate (PMA) (8). The ability of a β-PKC specific inhibitor to reduce ligand-initiated O\textsubscript{2}\textsuperscript{-} generation also indicated that β-PKC is essential for activation of O\textsubscript{2}\textsuperscript{-} generation (16). However, these studies did not distinguish between a role for βI-PKC or βII-PKC, isotypes that are identical except for the C-terminal V5 variable region (9, 10). The antisense oligonucleotide targeted the transcriptional start site, which is common to both these isoforms, and the inhibitor inhibited both βI-PKC and βII-PKC (8, 16).

Formation of a signaling complex that can target β-PKC to substrates such as p47\textsuperscript{phox} and p47\textsuperscript{phox} to the cell membrane is essential for specificity and efficiency of signal transduction (17). However β-PKC plays a role in signaling for multiple cell responses. β-PKC is essential for both proliferation (18) and for O\textsubscript{2}\textsuperscript{-} generation in HL60 cells (8), events that occur at the nucleus and plasmalemma, respectively. β-PKC also associates with the cytoskeleton (19). Therefore spatial considerations are a key element in defining a role for β-PKC in signal transduction for a particular response. β-PKC must be directed to different locations in the cell for each function, suggesting a role for scaffold proteins or PKC-binding proteins in β-PKC-based signaling for activation of O\textsubscript{2}\textsuperscript{-} generation (20–22).

Receptor for Activated C Kinase (RACKs) are a family of cytoskeleton and membrane-associated anchor molecules that bind activated, Ca\textsuperscript{2+}/DG-dependent PKC isotypes α-, β-, and γ-PKC as well as phospholipase Cγ (23–30). PKC isotypes

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1 The abbreviations used are: dHL60 cells, HL60 cells differentiated to a neutrophil phenotype; fMet-Leu-Phe, N-formyl-methionyl-leucyl-phenylalanine; O\textsubscript{2}\textsuperscript{-}, superoxide anion; PKC, protein kinase C; PAGE, polyacrylamide gel electrophoresis; PMA, phorbol myristate acetate; DMRIE-C, 1,2-dimyrisoetoxypropyl-3-dimethylhydroxyethylammonium-undecylammonium; poly(vinylidene difluoride); BSA, bovine serum albumin; DG, diglyceride; PVDF, polyvinylidene difluoride; AS, anti-sense; MS, missense; rhβII, recombinant human βII.
possess a pseudo-Rack binding site in the Ca\(^{2+}\) binding domain of \(\alpha, \beta, \) and \(\gamma\)-PKC (28). The conformational change in PKC induced by cofactors frees the RACK binding site and-rack. Thus cofactors simultaneously activate and target PKC isoforms. A peptide based on a sequence in annexin I disrupts the binding of PKC to RACK (24–27). When peptide I is injected into Xenopus oocytes, it inhibits insulin-induced translocation of \(\beta\)-PKC and oocyte maturation (25, 26).

RACK1 is a binding protein for \(\beta\)-II-PKC (31). In this study we have assessed the roles of the PKC isoforms \(\beta\)-PKC and \(\beta\)-II-PKC in O\(_2\) generation and, secondly, the role of \(\beta\)-II-PKC in O\(_2\) generation.

Generation and, secondly, the role of \(\beta\)-II-PKC

A method of electroporation was chosen for assay of molecules in the presence or absence of 200 \(\mu\)M peptide I. Previous studies using \(\beta\)-II-PKC and \(\beta\)-PKC were synthesized by the PENN Nucleic Acid Facility as the phosphorothioate.


generated \(\beta\)-II-PKC and \(\beta\)-II-PKC as part of a down-regulation step in O\(_2\) generation.

**MATERIALS AND METHODS**

**HL60 Cell Culture**—Human promyelocytic HL60 leukemia cells were obtained from the American Type Culture Collection. The cells were grown in suspension culture in RPMI 1640 medium supplemented with 2 mM l-glutamine, 1% minimal essential medium vitamin solution, 1% nonessential amino acids, 0.1% gentamicin, and 10% heat-inactivated fetal bovine serum. The cell cultures were maintained at 37 °C in a 5% CO\(_2\)-humidified atmosphere. The initial culture was positive for neutrophil-like phenotype before treatment with the oligonucleotide.

