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The adaptor protein p40^{phox} as a positive regulator of the superoxide-producing phagocyte oxidase

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Activation of the superoxide-producing phagocyte NADPH oxidase, crucial in host defense, requires the cytosolic proteins p67^{phox} and p47^{phox}. They translocate to the membrane upon cell stimulation and activate flavocytochrome b_{558}, the membrane-integrated catalytic core of this enzyme system. The activators p67^{phox} and p47^{phox} form a ternary complex together with p40^{phox}, an adaptor protein with unknown function, comprising the PX/PB2, SH3 and PC motif-containing domains: p40^{phox} associates with p67^{phox} via binding of the p40^{phox} PC motif to the p67^{phox} PB1 domain, while p47^{phox} directly interacts with p67^{phox} but not with p40^{phox}. Here we show that p40^{phox} enhances membrane translocation of p67^{phox} and p47^{phox} in stimulated cells, which leads to facilitated production of superoxide. The enhancement cannot be elicited by a mutant p40^{phox} carrying the D289A substitution in PC or a p67^{phox} with the K355A substitution in PB1, each being defective in binding to its respective partner. Thus p40^{phox} participates in activation of the phagocyte oxidase by regulating membrane recruitment of p67^{phox} and p47^{phox} via the PB1–PC interaction with p67^{phox}.

Keywords: NADPH oxidase/p40^{phox}/p67^{phox}/PB1 domain/PC motif

Introduction

Professional phagocytes, including neutrophils and macrophages, play a crucial role in host defense against microbial infection. During phagocytosis of invading microorganisms or upon cell stimulation with soluble agents, phagocytes produce superoxide (O_2^-), a precursor of microbicidal oxidants, by catalysis of an activated NADPH oxidase (Sumimoto *et al*., 1997; Babior, 1999; Clark, 1999; Nauseef, 1999). The soluble stimulants include phorbol myristate acetate (PMA) and chemoattractants, the latter of which bind to their own receptors to activate the oxidase via the trimeric GTPase G_i (Bokoch, 1995). The significance of the oxidase in host defense is exemplified by recurrent and life-threatening infections that occur in patients with chronic granulomatous disease (CGD), whose phagocytes are defective in the superoxide-producing activity (Roos *et al*., 1996).

The redox core of the phagocyte NADPH oxidase is a membrane-spanning flavocytochrome b_{558}, comprising the two subunits gp91^{phox} and p22^{phox}. The cytochrome is catalytically inactive in resting cells but acquires the capacity to transfer electrons from NADPH to molecular oxygen upon cell stimulation, leading to superoxide production (Roos *et al*., 1996; Babior, 1999). The activation of cytochrome b_{558} requires stimulus-induced membrane translocation of cytosolic proteins, namely the small GTPases Rac and two SH3 domain-harboring oxidase factors p47^{phox} and p67^{phox} (Sumimoto *et al*., 1997; Babior, 1999; Clark, 1999; Nauseef, 1999). In CGD neutrophils lacking cytochrome b_{558}, neither p47^{phox} nor p67^{phox} can be recruited to the membrane upon cell stimulation (Heyworth *et al*., 1991). In p47^{phox}-deficient phagocytes, membrane targeting of p67^{phox} does not occur, whereas p47^{phox} is independently targeted in p67^{phox}-defective cells (Heyworth *et al*., 1991; Dusi *et al*., 1996). Thus p47^{phox} appears to directly interact with cytochrome b_{558} and recruits p67^{phox} to the membrane. Indeed the SH3 domains of p47^{phox} bind to a proline-rich region (PRR) of p22^{phox}, the small subunit of cytochrome b_{558} (Leto *et al*., 1994; Sumimoto *et al*., 1994). Although the SH3 domains are normally masked via an intramolecular interaction, the conformation of p47^{phox} becomes changed upon cell stimulation to render the domains in a state accessible to p22^{phox}, which leads to the activation of the NADPH oxidase (Sumimoto *et al*., 1994; Ago *et al*., 1999). The P156Q substitution in the p22^{phox} PRR, a mutation that has occurred in a case of CGD (Dinauer *et al*., 1991), results in not only impaired interaction between p22^{phox} and p47^{phox} *in vitro* (Sumimoto *et al*., 1994), but also defective translocation of p47^{phox} to the membrane *in vivo* (Leusen *et al*., 1994). It is also known that p67^{phox} constitutively associates with p47^{phox} via a tail-to-tail interaction: the C-terminal SH3 domain of p67^{phox} directly binds to a PRR in the C-terminal region of p47^{phox} (Leusen *et al*., 1994; Ito *et al*., 1996). On the other hand, membrane translocation of Rac is independent of the presence of cytochrome b_{558}, p47^{phox} and p67^{phox}: even in CGD neutrophils lacking one of these proteins, the GTPase is normally recruited to the membrane (Heyworth *et al*., 1994).

Another SH3 domain-containing oxidase factor, p40^{phox}, has been identified as a protein that constitutively associates with p67^{phox} in the cytosol of resting phagocytes (Someya *et al*., 1993; Wientjes *et al*., 1993; Tsunawaki *et al*., 1994). It comprises three portions: a PX (Phox)

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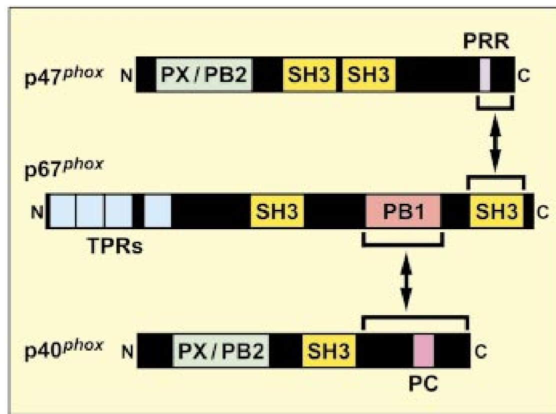

Fig. 1. Protein–protein interactions in a complex of the phagocyte NADPH oxidase factors p67*phox*, p47*phox* and p40*phox*. The NADPH oxidase proteins p67*phox*, p47*phox* and p40*phox* form a complex in resting phagocytes. The three proteins harbor multiple modular domains: p67*phox* comprises a domain containing four units of TPR (tetratricopeptide repeat), the first SH3 domain, PB1 domain and the second SH3 domain; p47*phox* contains PX or PB2 domain, two SH3 domains and the C-terminal PRR; and p40*phox* harbors PX/PB2 and SH3 domains, followed by PC motif. In the oxidase complex, p67*phox* directly binds to p47*phox* via a tail-to-tail interaction between the C-terminal SH3 of p67*phox* and the PRR of p47*phox*; and p67*phox* also directly binds to p40*phox* via a novel modular interaction between the PB1 domain of p67*phox* and the PC motif-containing region of p40*phox*. Thus p67*phox* tethers p47*phox* to p40*phox*.

