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Identification of an actin-binding site in p47^{phox} an organizer protein of NADPH oxidase

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Abstract **Actin has been reported to enhance the superoxide-generating activity of neutrophil NADPH oxidase in a cell-free system and to interact with p47^{phox}, a regulatory subunit of the oxidase. In the present study, we searched for an actin-binding site in p47^{phox} by far-western blotting and blot-binding assays using truncated forms of p47^{phox}. The amino-acid sequence 319–337 was identified as an actin-binding site, and a synthetic peptide of this sequence bound to actin. The sequence shows no homology to other known actin-binding motifs. It is located in the autoinhibitory region of p47^{phox} and includes Ser-328, a phosphorylation site essential for unmasking. Although a phosphorylation-mimetic p47^{phox} mutant bound to actin with a lower affinity than the wild type, the same mutant interacted with filamentous actin more efficiently than the wild type. A mutant peptide p47^{phox} (319–337, Ser328Glu) bound to filamentous actin more tightly than to monomer actin. These results suggest that p47^{phox} moves to cortical actin when it becomes unmasked in the cells.**

Keywords: Superoxide; NADPH oxidase; p47^{phox}; β-Actin; Protein–protein interaction; Actin-binding motif; Actin polymerization

1. Introduction

p47^{phox} is a regulatory subunit of phagocyte NADPH oxidase, which produces superoxide (O_2^-) during phagocytosis and plays a crucial role in host defense [1]. The oxidase is dormant in resting cells, and becomes activated upon cell stimulation through assembly of its catalytic and regulatory subunits [2,3]. The catalytic core protein is the membrane flavocytochrome b_{558}, consisting of p22^{phox} and gp91^{phox} (Nox2), while the regulatory subunits are p47^{phox}, p67^{phox}, rac and p40^{phox}, which are translocated from the cytosol to the membrane. Although p47^{phox} was first discovered in phagocytes, subsequent studies have revealed that it is also expressed in non-phagocytic cells. For example, p47^{phox} activates Nox2 in vascular endothelial cells with other regulatory subunits [4]; p47^{phox} also activates Nox1, a homolog of Nox2 [5], in vascular smooth muscle cells [6]. The reactive oxygen species produced in these cells may have other function than host defense, such as redox regulation or cell proliferation [4,7].

p47^{phox} is assumed to play as an adapter for organization of the oxidase complex, since it reduces the required concentrations of other subunits [8,9] and stabilizes the oxidase [10,11]. It has been established that p47^{phox} is intramolecularly masked through interactions among its PX domain, SH3 domains and autoinhibitory region (AIR) [3]. After becoming unmasked by phosphorylation, p47^{phox} interacts with p22^{phox}, a subunit of flavocytochrome b_{558}, thereby leading to activation of the oxidase [12,13]. In addition, the PX domain has been suggested to interact with the plasma membrane through phosphoinositides [3]. These concepts were recently confirmed by structural studies [14].

Actin was originally discovered as a component of myofibrils present in muscle cells (α-actin). The protein is also known to be a component of the cytoskeleton in non-muscle cells (mainly β-actin). In human neutrophils, actin is abundantly present (~30 μM) and one-third of it is polymerized in the resting state [15]. Upon cell activation, more actin molecules become polymerized into filamentous actin (F-actin). Several studies have demonstrated interactions between the cytoskeleton and cytosolic components of NADPH oxidase [16,17]. Furthermore, we previously found that actin enhances the activation of neutrophil NADPH oxidase [18], and actin-depolymerizing agents facilitated its deactivation in a cell-free system [19]. Clements et al. [20] demonstrated that inhibition of actin polymerization modulates the respiratory burst of neutrophils. Despite these lines of evidence, neither the role of actin filaments in regulating NADPH oxidase nor the mechanisms for these phenomena have been well defined.

In an attempt to clarify these points, we focused on the protein–protein interaction between p47^{phox} and actin which we previously found [21]. We searched for an actin-binding site in the p47^{phox} molecule, and based on the obtained result, we then examined whether the interaction between p47^{phox} and actin is influenced by the conformational change of p47^{phox} by unmasking or that of actin by polymerization.

