Activation domain in P67^{phox} regulates the steady state reduction of FAD in gp91^{phox}

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An activation domain in p67^{phox} (residues 199-210) is critical for regulating NADPH oxidase activity in cell-free system [10] To determine the steady state reduction of FAD, thioacetamide-FAD was reconstituted in gp91^{phox}, and the fluorescence of its oxidised form was monitored. Omission of p67^{phox} decreased the steady state reduction of the FAD from 28% to 4%, but omission of p47^{phox} had little effect. A series of the truncated forms of p67^{phox} were expressed in *E.coli* to determine the domain in p67^{phox} which is essential for regulating the steady state of FAD reduction. The minimal length of p67^{phox} for for regulating the steady state of FAD reduction is shown to be 1-210 using a series of truncation mutants which indicates that the region 199-210 is also important for regulating electron flow within flavocytochrome b$_{558}$. The deletion of this domain not only decreased the superoxide generation but also decreased the steady state of FAD reduction. Therefore, the activation domain on p67^{phox} regulates the reductive half-reaction for FAD, consistent with a dominant effect on hydride/electron transfer from NADPH to FAD.

Key words: the activation domain on p67^{phox}; the steady state of FAD reduction.

Introduction

Neutrophiles and macrophages produce superoxide and other reactive oxygen species that participate in intracellular killing of phagocytized microorganisms [2,5]. Superoxide generation is catalyzed by NADPH oxidase which consists of both cytosolic factors (p47^{phox} and p67^{phox}) and plasma membrane associated flavocytochrome b$_{558}$. In cell resting state, cytosolic factors p47^{phox}, p67^{phox} exist in the cytosol as a complex along with a third component,

p40^{phox}, which appears to stabilize a 240 kDa complex of cytosolic factors [9, 21]. Upon activation, in response to microorganisms or to a variety of soluble agonists, cytosolic factors p47^{phox}, p67$^{phox\ 2}$, and possibly p40^{phox} translocate to membrane where they bind directly or indirectly with flavocytochrome b$_{558}$ [7, 18]. The small GTP-binding protein, Rac, translocates to membrane independently of the other cytosolic components [8, 11], and thereby assembled complex catalyzes the reduction of oxygen to superoxide.

Flavocytochrome b$_{558}$ is a membrane-associated heterodimer (p22^{phox} and gp91^{phox}) that contains putative binding sites for NADPH, FAD, and heme [16, 19] and considered to be redox center of the NADPH oxidase. Three cytosolic components (p47^{phox}, p67^{phox}, and small GTPase Rac) are considered to be regulatory subunits of NADPH oxidase. A great deal of current research involves understanding the protein-protein interactions among the components of NADPH oxidase complex, and how these change with the activation state. Supporting the importance of these interactions, individuals with genetic deficiencies or mutations in p47^{phox}, p67^{phox}, or one of the subunits of cytochrome b$_{558}$ (gp91^{phox} and p22^{phox}) exhibit chronic granulomatous disease [5], which is characterized by the inability of phagocytic leukocytes (neutrophils, eosinophils, monocytes, and macrophages) to generate active oxygen species which are necessary for killing of phagocytized pathogens (reviewed in 11).

NADPH oxidase activity can be reconstituted *in vitro* using purified cytosolic factors p47^{phox}, p67^{phox}, GTPgS preloaded Rac, and phospholipid-reconstituted flavocytochrome b$_{558}$ along with an anionic amphiphiles such as arachidonate [1, 17].

Based on chemical precedent and structural models of the enzyme [22], the pathway for electron flow within flavocytochrome b$_{558}$ has been proposed in Scheme I.

$$NADPH \rightarrow FAD \rightarrow Heme\ A \rightarrow Heme\ B \rightarrow O_2 \quad Scheme\ I$$
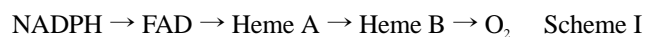
Our recent study identified an activation domain in p67^{phox}

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that is essential for NADPH oxidase activity [10]. Deletion of this region within residues 199~210 completely eliminated NADPH oxidase activity.