**Oligonucleotide Synthesis and Sequences**—An antisense oligonucleotide was designed against the translation start site of human RACK1 using the commercial primer analysis software Oligo (National Bio- science, Inc.). A 20-mer sequence was chosen for self-complementarity and was optimized for maximal \(T_m\) to promote high affinity binding to mRNA; a \(T_m\) of 64.6 °C was calculated at 150 mM salt and 37 °C. The 20-mer oligonucleotides had the following sequences: RACK1 antisense (RACK1 AS): 5'-TGG ACC AGG AGT CAT CTT C-3'; RACK1 sense: 5'-G CAG ATG ACC CTT CGT GGC A-3'. A cloned sequence of these oligonucleotides was confirmed by searching the GenBank\(^{\text{TM}}\) data base. For depletion of \(\beta\)-PKC, a 19-mer oligonucleotide having the sequence \(\beta\)-PKC antisense (\(\beta\)AS), 5'-AGC CGG GTC AGC CAT CTT G-3', and a scrambled missense oligonucleotide \(\beta\)-PKC missense (\(\beta\)MS) were used as previously described (8). Antisense and scrambled control oligonucleotides to RACK1 and \(\beta\)-PKC were synthesized by the PENN Nucleic Acid Facility as the phosphorothioate derivatives and purified by high performance liquid chromatography.

**Treatment of Cells with Oligonucleotides**—Delivery of the oligonucleotides was enhanced with the cationic lipid 1,2-dimyristoylphosphatidyl-

**Electroporation of HL60 Cells**—A method of electroporation was chosen that allows efficient incorporation of molecules of molecular mass <1000 Da and transient passage of molecules >10000 Da. We chose a one-pulse protocol to optimize preservation of intracellular metabolites.

**Statistical Analysis**—Results were expressed as mean ± S.E. (n). Data were analyzed by Student’s t test.

**Reagents**—Cytochalasin B, cytochrome c, protease inhibitors (leupeptin, soybean trypsin inhibitor, and aprotinin), BSA, PMA, \(\beta\)-Met-Leu-Phe, and phenylmethylsulfonyl fluoride were purchased from Sigma. PMA was stored as a concentrated stock solution in Me\(_2\)SO and diluted with Hepes Buffer before use. \(\beta\)-Met-Leu-Phe was stored as a stock solution in ethanol and diluted in buffer before use. Pepsin, soybean trypsin inhibitor, and aprotinin), BSA, PMA, \(\beta\)-Met-Leu-Phe, and phenylmethylsulfonyl fluoride were purchased from Sigma.

**Western Blots**—Lysates of dHL60 cells (10 \(\times\) 10\(^6\) cells/sample) were prepared at the cells heating at 95 °C for 5 min in 2× SDS-PAGE sample buffer. The samples were briefly sonicated (12 s) to reduce viscosity. The dHL60 cell lysates were run on a 4–12% gradient SDS-PAGE, transferred to a PVDF membrane, and blocked for 1 h at room temperature before incubation with Tris-buffered saline (pH 7.5) containing 0.1% Tween 20 and 1% BSA, 3% casein. To identify the different PKC isoforms, the membrane was incubated with a panel of PKC antibodies followed by incubation with peroxidase-conjugated goat anti-rabbit IgG. For detection of RACK1, the membrane was incubated with a monoclonal antibody to RACK1, followed by incubation with peroxidase-conjugated goat anti-mouse IgM. Immunoreactive bands were visualized by Pierce SuperSignal Ultra chemiluminescence substrate. The software SigmaScan (Jandel/SPSS) was used for densitometric analysis. The generation of superoxide anion (O\(_2^\cdot\)) by dHL60 cells was measured as superoxide dismutase inhibitable cytochrome c reduction by either a continuous recording method (33) or endpoint analysis. Cells were activated by 1 \(\mu\)M Met-Leu-Phe in the presence of 5 \(\mu\)g/ml cytochalasin B or by 1 \(\mu\)g/ml PMA in the absence of cytochalasin B.

**Immunoprecipitation of \(\beta\)-II-PKC and \(\beta\)-PKC**—dHL60 cells (50 \(\times\) 10\(^6\) cells/ml) were stimulated with either buffer alone or Met-Leu-Phe (1 \(\mu\)M) for 1 min. The reaction was stopped by the addition of cold immunoprecipitation buffer. Immunoprecipitation buffer consisted of 10 mM Hepes (pH 7.4) containing 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 2 mM phenylmethanesulfonfyl fluoride, 0.2% Nonidet P-40, 0.027 trypsin inhibitory units/ml of aprotinin, 2 \(\mu\)g/ml leupeptin, and 5 mg/ml BSA. The samples were then vortexed for 20 min to solubilize the membrane fraction, and the supernatant was collected after microcentrifuging for 5 min. A rabbit polyclonal antibody to \(\beta\)-II-PKC or to \(\beta\)-PKC was added, and the samples were incubated for 1 h at 4 °C with shaking. The reaction tubes were then microcentrifuged for 30 s, and the supernatants were discarded.

