Activation of the superoxide-producing phagocyte NADPH oxidase requires co-operation between the tandem SH3 domains of p47^{phox} in recognition of a polyproline type II helix and an adjacent α-helix of p22^{phox}

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Activation of the superoxide-producing phagocyte NADPH oxidase, crucial for host defence, requires an SH3 (Src homology 3)-domain-mediated interaction of the regulatory protein p47^{phox} with p22^{phox}, a subunit of the oxidase catalytic core flavocytochrome b_{558}. Although previous analysis of a crystal structure has demonstrated that the tandem SH3 domains of p47^{phox} sandwich a short PRR (proline-rich region) of p22^{phox} (amino acids 151–160), containing a polyproline II helix, it has remained unknown whether this model is indeed functional in activation of the oxidase. In the present paper we show that the co-operativity between the two SH3 domains of p47^{phox}, as expected from the model, is required for oxidase activation. Deletion of the linker between the p47^{phox} SH3 domains results not only in a defective binding to p22^{phox} but also in a loss of the activity to support superoxide production. The present analysis using alanine-scanning mutagenesis identifies Pro^{152}, Pro^{156} and Arg^{158} in the p22^{phox} PRR as residues indispensable for the interaction with p47^{phox}. Pro^{152} and Pro^{156} are recognized by the N-terminal SH3 domain, whereas Arg^{158} contacts with the C-terminal SH3 domain. Amino acid substitution for any of the three residues in the p22^{phox} PRR abrogates the superoxide-producing activity of the oxidase reconstituted in intact cells. The bis-SH3-mediated interaction of p47^{phox} with p22^{phox} thus functions to activate the phagocyte oxidase. Furthermore, we provide evidence that a region C-terminal to the PRR of p22^{phox} (amino acids 161–164), adopting an α-helical conformation, participates in full activation of the phagocyte oxidase by fortifying the association with the p47^{phox} SH3 domains.

Key words: bis-SH3 domain, flavocytochrome b_{558}, NADPH oxidase, proline-rich region, p22^{phox}, p47^{phox}.

INTRODUCTION

Professional phagocytes such as neutrophils and macrophages play a crucial role in the first line of innate immune defence against invading microbes. One of the mechanisms for microbe elimination is via production of ROS (reactive oxygen species). The phagocyte NADPH oxidase, which is dormant in resting cells, becomes activated during phagocytosis to produce superoxide, a precursor of powerful microbicidal ROS [1–5]. The significance of the oxidase in host defence is exemplified by recurrent and life-threatening infections that occur in patients with CGD (chronic granulomatous disease) because of the lack of the superoxide-producing system in phagocytes. Because inappropriate or excessive production of ROS, on the other hand, results in inflammatory disorders, the activity of the phagocyte oxidase should be strictly regulated. It is thus important to understand in detail the molecular mechanism of oxidase regulation.

The catalytic core of the oxidase is flavocytochrome b_{558}, comprising the two membrane-integrated proteins gp91^{phox} and p22^{phox}; the subunit association is essential for stabilization of both proteins [1–5]. Whereas gp91^{phox} harbours a complete electron-transferring apparatus from NADPH to molecular oxygen for superoxide production, p22^{phox} is not directly involved in the electron transfer, but participates in oxidase regulation by serving as an anchoring site for regulatory proteins. Activation of the phagocyte oxidase requires the specific regulatory proteins p47^{phox} and p67^{phox}, and the small GTPase Rac; these exist in the cytoplasm of resting phagocytes and translocate upon cell stimulation to the membrane to interact with flavocytochrome b_{558}. The interaction allows gp91^{phox} to transport electrons, leading to superoxide production. In this process, p47^{phox} plays a central role: p47^{phox} by itself moves to the membrane to interact directly with cytochrome b_{558}, whereas p67^{phox} is recruited via its association with p47^{phox} [6]. In contrast, Rac is independently targeted to the membrane [7,8] and thus participates in the oxidase assembly [9–13].

p47^{phox} harbours two SH3 (Src homology 3) domains, which are arranged in tandem [p47-(SH3)$_2$; see Figure 1A] and are capable of interacting with a PRR (proline-rich region) in the C-terminus of p22^{phox} [14–17]. This interaction is crucial for both membrane recruitment of p47^{phox} and oxidase activation. For example, substitution of Gln for Pro^{156} (P156Q) in the p22^{phox} PRR, a mutation found in a patient with CGD [18], abrogates the interaction with p47^{phox} and the translocation of p47^{phox} [14–17]. p47-(SH3)$_2$ are normally masked by an intramolecular association with the AIR (auto-inhibitory region) that exists between the SH3(C) (C-terminal SH3 domain) of p47^{phox} [p47-SH3(C)] and the PRR

Abbreviations used: AIR, auto-inhibitory region; CBB, Coomassie Brilliant Blue; CGD, chronic granulomatous disease; CHO, Chinese-hamster ovary; GST, glutathione S-transferase; MBP, maltose-binding protein; p47-F, full-length p47^{phox}; p47-ΔC, amino acid residues 1–286 of p47^{phox}; p47-(SH3)$_2$, tandem SH3 (Src homology 3) domains of p47^{phox}; PPII, polyproline II; PRR, proline-rich region; ROS, reactive oxygen species; SH3(C), C-terminal SH3 domain; SH3(N), N-terminal SH3 domain; SOD, superoxide dismutase.

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(see Figure 1A) [19,20]; via the PRR, p47^{phox} associates with p67^{phox} [21]. Upon cell stimulation, p47^{phox} undergoes phosphorylation on multiple serines in the AIR [22–27], an event that induces a conformational change of this protein to render the SH3 domains in a state accessible to the target p22^{phox} [19,26]. The SH3-mediated interaction between p47^{phox} and p22^{phox} is strictly regulated so that it functions as a switch of phagocyte oxidase activation.

With hundreds of SH3 domains in the human genome, binding selectivity is a key issue in understanding the molecular basis of SH3-domain interactions [28–32]. Typical SH3 domains interact directly with a PRR containing a conserved PxxP motif (x is any amino acid) in a PPII (polyproline type II) helix conformation. Because most SH3–PxxP interactions are of relatively low affinity, ligand peptides show high cross-reactivity with several SH3 domains [29–32]. To obtain high affinity and selectivity, SH3 domains are considered to usually make additional contacts with regions outside the proline-rich core, albeit there exist only a few known examples in which an SH3 domain simultaneously interacts with PxxP and extra-PxxP regions of a natural ligand [21,33–35].

It is known that the SH3(N) (N-terminal SH3 domain) of p47^{phox} [p47-SH3(N)] alone can bind directly to the ten-amino-acid proline-rich core of p22^{phox} (amino acids 151–160; PPSNPPPRPP), while p47-SH3(C) by itself fails to interact with p22^{phox} [17,36]. Consistent with the former observation, the amino acid substitution of Arg for Trp^{193} (W193R) in p47-SH3(N), the invariant residue among SH3 domains, results in a complete loss of the activity to support oxidase activation [17,36,37]. On the other hand, a mutant p47^{phox} carrying the corresponding W263R substitution in SH3(C) has a significant, but extremely weak, activity [36,37], suggesting that p47-SH3(C) also plays a role. Recent analysis of a crystal structure of p47-(SH3)$_2$ complexed with a proline-rich peptide of p22^{phox} [38] has revealed that the two SH3 domains bind to the PRR in a PPII helix conformation at the same time, thereby conferring high affinity and specificity, although the SH3(N) forms an intertwined dimer in the crystal. The proposed target recognition via the bis-SH3 domain appears to be a novel mode of improvement of affinity and selectivity in SH3-mediated interactions. However, no functional analysis has been performed and thus it has remained unknown whether the bis-SH3-mediated recognition indeed functions in activation of the phagocyte oxidase, especially *in vivo*.

