INTRODUCTION

Rac is a member of the Rho subfamily of Ras-related small GTPases, which includes Cdc42 and RhoA [1, 2]. The Rho subfamily proteins regulate various cellular functions such as actin cytoskeletal organization and cell proliferation by serving as a molecular switch, cycling between an inactive GDP-bound and an active GTP-bound state [1, 2]. In the GTP-bound state, Rho GTPases interact and activate with effector proteins via the conserved switch I region and/or switch II regions. Rac has three isoforms 1, 2, and 3; Rac1 is ubiquitously expressed, whereas Rac2 expression is mostly restricted to cells of haematopoietic origin and Rac3 is most abundant in the brain. Among human leucocytes, neutrophils almost exclusively express Rac2, whereas Rac1 is the major isoform in monocytes and macrophages [1–4]. In these ‘professional’ phagocytes, Rac plays an essential role in activation of the superoxide-producing NADPH oxidase [5–8].

The phagocyte NADPH oxidase is dormant in resting phagocytes but becomes activated during phagocytosis to generate superoxide, a precursor of the microbicidal ROS (reactive oxygen species) [5–8]. The significance of this enzyme in host defence is evident from the fact that recurrent and life-threatening species) [5–8]. The enzyme in host defence superoxide production by gp91 \( \beta \)-245, \( \beta \) polypeptide; also known as Cybb), the catalytic core of the superoxide-producing NADPH oxidase in phagocytes. Rac1 also contributes to activation of the non-phagocytic oxidases Nox1 (NADPH oxidase 1) and Nox3 (NADPH oxidase 3), each related closely to gp91 \( \beta \)-245/Nox2. It has remained controversial whether the insert region of Rac (amino acids 123–135), unique to the Rho subfamily proteins, is involved in gp91 \( \beta \)-245/Nox2 activation. In the present study we show that removal of the insert region from Rac1 neither affects activation of gp91 \( \beta \)-245/Nox2, which is reconstituted under cell-free and whole-cell conditions, nor blocks its localization to phagosomes during ingestion of IgG-coated beads by macrophage-like RAW264.7 cells. The insert region of Rac2 is also dispensable for gp91 \( \beta \)-245/Nox2 activation at the cellular level. Although Rac2, as well as Rac1, is capable of enhancing superoxide production by Nox1 and Nox3, the enhancements by the two GTPases are both independent of the insert region. We also demonstrate that Rac3, a third member of the Rac family in mammals, has an ability to activate the three oxidases and that the activation does not require the insert region. Thus the insert region of the Rac GTPases does not participate in regulation of the Nox family NADPH oxidases.

Key words: macrophage, NADPH oxidase, Nox family, Rac GTPase family, superoxide.
acids 120–140 replaced by the corresponding sequence from H-Ras (Figure 1B). We show that this protein fully activates gp91^phox/Nox2 in both cell-free and whole-cell conditions. In macrophage-like RAW264.7 cells, the mutant Rac1, carrying the replacement insert region, is normally targeted to phagosomes, a process which is crucial for oxidase activation during phagocytosis. The three Rac GTPases, Rac1–3, are also capable of participating in activation of the non-phagocytic oxidases Nox1 and Nox3 independently of the insert region. Thus the insert region of Rac is dispensable for activation of superoxide-producing NADPH oxidases.

**EXPERIMENTAL**

**Plasmid construction**

The cDNAs encoding Rac1, Rac2, Cdc42, Nox1, gp91^phox/Nox2, Nox3, p47^phox, p67^phox, Nox1 and Nox3 were prepared as described previously in [14,17,18,21]. The cDNA for Rac3 was prepared by PCR using a human brain cDNA library (Stratagene) as a template. The ΔIns1 mutant Rac1, Rac2, and Rac3 proteins were generated by replacement of the insert region, amino acids 120–140, by the corresponding sequence from loop eight of H-Ras (amino acids 121–128; Ala-Ala-Arg-Thr-Val-Glu-Ser-Arg). This swap was equivalent to those previously made in Cdc42 and RhoA to minimize disruption of the global structure [32–34]. The following mutant Rac proteins used in the present study were constructed as reported previously in [25–31]: Rac1-ΔIns2 and Rac2-ΔIns2, with both the deletion of amino acids 124–135 and the P136A substitution [25–27]; Rac1-ΔIns3 and Rac2-ΔIns3, with the deletion of amino acids 124–135 [28]; and Rac1-ΔIns4 and Rac2-ΔIns4, with the replacement of amino acids 123–133 with an alanine residue [29]. Mutations leading to the indicated amino acid substitutions were introduced by PCR-mediated site-directed mutagenesis.

The cDNAs were ligated into the following expression vectors: pGEX-6P (GE Healthcare Biosciences) for expression of proteins fused to GST (glutathione transferase) in *Escherichia coli*; pcDNA3 (Invitrogen) for expression of Nox proteins in mammalian cells; pEF-BOS for expression of FLAG- or Myc-tagged proteins in mammalian cells; pEGFP-C1 (Clontech) for expression as an N-terminally GFP (green fluorescent protein)-tagged protein in mammalian cells [*14,17,18,21,*35]. All of the constructs were sequenced for confirmation of their identities.