**Statistical Analysis**—Results were expressed as mean ± S.E. (n). Data were analyzed by Student’s t test.

**Reagents**—Cytochalasin B, cytochrome c, protease inhibitors (leupeptin, soybean trypsin inhibitor, and aprotinin), BSA, PMA, \(\beta\)-Met-Leu-Phe, and phenylmethylsulfonyl fluoride were purchased from Sigma. PMA was stored as a concentrated stock solution in Me\(_2\)SO and diluted with Hepes Buffer before use. \(\beta\)-Met-Leu-Phe was stored as a stock solution in ethanol and diluted in buffer before use. Pepsin, soybean trypsin inhibitor, and aprotinin), BSA, PMA, \(\beta\)-Met-Leu-Phe, and phenylmethylsulfonyl fluoride were purchased from Sigma.
Generation in βII-PKCI and RACK1 in Signaling for Superoxide Anion Generation

RESULTS

A Role for βII-PKCI in Ligand-initiated O₂⁻ Generation—A clone of HL60 cells that contained the βII-PKCI isoform of PKC as well as α-PKC, δ-PKC, and ζ-PKC was selected and probed for immunoreactivity to PKC antibodies (Fig. 1A). In comparison to the parent cell line, which contains β-PKC, the β-PKCI protein null line contained no detectable amount of β-PKCI (Fig. 1A). In contrast, both β-PKCI-positive and β-PKCI null cell lines contained equivalent amounts of α-PKC, δ-PKCI, and ζ-PKCI. Previous studies in which both β-PKCI and β-PKCI were depleted by an antisense strategy demonstrated a role for β-PKCI in activation of the NADPH oxidase in HL60 cells (8). To discriminate between roles for β-PKCI and β-PKCI in ligand-initiated activation of O₂⁻ generation, we compared β-leucine-Phe-activated O₂⁻ generation in β-PKCI null and β-PKCI-positive HL60 cells. Generation of O₂⁻ triggered by 1 μM β-fMet-Leu-Phe was 11.3 ± 1.9 (n = 12) nmol/10⁶ cells/10 min in β-PKCI null HL60 cells, a rate that was not significantly different from the rate of 11.2 ± 1.8 (n = 8) nmol/10⁶ cells/10 min observed in β-PKCI-positive HL60 cells (Fig. 1A). Generation of O₂⁻ in response to 1 μg/ml PMA was also similar in β-PKCI null and β-PKCI-positive HL60 cells (Fig. 1B). In β-PKCI-positive HL60 cells, PMA triggered generation of 22.3 ± 4.1 (n = 5) nmol of O₂⁻/10⁶ cells/10 min, whereas in β-PKCI cells, PMA triggered generation of 21.2 ± 1.7 (n = 6) nmol of O₂⁻/10⁶ cells/10 min (Fig. 1B). Therefore, β-PKCI was not essential for optimal PMA- or β-fMet-Leu-Phe-initiated generation of O₂⁻ in HL60 cells.

Inhibition of O₂⁻ Generation in βII-PKCI-depleted β-PKCI Null HL60 Cells—β-PKCI null HL60 cells were treated for 2 days with 400 nM of an antisense oligonucleotide to β-PKCI (βPKCI AS) or with 400 nM control missense oligonucleotide to β-PKCI (βPKCI MS) as described under "Materials and Methods." Treatment with βPKCI AS resulted in a reduction in the levels of β-PKCI and β-PKCI in ligand-activated generation of O₂⁻ in HL60 cells. Generation of O₂⁻ in β-PKCI null cells pretreated with 400 nM β-fMet-Leu-Phe plus 5 μg/ml cycloheximide B or by 1 μg/ml PMA. Data shown were the mean of 8 experiments for β-PKCI null cells and 12 experiments for β-PKCI-positive HL60 cells and are expressed as nmol of O₂⁻/10⁶ cells/10 min.

KGDYEKILVALCGGN, was purchased from Coast Scientific.

Anti-peptide polyclonal antibodies to α-PKC, β-PKCI, γ-PKCI, and δ-PKC and peroxidase-conjugated goat anti-rabbit IgG and peroxidase-conjugated goat anti-mouse IgG were obtained from Santa Cruz Biotechnology. Peroxidase-conjugated anti-mouse IgM was obtained from Kirkegaard and Perry Laboratories. Mouse monoclonal antibodies to δ-PKC, ζ-PKC, and RACK1 and a rabbit polyclonal antibody to p47-phox were purchased from Transduction Laboratories. Protein A-agarose was obtained from Life Technologies, Inc.
Generation by generation in response to generation decreased significantly from a rate of generation triggered by 1 generation, was significantly decreased in cells depleted of O2. Reduced in cells depleted of O2, defined as the generation, was reduced in cells depleted of O2 nmol of O2/min.