domain [also known as PB2 (Phox and Bem 2) domain] at the N-terminus, an SH3 domain in the middle and the C-terminal region containing a PC (Phox and Cdc) motif (Ponting, 1996; Sumimoto *et al*., 1997; Mizuki *et al*., 1998; Nakamura *et al*., 1998; Hiroaki *et al*., 2001) (Figure 1). The PC motif is present in a variety of signaling proteins such as the yeast polarity protein Cdc24p, the protein kinase MEK5 and Zip, a protein that interacts with the ζ isoform of protein kinase C, and serves as a target of PB1 (Phox and Bem 1) domain, thereby mediating modular protein–protein interactions (Ito *et al*., 2001; Terasawa *et al*., 2001). The protein p67*phox* contains a PB1 domain between the two SH3 domains, which recognizes the PC motif of p40*phox* and plays an essential role in interaction between p67*phox* and p40*phox* (Ito *et al*., 2001). Thus, in a ternary oxidase complex of resting phagocytes, p67*phox* is thought to tether p47*phox* to p40*phox*: the C-terminal SH3 domain binds to the PRR of p47*phox* and the PB1 domain directly interacts with the PC motif of p40*phox* (Figure 1).

The role of p40*phox* in the phagocyte NADPH oxidase activation has remained largely elusive, although it probably resides in a complex of the oxidase as described above. The oxidase can be activated in a cell-free system reconstituted with cytochrome b_{558}, cytosolic proteins (p47*phox*, p67*phox* and Rac in the GTP-bound form) and anionic amphiphiles such as arachidonate and sodium dodecyl sulfate (Bromberg and Pick, 1985; Hata *et al*., 1998). It is well known that the cell-free activation does not require p40*phox*. The effect of the addition of p40*phox* to the cell-free system, however, seems controversial: Cross (2000) has shown that p40*phox* only slightly elevates the oxidase activity under cell-free conditions, while other investigators have reported that p40*phox* represses the

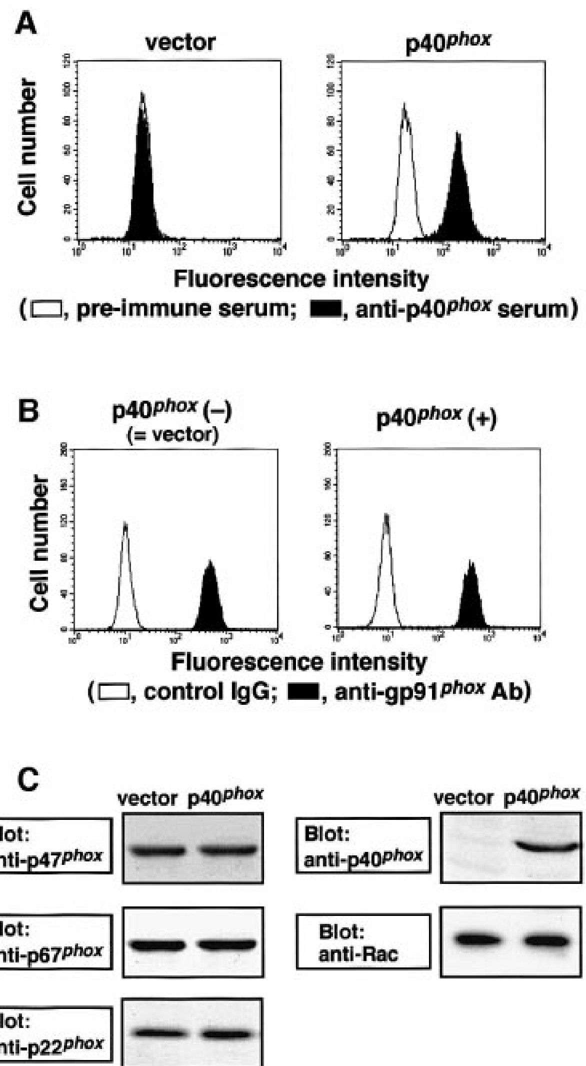

Fig. 2. Stable expression of p40*phox* in K562 cells transfected with pREP4 encoding the full-length of this protein. (**A**) Expression of p40*phox* in the K562 cells. Parent K562 cells stably expressing gp91*phox*, p67*phox* and p47*phox* were transfected with pREP4 encoding the full-length p40*phox* (p40*phox*) or vector alone (vector). The cells were stained with anti-p40*phox* serum (filled histogram) or pre-immune serum (open histogram) and analyzed by flow cytometry. For details see Materials and methods. (**B**) Expression of functional gp91*phox*. The cells were stained with the monoclonal antibody 7D5 to detect gp91*phox* in functional cytochrome b_{558} (filled histogram) or control IgG (open histogram) and analyzed by flow cytometry. (**C**) Expression of p47*phox*, p67*phox*, p40*phox* and p22*phox*. The lysates of the K562 cells were analyzed by immunoblot with an anti-p47*phox*, an anti-p67*phox*, an anti-p40*phox* or an anti-p22*phox* antibody, as described in Materials and methods.

oxidase activation at high concentrations (Sathyamoorthy *et al*., 1997). Although the cell-free system has greatly contributed to understanding of the molecular mechanism for activation of the NADPH oxidase, results obtained using this system sometimes seem inconsistent with those using intact cells. For instance, p67*phox* lacking the C-terminal SH3 domain is incapable of activating the NADPH oxidase when it is expressed in cells, while the mutant p67*phox* almost fully activates the oxidase under cell-free conditions (de Mendez *et al*., 1994).

Here we describe the role of p40*phox* in activation of the phagocyte NADPH oxidase at the cellular level. This

