On the Mechanism of Inhibition of the Neutrophil Respiratory Burst Oxidase by a Peptide from the C-Terminus of the Large Subunit of Cytochrome b_{558}[†]

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ABSTRACT: A peptide (RGVHFIF) from near the carboxyl terminus (residues 559−565) of gp91-*phox*, the large subunit of cytochrome b_{558}, was previously shown to inhibit activation of the respiratory burst oxidase [Kleinberg, M. E., Malech, H. L., & Rotrosen, D. (1990) *J. Biol. Chem. 265*, 15577−15583]. The peptide has been proposed to compete with gp91-*phox* binding to p47-*phox*, one of the cytosolic oxidase components. In the present studies, we have used a semirecombinant system consisting of recombinant cytosolic factors (p47-*phox*, p67-*phox*, and Rac1) along with isolated plasma membrane to investigate the mechanism by which the peptide inhibits oxidase activation. In an *in vitro* translocation model, the peptide inhibited arachidonate-activated translocation of both p47-*phox* and p67-*phox* to the plasma membrane. The kinetic mechanism of inhibition was examined. Inhibition was noncompetitive or mixed with respect to not only Rac and p67-*phox* but also to p47-*phox*. We suggest that the peptide, rather than competing for cytochrome−p47-*phox* interactions, inhibits indirectly, perhaps by binding to and altering the conformation of cytochrome b_{558}.

The major host defense against microbial infection is provided by polymorphonuclear leukocytes (neutrophils). In response to microorganisms or soluble stimuli, these cells initially generate superoxide and, secondarily, other reactive oxygen intermediates in what has been termed the respiratory burst [reviewed in Lambeth (1988)]. Superoxide generation is initiated by the NADPH-dependent respiratory burst oxidase. The enzyme consists of both cytosolic and plasma membrane components. The cytosolic proteins include p47-*phox*, p67-*phox*, and a small GTP-binding protein (Rac1 and/or Rac2), while the membrane contains flavocytochrome b_{558}, which consists of both large and small subunits and contains the NADPH-binding site. p47-*phox* and p67-*phox* assemble in a 1:1:1 ratio with cytochrome (Uhlinger et al., 1993) in response to activating stimuli to form the active complex capable of catalyzing the NADPH-dependent reduction of molecular oxygen to superoxide. Rac probably associates also with the cytochrome and/or the other protein components. The importance of a functional oxidase is illustrated by the genetic condition chronic granulomatous disease (CGD) in which victims experience recurrent infections due to defective oxidase activity.

Several types of studies have implicated p47-*phox* as an early reactant in oxidase activation. In cells from CGD patients lacking either p47-*phox* or p67-*phox*, p47-*phox* association with the membrane is dependent upon the presence of the cytochrome, and occurs in the absence of p67-*phox*. However, p67-*phox* translocation requires the presence of p47-*phox* (Nauseef et al., 1991; Heyworth et al., 1991). In cell-free activation studies, a kinetic approach (Kleinberg et al., 1990) has also suggested that p47-*phox* binding precedes that of p67-*phox*, and a direct interaction of the former with the cytochrome has been proposed. More recently, we have shown that the interaction of both p47-*phox* and p67-*phox* with the oxidase complex is a mutually facilitated process, with the binding of either protein affecting the binding of the other (Uhlinger et al., 1994).

Peptides have been used as competing probes to investigate the interaction of specific components. Various peptides from the C-terminus of both the large (gp91-*phox*) (Kleinberg et al., 1990) and small (p22-*phox*) (Nakanishi et al., 1992) cytochrome subunits inhibit oxidase activity. The most thoroughly studied of these is that from gp91-*phox*. The smallest inhibiting structure is RGVHFIF, corresponding to residues 559−565 (Kleinberg et al., 1992). This peptide inhibits activation, but once activation has occurred, it fails to inhibit (Rotrosen et al., 1990). This has suggested that assembly rather than activity *per se* is affected, as recently confirmed in a cell-free system using cytosol as the source of translocating p47-*phox* and p67-*phox* (Park et al., 1992).