FIG. 3. Effect of antisense depletion of β-PKC on O2 generation in β-PKC null dHL60 cells. O2 generation by β-PKC null dHL60 cells depleted of β1-PKC was determined in missense (βPKC MS)- and antisense (βPKC AS)-pretreated dHL60 cells as described in Fig. 1B. A, O2 generation triggered by 1 μM fMet-Leu-Phe plus 5 μg/ml cytochalasin B (n = 6) and 1 μg/ml PMA (n = 7). Data shown are expressed as nmol of O2/10^6 cells/10 min. B, Vmax of O2 generation triggered by 1 μM fMet-Leu-Phe plus 5 μg/ml cytochalasin B and 1 μg/ml PMA. Data shown are expressed as nmol of O2/10^6 cells/minute.

(Fig. 3A). The kinetics of fMet-Leu-Phe-activated O2 generation are characterized by a rapid initial rate continuing for ~2 min followed by a slow rate of generation that ceases by 5–10 min. In contrast, PMA triggers a sustained generation of O2. The Vmax of fMet-Leu-Phe-induced O2 generation, defined as the maximal rate of O2 generation, was reduced in cells depleted of β-PKC. Calculation of the Vmax demonstrated that in control βPKC MS-treated cells activated by 1 μM fMet-Leu-Phe, the Vmax was 2.70 ± 0.44 (n = 6) nmol/min/10^6 cells; the Vmax of β-PKC-depleted cells treated with βPKC AS was significantly reduced to 1.41 ± 0.74 (n = 6) nmol/min/10^6 cells (53.0 ± 7.2% control βPKC MS-treated cells (p < 0.01) (Fig. 3B).

Similarly, activation of control βPKC MS-pretreated cells by 1 μg/ml PMA triggered generation of 16.6 ± 1.5 (n = 7) nmol of O2/10^6 cells/10 min, an amount that was significantly greater than the generation of 8.2 ± 1.3 (n = 7) nmol of O2/10^6 cells/10 min observed in dHL60 cells depleted of β-PKC by treatment with βPKC AS (48.7 ± 6.2% control, p < 0.01) (Fig. 3A). The Vmax of PMA-induced O2 generation was also significantly decreased in β-PKC-depleted cells as compared with controls. The Vmax of O2 generation decreased significantly from a rate of 3.12 ± 0.37 (n = 7) nmol/min/10^6 cells in control βPKC MS-treated cells to a Vmax of 2.06 ± 0.27 (n = 8) nmol/min/10^6 cells in β-PKC-depleted cells (66.1 ± 8.2% control, p < 0.01) (Fig. 3B). Thus, depletion of β1-PKC in β-PKC null dHL60 cells resulted in inhibition of the rate of O2 generation in response to both fMet-Leu-Phe and PMA, indicating an essential role for the β1 isotype but not the β2 isotype of PKC in signaling for O2 generation.

Cofactor-dependent Binding of rhβII-PKC to Endogenous Proteins from dHL60 Cells—β-PKC is capable of phosphorylating multiple proteins in vitro (15). However, in the intact cell, scaffold proteins may provide added substrate specificity by targeting the kinase to a particular cellular location. RACK1 is a scaffold or escort protein that selectively binds to β1-PKC, and may provide added substrate specificity by targeting the kinase to a particular cellular location. RACK1 is a scaffold or escort protein that selectively binds to β1-PKC, and may provide added substrate specificity by targeting the kinase to a particular cellular location. RACK1 is a scaffold or escort protein that selectively binds to β1-PKC, and may provide added substrate specificity by targeting the kinase to a particular cellular location. RACK1 is a scaffold or escort protein that selectively binds to β1-PKC, and may provide added substrate specificity by targeting the kinase to a particular cellular location.
kDa corresponding to the endogenous βII-PKC was observed (Fig. 4A, lane 1). rhβII-PKC in the absence of cofactors bound strongly only to a protein of 19 kDa (Fig. 4A, lane 2). However, when rhβII-PKC was added in the presence of the cofactors PS, DG, and Ca^{2+}, additional binding of rhβII-PKC to bands of 29, 32, 36, 39, 47, and 55 kDa was observed (Fig. 4A, lane 3). Probing with an antibody to RACK1 (Fig. 4A, lane 4) showed a strong band at 36 kDa, demonstrating the presence of RACK1 in dHL60 cells. Quantitation by densitometry demonstrated that the band at 80 kDa corresponding to endogenous βII-PKC was not affected by the presence of cofactors and had a density of 81.3 ± 3.1 (n = 3) DU in the absence of cofactors and of 82.7 ± 0.7 DU in the presence of cofactors (Fig. 4B). In contrast, binding of rhβII-PKC to the 36-kDa band, which had the same molecular mass as RACK1, was significantly enhanced in the presence of cofactors (Fig. 4B). The density of the 36-kDa band was 34.7 ± 3.1 (n = 3) DU in the absence of cofactors and significantly enhanced to 77.3 ± 4.7 (n = 3) DU in the presence of cofactors (p < 0.005 paired Student’s t test). In addition, binding of rhβII-PKC to a band of 47 kDa, a candidate for p47^{phox}, was enhanced by the presence of cofactors. Binding of rhβII-PKC to the 47-kDa band was 23.0 ± 1.4 (n = 3) DU in the absence of cofactors and 60.7 ± 6.9 DU in the presence of cofactors (p < 0.05, paired t test). These results demonstrated that RACK1 is present in dHL60 cells and that βII-PKC can bind to numerous HL60 proteins in a cofactor-dependent fashion.