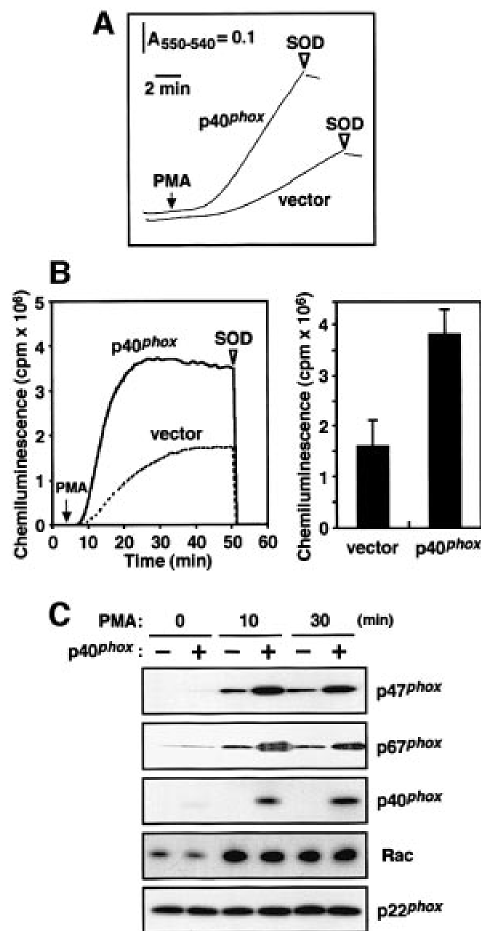

Fig. 3. p40^{phox}-enhanced superoxide production and membrane translocation of p47^{phox} and p67^{phox} upon cell stimulation with PMA. (**A**) Superoxide production by the K562 cells with stable expression of p40^{phox} or without p40^{phox} was measured as reduction of cytochrome *c*. The cells (5.0×10^5 cells/ml) were stimulated with PMA (200 ng/ml) (arrow), and the superoxide-producing activity was measured by determining the rate of ferricytochrome *c* reduction at 550 to 540 nm. The reduction was stopped by addition of superoxide dismutase (SOD) (50 µg/ml) (arrowhead). (**B**) Superoxide production by the K562 cells with p40^{phox} or without p40^{phox} was measured as change of chemiluminescence. The K562 cells (5.0×10^3 cells) were stimulated with PMA (200 ng/ml), and the chemiluminescence change was continuously monitored with DIOGENES. SOD (50 µg/ml) was added where indicated (arrowhead). For details see Materials and methods. (**C**) Membrane translocation of p47^{phox}, p67^{phox}, p40^{phox} and Rac upon PMA stimulation. The K562 cells with or without p40^{phox} (1.0×10^7 cells) were incubated with PMA (200 ng/ml) for the indicated time. After the incubation, the cells were sonicated and ultracentrifuged as described in Materials and methods. The membrane fractions were analyzed by immunoblot with the antibody against p47^{phox}, p67^{phox}, p40^{phox}, Rac or p22^{phox}. The data are representative of results from five independent experiments.

protein enhances membrane translocation of the cytosolic activators p67^{phox} and p47^{phox}, and subsequent activation of the NADPH oxidase by 2- to 3-fold, when cells are stimulated with PMA. The enhancements are totally dependent on the binding of p40^{phox} to p67^{phox}, which is mediated via the PB1–PC interaction. Intriguingly, in cells treated with a G_i-activating peptide instead of PMA, p40^{phox} serves as a much more efficient activator of the oxidase, thereby underscoring its importance in a physiological context that cannot be reproduced under cell-free conditions.

Results

Expression of p40^{phox} leads to enhanced production of superoxide in PMA-stimulated cells

To investigate the role of p40^{phox} in activation of the phagocyte NADPH oxidase, we transfected the plasmid vector pREP4 encoding the full-length cDNA of p40^{phox} or vector alone to K562 cells that express functional cytochrome b_{558}, p47^{phox} and p67^{phox} (Ago *et al.*, 1999; Koga *et al.*, 1999), and cloned seven independent transformants, each expressing p40^{phox} at the same level (for details see Materials and methods). The transformants stably express p40^{phox} as estimated by flow cytometric analysis (Figure 2A). The p40^{phox}-expressing cells harbored the same amounts of functional gp91^{phox} as the p40^{phox}-deficient cells, containing the vector pREP4 that did not encode this protein (Figure 2B). In addition, analyses by immunoblot (Figure 2C) and flow cytometry (data not shown) revealed that no difference existed between these cells in expression of the proteins p22^{phox}, p47^{phox}, p67^{phox} and the small GTPase Rac.

When the K562 cells expressing p40^{phox} were stimulated with PMA, they produced a large amount of superoxide, which was estimated by superoxide dismutase (SOD)-inhibitable cytochrome *c* reduction (Figure 3A). We also tested four other clones (data not shown) and found that the cells exhibited essentially the same superoxide-producing activity of 4.2 ± 0.14 nmol/min per 10^6 cells ($n = 5$). On the other hand, the activity of the cells without p40^{phox} was 1.8 ± 0.12 nmol/min per 10^6 cells ($n = 5$ independent clones) (Figure 3A; data not shown). Using another method, SOD-inhibitable chemiluminescence, we also tested the effect of p40^{phox} in activation of the phagocyte NADPH oxidase (Figure 3B). In response to PMA, seven independent clones of the p40^{phox}-expressing K562 cells all produced 2- to 3-fold more superoxide than the cells lacking p40^{phox} (Figure 3B). Thus expression of p40^{phox} leads to enhanced production of superoxide in PMA-stimulated cells, raising the possibility that p40^{phox} facilitates the assembly of the phagocyte oxidase factors.

p40^{phox} facilitates membrane translocation of p47^{phox} and p67^{phox} but not of Rac upon cell stimulation

It is well established that stimulus-elicited translocation of p47^{phox} and p67^{phox} from the cytosol to the membrane is required for activation of the phagocyte NADPH oxidase (Roos *et al.*, 1996; Clark, 1999; Nauseef, 1999). Since p40^{phox} is constitutively associated with p67^{phox} and enhances the oxidase activation as shown above (Figure 3A and B), it seems likely that p40^{phox} affects the membrane recruitment of p47^{phox} and p67^{phox}. To test this possibility, we fractionated the membrane of the K562 cells with or without p40^{phox} and estimated the amounts of p47^{phox} and p67^{phox}. When the p40^{phox}-lacking cells were stimulated with PMA, both p47^{phox} and p67^{phox} translocated to the membrane in a time-dependent manner (Figure 3C). In the cells expressing p40^{phox}, this protein as well as p47^{phox} and p67^{phox} was targeted upon cell stimulation to the membrane (Figure 3C). The amounts of p47^{phox} and p67^{phox} at the membrane were increased by 2- to 3-fold, compared with those of the p40^{phox}-deficient