Most of these previous studies used whole cytosol rather than isolated components. Thus, the fundamental hypothesis—that the peptide competes for p47-*phox* binding—was not testable since individual components could not be varied. We have previously developed a "semi-recombinant" cell-free system consisting of isolated plasma membranes as a source of the cytochrome along with purified recombinant p47-*phox*, p67-*phox*, and Rac1 (Uhlinger et al., 1992; Kreck et al., 1994). Herein, we investigated the effect of RGVHFIF on cell-free translocation of p47-*phox* and on the steady-state kinetics when cytosolic factors are varied. We confirm the RGVHFIF inhibits the assembly of the respiratory burst oxidase, but that its mechanism of inhibition may be more complicated than previously supposed.

EXPERIMENTAL PROCEDURES

Materials. Hespan (6.2% hetastarch in 0.9% NaCl) was obtained from DuPont Pharmaceuticals (Wilmington, DE). Lymphocyte separation medium (6.2% Ficoll, 9.4% sodium diatrizoate) was purchased from Organon Teknika Corp. (Durham, NC). NADPH, cytochrome *c* (type IV:horse

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heart), GTPγS, diisopropyl fluorophosphate (DFP), glutathione−agarose (glutathione insolubilized on cross-linked 4% beaded agarose), thrombin (human) and isopropyl thio-β-D-galactopyranoside were obtained from Sigma. The peptides MPO (154−166) (KSSGCAYQDVGVT) and RGVHFIF (residues 559−565 of the p91-*phox*) were synthesized using solid-phase methods by the Emory Microchemical Facility. The peptides were purified by reverse-phase HPLC, and the compositions were confirmed by amino acid analysis.

Isolation of Human Neutrophils and Preparation of Plasma Membranes. Human neutrophils were isolated from peripheral blood from healthy adult donors as described (Pember et al., 1983). Informed consent was obtained from all donors. The cells were incubated with 4 mM DFP and disrupted by nitrogen cavitation. Plasma membranes were recovered after centrifugation through sucrose step gradients as described (Burnham et al., 1990) and pelleted by centrifugation at 400000*g* for 90 min, using a Beckman TL-100 ultracentrifuge. The pellet was resuspended in 50 mM KCl, 1.5 mM NaCl, 5 mM PIPES (pH 7.0), 2.0 mM $MgCl_2$, 0.34 M sucrose, and 1 mM EGTA to a concentration of 3−5 mg/mL and stored at −80 °C.

Expression and Purification of Rac1(C189S), p47-phox, and p67-phox. Rac1(C189S) was cloned and expressed in *E. coli* as a glutathione-*S*-transferase fusion protein using the PGEX-2T vector (Amrad Corp.) and was purified to 99% homogeneity using thrombin cleavage from a glutathione affinity matrix as described (Kreck et al., 1994). We have previously shown that this form of Rac is highly active in the cell-free system. Recombinant p47-*phox* and p67-*phox* were expressed, identified immunochemically, and purified isolated from recombinant baculovirus-infected Sf9 insect cells as detailed in Uhlinger et al. (1992). Proteins were greater than 95% pure, as assessed by Coomassie staining following SDS−polyacrylamide gel electrophoresis. All cells were treated with 4 mM diisopropyl fluorophosphate, 1 mM phenylmethanesulfonyl fluoride, 2 μM each of pepstatin, leupeptin, and aprotinin, and finally 1 mM each of EDTA and EGTA, as detailed in the above references, to guard against proteolysis and the attendant potential problems in the interpretation of the kinetic experiments performed in this study.