Ligand-initiated Association of p47^{phox} with βII-PKC, but Not with RACK1—Coimmunoprecipitation was next used as a tool to determine whether βII-PKC, RACK1, and p47^{phox} formed a signaling complex in activated dHL60 cells. To determine whether p47^{phox} and RACK1 were associated with βII-PKC in activated cells, βII-PKC was immunoprecipitated from resting buffer-treated dHL60 cells and from cells activated for 1 min with 1 μM fMet-Leu-Phe. Western blots with an antibody to βII-PKC followed by densitometry of the immunoprecipitates demonstrated that approximately equivalent amounts of βII-PKC were derived from resting and activated cells, 1987 ± 207 (n = 4) DU from resting cells as compared with 1895 ± 142 DU in fMet-Leu-Phe-activated dHL60 cells (Fig. 5A). Probing of the immunoprecipitates with an antibody to p47^{phox} demonstrated a significant increase in the association of p47^{phox} with βII-PKC, from a level of 424 ± 204 (n = 4) DU in resting cells to a level of 1133 ± 314 DU in cells activated by fMet-Leu-Phe (p < 0.03) (Fig. 5A). Probing the βII-PKC immunoprecipitates with an antibody to RACK1 also revealed that activation of the dHL60 cells with fMet-Leu-Phe triggered a significant increase in association of RACK1 with the βII-PKC, from 201 ± 115 (n = 3) DU in resting cells to 1105 ± 224 DU in fMet-Leu-Phe-activated cells (p < 0.04) (Fig. 5A). Furthermore, activation of dHL60 cells by fMet-Leu-Phe triggers enhanced association of βII-PKC with the scaffold protein RACK1 as well as with p47^{phox}.

To determine whether fMet-Leu-Phe triggered enhanced association of RACK1 with p47^{phox}, concomitant with the enhanced association of RACK1 with βII-PKC, p47^{phox} was immunoprecipitated from resting and activated dHL60 cells. Western blots of immunoprecipitates of p47^{phox} followed by densitometry demonstrated equivalent levels of p47^{phox} in resting cells and in cells stimulated for 1 min with 1 μM fMet-Leu-Phe (Fig. 5B). Densitometry of the Western blots showed a level of p47^{phox} of 1588 ± 293 (n = 3) DU in resting cells as compared with a level of 1519 ± 284 (n = 3) DU in cells activated by 1 μM fMet-Leu-Phe. Measurement of the level of βII-PKC associated with the p47^{phox} in these immunoprecipitates demonstrated a significant increase in association of βII-PKC with p47^{phox} (Fig. 5B). The level of βII-PKC observed in resting cells was 451 ± 154 (n = 3) DU as compared with an enhanced level of 1212 ± 323 (n = 3) DU in cells activated by fMet-Leu-Phe (213 ± 36% control, p < 0.05) (Fig. 5B). Therefore, immunoprecipitation of p47^{phox} or of βII-PKC demonstrated that cell activation by fMet-Leu-Phe triggered enhanced association of βII-PKC with its substrate p47^{phox}. In contrast, probing the p47^{phox} immunoprecipitates with an antibody to RACK1 demonstrated no significant association of RACK1 with p47^{phox} (Fig. 5B). The level of RACK1 associated with the p47^{phox} immunoprecipitate from resting cells was 21 ± 11 (n = 3) DU, whereas the level in fMet-Leu-Phe-activated cells was 42 ± 26 (n = 3) DU, a difference that was not statistically significant. Therefore, although fMet-Leu-Phe triggered an increase in association of βII-PKC with the substrate p47^{phox}, no increase in association of RACK1 with the p47^{phox} or p47^{phox} associated βII-PKC was observed. We therefore questioned whether RACK1 played a role in signaling for activation of O_{2}^{−} generation, a process that is dependent on βII-PKC.