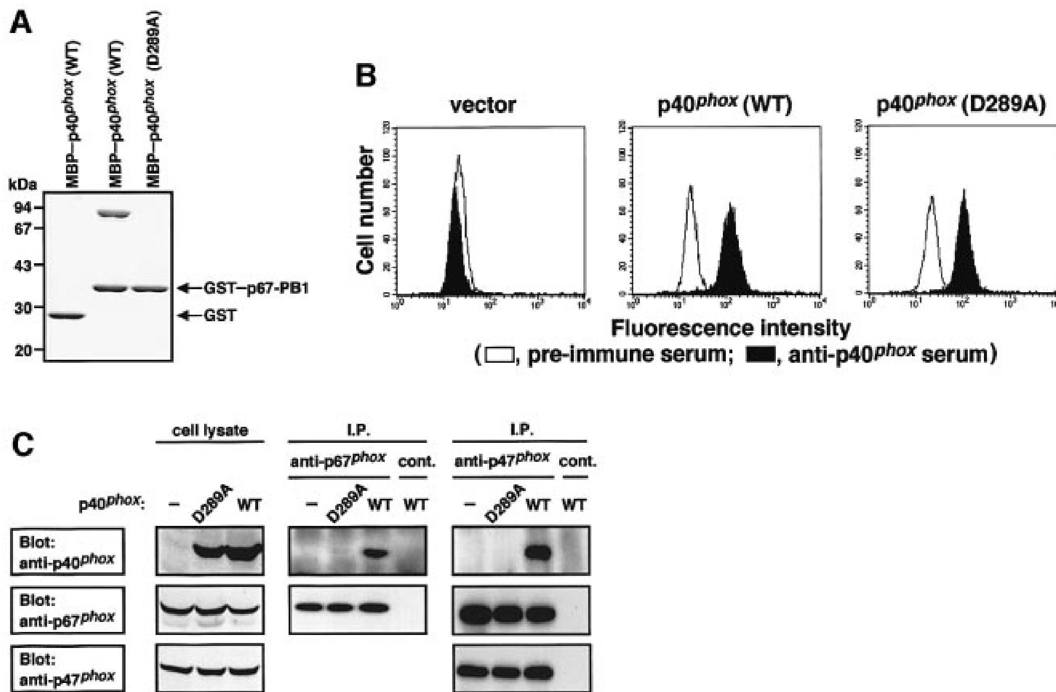

Fig. 4. Effect of the D289A substitution in the PC motif of p40^{phox} on its interaction with p67^{phox} both *in vivo* and *in vitro*. (**A**) Direct interaction of p40^{phox} with p67^{phox}. GST-tagged PB1 domain of p67^{phox} (GST–p67-PB1) or GST alone was incubated with MBP–p40^{phox} (WT) or MBP–p40^{phox} (D289A) and proteins were pulled down with glutathione–Sepharose-4B. The precipitated proteins were subjected to SDS–PAGE, followed by staining with CBB, as described, see Materials and methods. Positions for marker proteins are indicated in kDa. (**B**) Expression of p40^{phox} (D289A) in K562 cells. K562 cells stably expressing gp91^{phox}, p67^{phox} and p47^{phox} were transfected with pREP4 encoding p40^{phox} with the D289A substitution, p40^{phox} (D289A). The K562 cells were stained with anti-p40^{phox} serum (filled histogram) or pre-immune serum (open histogram) and analyzed by flow cytometry. (**C**) Interaction of p40^{phox} with p67^{phox} in the K562 cells. The cell lysates of the K562 cells were analyzed by immunoprecipitation with an anti-p67^{phox} or control IgG (cont.) (left panel), or an anti-p47^{phox} antibody or control IgG (cont.) (right panel) followed by immunoblot with the indicated antibody, as described under Materials and methods.

cells (Figure 3C). On the other hand, p40^{phox} did not affect PMA-elicited membrane targeting of Rac (Figure 3C). Thus the enhancement of the oxidase activation by p40^{phox} is likely to be due to the p40^{phox}-induced facilitation of membrane translocation of p47^{phox} and p67^{phox}.

p40^{phox} carrying the D289A substitution is incapable of binding to p67^{phox} and fails to enhance superoxide production and membrane translocation of p47^{phox} and p67^{phox}

We next tested whether the interaction of p40^{phox} with p67^{phox} participates in the p40^{phox}-enhanced translocation of p47^{phox} and p67^{phox}. As we have previously shown, this interaction is mediated via a direct binding of the p40^{phox} PC motif to the p67^{phox} PB1 domain (Nakamura *et al.*, 1998; Ito *et al.*, 2001). Consistent with previous observations, the maltose-binding protein (MBP)-tagged full-length protein of p40^{phox}, MBP–p40^{phox} (WT), directly interacted with the p67^{phox} PB1 domain as a glutathione *S*-transferase (GST) fusion (GST–p67-PB1) as analyzed by a pull-down assay (Figure 4A). To confirm the role for the PC motif of p40^{phox}, we purified a mutant p40^{phox} carrying the amino acid substitution of Ala for Asp289, an invariant residue among PC motifs (Nakamura *et al.*, 1998); the corresponding residue in the PC motif of the yeast polarity protein Cdc24p plays an essential role in its binding to the PB1 domain of Bem1p (Ito *et al.*, 2001). As shown in Figure 4A, the D289A substitution resulted in a complete loss of the interaction of p40^{phox} with p67^{phox}.

To examine the effect of the D289A substitution in the PC motif at the cellular level, we prepared seven clones of the K562 cells stably expressing p40^{phox} (D289A). The amount of p40^{phox} as well as those of other oxidase proteins including gp91^{phox}, p22^{phox}, p47^{phox} and p67^{phox} in the p40^{phox} (D289A)-expressing cells was identical to that in the cells expressing the wild-type p40^{phox} (WT) (Figure 4B and C; data not shown). An immunoprecipitation assay demonstrated that p67^{phox} binds to p40^{phox} (WT) but not to p40^{phox} (D289A) *in vivo* (Figure 4C), indicating that the binding of p40^{phox} to p67^{phox} is mediated solely via the PC motif even at the cellular level. Although p47^{phox} was involved in a complex with p40^{phox} and p67^{phox} (Figure 4C), it should be noted that the interaction between p47^{phox} and p67^{phox} occurs in a manner independent of the presence of p40^{phox} (Figure 4C).

When cells were stimulated with PMA, p40^{phox} (D289A) was incapable of enhancing the activation of the NADPH oxidase: the amount of superoxide produced by the cells expressing p40^{phox} (D289A) was similar to that by the cells without p40^{phox} (Figure 5A). The amounts of p67^{phox} and p47^{phox} in the membrane of the p40^{phox} (D289A)-expressing cells were half-to-one-third of those of the p40^{phox} (WT)-expressing ones. In addition, p40^{phox} (D289A) did not translocate to the membrane in response to PMA (Figure 5B), suggesting that its localization is totally dependent on the interaction with p67^{phox}. The present observations thus indicate that membrane translocation of p47^{phox} and p67^{phox} is facilitated by the binding

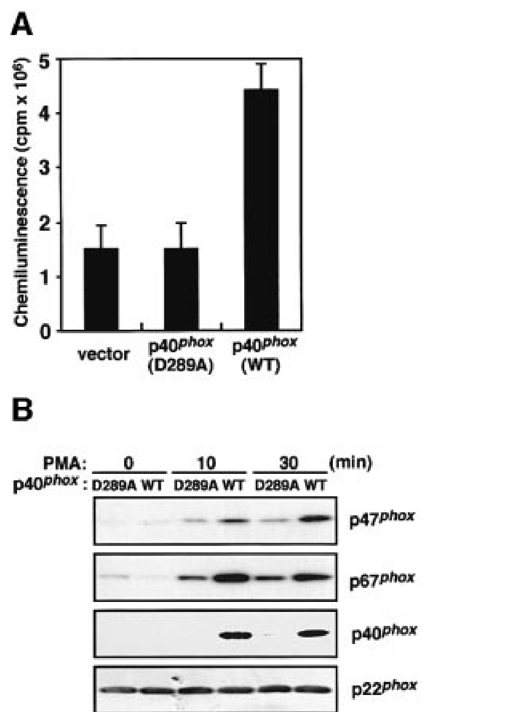

Fig. 5. Effect of the D289A substitution in the PC motif of p40^{phox} on superoxide production and membrane translocation of p47^{phox} and p67^{phox} upon PMA stimulation. (**A**) Superoxide production by the K562 cells with p40^{phox} (WT) or p40^{phox} (D289A) was measured as change of chemiluminescence. The K562 cells (5.0 $\times$ 10^3 cells) were stimulated with PMA (200 ng/ml) and the chemiluminescence change was measured. Each histogram indicates the average from five independent experiments, with bars representing SD. (**B**) Membrane translocation of p47^{phox}, p67^{phox} and p40^{phox} upon PMA stimulation. The K562 cells stably expressing p40^{phox} (WT) or p40^{phox} (D289A) (1.0 $\times$ 10^7 cells) were stimulated with PMA (200 ng/ml) for the indicated time and the amounts of p47^{phox}, p67^{phox} and p40^{phox} in the membrane fractions were analyzed by immunoblot as described under Materials and methods.