**Cell-free activation of the phagocyte NADPH oxidase**

The membrane fraction of human neutrophils was prepared as described previously in [*10,36,*]. GST-tagged full-length p47^phox-TPR (amino acids 1–390) and the p67^phox-N-terminal region (amino acids 1–242) were purified using glutathione–Sepharose 4B columns (GE Healthcare), as described previously in [*10,*]. Rac1, Rac1-ΔIns1(C189S), Rac1-ΔIns2(C189S), Rac1-ΔIns3(C189S) and Rac1-ΔIns4(C189S) were expressed as GST-fusion proteins and purified using glutathione–Sepharose 4B columns, followed by digestion with PreScission protease (GE Healthcare), according to the manufacturer’s protocol. These five Rac1 proteins contain the C189S substitution to increase the stability of the proteins during storage [*10,25,*]. The neutrophil membrane (3.4 μg/ml) was mixed with 200 nM GST–p47^phox-TPR, 200 nM GST–p67^phox-TPR, the indicated concentration of Rac1(C189S), Rac1-ΔIns1(C189S), Rac1-ΔIns2(C189S), Rac1-ΔIns3(C189S) or Rac1-ΔIns4(C189S) and was preloaded with GTP[S] (guanosine 5’-[γ-thio]triphosphate) as reported previously in [*10,*]. The mixture was incubated for 2.5 min at 25°C with 100 μM SDS in 100 mM potassium phosphate, pH 7.0, containing 75 μM cytochrome c, 1.0 mM FAD, 1.0 mM EGTA and 1.0 mM NaN₃.
The reaction was then initiated by the addition of 1.0 mM NADPH. The NADPH-dependent superoxide-producing activity was measured by determining the rate of SOD (superoxide dismutase)-inhibitable cytochrome c reduction at 550–540 nm using a Hitachi 557 dual-wavelength spectrophotometer [10,36]. The superoxide-producing activity was represented as mol of superoxide produced/s per mol of cytochrome c. Positions for marker proteins are indicated in kDa. The Rac1 proteins described for A were used in a cell-free activation system of the phagocyte NADPH oxidase. The oxidase was activated in the presence of the indicated concentration of the Rac1 protein. The NADPH-dependent superoxide-producing activity was represented by the rate of SOD-inhibitable cytochrome c reduction, as described in the Experimental section. Each graph represents the means± S.D. of data from three independent experiments.

Activation of Nox1–3 in HeLa and HEK (human embryonic kidney)-293 cells

Experiments for activation of Nox1–3 in HeLa and HEK-293 cells were performed as described previously in [14,17,18,21]. The plasmids used to express the proteins were: pcDNA3-Nox1, pcDNA3-gp91phox/Nox2, or pcDNA3-Nox3; pEF-BOS-Myc-Rac1 (Q61L); pEF-BOS-Myc-Rac1–ΔIns1 (Q61L); pEF-BOS-Myc-Rac1–ΔIns2 (Q61L); pEF-BOS-Myc-Rac1–ΔIns3 (Q61L); pEF-BOS-Myc-Rac1–ΔIns4 (Q61L); pEF-BOS-Myc-Rac2–ΔIns1 (Q61L); pEF-BOS-Myc-Rac2–ΔIns2 (Q61L); pEF-BOS-Myc-Rac2–ΔIns3 (Q61L); pEF-BOS-Myc-Rac2–ΔIns4 (Q61L); pEF-BOS-Myc-Rac3–ΔIns1 (Q61L); pEF-BOS-Myc-Rac3–ΔIns2 (Q61L); pEF-BOS-Myc-Rac3–ΔIns3 (Q61L); pEF-BOS-Myc-Rac3–ΔIns4 (Q61L); pEF-BOS-FLAG-p47phox; pEF-BOS-FLAG-Nox1; pEF-BOS-Myc-p67phox; and pEF-BOS-Myc-Nox1 as indicated. The Rac proteins contain the Q61L substitution, a mutation that constitutively renders the GTPase in an active GTP-bound state. Cells were transfected using Lipofectamine (Invitrogen). Total lysates of HeLa and HEK-293 cells were used for estimation of expression of Rac, p47phox or Nox1 and p67phox or Noxal. The lysates were subjected to SDS/PAGE (13 % gels), transferred on to a (PDVF) (Millipore), and were probed with anti-Myc monoclonal antibody (Roche Diagnostics) or an anti-FLAG monoclonal antibody (Sigma–Aldrich). The Western blots were developed using ECL-plus (Amersham Biosciences) for visualization of the antibodies, as described previously in [14,17,18,21].