Effect of Inhibitor Peptide, Peptide I, on fMet-Leu-Phe Triggered O_{2}^{−} Generation—Peptide I, KGDYEKILVACGGN, which is derived from the annexin I and 14-3-3 sequences, was used to probe a possible role for RACK1 interaction with βII-PKC in activation of O_{2}^{−} generation. Peptide I inhibits the binding of RACK to PKC and the translocation of β-PKC to the membrane in other cell types (24–27). We first tested the ability of peptide
I to inhibit the binding of rhβII-PKC to endogenous dHL60 proteins using an overlay assay (see Fig. 4). Lysates of βII-PKC null dHL60 cells were separated on SDS-PAGE and transferred to PVDF membranes, and the membranes were incubated with rhβII-PKC in the presence of the PKC cofactors PS, DG and Ca\(^{2+}\) and in the presence or absence of 10 μM peptide I. In the presence of 10 μM peptide I, there was a selective inhibition of the ability of βII-PKC to bind to a band of 36 kDa that was immunoreactive to RACK1 antibody. The density of the 36-kDa band was 40.3 ± 11.4 (n = 4) DU in the absence of peptide I and significantly decreased to 23.0 ± 7.2 (n = 4) DU in the presence of 10 μM peptide I (61.0 ± 7.1% control, p < 0.005). In contrast, the 80-kDa band, which represents endogenous βII-PKC, was 67.0 ± 3.6 (n = 4) DU in the absence of peptide I and 66.8 ± 3.8 (n = 4) DU in the presence of peptide I. Therefore, Peptide I inhibits binding of rhβII-PKC to a 36-kDa band that was immunoreactive to a RACK1 antibody.

Peptide I was then used to probe a role for βII-PKC binding to RACK1 in signaling for O\(_{2}\)\(^{-}\) generation. To probe a possible role for RACK1 interaction with βII-PKC in activation of O\(_{2}\)\(^{-}\) generation, cells were electroporated in the presence of buffer or peptide I. Electroporation of dHL60 cells in the presence of 200 μM peptide I, which gives a final intracellular concentration of peptide I of −10 μM, caused a significant increase in O\(_{2}\)\(^{-}\) generation triggered by 1 μM fMet-Leu-Phe (Fig. 6). fMet-Leu-Phe-triggered O\(_{2}\)\(^{-}\) generation of 13.4 ± 3.0 (n = 6) nmol/10\(^{6}\) cells/10 min in buffer-treated cells; in peptide I-treated cells fMet-Leu-Phe-triggered O\(_{2}\)\(^{-}\) generation was increased to 170.2 ± 35.3% control (n = 4, p < 0.02) (Fig. 6). Similarly, when O\(_{2}\)\(^{-}\) generation was triggered by 1 μg/ml PMA, peptide I enhanced O\(_{2}\)\(^{-}\) generation from a rate of 21.7 ± 3.5 (n = 6) nmol/10\(^{6}\) cells/10 min in buffer-treated cells to a rate that was 121.7 ± 14.6 (n = 6) βII% control in peptide I-treated cells (p < 0.05) (Fig. 6).

Thus peptide I, which inhibits the binding of RACK1 to βII-PKC and translocation of β-PKC to the membrane, enhanced rather than inhibited ligand-initiated O\(_{2}\)\(^{-}\) generation in βII-PKC null dHL60 cells.

**Depletion of RACK1 by Antisense Treatment**—The use of peptides to probe a role for RACK1, particularly in electroporated cells, has the potential for nonspecific effects. Depletion of RACK1 by an antisense strategy is a more specific probe in assessing a role for RACK1 in signaling for activation of the NADPH oxidase.

βII-PKC null dHL60 cells were treated for 2 days with 500 nM phosphorothioate antisense oligonucleotide to RACK1 (RACK1 AS) or with 500 nM control missense phosphorothioate oligonucleotide to RACK1 (RACK1 MS) as described under “Materials and Methods.” Treatment with RACK1 AS resulted in a reduction in the level of RACK1 to 1240 ± 273 (n = 8) as compared with a level of 1949 ± 452 (n = 8) control RACK1 MS-treated cells (57.9 ± 5.5% control, p < 0.01) (Fig. 7). In contrast, when the blasts were probed with an antibody to βII-PKC, no difference in immunoreactivity to βII-PKC was observed between the RACK1 AS- and RACK1 MS-pretreated cells (Fig. 7). Therefore, the RACK1 AS treatment selectively depletes dHL60 cells of RACK1.