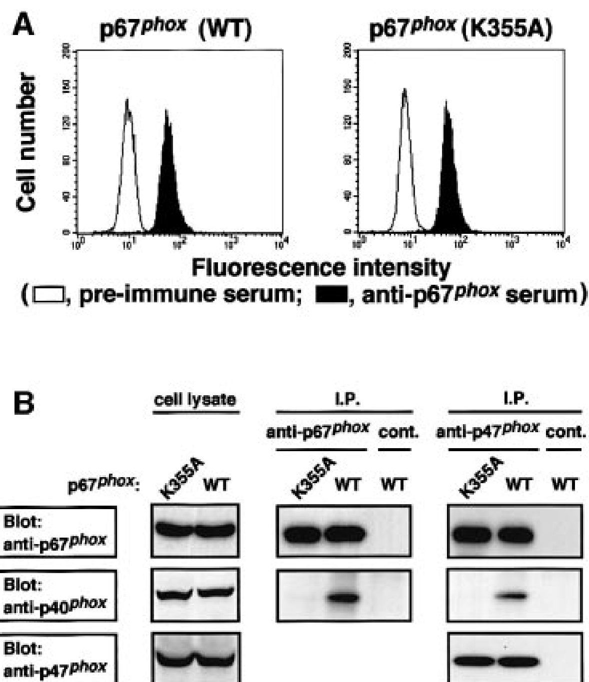

Fig. 6. Effect of the K355A substitution in the PB1 domain of p67^{phox} on its interaction with p40^{phox}. (**A**) Expression of p67^{phox} in K562 cells. K562 cells stably expressing gp91^{phox}, p47^{phox} and p40^{phox} were transfected with pREP4 encoding p67^{phox} (WT) or p67^{phox} (K355A). The K562 cells were stained with anti-p67^{phox} serum (filled histogram) or pre-immune serum (open histogram), and analyzed by flow cytometry. (**B**) *In vivo* interaction of p67^{phox} with p40^{phox} in the K562 cells. The cell lysates of the K562 cells were analyzed by immunoprecipitation with the anti-p67^{phox} or control IgG (cont.) (left panel), or the anti-p47^{phox} antibody or control IgG (cont.) (right panel) followed by immunoblot (Blot) with the indicated antibody.

of p40^{phox} to p67^{phox}, which leads to enhanced activation of the NADPH oxidase.

p67^{phox} carrying the K355A substitution is incapable of interacting with p40^{phox}, and fails to enhance superoxide production and membrane translocation of p47^{phox} and p67^{phox}

To further study the importance of the interaction between p40^{phox} and p67^{phox}, we used a mutant p67^{phox} with the substitution of Ala for Lys355 (Figure 6A), a conserved residue among PB1 domains (Ito *et al*., 2001; Terasawa *et al*., 2001). In an *in vitro* pull-down assay, GST–p67-PB1 (K355A) was incapable of interacting with MBP–p40^{phox}, while the wild-type PB1 domain fully bound to p40^{phox} (data not shown). Similarly, p67^{phox} (K355A) could not associate with p40^{phox} *in vivo* (Figure 6B). On the other hand, both p67^{phox} (WT) and p67^{phox} (K355A) were co-immunoprecipitated with p47^{phox} (Figure 6B), indicating that p40^{phox} binds to p47^{phox} indirectly but via p67^{phox} (see Figure 1).

We next investigated the effect of the K355A substitution in the p67^{phox} PB1 domain on superoxide production and membrane translocation of p47^{phox} and p67^{phox}. As shown in Figure 7A, the amounts of p47^{phox} and p67^{phox} in the membranes of the p67^{phox} (K355A)-expressing cells were half-to-one-third of those of the p67^{phox} (WT)-expressing cells. In the cells expressing p67^{phox} (K355A), p40^{phox} failed to translocate to the membranes. As shown in Figure 7B, the amount of superoxide produced by the cells expressing p67^{phox} (K355A) was half-to-one-third of that by those expressing p67^{phox} (WT). In response to PMA, the cells with p67^{phox} (K355A) produced the same low amount of superoxide irrespective of the presence of p40^{phox} (Figure 7B). These observations further supported the idea that the p40^{phox}–p67^{phox} interaction enhances the membrane translocation of p47^{phox} and p67^{phox}, thereby facilitating the assembly of the active oxidase complex.

p40^{phox} greatly enhances superoxide production in cells stimulated with the muscarinic receptor peptide m4I3C(14), an agent that can activate the trimeric GTPase G_i

It is well documented that, in neutrophils, chemoattractants such as *N*-formyl peptides induce superoxide production via the trimeric GTPase G_i (Bokoch, 1995). The chemotactic receptor, however, is not expressed in K562 cells. To further clarify the role of p40^{phox} in the oxidase activation, we used the muscarinic receptor peptide m4I3C(14) as a stimulant.

In response to m4I3C(14), K562 cells expressing p40^{phox} produced superoxide (Figure 8A). The superoxide production, albeit 10-fold less than that by PMA-stimulated cells, is likely to be catalyzed by the phagocyte NADPH oxidase, since the peptide did not act on parental K562 cells that lack oxidase proteins (data not shown) or