In the present study, we observed that the activation domain is also essential for regulating electron flux in the complex. We propose that the activation domain on p67^{phox} directly activates a particular step in the electron transfer pathway depicted above in Scheme I. We provide evidence that the activation domain on p67^{phox} regulates the reduction of FAD by NADPH, consistent with the regulation of the NADPH→FAD hydride/electron transfer reaction.

Materials and Methods

Preparation of plasma membrane, cytochrome b$_{558}$ and recombinant proteins:

Plasma membranes were isolated as described by Burnham et al. [4]. Further purification steps were done for isolating cytochrome b$_{558}$ from plasma membrane as described previously [14]. Rac cDNA cloned in pGEX-2T was expressed in DH5a cells as a GST fusion form, and purified by using glutathione-Sepharose followed by thrombin cleavage as described by Kreck et al. [12]. Recombinant proteins p47^{phox} and wild-type p67^{phox} were expressed in insect cells (sf9 cell) as described previously [10]. A series of truncated p67^{phox} and their mutants were expressed in *E. coli*, were purified with glutathione-Sepharose followed by glutathione elution as described previously [10], and were dialyzed to remove free glutathione. Protein concentrations were determined according to Bradford [3]. The purity of the proteins were confimed by SDS-PAGE and Coomassie Blue staining.

Truncations of p67^{phox}:

A series of truncated p67^{phox} clones were obtained by PCR using p67^{phox} DNA cloned in pGEX-2T as the template. For all PCR reactions, the forward primer (CGT**GGATCC**<u>ATG</u>TCCCTGGTGGAG GCC) was designed to anneal to 5 end of p67^{phox} sequence and to introduce a BamHI site (shown in bold) and the initiation codon (underlined). For each truncation, the reverse primer (e.g. for p67^{phox}(1-210) mutant, GAT**GAATTC**<u>TTA</u>ATCCACCACAGATGC) was designed to anneal to the p67^{phox} sequence immediately 5 to the region to be truncated, and to introduce the stop codon (underlined) and a EcoRI site (shown in bold). These PCR products were ligated into the BamHI and EcoRI sites of pGEX-2T vector, and were transformed into DH5a for expression of the protein. The PCR products were sequenced to verify that no unexpected mutations were introduced by PCR and to confirmed the truncations.

NADPH oxidase activity assay:

Superoxide generation was measured by SOD-inhibitable reduction of cytochrome c as described by Burnham et al. [4] using a Thermomax Kinetic Microplate reader (Molecular Devices, Menlo Park, CA). Rac was preloaded with 5-fold molar excess of GTPgS for 15 min at room temperature in the absence of MgCl$_2$ as described previously [12]. For the standard assay condition, the cell-free reaction mixtures include 60 nM flavocytochrome b$_{558}$ that had been reconstituted with FAD or FAD analog and phospholipids, 800 nM p47^{phox}, 900 nM p67^{phox}, 450 nM Rac, 10 mM GTPgS, and 200-240 mM arachidonate in a total of 50 ml. Three 10 ml aliquots of each reaction mixture were transferred to 96-well assay plates and preincubated for 5 min at 25°C. For each well, 240 ml of substrate cocktail containing 200 mM NADPH and 80 mM cytochrome c in buffer A (100 mM KCl, 3 mM NaCl, 4 mM MgCl$_2$, 1 mM EGTA, and 10 mM PIPES, pH 7.0), was added to initiate superoxide generation. NADPH oxidase activity was measured by monitoring absorbance change at 550 nm. An extinction coefficient at 550 nm of 21 mM^{-1} cm^{-1} was used to calculate the quantity of cytochrome c reduced [13].