Assay for NADPH Superoxide Generating Activity. Superoxide generation was measured by superoxide dismutase inhibitable reduction of cytochrome *c* as described previously (Burnham et al., 1990), using a Thermomax Kinetic Microplate reader (Molecular Devices, Menlo Park, CA). Rac1-(C189S) was preloaded with GTPγS by preincubation for 15 min, at room temperature, with a 2-fold molar excess of GTPγS in Mg^{2+}-free buffer. The semirecombinant cell-free reaction mixtures included 10 μg of plasma membrane protein, the indicated concentrations of recombinant p47-*phox*, p67-*phox*, and pre-loaded Rac1(C189S), 10 μM GTPγS and 160−224 μM arachidonate in a total volume of 50 μL. The peptide, or vehicle control, was added to the reaction mixture prior to addition of any cytosolic components or agonists. The concentration of arachidonate yielding optimal activity was determined by titration prior to the experiment, and differed within this range among plasma membrane preparations. Three 10 μL aliquots of each reaction mixture were transferred to 96 well assay plates (Corning) and preincubated for 5 min at 25 °C. A solution (240 μL) containing 200 μM NADPH and 80 μM cytochrome *c* in 100 mM KCl, 3 mM NaCl, 4 mM $MgCl_2$, 1 mM EGTA, and 10 mM PIPES, pH 7.0 (buffer A), was added to initiate superoxide generation. An extinction coefficient at 550 nm of 21 mM^{-1} cm^{-1} was used to calculate the quantity of cytochrome *c* reduced (Van Gelder & Slater, 1962). For a given incubation, values for a minimum of three kinetic plots were averaged. A minimum of three separate incubations were averaged to provide the reported standard errors.

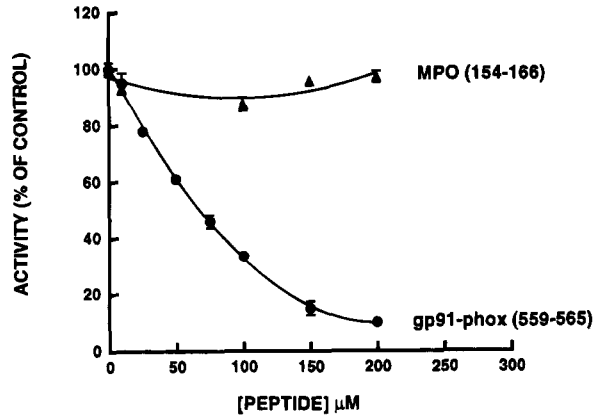

FIGURE 1: Inhibition of superoxide genration in the semirecombinant cell-free system by RGVHFIF but not control peptide. The initial rate of superoxide generation was monitored continuously by NADPH-dependent cytochrome *c* reduction as described under Experimental Procedures. The assay contained 10 μM GTPγS, 1 μM GTPγS-preloaded Rac1(C189S), 1.3 μM p47-*phox*, 1.3 μM p67-*phox*, 10 μg of neutrophil plasma membrane, 224 μM arachidonic acid, and the indicated concentration of peptide, either RGVHFIF [gp91-*phox*(559−565)] or MPO(154−166). Each point is the average rate from triplicate kinetic traces. The experiment shown is representative of four.

Translocation of Cytosolic Factors. Translocation is defined operationally as a stable association with the plasma membrane fraction which survives membrane reisolation in a discontinuous sucrose gradient. Experiments were carried out as described previously (Tyagi et al., 1992; Uhlinger et al., 1993). Unless indicated otherwise, a 50 μL incubation mixture contained 10 μg of plasma membrane (about 5−10 pmol of cytochrome b_{558}), 10 μM GTPγS, 1.3 μM p47-*phox*, and 1.3 μM p67-*phox*. Each translocation experiment included 3000−7000 cpm of one of the cytosolic factors, either recombinant [^{35}S]methionine-labeled p47-*phox* or p67-*phox* protein. Unless otherwise noted, the mass of the radiolabeled proteins was negligible compared with that of the added expressed proteins. The data are presented as percent translocation which is defined as (counts in the gradient interface/sum of the counts in all the phases) × 100. Since the specific activities of the labeled proteins are known, one can convert from percent translocation to picomoles of each protein translocated.