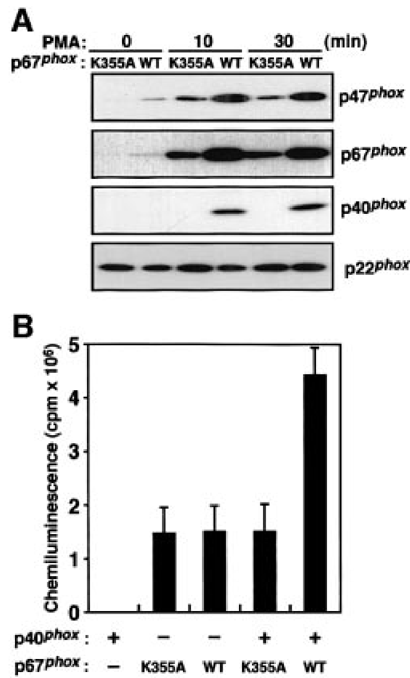

Fig. 7. Effect of the K355A substitution in the PB1 domain on membrane translocation of p47*phox* and p67*phox* and superoxide production upon PMA stimulation. (**A**) Membrane translocation of p47*phox*, p67*phox* and p40*phox* upon PMA stimulation. The K562 cells stably expressing p67*phox* (WT) or p67*phox* (K355A) (1.0×10^7 cells) were stimulated with PMA (200 ng/ml) for the indicated time and the amounts of p47*phox*, p67*phox* and p40*phox* in the membrane fractions were analyzed by immunoblot. (**B**) Superoxide production by the K562 cells with p67*phox* (WT) or p67*phox* (K355A) was measured as change of chemiluminescence. The K562 cells (5.0×10^3 cells) were stimulated with PMA (200 ng/ml) and the chemiluminescence change was measured. The chemiluminescence change by K562 cells without the expression of p40*phox* and with p67*phox* (WT) or p67*phox* (K355A) was also measured. Each histogram indicates the average from five independent experiments, with bars representing SD.

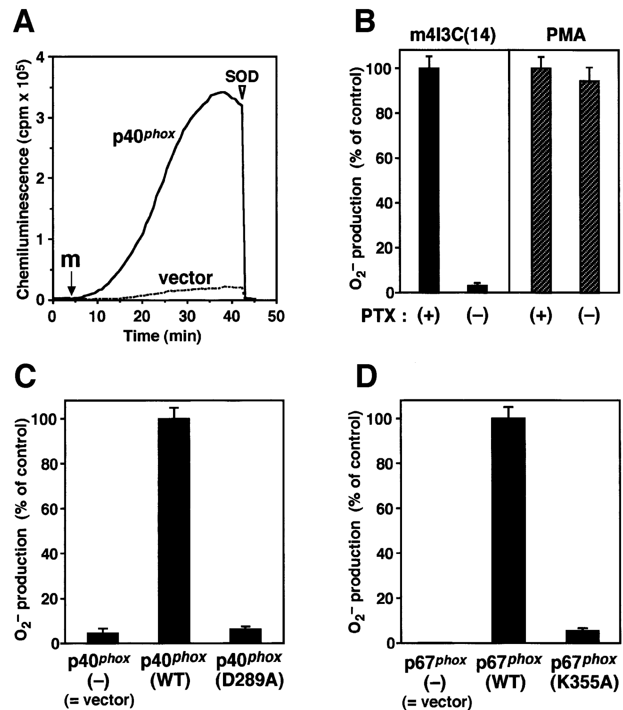

Fig. 8. p40*phox*-enhanced superoxide production upon cell stimulation with the muscarinic receptor peptide m4I3C(14). (**A**) Superoxide production by the K562 cells with stable expression of p40*phox* or without p40*phox*. The K562 cells (5.0×10^3 cells) were stimulated with the muscarinic receptor peptide m4I3C(14) (m) (200 μM) and the chemiluminescence change was continuously monitored with DIOGENES, and SOD (50 μg/ml) was added where indicated (arrowhead). For details see Materials and methods. (**B**) Effect of *pertussis* toxin (PTX) on superoxide production upon stimulation with m4I3C(14) or PMA. The K562 cells (5.0×10^3 cells) were pretreated with PTX (18 μg/ml) and stimulated with m4I3C(14) (200 μM) or PMA (200 ng/ml) and superoxide production was measured as change of chemiluminescence. Each histogram indicates the average from five independent experiments, with bars representing SD. (**C**) Effect of the D289A substitution in the PC motif of p40*phox* on superoxide production upon stimulation with m4I3C(14). The K562 cells with p40*phox* (WT) or p40*phox* (D289A) or without p40*phox* (5.0×10^3 cells) were stimulated with 200 μM m4I3C(14) and superoxide production was measured as change of chemiluminescence. Each histogram represents the average from five independent experiments, with bars representing SD. (**D**) Effect of the K355A substitution in the PB1 domain of p67*phox* on superoxide production upon stimulation with m4I3C(14). The K562 cells with p67*phox* (WT) or p67*phox* (K355A) or without p67*phox* (5.0×10^3 cells) were stimulated with 200 μM m4I3C(14), and superoxide production was measured as change of chemiluminescence. Each histogram indicates the average from five independent experiments, with bars representing SD.

cells expressing all oxidase factors except p67*phox* (see later). Furthermore, we did detect, although qualitatively, the peptide-induced membrane translocation of the cytosolic oxidase proteins (data not shown). The treatment of the K562 cells with *pertussis* toxin (PTX) resulted in a loss of the m4I3C(14)-induced superoxide production, while the toxin did not affect that induced by PMA (Figure 8B). The peptide thus activates the oxidase via G_i.

Intriguingly, in response to m4I3C(14), K562 cells expressing p40*phox* produced superoxide ~20-fold as much compared with the cells lacking this adaptor protein (Figure 8C). In addition, the peptide served as a weak agonist when cells expressed p40*phox* (D289A) but not the wild-type p40*phox* (Figure 8C). Consistent with this, only a small amount of superoxide was generated in cells expressing p67*phox* (K355A) instead of the wild-type p67*phox*, while no superoxide production was detected in cells lacking p67*phox* (Figure 8D). Thus, in cells stimulated with the G_i activator m4I3C(14), p40*phox* greatly enhances activation of the phagocyte NADPH oxidase, which is dependent on its interaction with p67*phox*.

Discussion

Although p40*phox* is known as a protein that constitutively associates with the phagocyte oxidase activator p67*phox*, the role of p40*phox* in the oxidase activation has remained elusive. In the present study, we show that p40*phox* facilitates the activation of the phagocyte NADPH oxidase at the cellular level (Figures 3 and 8). The facilitation is thought to be caused by enhancing the stimulus-induced recruitment of p67*phox* and p47*phox* to the membrane, an essential step for the oxidase activation, without affecting the membrane translocation of the small GTPase Rac (Figure 3). We also demonstrate that both enhanced recruitment of the oxidase activators and facilitated activation of the oxidase are totally dependent on the binding of p40*phox* to p67*phox*, which is mediated via a novel modular interaction between the p40*phox* PC motif and the p67*phox* PB1 domain (Figures 5, 7 and 8).