Spectrophotometric and fluorometric assays:

Heme content was determined by reduced minus oxidized difference spectroscopy at 424~440 nm using an extinction coefficient of 161 mM^{-1}cm^{-1} [6]. The flavin content of FAD analog-reconstituted cytochrome b$_{558}$ was estimated fluorimetrically. Fluorescence spectra were recorded with a Hitachi model F-3000 spectrofluorimeter. Fluorescence changes at 525 nm induced by NADPH-FAD analog oxidoreduction during cell-free NADPH oxidase activation occurred slowly for about 5 min, and the total fluorescence change due to the complete reduction of the FAD analog was measured by adding a few crystals of sodium dithionate. To calculate the percent reduction of the FAD analog at steady state, the fluorescence change at 525 nm attributable to NADPH oxidation was subtracted from that due to oxidoreduction of NADPH and the FAD analog. The time course of heme reduction was derived from the absorbance changes at 558 minus 540 nm, using an extinction coefficient of 21.6 mM^{-1}cm^{-1} [6].

Results

Effect of cytosolic factors on the reduction of fad and heme:

The steady state reduction levels were calculated based on the percent fluorescence bleaching achieved at 5 min, correcting for the decrease in fluorescence contributed by NADPH oxidation. Based on this calculation the fraction reduction of flavin after steady state has been achieved is $28 \pm 3\%$ (Table 1). In contrast to flavin reduction, addition of NADPH produced < 2% steady state reduction of heme based on absorbance changes at 558 nm minus 540 nm

Table 1. *Effects of cytosolic factors on NADPH oxidase activity and on the steady state reduction of FAD and heme* 8-Thioacetamido-FAD was reconstituted into purified cytochrome b$_{558}$ in the presence of phospholipids as described in "Materials and Methods". NADPH-dependent superoxide generation was monitored in the presence or absence of p47^{phox} and p67^{phox}.

Components	NADPH oxidase activity	Steady state reduction level	Heme
	(nmol/min/nmol of heme)	FAD analog	
Complete	320 $\pm$ 50	28 $\pm$ 3%	<2%
-p47^{phox}	210 $\pm$ 30	21 $\pm$ 2%	<2%
-p67^{phox}	0	4+1%	<2%

(Table 1). The steady state percent reduction of the FAD analog and heme was determined as above in the complete system or in the absence of either p47^{phox} or p67^{phox} (Table 1). When p47^{phox} was omitted, there was still significant reduction of FAD (21% compared with 28%). However, when p67^{phox} was omitted, FAD was almost completely oxidised (Table 1). The steady state of reduction of FAD correlated with the rate of superoxide generation under the same conditions, indicating a functional relationship between FAD reduction and superoxide generation (Table 1). In contrast, heme was completely oxidised regardless of the presence of the cytosolic factors (Table 1).

Expression of truncated p67^{phox}:

A series of truncated mutant p67^{phox} (Fig. 1) was generated to determine the region which is important for regulating the steady state FAD reduction. As shown in Fig. 2, p67^{phox} (1-246) partially (approximately 50% of V$_{max}$) activates flavocytochrome b$_{558}$ which is consistent with previous observation [10]. Further truncated mutants p67^{phox} (1-235), p67^{phox} (1-221), p67^{phox} (1-216), and p67^{phox} (1-210) thoroughly regain their abilities for activating NADPH oxidase almost same as wild-type p67^{phox} (Fig. 2). Further
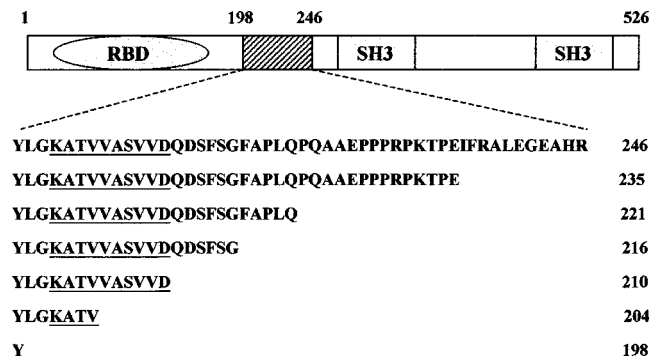