In the present paper we demonstrate that the co-operativity between the two SH3 domains of p47^{phox}, which is expected from the bis-SH3-mediated recognition model, participates in both interaction with p22^{phox} and activation of the phagocyte oxidase. The present functional analysis identifies Pro^{152} and Arg^{158} in the p22^{phox} PRR, besides Pro^{156}, as residues crucial for this mode of recognition. Importantly, each of the three residues plays an indispensable role in the oxidase activation *in vivo*. Thus the bis-SH3-mediated interaction functions to activate the phagocyte oxidase. In addition to the PRR, we show that its C-terminally flanking region in p22^{phox} (amino acids 161–164), adopting an α-helix in the p47^{phox}–p22^{phox} complex in solution [39], is also involved in full activation of the phagocyte oxidase by fortifying the interaction with the p47^{phox} SH3 domains.

EXPERIMENTAL

Plasmid construction

The DNA fragments encoding the full-length of p47^{phox} (p47-F; amino acid residues 1–390), p47-ΔC (amino acid residues 1–286 of p47^{phox}), p47-(SH3)$_2$; (amino acid residues 151–286), SH3(N) (amino acid residues 151–219) and SH3(C) (amino acid residues 223–286) were amplified from a cloned cDNA encoding human p47^{phox} by PCR using specific primers [17,19]. Similarly, the DNA fragments that encode the full-length of p22^{phox} (amino acid residues 1–195), p22-(132–160), p22-(132–165), p22-(132–195), p22-(150–195) and p22-(151–160) were constructed from a cloned cDNA encoding human p22^{phox} by PCR using specific primers [17,19]. Mutations leading to the indicated amino acid substitutions were introduced by PCR-mediated site-directed mutagenesis. The cDNAs encoding p47-ΔC lacking amino acid residues 219–222 or 217–224, or those encoding p47-(SH3)$_2$ lacking amino acid residues 220–221, 219–222, 218–223, 217–224 or 216–225, or containing two additional serine residues at the positions between 220 and 221, were also prepared with PCR-mediated site-directed mutagenesis. All of the constructs were sequenced for confirmation of their identities.

In vitro pull-down binding assays using purified proteins

For expression in *Escherichia coli*, cDNA fragments were ligated to the following vectors: pGEX-2T (Amersham Biosciences) for GST (gluthathione S-transferase)-fusion protein; pMALc2 (New England BioLab) for MBP (maltose-binding protein)-fusion protein; and pProEX-HTb (Invitrogen) for His$_6$-tagged protein. GST-, MBP- or His$_6$-tagged proteins were expressed in *E. coli* strain DH5 and purified by glutathione–Sepharose-4B (Amersham Biosciences), amylose resin (New England BioLab) or His-bind resin (Novagen) respectively, according to the manufacturers' instructions. For *in vitro* pull-down binding assays, a pair of a GST-fusion proteins and an MBP-fusion protein, or a GST- and a His$_6$-tagged protein were mixed in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 and 1.4 mM KH_2PO_4, pH 7.4) containing 1 % Triton X-100 and incubated for 30 min at 4 °C. For the pull-down assay of a GST-fusion protein, a slurry of glutathione–Sepharose-4B beads was added to the mixture and then incubated for 30 min at 4 °C. After washing three times with PBS, GST-fusion and its interacting proteins were eluted from glutathione–Sepharose-4B beads by 20 mM Tris/HCl (pH 8.0) containing 10 mM glutathione. The eluates were subjected to SDS/10 % PAGE, followed by protein staining with CBB (Coomassie Brilliant Blue). To estimate the binding ratio of p22-(151–160) to p47-(SH3)$_2$, amounts of the stained proteins on the gel were quantified by the image analyser LAS1000 (Fuji).

Cell-free activation of the phagocyte NADPH oxidase

The membrane fraction of human neutrophils was prepared as previously described [9,17,19,26]. The membranes (0.43 μg/ml) were mixed with: 90 nM GST-fused full-length p67^{phox} (GST–p67-F); 90 nM His$_6$-tagged Rac2 carrying the Q61L substitution, namely His–Rac2 (Q61L) [9]; and the indicated concentration of GST–p47-ΔC or its mutant protein lacking amino acids 219–222 or 217–224. The mixture was incubated with 100 μM SDS for 2.5 min at 25 °C in 100 mM potassium phosphate (pH 7.0), containing 75 μM cytochrome *c*, 1.0 μM FAD, 1.0 mM EGTA, 1.0 mM $MgCl_2$, 100 μM GTPγS and 1.0 mM NaN_3, and the reaction was initiated by the addition of 1.0 mM NADPH. The NADPH-dependent superoxide production was measured by determining the rate of SOD (superoxide dismutase)-inhibitable ferricytochrome *c* reduction at 550–540 nm using a Hitachi 557 dual-wavelength spectrophotometer [9,17,19,26]. The superoxide-producing activity was represented as mol of superoxide produced/s per mol of cytochrome b_{558} haem; the haem content was calculated from the reduced absorption value minus the oxidized absorption value at 558 nm [40].

Whole-cell activation of the phagocyte NADPH oxidase in CHO (Chinese-hamster ovary) cells

Experiments for activation of the phagocyte NADPH oxidase in a whole-cell system were performed as described previously [41] with minor modifications. The cDNAs encoding the wild-type and mutant proteins of p22^{phox}, the wild-type p47^{phox}, and the wild-type p67^{phox} were ligated to the expression vector pEF-BOS [42], whereas the cDNA of the wild-type gp91^{phox} was ligated to pcDNA3.0 (Invitrogen). The CHO cells were transfected with pEF-BOS–p47^{phox}, pEF-BOS–p67^{phox}, pcDNA3.0–gp91^{phox} and pEF-BOS encoding various forms of p22^{phox}. After culture for 30 h at 37 °C, adherent cells were harvested by incubating with trypsin/EDTA for 1 min at 37 °C, and washed with Hepes-buffered saline (120 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 17 mM Hepes, pH 7.4).

For detection of p22^{phox}, CHO cells (1×10^5 cells) were lysed by sonication, and the sonicates were analysed by SDS/12 % PAGE. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore) and probed with anti-p22^{phox} antibodies (Santa Cruz Biotech). The blots were developed using ECL-plus (Amersham Biosciences) to visualize the antibodies. Expression of gp91^{phox} was estimated by using 7D5 (from Professor Michio Nakamura, Nagasaki University), a monoclonal antibody that recognizes gp91^{phox} functionally complexed with p22^{phox} [43,44], as described below. Transfected cells were fixed for 15 min at 25 °C in 3.7 % (w/v) formaldehyde. The fixed cells were washed four times with PBS and blocked with PBS containing 3 % (w/v) BSA for 60 min. The sample was subsequently incubated with 7D5 for 1 h at 25 °C, and probed with Alexa Flour®-488-labelled goat anti-(mouse IgG) antibodies (Molecular Probes) as secondary antibodies. Images were visualized with an LSM5 PASCAL confocal laser scanning microscope (Carl Zeiss).