One of the reasons why the role of p40*phox* has been obscure for a long time is due to the fact that p40*phox* is

dispensable for NADPH oxidase activation under both cell-free and whole-cell conditions (de Mendez *et al*., 1994; Sathyamoorthy *et al*., 1997). In addition, it has been reported that the presence of p40^{phox} marginally affects the oxidase activity in a cell-free activation system: this protein only slightly enhances the activation (Cross, 2000). Although studies under cell-free conditions have shed light on the understanding of the molecular mechanism for the oxidase activation, results obtained in such studies sometimes seem discrepant with those observed in a whole-cell system. For instance, p67^{phox} binds to p47^{phox} via the C-terminal SH3 domain of p67^{phox}, a domain which is required for the oxidase activation in a whole-cell system (de Mendez *et al*., 1994) but not for the cell-free activation (de Mendez *et al*., 1994; Leusen *et al*., 1995; Hata *et al*., 1998). Here we show, using cells stably expressing p40^{phox}, that p40^{phox} enhances activation of the NADPH oxidase. When cells are stimulated with PMA, a potent activator of protein kinase C, expression of p40^{phox} leads to a 2- to 3-fold enhancement (Figure 3). On the other hand, the oxidase activation is much more drastically (~20-fold) facilitated by p40^{phox} in response to the muscarinic receptor peptide m4I3C(14), that acts as an activator of G_i (Figure 8). The peptide probably functions as a more physiological stimulant, since PTX blocks the peptide-induced superoxide production but not the PMA-elicited one (Figure 8). It seems probable that, in *in vivo* activation of the oxidase, p40^{phox} plays a role that is much more important than expected from the results of experiments with PMA. Alternatively, the significance of p40^{phox} could be dependent on the types of stimulants or signaling pathways for the oxidase activation. In either case, p40^{phox} is deeply involved in activation of the phagocyte NADPH oxidase.

Although no case has been thus far reported of CGD with the defect of p40^{phox}, it is conceivable that its defect causes less severe forms of compromised host defense and hence has escaped the clinical screening for CGD. Alternatively, since the phagocyte oxidase is known to participate not only in host defense but also inflammation, an aberrance in inflammatory response, rather than vulnerability to infection, may be manifested in the cases with defect of p40^{phox}. It is thus intriguing to search single nucleotide polymorphisms or mutations in the p40^{phox} gene *NCF4* showing significant association with those displaying these symptoms. However, one should note that extra-phagocytic roles for p40^{phox} and hence its involvement in other diseases are plausible, because mouse *Ncf4* gene is also expressed in T cells and neurons, with no expression of p67^{phox} (Mizuki *et al*., 1998). In any case, pathophysiological roles for p40^{phox} remain largely elusive.

In contrast with the present results, Sathyamoorthy *et al*. (1997) have reported that transient expression of p40^{phox} leads to a repressed activation of the NADPH oxidase when cells are stimulated with PMA. The reason for this discrepancy is presently unknown. They have also shown that expression of the SH3 domain of p40^{phox} by itself represses the oxidase activation more efficiently than the full-length p40^{phox} does (Sathyamoorthy *et al*., 1997). Since the SH3 domain of p40^{phox} is capable of binding to p47^{phox} but to a much lesser extent than the C-terminal SH3 domain of p67^{phox} (Ito *et al*., 1996), overexpression of the p40^{phox} SH3 domain may compete with the SH3 domain of p67^{phox} to prevent p67^{phox} from interacting with p47^{phox}, thereby inhibiting the oxidase activation.

The adaptor protein p40^{phox} is present in a complex containing not only p67^{phox} but also p47^{phox}. In this complex, p40^{phox} directly binds to p67^{phox} via the PB1–PC interaction, while p40^{phox} interacts with p47^{phox} in an indirect manner: p67^{phox} tethers p40^{phox} to p47^{phox} (Figure 1). If the association between p40^{phox} and p67^{phox} is absent, p40^{phox} and p47^{phox} cannot reside in the same complex (Figures 4 and 6). Thus membrane translocation of p47^{phox} is not enhanced by p40^{phox} under conditions where the association between p40^{phox} and p67^{phox} is specifically disrupted (Figures 5 and 7). On the other hand, the membrane translocation of p40^{phox} requires p67^{phox}. When a mutant p67^{phox} (K355A), that cannot interact with p40^{phox}, is expressed instead of the wild-type p67^{phox}, p40^{phox} is incapable of translocating to the membrane upon cell stimulation (Figure 7). This is consistent with a previous observation that, in neutrophils of a p67^{phox}-deficient CGD patient, p40^{phox} is not targeted to the membrane in response to cell stimuli (Dusi *et al*., 1996). Even in these cells, stimulus-induced translocation of p47^{phox} can still be observed (Dusi *et al*., 1996). Taken together, p47^{phox} plays an essential role in membrane recruitment of the p67^{phox}–p47^{phox}–p40^{phox} complex, an event which is strongly enhanced by p40^{phox}.

This study clearly demonstrates that p40^{phox} facilitates membrane targeting of the essential oxidase factors p67^{phox} and p47^{phox}, which requires the interaction of p40^{phox} with p67^{phox}. The molecular mechanism whereby p40^{phox} functions, however, is not fully understood at present. In the N-terminal region, p40^{phox} harbors the PX/PB2 domain (Figure 1), a module that interacts with phosphoinositides (Ago *et al*., 2001; Kanai *et al*., 2001; Ellson *et al*., 2001b). The PX domain of p40^{phox} specifically binds to phosphatidylinositol-3-phosphate [PtdIns(3)P] *in vitro*, and the domain expressed as a fusion to green fluorescent protein localizes to early endosomes where this phosphoinositide is enriched (Ago *et al*., 2001; Kanai *et al*., 2001; Ellson *et al*., 2001b). It is possible that the phospholipid-binding activity of p40^{phox} is involved in its membrane localization. In this context, it is intriguing to note a current report showing that PtdIns(3)P is enriched in phagosomes (Ellson *et al*., 2001a), where the NADPH oxidase is activated. It has been also reported that p40^{phox} can interact with coronin, an actin-binding protein (Grogan *et al*., 1997). Interaction of p40^{phox} with cytoskeletal elements may participate in a process of membrane translocation of this protein. In addition, stimulus-induced phosphorylation of p40^{phox} (Bouin *et al*., 1998) possibly plays a role, an event which has escaped analysis in a cell-free system. These possibilities should be tested in future studies.

The PB1 domain and PC motif prevail in a variety of proteins and mediate protein–protein interactions: the PB1 domain of Bem1p, scd2, atypical protein kinase Cζ or p67^{phox} recognizes and binds to the PC motif of Cdc24p, scd1, ZIP or p40^{phox}, respectively (Ito *et al*., 2001). In the budding yeast *Saccharomyces cerevisiae*, the interaction between Bem1p and Cdc24p is essential for polarity establishment of cells (Ito *et al*., 2001; Butty *et al*., 2002). The present study provides another example of functional

interactions mediated via these modules, i.e. the binding of p40^{phox} to p67^{phox} plays a significant role in activation of the phagocyte NADPH oxidase, an enzyme involved in host defense. Further studies are required to clarify the importance of other PB1–PC interactions.

Materials and methods

Plasmid constructions

cDNA fragments encoding p40^{phox}, p47^{phox} and p67^{phox} were prepared as described previously (Hata *et al*., 1998; Nakamura *et al*., 1998; Ago *et al*., 1999) and cloned into the vectors pREP (Invitrogen), pGEX-4T1 (Amersham Pharmacia Biotech) and pMALc2 (New England Biolabs). Mutations leading to the indicated amino acid substitutions were introduced by PCR-mediated site-directed mutagenesis. All the constructs were sequenced to confirm their identities.