Fig 1. Truncation of p67^{phox} and its effect on NADPH oxidase activation. Various domains of p67^{phox}, including two SH3 (src homologous region 3) domains, a Rac-binding domain (RBD), and the region from amino acid residues 198 to 246 (hatched). This region is expanded to show the amino acid sequence and residue number. The activation domain is underlined
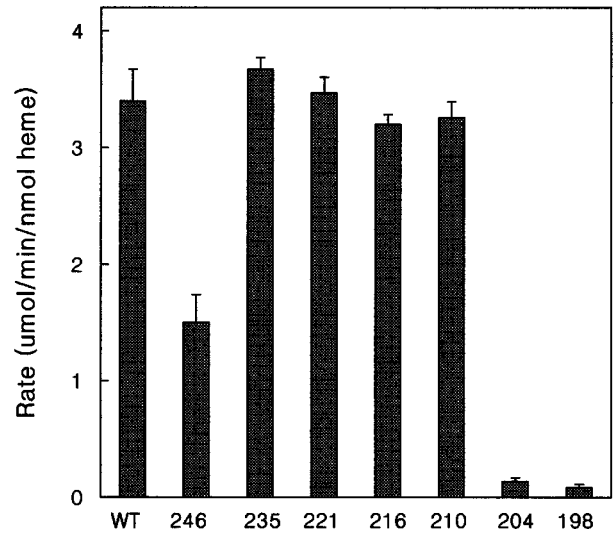

Fig 2. NADPH oxidase activation by truncated p67^{phox}. Superoxide generation was measured as described under Materials and Methods. The reaction mixture was consisted of 60 nM flavocytochrome b$_{558}$ that had been reconstituted with FAD and phospholipids. In cubations contained 600 nM p47^{phox}, 450 nM Rac1, 900 nM of truncated p67^{phox}, and 0.2 mM arachidonate. Error bars show the standard error of the mean (n = 3).
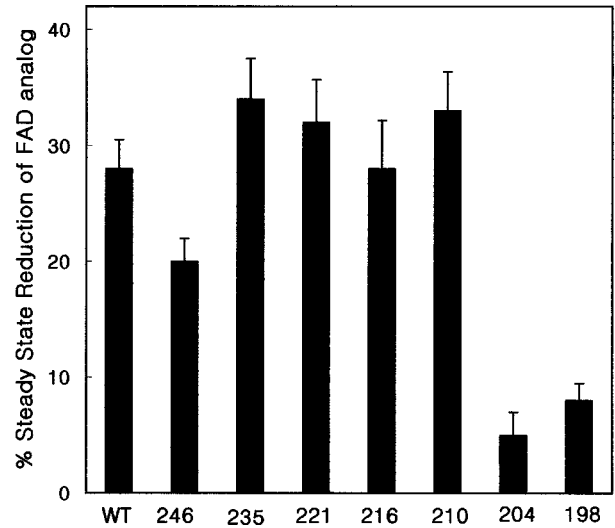

Fig 3. Effect of truncated p67^{phox} on the steady state reduction of FAD. The reaction mixture was consisted of 60 nM of FAD analog reconstituted flavocytochrome b$_{558}$, 800 nM p47^{phox}, 450 nM Rac1, 900 nM of truncated p67^{phox}, and 0.2 mM arachidonate. The fluorescence emission spectrum of 525 nm (excitation wavelength, 475 nm) was recorded as described in Materials and Methods. Error bars show the standard error of the mean (n = 3).