Kinetic Data Analysis. Michaelis−Menten equation kinetic parameters were determined using a nonlinear least-squares regression fit of the data, programmed in Sigma Plot.

RESULTS

Inhibition of Superoxide Generation by RGVHFIF. We first confirmed that the peptide was inhibitory in the semirecombinant cell-free system, using neutrophil plasma membrane plus recombinant Rac1(C189S), p47-*phox*, and p67-*phox* activated with arachidonate plus GTPγS. Results are shown in Figure 1. The data are expressed as a percent

Table 1: Effect of Cytochrome b_{558} Peptide RGVHFIF on Membrane Translocation of p47-*phox* and p67-*phox*

agonist[a]	peptide	% translocation of p47-*phox*	pmol
−	none	0	0
+	none	19.1 ± 1.2	12.8
+	RGVHFIF	14.5 ± 0.6	9.7
+	MPO peptide	18.7 ± 0.4	12.5
agonist	**peptide**	**% translocation of p67-*phox***	**pmol**
−	none	0	0
+	none	10.9 ± 1.5	7.3
+	RGVHFIF	6.5 ± 0.4	4.3
+	MPO peptide	11.2 ± 0.7	7.5

[a] GTPγS and arachidonate were used as agonists in all groups. The reaction mixtures were as described (Experimental Procedures, Figure 1) with either radiolabeled p47-*phox* or p67-*phox* and 150 μM peptide as indicated. Values shown are the average and standard error of four determinations.

of control superoxide-generating activity using vehicle alone. The IC_{50} for the cytochrome peptide was 80 μM, while a control MPO peptide had no effect. A carboxy-terminal peptide of p47-*phox* (378−390) (CSESTKRKLASAV) also had no effect on superoxide generation (data not shown).

Effect of RGVHFIF on Translocation of Cytosolic Factors. The effect of the peptides on the translocation of radiolabeled recombinant cytosolic factors to the plasma membrane fraction in the cell-free system is illustrated in Table 1. The control MPO peptide did not affect the translocation of either component while the RGVHFIF peptide partially inhibited the translocation of both p47-*phox* and p67-*phox* to the plasma membrane. We have previously shown that under the same conditions approximately 5−7 pmol of p47-*phox* was translocated to the plasma membrane in a manner which required the presence of cytochrome b_{558} (Uhlinger et al., 1993), with a similar amount associating in a nonspecific manner. In this study, 3.1 pmol of p47-*phox* translocation was inhibited by the peptide. For p67-*phox*, essentially all of the translocation is cytochrome-dependent, and of this 3.0 pmol was inhibited by RGVHFIF. Thus, the effect of the peptide was to block the same amount of both p47-*phox* and p67-*phox* translocation. Because p67-*phox* translocation requires prior association of p47-*phox* (Heyworth et al., 1991; Uhlinger et al., 1993), these results are consistent with a single inhibitory effect at the level of p47-*phox* association with the cytochrome. The apparent discrepancy between the concentration of peptide required for 90% inhibition of superoxide generation and the lesser affect on translocation of cytosolic factors may be due to the fact that in addition to blocking the association of p47-*phox* and p67-*phox*, the peptide also somehow allows nonproductive or nonactive complexation to occur.