Expression of p40^{phox} in K562 cells containing cytochrome b_{558}, p47^{phox} and p67^{phox}

The doubly transduced K562 cells that stably express both cytochrome b_{558} and p67^{phox} were prepared using the monoclonal antibody 7D5 to detect gp91^{phox} in functional cytochrome b_{558} (Yamauchi *et al*., 2001) and anti-p67^{phox} polyclonal antibodies raised to a C-terminal peptide of this protein (Imajoh-Ohmi *et al*., 1992), as previously described (Ago *et al*., 1999). The cells were electroporated in the presence of the full-length cDNA of p47^{phox} in pREP9. After incubation with G418 (1.0 mg/ml), the cells were cloned by limiting dilution and checked by flow cytometry with an anti-p47^{phox} monoclonal antibody (Transduction Laboratories). The stable transformants were further transfected with pREP4 encoding the wild-type p40^{phox} or a mutant one carrying the D289A substitution. The transfected cells were cloned by limiting dilution in the presence of hygromycin B (100 μg/ml) and expression of p40^{phox} was confirmed by flow cytometry with anti-p40^{phox} polyclonal antibodies raised to an N-terminal peptide of this protein (Tsunawaki *et al*., 1994).

Expression of p67^{phox} in K562 cells containing cytochrome b_{558}, p47^{phox} and p40^{phox}

The doubly transduced K562 cells that stably express both functional cytochrome b_{558} and p47^{phox} were prepared as previously described (Koga *et al*., 1999). The cells were electroporated in the presence of pREP9 (Invitrogen) encoding the full-length cDNA of p40^{phox} or vector alone. After incubation with G418 (1.0 mg/ml), the K562 cells were cloned by limiting dilution. The stable transformants were further transfected with pREP10 encoding the wild-type p67^{phox} or a mutant one carrying the K355A substitution. The transfected cells were cloned by limiting dilution in the presence of hygromycin B (100 μg/ml), and expression of p67^{phox} was confirmed by flow cytometry with the anti-p67^{phox} antibodies.

Flow cytometric analysis

Cells were washed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4 pH 7.4), and fixed with 2.0% paraformaldehyde for 1 h at 25°C. For detection of cytosolic proteins, the fixed cells were permeabilized with PBS including 0.1% Triton X-100 and 2.0% bovine serum albumin (BSA). The cells were incubated with the indicated antibody, washed twice with 2.0% BSA in PBS, and indirectly labeled with fluorescein isothiocyanate-conjugated goat-anti mouse or rabbit IgG (BioSource International). Cells were washed twice with PBS and analyzed by FACS scan.

Activation of the phagocyte NADPH oxidase

Superoxide production was determined as SOD-inhibitable chemiluminescence, as previously described (Ago *et al*., 1999; Koga *et al*., 1999). Cells in 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) were stimulated at 37°C with PMA (200 ng/ml), and the reaction was terminated by the addition of SOD (50 μg/ml). The chemiluminescence was counted with an enhancer-containing luminol-based detection system (DIOGENES; National Diagnostics) using a luminometer (Auto Lumat LB953; EG&G Berthold).

Alternatively, the superoxide-producing activity was measured by determining the rate of SOD-inhibitable cytochrome *c* reduction at 550–540 nm using a dual-wavelength spectrophotometer (Hitachi 557) (Sumimoto *et al*., 1994).

Translocation of p47^{phox}, p67^{phox} and p40^{phox} to the membrane in stimulated K562 cells

Membrane translocation of cytosolic oxidase factors were determined by the method of Leusen *et al*. (1994) with minor modifications. Briefly, cells were stimulated for the indicated time at 37°C with PMA (200 ng/ml). The incubation was terminated by the addition of ice cold PBS. The cells were resuspended in 3 ml of ice cold buffer A (75 mM NaCl, 170 mM sucrose, 1 mM $MgCl_2$, 0.5 mM EGTA, 10 μM ATP, 2 mM NaN_3, 5 μM GTPγS, 100 μg/ml of *p*-amidinophenyl methanesulfonyl fluoride hydrochloride and 20 mM HEPES pH 7.0) and lyzed by sonication. The sonicate was layered on a discontinuous sucrose gradient consisting of 4 ml of 40% (w/v) sucrose and 3 ml of 15% (w/v) sucrose with 1 mM $MgCl_2$, 40 mM NaCl, 0.5 mM EGTA and 5 μM 5′-3-*O*-(thio)-triphosphate (GTPγS). After ultracentrifugation for 45 min at 100 000 *g*, membrane-associated proteins (1.0 μg) were analyzed by immunoblot with the indicated antibody, which were developed using ECL-plus (Amersham Pharmacia Biotech).

An in vitro binding assay using purified recombinant proteins

The PB1 domain of p67^{phox} (amino acids 335–427) and the protein with the K355A substitution were expressed as GST fusion proteins in *Escherichia coli* and purified by glutathione–Sepharose-4B (Amersham Pharmacia Biotech) (Ago *et al*., 1999; Koga *et al*., 1999). MBP-tagged full-length p40^{phox} and the protein with the D289A substitution were also expressed and purified by amylose resin (New England BioLab). For *in vitro* pull-down binding assays, a pair of a GST fusion (250 μg) and an MBP-tagged protein (500 μg) were incubated for 30 min in 1 ml PBS containing 0.5% Triton X-100. Proteins were precipitated with glutathione–Sepharose-4B and eluted with 5 mM glutathione. The eluates were subjected to SDS–PAGE and stained with Coomassie Brilliant Blue (CBB).

An in vivo binding assay

Cells were lyzed with 1 ml of a lysis buffer (1% Triton X-100, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA and 40 mM HEPES pH 7.4). The lysate was precipitated with the anti-p47^{phox} or anti-p67^{phox} antibody in the presence of protein G–Sepharose (Amersham Pharmacia Biotech). After washing with the lysis buffer, precipitated proteins were analyzed by immunoblotting.

Preparation of the muscarinic receptor peptide m4I3C(14)

m4I3C(14) is a 14-residue peptide fragment corresponding to the C-terminal portion of the third intracellular loop and the N-terminal portion of the sixth transmembrane helix of human m4 muscarinic acetylcholine receptor (Bonner *et al*., 1987) (residues 393–406: RERKVTRTIFAILL). This peptide, like mastoparan (Ross and Higashijima, 1994), activates trimeric G_i reconstituted in phospholipid vesicles in a similar manner as the parental m4 muscarinic acetylcholine receptor (K.Wakamatsu, in preparation) yet does not show any cell-damaging activity as observed for mastoparan (Nakajima *et al*., 2000). The peptide was synthesized by a standard fluoren-9-ylmethoxycarbonyl-based solid-phase method. The purity and the identity of the synthesized peptide were confirmed by analytical HPLC and time-of-flight mass spectroscopy.

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