**Depletion of RACK1 Enhances O\(_{2}\)\(^{-}\) Generation by dHL60 Cells**—A role for RACK1 in signaling for activation of the NADPH oxidase and generation of O\(_{2}\)\(^{-}\) was examined using βII-PKC null dHL60 cells depleted of RACK1 by antisense pretreatment. O\(_{2}\)\(^{-}\) generation triggered by 1 μM fMet-Leu-Phe in RACK1-depleted cells treated with RACK1 AS was significantly increased to a level of 16.7 ± 3.4 (n = 5) nmol/10\(^{6}\) cells/10 min as compared with a level of 10.7 ± 2.5 (n = 5) nmol/10\(^{6}\) cells/10 min in control RACK1 MS-treated dHL60 cells (175.0 ± 24.9% control, p < 0.03) (Fig. 8A). A similar increase in O\(_{2}\)\(^{-}\) generation in RACK1-depleted dHL60 cells was observed when the cells were activated by 1 μg/ml PMA. O\(_{2}\)\(^{-}\) generation triggered by 1 μg/ml PMA in RACK1 AS-treated cells was significantly increased to a level of 26.3 ± 1.9 (n = 5) nmol/10\(^{6}\) cells/10 min as compared with a level of 22.3 ± 2.5 (n = 5) nmol/10\(^{6}\) cells/10 min in control RACK1 MS-treated dHL60 cells (119.4 ± 6.4% control, p < 0.03) (Fig. 8A).

In addition, the V\(_{\text{max}}\), of ligand-induced O\(_{2}\)\(^{-}\) generation, defined as the maximal rate of O\(_{2}\)\(^{-}\) generation, was enhanced in cells depleted of RACK1 (Fig. 8B). The calculation of the V\(_{\text{max}}\) demonstrated that in RACK1 AS-treated cells activated by 1 μg/ml fMet-Leu-Phe, the V\(_{\text{max}}\) was significantly enhanced to a rate of 7.4 ± 0.8 (n = 5) nmol/min/10\(^{6}\) cells as compared with a rate of 5.1 ± 0.6 (n = 5) nmol/min/10\(^{6}\) in control cells treated with RACK1 MS (150.0 ± 45.8% control, p < 0.05) (Fig. 8B). Similarly, an increase in V\(_{\text{max}}\) was observed in RACK1-depleted dHL60 cells activated by 1 μg/ml PMA; however, the increase was not statistically significant. The V\(_{\text{max}}\) in RACK1 AS-treated cells activated by 1 μg/ml PMA was 5.1 ± 6.2 (n = 5) nmol/min/10\(^{6}\) cells as compared with a V\(_{\text{max}}\) of 3.6 ± 6.2 (n =
5) nmol/min/10^6 cells in control RACK1 MS-treated dHL60 cells. Therefore, ligand-initiated O_2^- generation was enhanced in cells depleted of RACK1. These findings are in agreement with studies using the inhibitory peptide, peptide 1, where inhibition of the interaction of RACK1 with βII-PKC also resulted in enhanced O_2^- generation (Fig. 6).

DISCUSSION

Phagocytic cells, such as HL60 cells differentiated to a neutrophilic phenotype, and neutrophils possess multiple forms of PKC isotypes including Ca^{2+}-dependent α-PKC, βⅠ-PKC, and βⅡ-PKC, Ca^{2+}-independent δ-PKC, and atypical ε-PKC. Assembly of an active NADPH oxidase is tightly controlled and involves association of the cytosolic components p47^{phox} and p67^{phox} with the plasma membrane-associated cytochrome b_{558} (1–3). Phosphorylation of p47^{phox} on multiple sites is an essential step in triggering translocation of p47^{phox} to the plasma-

mma, where it binds to the cytochrome b_{558} (34, 35). A selective role for β-PKC in activation of the NADPH oxidase and generation of O_2^- has previously been demonstrated using an antisense strategy (8) and by studies with a β-PKC-selective inhibitor (16).