Superoxide production by the cells was determined by SOD-inhibitable chemiluminescence with an enhancer-containing luminol-based detection system (DIOGENES; National Diagnostics) as previously described [9,27,41]. After the addition of the enhanced luminol-based substrate, the cells were stimulated with 200 ng/ml PMA. The chemiluminescence was assayed using a luminometer (Auto Lumat LB953; EG&G Berthold).

RESULTS

Role of the p47-(SH3)$_2$ in binding to the proline-rich core of p22^{phox}

Structural analysis has demonstrated that, in the p47-(SH3)$_2$–p22-PRR complex, the two SH3 domains of p47^{phox} sandwich the PRR of p22^{phox}, which probably confers high affinity and specificity [38]. According to this model, the two SH3 domains should function co-operatively in binding to p22^{phox} and probably in activation of the phagocyte oxidase. We first examined the co-operativity between the p47^{phox} SH3 domains in the interaction with p22^{phox} using a pull-down binding assay with purified proteins. As shown in Figure 1(B), MBP–p47-(SH3)$_2$ bound to the entire C-terminal cytoplasmic region of p22^{phox} (amino acids 132–195) expressed as a GST-fusion protein; it also interacted with the ten-amino-acid proline-rich core of p22^{phox} (amino acids 151–160; PPSNPPPRPP) but to a lesser extent. In contrast, MBP–p47-(SH3)$_2$ was not pulled down by GST alone (results not shown). MBP–p47-SH3(N) bound to both GST–p22-(132–195) and GST–p22-(151–160) much more weakly than MBP–p47-(SH3)$_2$ bound (Figure 1B), whereas p47-SH3(C) was incapable of interacting with p22^{phox} (Figure 1B). In addition, the W193R substitution in SH3(N), the invariant residue that is known to make a contact with a proline of SH3-target proteins, led to a completely impaired interaction with the proline-rich core of p22^{phox}, whereas the corresponding

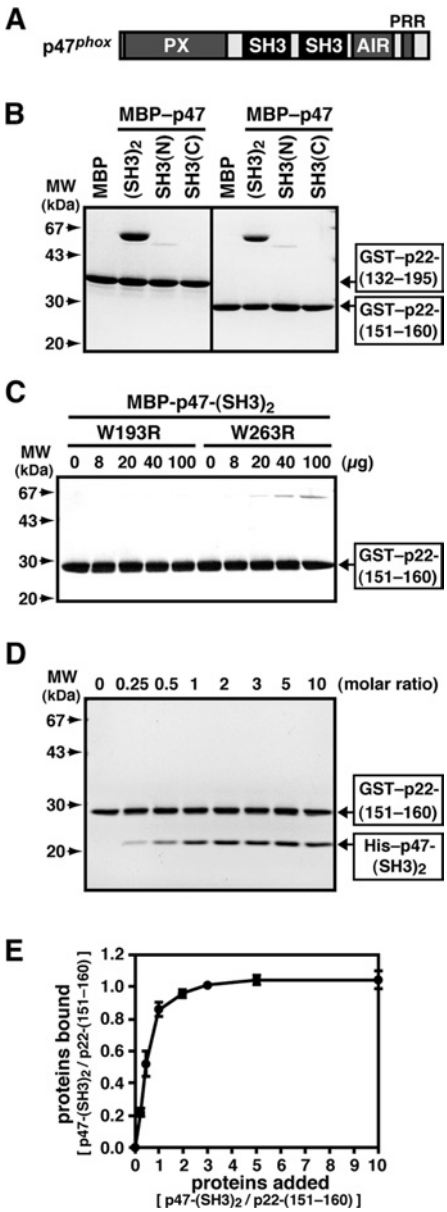

Figure 1 Interaction of the SH3 domains of p47^{phox} with the proline-rich core of p22^{phox}

The interaction of the SH3 domains of p47^{phox} with the p22^{phox} PRR was estimated by an *in vitro* pull-down assay using purified proteins. (**A**) A representation of the domain arrangement of p47^{phox}. PX, *phox* homology domain. (**B**) MBP alone, MBP–p47-(SH3)$_2$, MBP–p47-SH3(N), or MBP–p47-SH3(C) (8 μg) was incubated with 10 μg of GST–p22-(132–195) or GST–p22-(151–160) and separated by pull-down assay with gluthathione–Sepharose beads. The precipitated proteins were subjected to SDS/10 % PAGE, followed by staining with CBB. Molecular masses (MW) of marker proteins are indicated. (**C**) MBP–p47-(SH3)$_2$ carrying the W193R or W263R substitution (8 μg) was incubated with GST–p22-(151–160) (10 μg) and separated by pull-down assay with gluthathione–Sepharose beads. The precipitated proteins were subjected to SDS/10 % PAGE, followed by staining with CBB. Molecular masses (MW) of marker proteins are indicated. (**D**) GST–p22-(151–160) (100 nM) was incubated with His–p47-(SH3)$_2$ at the indicated molar ratio. The proteins were precipitated with glutathione–Sepharose beads and eluted from the beads with gluthathione. The eluates were subjected to SDS/10 % PAGE and stained with CBB. Molecular masses (MW) of marker proteins are indicated. (**E**) Intensities of the bands on the gel in (**D**) were quantified by the image analyser. The experiments were repeated three times with similar results.

substitution in SH3(C) (W263R) led to a severe but incomplete loss of the binding to p22^{phox} (Figure 1C). Thus, under the present experimental conditions, p47-SH3(N) primarily binds to the

ten-amino-acid proline-rich core of p22^{phox}, and p47-SH3(C) supports the interaction.

To confirm that the two SH3 domains of p47^{phox} bind to the single proline-rich core of p22^{phox} at the same time, we accessed the stoichiometry of the interaction by a pull-down assay using purified His_6-tagged p47-$(SH3)_2$ and GST-fused p22^{phox} (Figure 1D). As shown in Figure 1(E), increasing the concentration of His–p47-$(SH3)_2$ in a solution containing 100 nM GST–p22-(151–160) yielded a profile exhibiting a saturating plateau, consistent with the formation of a complex of 1:1 stoichiometry. The present experimental conditions thus allow the two SH3 domains of p47^{phox} to bind simultaneously to the ten-amino-acid proline-rich core of p22^{phox}. From the binding profile (Figure 1E), the K_D value was calculated at 0.2 μM, which is in good agreement with the values obtained by other methods such as isothermal titration calorimetry and a binding assay using a fluorescein-labelled p22^{phox} peptide [38]. We also tried to estimate the K_D value for the p47-SH3(N)–p22^{phox} interaction using the pull-down assay. Although the increase in the concentration of His–p47-SH3(N) resulted in an elevated binding to GST–p22-(151–160), the binding was not saturated even under the conditions where 50 times higher concentrations of His–p47-SH3(N) were used (results not shown); its K_D value was calculated at more than 5 μM, which agrees with results obtained by other methods [38].