Kinetic Analysis of Inhibition of NADPH Oxidase Activity by RGVHFIF with Respect to Recombinant Cytosolic Components. To investigate further the locus of inhibition by the cytochrome peptide, we carried out a kinetic analysis, varying the concentration of each cytosolic component at fixed concentrations of each of the other proteins, in the absence and presence of the peptide. If the peptide is binding exclusively to the cytochrome-interacting site on p47-*phox*, then an excess of p47-*phox* should out-compete peptide binding, resulting in a competitive pattern of inhibition with respect to p47-*phox*. If, however, the peptide inhibits by binding to other sites, then it will produce a noncompetitive or mixed pattern of inhibition with respect to p47-*phox*. As

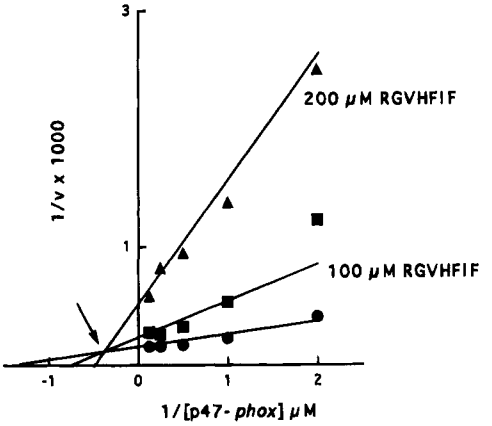

FIGURE 2: Kinetic mechanism of inhibition of the respiratory burst oxidase by RGVHFIF with respect to p47-*phox*. The reaction mixtures were as described (Experimental Procedures, Figure 1) with varying amounts of p47-*phox* as indicated on the *abscissa*. In addition, either 100 μM RGVHFIF (filled squares), 200 μM RGVHFIF (filled triangles), or no added peptide (filled circles) was added 5 min prior to initiating the assay with cytochrome *c* and NADPH. The kinetic data are presented in a Lineweaver−Burk format. Data points represent the mean of three determinations obtained in one experiment. Data are representative of three independent experiments.

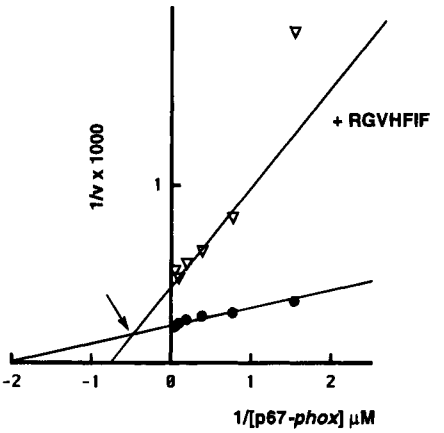

FIGURE 3: Kinetic mechanism of inhibition of the respiratory burst oxidase by RGVHFIF with respect to p67-*phox*. The reaction mixtures were as described (Experimental Procedures, Figure 1) with varying amounts of p67-*phox* as indicated on the *abscissa*. In addition, either 100 μM RGVHFIF (open triangles) or no added peptide (filled circles) was added 5 min prior to initiating the assay with cytochrome *c* and NADPH. The kinetic data are presented in a Lineweaver−Burk format. Data points represent the mean of three determinations obtained in one experiment. Data are representative of three independent experiments.

shown in the Lineweaver−Burk kinetic plot in Figure 2, the RGVHFIF peptide causes a mixed pattern of inhibition with respect to p47-*phox*. Thus, simple binding to the cytochrome interaction site (Kleinberg et al., 1990, 1994) does not fully explain the kinetics of inhibition.

A similar series of experiments were conducted in which the concentration of p67-*phox* or Rac1(C189S) was varied. As shown in Figure 3, RGVHFIF also shows a mixed pattern of inhibition with respect to p67-*phox*. The effects of the cytochrome peptide on the kinetic parameters with respect to Rac1(C189S) are shown in Figure 4. As indicated in the Lineweaver−Burk plot, the peptide produces classical noncompetitive inhibition with respect to the small GTPase. Thus, the peptide does not appear to compete directly with either Rac or p67-*phox* for binding to the cytochrome.