2. Materials and methods

2.1. Materials

Glutathione–Sepharose, CM–Sepharose, pGEX-6P and PreScission Protease were purchased from Amersham Biosciences (Piscataway, NJ). Oligonucleotide primers for mutagenesis were synthesized by Amersham Biosciences or Sigma Genosys (St. Louis, MO). A Quik Change mutagenesis kit was purchased from Stratagene (La Jolla, CA). Diisopropyl fluorophosphate was obtained from Wako Pure

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Abbreviations: O_2^-, superoxide; AIR, autoinhibitory region; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; F-actin, filamentous actin; G-actin, globular actin

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Chemicals (Osaka, Japan). A rabbit antibody against human actin (residues 20–33) and streptavidin–agarose CL-4B were purchased from Sigma–Aldrich (St. Louis, MO). An antibody against human $p47^{phox}$ (C-terminal 13mer peptide) was a kind gift from Dr. David Lambeth (Department of Pathology, Emory University School of Medicine, Atlanta, GA). Biotinylated peptides LSQDAYRRNSVRFLQQRRR and LSQDAYRRNEVRFLQQRRR (purity 97%) were synthesized by Thermo Electron (Ulm, Germany).

2.2. Construction of plasmids for truncated forms of $p47^{phox}$ and a triple mutant

A complementary DNA (cDNA) for human $p47^{phox}$ (in pVL1393) was a kind gift from Dr. David Lambeth (Department of Pathology, Emory University School of Medicine). The cDNA was amplified by PCR using specific 5′- and 3′-primers with attached *Eco*RI site linkers and then subcloned into pGEX-6P (*Eco*RI fragment). The $p47^{phox}$ cDNA in pGEX-6P was then mutated to introduce a stop codon at each designated site using a Quik Change mutagenesis kit. To construct a $p47^{phox}$ triple mutant (Ser303/304/328Glu), the $p47^{phox}$ cDNA was initially mutated to convert Ser-303 and -304 to Glu, and then further mutated to convert Ser-328 to Glu using a Quik Change mutagenesis kit. The sequences of all the truncated forms and the triple mutant were confirmed by dideoxynucleotide-based sequencing.

2.3. Expression and purification of the $p47^{phox}$ truncates and the triple mutant

Expression and purification of the truncated forms of $p47^{phox}$ were performed as previously described for full-length $p47^{phox}$ [11]. After expression, the cells were lysed in the presence of 0.2 mM diisopropyl fluorophosphate and purified with glutathione–Sepharose beads. Each truncated form was released from the glutathione-*S*-transferase beads by treatment with PreScission Protease at 4 °C for 4 h. After centrifugation, the precipitates were resuspended in the same buffer and incubated for an additional 12 h. The fractions containing each truncated form were combined, dialyzed against 20 mM potassium phosphate buffer (pH 7.0) and concentrated with Ultrafree Biomax 30k (Millipore, Bedford, MA). For the triple mutant or full-length $p47^{phox}$, the glutathione–Sepharose-purified fraction was applied to a CM–Sepharose column equilibrated with 20 mM sodium phosphate buffer (pH 7.0) containing 60 mM NaCl, and the column was eluted with a NaCl gradient (60–200 mM) in the same buffer. The fractions containing the protein were concentrated and dialyzed against 20 mM potassium phosphate buffer (pH 7.0).

2.4. Construction of a plasmid containing human β-actin

A cDNA for human β-actin (in pHF) was a kind gift from Dr. Laurence Kedes (Institute for Genetic Medicine, Southern California University School of Medicine, Los Angeles, CA). The cDNA was amplified by PCR using specific 5′ and 3′-primers with an attached *Bam*HI and *Eco*RI site, respectively, and then subcloned into pGEX-6P (*Bam*HI and *Eco*RI fragment).

2.5. Expression and purification of β-actin

The pGEX-6P containing the β-actin cDNA was transfected into *Escherichia coli* BL-21 and expressed at 20 °C for 20 h. Lysis and purification procedures were performed using our established procedures (Tamura et al., manuscript in preparation). Briefly, the cells were lysed by sonication in the absence of DNase I, which tightly binds to actin. The extracted protein was purified with glutathione–Sepharose beads, and then further purified by a polymerization–depolymerization method [22]. The precipitate was dissolved in actin monomer buffer (buffer G) (5 mM Tris–HCl, pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP, and 0.5 mM DTT) and dialyzed against the same buffer. The purified monomer actin (G-actin) was lyophilized and stored at −20 °C until use.