Effect of truncation of the linker region between the p47-$(SH3)_2$ in the interaction with p22^{phox}

To study further the co-operativity between the p47-$(SH3)_2$ in the interaction with p22^{phox}, we prepared mutant p47^{phox} proteins with various lengths of the linker region between the domains (amino acids 216–225; PDETEDPEPN) as MBP-fusions (Figure 2A) and investigated their activity to bind to p22^{phox}. As shown in Figure 2(B), a mutant p47^{phox} containing two additional serine residues at the position between 220 and 221 was as active as the wild-type. Similarly, a mutant protein lacking the two amino acids Glu^{220} and Asp^{221}, Δ(220–221), fully bound to p22^{phox} (Figure 2B). Further deletion in the linker region led to an impaired interaction with p22^{phox}: mutant p47^{phox} proteins without four and six amino acids, Δ(219–222) and Δ(218–223) respectively, associated with p22^{phox}, but to a slightly lesser extent than did the wild-type p47-$(SH3)_2$. On the other hand, a mutant protein lacking eight or ten amino acids, Δ(217–224) and Δ(216–225) respectively, only weakly bound to p22^{phox}, to an extent similar to that of p47-SH3(N) (Figure 2B). This shortening of the linker region prevents the tandem SH3 domains from simultaneously recognizing p22^{phox}.

Effect of truncation of the linker region between the p47-$(SH3)_2$ in activation of the phagocyte NADPH oxidase

To clarify the role of the co-operativity between the p47-$(SH3)_2$ in phagocyte oxidase activation, we next tested the activity of linker-deleted mutant p47^{phox} proteins to support superoxide production in a cell-free system, which was reconstituted with human neutrophil membranes rich in cytochrome b_{558} and the recombinant proteins p67^{phox} and Rac2 (Figure 3A). As shown in Figure 3(B), a mutant p47^{phox} lacking four amino acids, Δ(219–222), was capable of supporting superoxide production but to a lesser extent than the wild-type p47^{phox}. Furthermore, deletion of eight amino acids in the linker, Δ(217–224), resulted in a severe loss of superoxide production (Figure 3B). The effect of the deletions on oxidase activation (Figure 3B) is well correlated with that on the ability to bind to p22^{phox} (Figure 2B). Shortening of the linker region between the tandem SH3 domains prevents them from sandwiching p22^{phox}, which leads to defective activation of

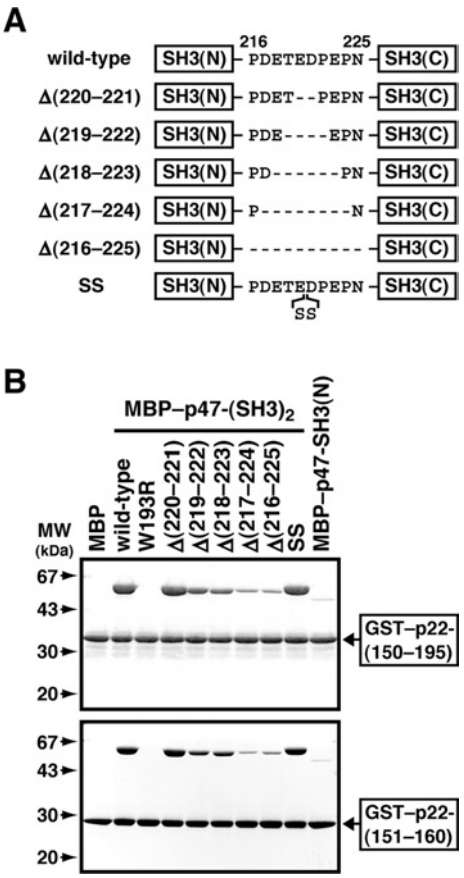

Figure 2 Role of the linker region between the SH3 domains of p47^{phox} in the binding to p22^{phox}

(**A**) Representation of the mutant p47^{phox} proteins used in the present study. They have various lengths of the linker region between the SH3(N) and SH3(C). (**B**) MBP alone, the wild-type p47-$(SH3)_2$ fused to MBP, or mutant MBP–p47-$(SH3)_2$ (8 μg) with the indicated length of the linker was incubated with 10 μg of GST–p22-(150–195) or GST–p22-(151–160) and separated by pull-down assay with gluthathione–Sepharose beads. The precipitated proteins were subjected to SDS/10 % PAGE followed by staining with CBB. Molecular masses (MW) of marker proteins are indicated. The experiments were repeated three times with similar results.

the phagocyte oxidase. Thus oxidase activation requires the bis-SH3-mediated recognition, in which the p47^{phox} SH3 domains co-operatively bind to the p22^{phox} PRR.

Role of each individual amino acid residue of the p22^{phox} PRR in the interaction with the p47^{phox} SH3 domains

Whereas structural analysis of p47-$(SH3)_2$–p22-PRR complexes revealed a number of contacts between p47-$(SH3)_2$ and residues of the proline-rich core of p22^{phox} (amino acids 151–160; PPSNPPPRPP) [38], functional analysis has been expected to provide information on relative contribution of each individual residue in the p22^{phox} PRR to the interaction with p47^{phox}. To obtain such information, we prepared a series of mutant proteins carrying the substitution of alanine for one of the residues and tested their activity to bind to p47-$(SH3)_2$. As shown in Figure 4(A), a mutant p22^{phox} with the alanine substitution for Pro^{152}, Pro^{156} or Arg^{158} was incapable of interacting with p47-$(SH3)_2$, indicative of crucial roles of the three residues. Whereas the K_D value for the wild-type p22^{phox} binding to p47-$(SH3)_2$ was estimated at 0.2 μM from the present data as described above (Figure 1E), similar experiments revealed that K_D values for mutant proteins carrying the P152A, P156Q or R158A substitution were more than 5 μM (results not

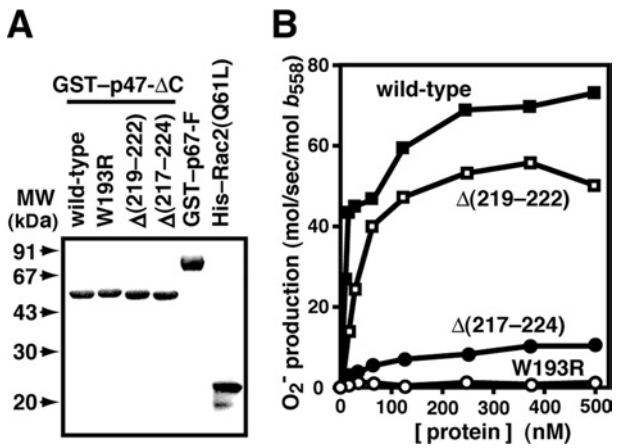

Figure 3 Role of the linker region between the two SH3 domains of p47^{phox} in cell-free activation of the phagocyte NADPH oxidase