truncated mutants, p67^{phox} (1-204) and p67^{phox} (1-198), dramatically reduces superoxide generation, which suggests that p67^{phox} (1-210) is the minimal-size active domain, and the region 199~210 of p67^{phox} is critical for activating flavocytochrome b$_{558}$ in cell-free oxidase reconstitution. Therefore, the activation domain is important for regulating

electron flow within flavocytochrome b_{558}, and data suggest that it does so by interacting directly with this catalytic component.
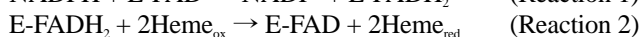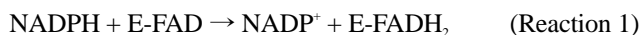
Role of the activation domain in p67^{phox} in FAD reduction:

As shown in Fig. 2 and 3, p67^{phox} derivatives which have an activation domain showed both higher rate of superoxide generation and higher level of steady state reduction of 8-thioacetamido-FAD. However, the truncation of the activation domain resulted in much lower rate of superoxide generation (Fig. 2) and a very low steady state reduction of 8-thioacetamido-FAD (Fig. 3). The activation domain is not involved in the interaction with Rac1 or p47^{phox} [10]. Therefore, the truncation of the activation domain suppresses the reduction of FAD by NADPH in flavocytochrome b_{558}.

Discussion

Based on the sequence homologies between p67^{phox} and the putative pyridine nucleotide-binding sites of NADPH-dependent enzymes, the 193~212 amino acid region of p67^{phox} was proposed as the one of the candidates for NADPH-binding site [20]. NADPH-binding site on the b subunit of flavocytochrome b_{558} (gp91^{phox}) was also postulated on the basis of sequence homologies; alignment of the amino acid sequence of gp91^{phox} with other flavoprotein revealed that five peptide segments in the 403~570 amino acid region of gp91^{phox} are likely to be NADPH-binding domain [16, 19]. The docking site of p67^{phox} on flavocytochrome b_{558} is still unknown. Therefore, one of the possible role of the 201~210 amino acid region is transfering NADPH from cytosol to the substrate binding site of gp91^{phox} to form [E-S] complex by opening the NADPH-binding site in gp91^{phox}.

A model has been proposed that attempts to explain individual roles for cytosolic factors during the protein assembly associated with activation of the respiratory burst (see Introduction). According to this model, it is p67^{phox} that directly regulates the rate-limiting transfer of electrons within the gp91^{phox} subunit through its activation domain within the 199~210 region. In the present study, we have investigated the influence of this region on regulating the rate of specific catalytic steps involved in transferring electrons from NADPH to O_2. The reductive half-reaction (Reaction 1) and reoxidative half-reaction (Reaction 2) with respect to FAD within gp91^{phox} are summarized as follows.

$$NADPH + E\text{-}FAD \rightarrow NADP^+ + E\text{-}FADH_2 \qquad \text{(Reaction 1)}$$
$$E\text{-}FADH_2 + 2Heme_{ox} \rightarrow E\text{-}FAD + 2Heme_{red} \qquad \text{(Reaction 2)}$$

We first used steady state kinetics to investigate whether the activation domain in p67^{phox} stimulates the reductive half-reaction (Reaction 1) or the reoxidative half-reaction (Reaction 2). If the former were the case then the p67^{phox} should increase the steady state reduction level of FAD, and truncations should lead to a more oxidized state. The opposite should be true if p67^{phox}x were functioning as an activator for Reaction 1. In addition, the heme should become more reduced. Thus, monitoring the steady state reduction of flavin and heme during turnover will distinguish between these two models.

We propose that the activation domain on p67^{phox} was critical for regulating FAD reduction, since the deletion of this domain not only decreased the superoxide generation but also decreased the steady state of FAD reduction. Thus, the activation domain on p67^{phox} regulates the reductive half-reaction for FAD (Reaction 1), consistent with a dominant effect on hydride/electron transfer from NADPH to FAD.

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