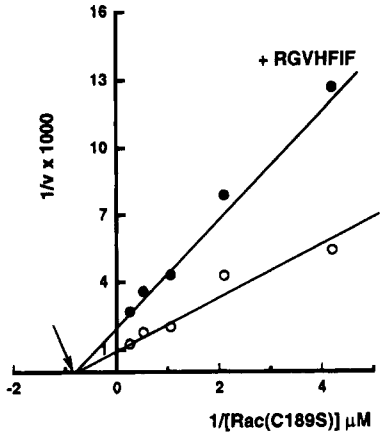

FIGURE 4: Kinetic mechanism of inhibition of the respiratory burst oxidase by RGVHFIF with respect to Rac1(C189S). The reaction mixtures were as described (Experimental Procedures, Figure 1) with varying amounts of GTPγS-preloaded Rac1(C189S) as indicated on the *abscissa*. In addition, either 50 μM RGVHFIF (filled circles) or no added peptide (open circles) was added 5 min prior to initiating the assay with cytochrome *c* and NADPH. The kinetic data are presented in a Lineweaver–Burk format. Data points represent the mean of three determinations obtained in one experiment. Data are representative of three independent experiments.

DISCUSSION

It now seems clear that activation of electron flow from NADPH to oxygen occurs through electron-transferring groups (FAD and heme) in cytochrome b_{558}, and that of electron flow is regulated by the association of p47-*phox*, p67-*phox*, and probably Rac. Nevertheless, the precise roles of these cytosolic regulatory proteins and the manner by which they influence electron flow remain unclear. A first step to understanding the roles of these proteins is mapping the specific protein–protein interactions among them. As described in the introduction, the *in vitro* (Park et al., 1992) and *in vivo* (Heyworth et al., 1991) dependence of p67-*phox* translocation on p47-*phox*, as well as the dependence of p47-*phox* translocation on cytochrome b_{558}, has been interpreted as indicating a direct interaction of p47-*phox* with cytochrome b_{558} (Kleinberg et al., 1990; Heyworth et al., 1991). The effect of RGVHFIF both on activity and on translocation of cytosolic factors has strengthened this view and has suggested a specific interaction of p47-*phox* with the C-terminus of gp91-*phox*.

The present studies suggest additional explanations for the peptide effects. While the bulk of the data suggest strongly that p47-*phox* binds directly to cytochrome b_{558}, the fact that the inhibition by peptide with respect to p47-*phox* is not strictly competitive calls into question the proposed specificity of the interaction of p47-*phox* with the gp91-*phox* C-terminus. The data from Figure 2 imply that a form of the oxidase exists which contains both p47-*phox* and the peptide, and that the latter form is less active or inactive. Possible physical explanations for the inhibition include intracytochrome binding of the peptide which modifies the conformation and/or α–β subunit orientation, and consequently its binding affinity for p47-*phox*. Such intramolecular effects of peptides have been proposed to account for inhibitory effects of receptor loop peptides on receptor–G protein interactions (Luttrell et al., 1993). Also, the peptide might bind in a nonspecific manner to p47-*phox* in such a way as to interfere with its optimal binding conformation. Such an explanation is consistent with chemical cross-linking studies that demonstrated cross-linking of a similar gp91-*phox* peptide to p47-*phox* (Nakanishi et al., 1992). Alternatively, the peptide may inhibit not only by competing with p47-*phox* binding but also by a second unknown mechanism.

Thus, while RGVHFIF can be used as a tool to block the translocation of p47-*phox* and p67-*phox* to the cytochrome, it is not possible to deduce interactions with specific regions of the cytochrome from these data. The carboxy terminus of the small subunit of cytochrome b_{558} contains a proline-rich region which might be involved in interaction with SH3 regions in p47-*phox*. Indeed, point mutations in this region render the cytochrome b_{558} completely inactive, presumably due to an inability of the oxidase to properly assemble (Dinauer et al., 1991). Thus, p47-*phox* may interact with the small rather than the large cytochrome subunit. The present studies caution against inferring specific interactions from peptide inhibition data unless competitive inhibition can be demonstrated.

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