(**A**) SDS/10 % PAGE analysis of purified GST-fusion proteins that were used in a cell-free system for activation of the phagocyte NADPH oxidase: wild-type p47-ΔC (amino acid residues 1–286); W193R, p47-ΔC carrying the W193R substitution; Δ(219–222), p47-ΔC lacking amino acid residues 219–222; Δ(217–224), p47-ΔC lacking amino acid residues 217–224; GST–p67-F, the full-length p67^{phox} fused to GST; and His–Rac2(Q61L), His_6-tagged Rac2 carrying the Q61L substitution. The proteins were subjected to SDS/10 % PAGE and stained with CBB. Molecular masses (MW) of marker proteins are indicated. (**B**) Various p47^{phox} proteins at the indicated concentrations were mixed with human neutrophil membranes (0.43 μg/ml), His–Rac2(Q61L) (90 nM), and GST–p67-F (90 nM) in the presence of FAD (1.0 μM) and GTPγS (100 μM), followed by incubation with 100 μM SDS for 2.5 min at 25 °C. The superoxide production was initiated by the addition of NADPH (1.0 mM) to the reaction mixture. The NADPH-dependent superoxide-generating activity was represented as mol of superoxide produced/s per mol of cytochrome b_{558} haem, as described in the Experimental section.

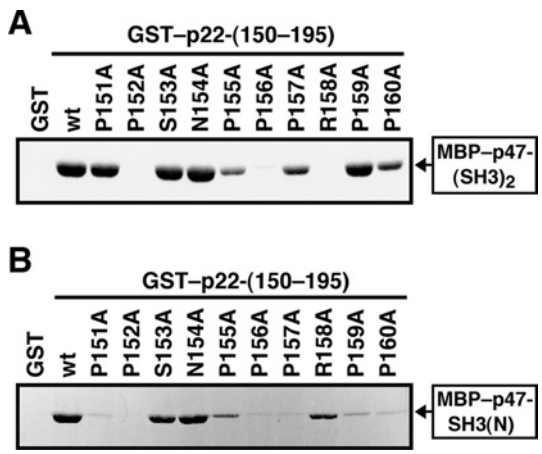

Figure 4 Roles of residues of the p22^{phox} proline-rich core in the interaction with p47^{phox}

(**A**) MBP–p47-$(SH3)_2$ (10 μg) was incubated with 8 μg of GST–p22-(150–195) wild-type (wt) or carrying the indicated amino acid substitution. The proteins were precipitated with glutathione–Sepharose beads and eluted from the beads with gluthathione. The eluates were subjected to SDS/10 % PAGE and stained with CBB. The experiments were repeated three times with similar results. (**B**) MBP–p47-SH3(N) (80 μg) was incubated with 20 μg of GST–p22-(150–195) wild type (wt) or carrying the indicated amino acid substitution. The proteins were precipitated with glutathione–Sepharose beads and eluted from the beads with gluthathione. The eluates were subjected to SDS/10 % PAGE and stained with CBB. The experiments were repeated three times with similar results.

shown). In addition, the substitution for Pro^{155}, Pro^{157} or Pro^{160} resulted in a partial loss of the binding activity. On the other hand, the replacement of Ser^{151}, Ser^{153}, Asn^{154} or Pro^{159} did not affect the interaction (Figure 4A), indicating that the side chains of these residues do not play a major role.

To know which SH3 domain of p47^{phox} recognizes the individual residues in the p22^{phox} PRR, we tested the effect of the substitutions in p22^{phox} on the interaction with p47-SH3(N). The binding of p22^{phox} to p47-SH3(N) was completely abolished by the substitution for Pro^{152}, and strongly reduced by that for Pro^{151}, Pro^{156}, Pro^{157}, Pro^{159} or Pro^{160} (Figure 4B). On the other hand, the replacement of Ser^{153}, Asn^{154} or Arg^{158} exhibited a small effect on the interaction with p47-SH3(N) (Figure 4B).

These findings suggest that, among the three residues crucial in the interaction with p47^{phox}, Pro^{152}/Pro^{156} and Arg^{158} are probably recognized in a distinct manner: Pro^{152} and Pro^{156} appear to be recognized mainly by p47-SH3(N), because the substitution for either proline residue led to a drastic loss of the interaction not only with p47-$(SH3)_2$ but also with p47-SH3(N); Arg^{158} probably makes a direct contact with p47-SH3(C), since the R158A substitution resulted in a completely impaired interaction with p47-$(SH3)_2$ but not with p47-SH3(N) (for details, see the Discussion). Among the residues that play a significant but minor role in the interaction with p47^{phox} (Figure 4B), Pro^{157} and Pro^{160} probably associate with p47-SH3(N), whereas Pro^{155} appears to bind mainly to p47-SH3(C) (Figure 4B).

Role of Pro^{152}, Pro^{156} and Arg^{158} in activation of the phagocyte NADPH oxidase

It is well established that gp91^{phox} tightly associates with p22^{phox} in phagocytic membranes, the association being essential for the stabilization of both proteins: one protein can not exist in the absence of the other [1–5]. To investigate the role of Pro^{152}, Pro^{156} and Arg^{158} in p22^{phox} activation of the phagocyte NADPH oxidase *in vivo*, we used the CHO cells to functionally reconstitute the phagocyte oxidase system by expressing the membranous and cytosolic oxidase factors [41]. Although the mRNA of p22^{phox} exists in a wide variety of cells, CHO cells scarcely express the message [41]. The phagocyte oxidase reconstitution in CHO cells, therefore, is totally dependent on ectopic expression of p22^{phox} [41].

We transfected CHO cells with cDNA encoding the wild-type p22^{phox} or a mutant protein with the P152A, P156Q or R158A substitution, and found that these proteins were expressed at essentially the same level (Figure 5A). The mutant proteins, as well as the wild-type one, seem to associate properly with gp91^{phox}: at the plasma membrane of the cells transfected with the wild-type or mutant p22^{phox} cDNA, gp91^{phox} was detected by 7D5, a monoclonal antibody that recognizes gp91^{phox} functionally complexed with p22^{phox} [43,44]. In contrast, the gp91^{phox} protein was not detected without expression of p22^{phox} (Figure 5B). Thus these mutant p22^{phox} proteins probably retain the conformational integrity. When the wild-type p22^{phox} was co-expressed with gp91^{phox}, p47^{phox} and p67^{phox} in CHO cells, superoxide was produced by the cells in response to PMA (Figure 5C). On the other hand, the superoxide production was not observed in the cells expressing a p22^{phox} carrying the P156Q substitution (Figure 5D), a mutation that occurs in a patient with CGD [18]. Similarly, the P152A or R158A substitution resulted in a complete loss of the superoxide-producing activity (Figures 5C and 5D). These findings clearly show that Pro^{152} and Arg^{158} in the proline-rich core of p22^{phox} participate in binding to p47^{phox} in intact cells, thereby playing a crucial role in activation of the phagocyte NADPH oxidase.