In some blot-binding assays, G-actin was polymerized by incubation in polymerization buffer (buffer F) (5 mM Tris–HCl, pH 8.0, 50 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, and 0.5 mM DTT) for 3 h at 4 °C and designated "F-actin".

2.6. Far-western blotting

Full-length or truncated forms of $p47^{phox}$ (0.1 nmol) were subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) in a 12% gel and the protein bands were blotted onto a BA85 nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). After checking proteins by Fast Green staining, the membrane was blocked with buffer A (20 mM Tris–HCl, pH 7.4, 0.3 M NaCl, and 0.05% Tween 20) and incubated with β-actin (2 μM) at 4 °C for 2 h. After washing with buffer A, the membrane was treated with actin IgG at 25 °C for 12 h, and detected with peroxidase-conjugated goat anti-rabbit IgG (Cappel Products, Irvine, CA). After color development with 4-chloro-1-naphthol, the blots were scanned using an Epson GT8700 scanner and analyzed with the NIH Image software.

2.7. Blot-binding assay

Full-length, truncated, or mutant forms of $p47^{phox}$ (0.1 nmol) were directly applied to a nitrocellulose membrane using a dot-blotting apparatus AE-6190 (Atto, Tokyo, Japan). After checking proteins by Fast Green, the membrane was blocked and treated as described for the far-western blotting.

2.8. Assay for O_2^- generation

Assay for O_2^- generation was performed as described previously [23]. The activation mixture contained $p47^{phox}$ or $p47^{phox}$ (S303/304/328E) (1.3 μM), $p67^{phox}$(1–210) (6 μM), racQ61L (6 μM), and purified cytochrome b_{558} [11] (0.1 μM) in 50 μl of buffer A containing 4mM $MgCl_2$, 10 μM FAD and 10 μM GTP. The mixture was incubated with 200 μM SDS for 5 min at 25 °C to activate NADPH oxidase. Four 10 μl aliquots of the activation mixture were transferred into the wells of a 96-well microplate and diluted with 240 μl of buffer B (20 mM potassium phosphate buffer, pH 7.0, 4 mM $MgCl_2$, and 10 μM FAD) containing 200 μM NADPH and 80 μM cytochrome *c*. Superoxide generation was measured by monitoring the cytochrome *c* reduction at 550 nm using a Spectra Classic microplate reader (Tecan, Zurich, Switzerland).

3. Results

3.1. Truncated forms of $p47^{phox}$

Our previous study revealed that p47N, $p47^{phox}$ (1–286), does not bind to actin, thereby suggesting that the C-terminal region of $p47^{phox}$ contains an actin-binding site [21]. To locate this site, we prepared various C-terminally truncated forms of $p47^{phox}$ and examined their interactions with actin. The structures of these truncated forms of $p47^{phox}$ are illustrated in Fig. 1A. p47Δ1 (1–337) contained nearly all of the AIR and terminated with a triad of arginine residues. p47Δ2 (1–330) terminated at the basic sequence RRNSVR, containing Ser-328 for phosphorylation. p47Δ3 (1–318) terminated at another basic sequence, RSRKR. p47Δ4 (1–298) terminated before the unique sequence PPRRSS, containing two serine residues for phosphorylation. Finally, p47N (1–286) lacked the whole AIR and the following region. An SDS–polyacrylamide gel after electrophoresis of the purified truncated forms of $p47^{phox}$ is shown in Fig. 1B. Each sample showed a single major band at the position expected for its molecular weight.

3.2. Interactions between the truncated forms of $p47^{phox}$ and actin

The interactions of the truncated forms of $p47^{phox}$ with β-actin were examined by far-western blotting. $p47^{phox}$ and its truncated mutants were electrophoresed in an SDS–polyacrylamide gel and blotted onto a nitrocellulose membrane. The membrane was incubated with human β-actin and the bound protein was detected by actin IgG. Fig. 2 shows typical results for this blotting analysis. The color development was analyzed in several experiments and expressed as the % intensity relative to that of full-length $p47^{phox}$ (Fig. 2B). p47Δ1 showed a similar intensity to full-length $p47^{phox}$, while