HL60 cells and neutrophils contain two β-PKC isotypes, βⅠ-PKC and βⅡ-PKC, which are derived by alternate splicing at the C terminus. Neither the antisense strategy, which targeted a sequence at the transcriptional start site, nor the β-PKC-selective inhibitor could discriminate between roles for these β-PKC isotypes. The present study demonstrated a specific role for βⅡ-PKC in signaling for activation of O_2^- generation using a β-PKC null subclone of dHL60 cells. The β-PKC HL60 cell subclone was negative for immunoreactivity to βⅠ-PKC but contained equivalent amounts of α-PKC, βⅠ-PKC, δ-PKC, and ε-PKC. Generation of O_2^- generation triggered by the chemotactic peptide fMet-Leu-Phe or the PKC activator PMA was not significantly different in βⅠ-PKC null cells as compared with the βⅠ-PKC-positive cells. Therefore the presence of β-PKC was not necessary for activation of O_2^- generation, and βⅡ-PKC was implicated in signaling for O_2^- generation. A role for βⅡ-PKC in signaling for the activation of O_2^- generation was demonstrated by antisense depletion of βⅡ-PKC. Both fMet-Leu-Phe and PMA-induced O_2^- generation were significantly inhibited in βⅠ-PKC null cells depleted of βⅡ-PKC. Therefore, βⅠ-PKC but not βⅠ-PKC is essential for signaling in the activation of the NADPH oxidase.

Spatial regulation of signaling elements is critical in the regulation of NADPH oxidase assembly and activation. Differential localization of PKC isotypes reflecting the many roles of PKC has been demonstrated in multiple cell types; PKC has been demonstrated in microfilaments, Golgi, endoplasmic reticulum, and nuclear and cell membranes (36–38). A role for β-PKC has been shown in ligand-initiated O_2^- generation of dHL60 cells and also in proliferation of HL60 cells (8, 16, 18). Indeed, βⅡ-PKC can translocate to the nucleus in K562 erythroleukemia cells (38); the V5 region of βⅡ-PKC binds to phosphatidyglycerol, a PKC activator in the nuclear membrane. In addition, β-PKC can translocate from cytosol to plasmalemma in response to elevated Ca^{2+} levels or to activation by fMet-Leu-Phe or PMA (3, 8, 39), where it could participate in signaling for cell membrane-associated events such as O_2^- generation. Scaffold or PKC binding proteins can localize PKC isotypes to discrete cell locations and to particular signaling cascades.

Scaffold proteins such as RACK, AKAP (A kinase anchoring protein), and adducins (20–22, 40–42) are proteins that bind to PKC isotypes and provide localization for greater specificity and efficiency of signaling. Scaffold proteins have been identified by (a) overlay assays, which use PKC to probe protein bands, (b) interaction cloning, and (c) the yeast two-hybrid genetic screen for protein-protein interactions. RACKs are cytoskeleton and membrane-associated proteins that bind phospholipase Cγ and activated forms of α/β-PKC in other cell types. Particular binding proteins may differentially target protein kinase C isoforms to defined cellular locations. In the present study, an overlay assay demonstrated cofactor-dependent binding of rhβII-PKC to a 36-kDa band that was immunoreactive to RACK1 as well as binding of rhβII-PKC to a 47-kDa band, a candidate for the substrate p47^{phox}. In dHL60 cells, immunoprecipitated βⅠ-PKC associated with p47^{phox} and with RACK1 upon cell activation by fMet-Leu-Phe. In contrast, when p47^{phox} was immunoprecipitated from dHL60 cells, fMet-Leu-Phe triggered association of βⅠ-PKC with p47^{phox} but no association of RACK1 with p47^{phox}. Therefore, although βⅠ-PKC associated with RACK1 in fMet-Leu-Phe-activated cells, the signaling complex of p47^{phox} and βⅠ-PKC was not associ-
beta-II-PKC and RACK1 in Signaling for Superoxide Anion Generation

Depletion of RACK1 in beta-II-PKC null dHL60 cells by an antisense strategy also produced enhanced O$_2^-$ generation triggered by fMet-Leu-Phe or by PMA. This finding confirmed that the interaction of RACK1 with beta-II-PKC was not essential in the signaling pathway for activation of NADPH oxidase. The enhanced O$_2^-$ generation upon RACK1 depletion indicates that RACK1 might serve to remove beta-II-PKC from the signaling complex required specifically for signaling activation of the NADPH oxidase. RACK1 might be viewed as diverting the beta-II-PKC to another pathway requiring beta-II-PKC as a signaling element. Indeed RACK1s have been shown to associate with the cytoskeleton, to bind selective PH domains (43), the beta-plex required specifically for signaling activation of the PKC molecule to RACK. Peptide I, a peptide based on a pseudo-RACK sequence in annexin I/14-3-3, interferes with the binding of the PKC molecule to RACK. Peptide I, a peptide based on a pseudo-RACK sequence in annexin I/14-3-3, interferes with the binding of the PKC molecule to RACK. Peptide I, a peptide based on a pseudo-RACK sequence in annexin I/14-3-3, interferes with the binding of the PKC molecule to RACK.

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