Role of the α-helical region C-terminal to the PRR in the interaction with p47-$(SH3)_2$

We next investigated the role of regions outside of the PRR of p22^{phox} in the interaction with p47^{phox}. The region N-terminal to

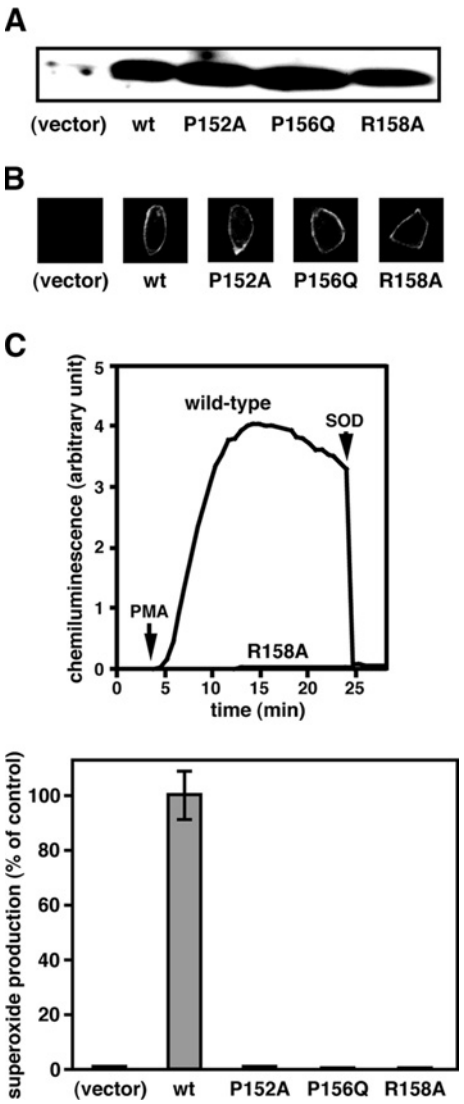

Figure 5 Role of Pro^{152} and Arg^{158} of p22^{phox} in whole-cell activation of the phagocyte NADPH oxidase

(**A**) CHO cells were transfected simultaneously with pcDNA3.0–gp91^{phox}, pEF-BOS–p47^{phox}, pEF-BOS–p67^{phox}, and pEF-BOS encoding the wild-type p22^{phox} (wt) or a mutant p22^{phox} carrying the P152A, P156Q or R158A substitution. Lysates of the cells transfected with the indicated form of p22^{phox} were subjected to SDS/12 % PAGE, followed by immunoblot with anti-p22^{phox} antibodies. See the Experimental section for details. These experiments were repeated more than three times with similar results. (**B**) The cells transfected with cDNA for the wild-type p22^{phox} (wt) or a mutant p22^{phox} carrying the P152A, P156Q or R158A substitution were fixed and stained with the anti-gp91^{phox} monoclonal antibody 7D5. The experiments were repeated more than three times with similar results. (**C**) The cells expressing the wild-type p22^{phox} or a mutant p22^{phox} carrying the R158A substitution (1×10^5 cells) were incubated for 5 min at 37 °C and then stimulated with PMA (200 ng/ml). Upper panel: chemiluminescence change was continuously monitored with DIOGENES, and SOD (50 μg/ml) was added where indicated (see the Experimental section for details). The experiments were repeated more than three times with similar results. Lower panel: the amount of superoxide produced by the cells expressing the indicated form of p22^{phox} was expressed as the percent activity relative to control cells transfected with pcDNA3.0–gp91^{phox}, pEF-BOS–p47^{phox}, pEF-BOS–p67^{phox} and pEF-BOS encoding the wild-type p22^{phox}. Values are means ± S.D. for three independent transfections.

the PRR does not seem to be involved in the interaction, because GST–p22-(150–195) bound to p47-(SH3)$_2$ to the same extent as GST–p22-(132–195) (Figure 6A). On the other hand, the addition of the C-terminal five amino acids (161–165) enhanced the interaction with p47-(SH3)$_2$ (Figure 6B), a finding that is consistent with a previous observation that the p22^{phox} peptide

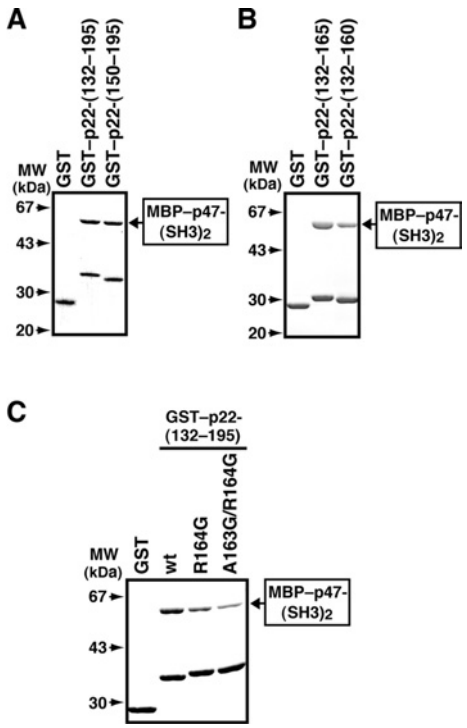

Figure 6 Role of a region C-terminal to the p22^{phox} PRR in the interaction with the p47^{phox} SH3 domains

(**A**) MBP–p47-(SH3)$_2$ (10 μg) was incubated with 8 μg of GST alone, GST–p22-(132–195) or GST–p22-(150–195). The proteins were precipitated with glutathione–Sepharose beads and eluted from the beads with gluthathione. The eluates were subjected to SDS/10 % PAGE and stained with CBB. The experiments were repeated three times with similar results. (**B**) MBP–p47-(SH3)$_2$ (10 μg) was incubated with 8 μg of GST alone, GST–p22-(132–160) or GST–p22-(132–165). The proteins were precipitated with glutathione–Sepharose beads and eluted from the beads with glutathione. The eluates were subjected to SDS/10 % PAGE and stained with CBB. The experiments were repeated three times with similar results. (**C**) MBP–p47-(SH3)$_2$ (10 μg) was incubated with 1.5 μg of GST alone or GST-fused protein of the p22^{phox} C-terminal region (132–195): the wild-type p22^{phox} (wt) or a mutant p22^{phox} carrying the R164G or A163G/R164G substitution. The proteins were precipitated with glutathione–Sepharose beads and eluted from the beads with gluthathione. The eluates were subjected to SDS/10 % PAGE and stained with CBB. The experiments were repeated three times with similar results. Molecular masses (MW) of marker proteins are indicated.

consisting of amino acids 151–165 binds to p47^{phox} more strongly than the peptide of 149–163 [45].

The region of amino acids 161–164 of p22^{phox} (AEAR) adopts an α-helical structure in a solution structure of p47-(SH3)$_2$ complexed with a p22^{phox} peptide, as revealed by our NMR analysis [39]. To investigate the role of the α-helix in the interaction with p47-(SH3)$_2$, we replaced Arg^{164} with Gly, a residue known to destabilize α-helical structures. As shown in Figure 6(C), the R164G substitution led to a decreased binding to p47-(SH3)$_2$. The binding was further impaired by the additional replacement of Ala^{163} with Gly (A163G/R164G) (Figure 6C). Thus α-helical conformation of the region C-terminal to the p22^{phox} PRR (amino acids 161–164) is likely to be involved in full interaction with p47-(SH3)$_2$.

Role of the α-helical region C-terminal to the p22^{phox} PRR in oxidase activation

To understand the role of the p22^{phox} α-helix of amino acids 161–164 in activation of the phagocyte NADPH oxidase, we transfected CHO cells with cDNA that encodes the wild-type p22^{phox} or a mutant protein carrying the R164G or A163G/R164G

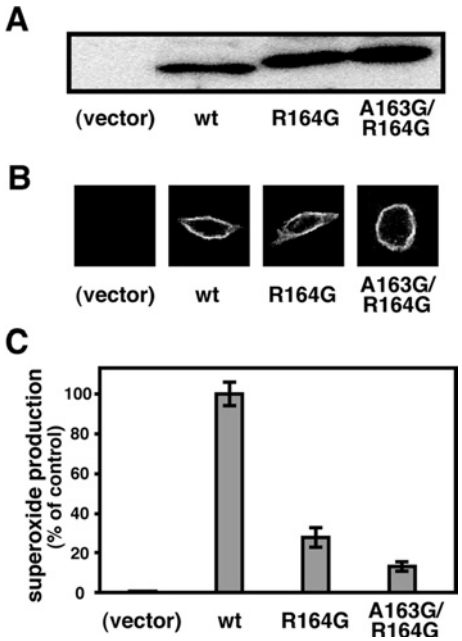

Figure 7 Role of an α-helix C-terminal to the p22^{phox} PRR in whole-cell activation of the phagocyte NADPH oxidase

(**A**) CHO cells were transfected simultaneously with pcDNA3.0–gp91^{phox}, pEF-BOS–p47^{phox}, pEF-BOS–p67^{phox}, and pEF-BOS encoding the wild-type p22^{phox} (wt) or a mutant p22^{phox} carrying the R164G or A163G/R164G substitution. Lysates of the cells transfected with the indicated form of p22^{phox} were subjected to SDS/12% PAGE, followed by immunoblot with anti-p22^{phox} antibodies (see the Experimental section for details). The experiments were repeated more than three times with similar results. (**B**) Cells transfected with cDNA for the wild-type p22^{phox} (wt) or a mutant p22^{phox} carrying the R164G or A163G/R164G substitution were fixed and stained with the anti-gp91^{phox} monoclonal antibody 7D5. The experiments were repeated more than three times with similar results. (**C**) Cells transfected with cDNA for the wild-type p22^{phox} (wt) or a mutant p22^{phox} carrying the R164G or A163G/R164G substitution were incubated for 5 min at 37 °C and then stimulated with PMA (200 ng/ml). Chemiluminescence change was continuously monitored with DIOGENES. The amount of superoxide by the cells expressing the indicated form of p22^{phox} was expressed as the percentage activity relative to control cells transfected with pcDNA3.0–gp91^{phox}, pEF-BOS–p47^{phox}, pEF-BOS–p67^{phox} and pEF-BOS encoding the wild-type p22^{phox}. Values are means ± S.D. for three independent transfections.

substitution. These mutant p22^{phox} proteins were expressed to the same extent as the wild-type one at the protein level (Figure 7A) and formed a proper complex with gp91^{phox} at the plasma membrane (Figure 7B). As shown in Figure 7(C), CHO cells expressing a mutant p22^{phox} with the R164G substitution produced a small amount of superoxide, compared with that produced by cells expressing the wild-type p22^{phox}. In addition, the double substitution A163G/R164G led to a further decrease in superoxide production (Figure 7C). These findings indicate that the α-helix C-terminal to the core PRR of p22^{phox} participates in full activation of the phagocyte NADPH oxidase.

DISCUSSION

In the present study, we showed that activation of the phagocyte NADPH oxidase required co-operative binding of the two SH3 domains of p47^{phox} to the ten-amino-acid PRR of p22^{phox} (amino acids 151–160). The target recognition by the bis-SH3 domain was proposed originally on the basis of a crystal structure of p47-(SH3)$_2$ in complex with a p22^{phox}-derived peptide: the tandem SH3 domains share an interface, which gives rise to a shallow groove that constitutes the peptide-binding surface and is lined with residues of the conserved SH3 domain ligand-binding surfaces [38]. Although p47-(SH3)$_2$ exists as an intertwined dimer in the crystal [38], this target-recognition model is supported by the present functional analysis using various mutant proteins of p47^{phox} and p22^{phox} (Figures 1–5; and see below). Furthermore, we also demonstrated that α-helical conformation of the region C-terminal to the p22^{phox} PRR (amino acids 161–164) is involved in full activation of the phagocyte oxidase (Figures 6 and 7).

The conclusion that the co-operation between the two SH3 domains in binding to p22^{phox} plays a crucial role in phagocyte oxidase activation is drawn from several lines of evidence obtained in the present study. First, deletion of the flexible linker connecting the SH3 domains of p47^{phox} led to a drastic decrease in both the interaction with p22^{phox} and oxidase activation (Figures 2 and 3). The shortening of distance between the SH3 domains probably prevents their ligand-binding surfaces from being arranged such that they can make contacts with the p22^{phox} PRR at the same time. Secondly, the co-operative role of p47-SH3(C) is also supported by the finding that the interaction of p47-(SH3)$_2$ with the p22^{phox} PRR is profoundly suppressed by W263R substitution in the SH3(C) (Figure 1), a mutation which is known to result in a significant loss of the ability to support activation of the NADPH oxidase [36,37]. The significance of p47-SH3(C) is consistent with the finding that a p47^{phox} peptide encompassing Trp^{263} (amino acids 253–267) moderately inhibits cell-free activation of the oxidase [46]. Finally, oxidase activation is abolished when alanine is substituted not only for the p47-SH3(N)-interacting residue Pro^{152} or Pro^{156} in the p22^{phox} PRR, but also for Arg^{158}, a residue that makes a close contact with p47-SH3(C) but not with p47-SH3(N) (Figure 4; and see below).

A number of direct contacts between residues of the p22^{phox} PRR and those of the p47^{phox} SH3 domains have been revealed by analysis of the crystal structure of p47-(SH3)$_2$–p22^{phox}-PRR complexes [38]. However, it remained unknown to what extent each individual residue in the p22^{phox} PRR contributed to the interaction with p47^{phox} and oxidase activation, except that replacement of Pro^{156} by glutamine, a mutation found in a patient with CGD [18], resulted in a complete loss of the interaction [14,15]. The present analysis using alanine-scanning mutagenesis showed the contribution of each individual residue in the p22^{phox} PRR. In addition to Pro^{156}, Pro^{152} and Arg^{158} are essential for binding to p47-(SH3)$_2$, whereas Pro^{155}, Pro^{157} and Pro^{160} play a modest role (Figure 4). In contrast, the side chains of Ser^{153} and Asn^{154} do not appear to be involved, since mutations of these residues to alanine have no effect (Figure 4). These findings agreed with those provided by the structural analysis of the p47^{phox}–p22^{phox} complexes [38], in which both Ser^{153} and Asn^{154} point away from the binding surfaces of the p47^{phox} SH3 domains, making no stabilizing contacts. Although Pro^{151} and Pro^{159} probably bind to p47-SH3(N) as indicated by the structural analysis [38] and the present binding assay (Figure 4B), these residues do not seem to make a major contribution to the interaction with p47-(SH3)$_2$ (Figure 4A). Among the three essential residues, Pro^{152} and Pro^{156} appear to make a direct contact with p47-SH3(N) whereas Arg^{158} associates with p47-SH3(C) (Figure 8): the alanine substitution for Pro^{152} or Pro^{156} abrogates the binding to p47-SH3(N) (Figure 4B) as well as that to p47-(SH3)$_2$ (Figure 4A); on the other hand, the substitution for Arg^{158} affects the interaction with p47-SH3(N) to a much lesser extent (Figure 4B). Consistent with these findings, Pro^{152} and Pro^{156} of p22^{phox} in the complex with p47^{phox} engage in van der Waals interactions with Trp^{193} of p47-SH3(N), whereas Arg^{158} makes salt bridges with Asp^{243} and Glu^{244} on p47-SH3(C) [38]. The present study further demonstrates that the substitutions for Pro^{152} and for Arg^{158}, as well as those for Pro^{156}, completely abrogate *in vivo* activation of the phagocyte NADPH oxidase without affecting the protein integrity (Figure 5). Taken together

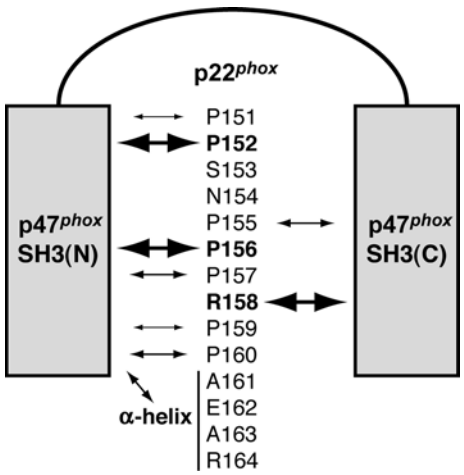

Figure 8 A model for interaction of the p47^{phox} bis-SH3 domain with p22^{phox}, which is essential for activation of the phagocyte NADPH oxidase

The p47^{phox} bis-SH3 domain, comprising SH3(N) and SH3(C), binds to the PRR (amino acids 151–160) and its C-terminal α-helical region (amino acids 161–164) of p22^{phox}. Among residues in p22^{phox}, Pro^{152}, Pro^{156} and Arg^{158} are essential for the interaction with p47^{phox} (as indicated by the bold text and largest arrows) and activation of the phagocyte NADPH oxidase: Pro^{152} and Pro^{156} make a direct contact with p47-SH3(N), whereas Arg^{158} associates with p47-SH3(C). Although Pro^{155}, Pro^{157} and Pro^{160} play a modest role (indicated by medium-sized arrows), Ser^{153} and Asn^{154} are not involved in the interaction with the p47^{phox} SH3 domains. The α-helix C-terminal to the p22^{phox} PRR binds to p47-SH3(N). This association also participates in oxidase activation.

with the above-mentioned co-operation between the two SH3 domains of p47^{phox}, we conclude that the bis-SH3-mediated target recognition is crucial for the oxidase activation both *in vivo* and *in vitro*.

In the present paper we also provide evidence that the C-terminal extension of the p22^{phox} PRR plays an important role in activation of the phagocyte NADPH oxidase. The extended region of amino acids 161–164 adopts an α-helical conformation in the complex of p47-$(SH3)_2$ with the p22^{phox}-derived peptide of amino acids 149–168 in solution, as shown in our NMR analysis [39]. On the other hand, the structure of this region is not determined in the crystal structure of p47-$(SH3)_2$ that is complexed with the p22^{phox} peptide of amino acids 149–166 [38]. Although the reason for this is presently unclear, it may be due to the difference in conformation between the solution and the crystal or the difference in length between the p22^{phox}-derived peptides used. The α-helix C-terminal to the PRR in p22^{phox} appears to participate in binding to p47-$(SH3)_2$ via hydrophobic interactions with p47-SH3(N) [39]. The interactions are considered to be important because comparison of the p47^{phox}-binding activity between p22-(132–160) and p22-(132–165) reveals that the addition of the α-helical region to the PRR increases the affinity for p47-$(SH3)_2$ (Figure 6), which is in agreement with a previous observation that the p22^{phox} peptide consisting of amino acids 151–165 binds to p47^{phox} more strongly than the peptide of 149–163 [45]. Replacement of Ala^{163} and Arg^{164} with glycine, a residue that is expected to destabilize α-helical structures, results in an incomplete but significant loss of the ability to bind to p47^{phox} (Figure 6) and to support superoxide production (Figure 7). These mutations, however, do not seem to affect the protein integrity of p22^{phox}: these mutant proteins expressed ectopically exist in cells to the same extent as the wild-type one (Figure 7A), and are targeted to the plasma membrane to form a properly arranged complex with gp91^{phox} (Figure 7B). Thus the α-helical structure of the region adjacent to the PRR of p22^{phox} is required for full activation of the phagocyte NADPH oxidase.

In general, the interaction of an SH3 domain with its proline-rich target in a PPII helix conformation tends to be fairly weak, with typical K_Ds in the μM range [29–32], whereas affinity and specificity can be enhanced by additional contacts outside the proline-rich core forming the PPII helix, as reported in some SH3-mediated interactions [21,33–35]. By contrast, in the interaction between p47^{phox} and p22^{phox}, the high affinity and high specificity are largely conferred by co-operative binding of the two SH3 domains of p47^{phox} to the single PRR of p22^{phox}, which plays a crucial role in activation of the phagocyte NADPH oxidase, as shown in the present study (Figure 8). Similar co-operativity between the p47^{phox} SH3 domains also participates in keeping them in an inactive state via an intramolecular interaction with AIR, which lies C-terminal to the tandem SH3 domains [19,38,47,48]. Besides the bis-SH3-mediated recognition with the proline-rich core, the interaction of p47^{phox} with p22^{phox} is further facilitated by a contact of SH3(N) with an α-helix, immediately adjacent to the PPII region; this additional contact contributes to full activation of the phagocyte oxidase (Figure 8). It is presently obscure how this highly specific, strong SH3-mediated interaction leads to triggering of electron transfer by gp91^{phox}, a complete electron-transporting apparatus containing NADPH-, FAD-, and haem-binding sites [1–5]. Once the active oxidase complex is formed, gp91^{phox} transfers electrons from NADPH to molecular oxygen, thereby generating superoxide [12,49–52]. The strong binding to p22^{phox} may allow p47^{phox} to stably interact with gp91^{phox}, the partner of p22^{phox} in flavocytochrome b_{558}, which might facilitate electron flow. Alternatively, because p47^{phox} associates with p67^{phox} via a tail-to-tail interaction [21], p47^{phox} in activated cells may serve solely as an adaptor molecule that forms a bridge between p67^{phox} and p22^{phox}, thereby stabilizing the attachment of p67^{phox} to gp91^{phox}, an event that is considered to induce electron transport [10,12,52]. Elucidation of the precise architecture of the membrane-assembled, active oxidase complex consisting of gp91^{phox}, p22^{phox}, p47^{phox}, p67^{phox} and Rac awaits further studies.

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