The superoxide-producing activity of transfected cells was determined by SOD-inhibitable chemiluminescence with an enhancer-containing luminol-based detection system (Diogenes; National Diagnostics), as described previously in [14,17,18,21]. Cells were suspended, at the concentration of 8 × 10^5 cells/ml, in Hepes-buffered saline (17 mM Hepes, pH 7.4, containing 120 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM MgCl2, and 0.5 mM CaCl2) and were pre-incubated for 5 min at 37°C. After the addition of the enhanced luminol-based substrate Diogenes, cells (1.0 × 10^6 cells/ml) were stimulated with 200 ng/ml PMA. The chemiluminescence was assayed at 37°C using a luminometer (Auto Lumat LB953; EG&G Berthold).

Localization of Rac during phagocytosis

Mouse macrophage-like RAW264.7 cells were maintained in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10 % fetal bovine serum. For expression of GFP–Rac1(Q61L) and GFP–Rac1–ΔIns1(Q61L), transfection was performed with Cell Line Nucleofector Kit V (Amarsa) according to the manufacturer’s instructions, and the cells were incubated for 4 h on 35-mm-diameter glass-bottom dishes (MatTek). The culture medium was then replaced with Hepes-buffered saline and rabbit IgG-opsonized beads, prepared as described previously in [35], were added to each dish for 5 min at 37°C. The cells were fixed in 1.2 % formaldehyde and analysed with a laser-scanning confocal microscope LSM 510 (Carl Zeiss).

RESULTS

The role of the insert region of Rac1 in activation of the phagocyte NADPH oxidase gp91phox/Nox2 in a cell-free system

To minimize disruption of the global structure, we constructed a chimeraic mutant in which the insert region of Rac (amino acids 120–140) is replaced by a short sequence from loop eight in H-Ras (amino acids 121–128), called Rac–ΔIns1 (Figure 1B). This replacement was equivalent to those made in Cdc42 and RhoA, which have been frequently used in studies on the roles of the insert region in these two GTPases [32–34]. We then compared the ability of recombinant Rac1 and Rac1–ΔIns1 (Figure 2A), to activate the phagocyte NADPH oxidase gp91phox/Nox2 in a cell-free activation system. As shown in Figure 2(B), GTP-loaded Rac1–ΔIns1 is as active as wild-type Rac1 in supporting superoxide production by gp91phox/Nox2 at all concentrations.
The role of the insert region of Rac1 and Rac2 in a whole-cell system for activation of the phagocyte NADPH oxidase gp91phox/Nox2

We next tested the effect of mutation of the Rac1 insert region in a whole-cell system for oxidase activation. As shown in Figure 3(A), when expressed in HeLa cells, Rac1-ΔIns1 with the Q61L substitution [Rac1-ΔIns1(Q61L)], a mutation that constitutively renders the GTPase in an active GTP-bound state, was capable of activating the phagocyte oxidase in response to PMA, a potent stimulant of gp91phox/Nox2 at the cellular level. Superoxide production supported by Rac1-ΔIns1(Q61L) was rather higher than that by Rac1(Q61L), which retained the insert region, at any protein levels tested (Figure 3A). Similarly, activation of gp91phox/Nox2 by Rac2 was not affected by the truncation of the insert region (Figure 3B). Essentially the same results were obtained when the phagocyte oxidase was reconstituted in HEK-293 cells (results not shown). Thus the insert region appears to be dispensable for oxidase activation.

As Rac is also involved in direct activation of signalling molecules, such as the protein kinase Pak [p21 protein (Cdc42/Rac)-activated kinase 1], it is possible that Rac1-ΔIns1(Q61L) indirectly activates gp91phox/Nox2. To exclude this possibility, we substituted a cysteine residue for Tyr40 in the switch I region;
Figure 4 Activation of the phagocyte NADPH oxidase gp91phox/Nox2 with insert-region-deleted Rac proteins in a whole-cell system

(A) Myc–Rac1(G61L), Myc–Rac1–ΔIns1(G61L), Myc–Rac1–ΔIns2(G61L), Myc–Rac1–ΔIns3(G61L) or Myc–Rac1–ΔIns4(G61L) were co-expressed with gp91phox/Nox2, Myc–p67phox and FLAG–p47phox in HeLa cells. Concentrations of transfected plasmid are indicated. (B and C) Myc–Rac2(G61L), Myc–Rac2–ΔIns1(G61L), Myc–Rac2–ΔIns2(G61L), Myc–Rac2–ΔIns3(G61L) or pEF-BOS-Myc–Rac2–ΔIns4(G61L) were co-expressed with gp91phox/Nox2, Myc–p67phox and pEF-BOS-Flag–p47phox in HeLa cells. Concentrations of transfected plasmid are indicated. (D) HeLa cells were transfected with 1.0 μg of pEF-BOS encoding Myc–Rac1(G61L) or the Myc–Rac1–ΔIns1(G61L) and co-expressed with gp91phox/Nox2, Myc–p67phox and FLAG–p47phox. Cells were incubated for 5 min at 37°C, and then stimulated with (A–C) 200 ng/ml PMA or (D) 100 μM arachidonic acid. Chemiluminescence change was continuously monitored with Diogenes. Each graph represents the means ± S.D. of the chemiluminescence values measured for 10 min and obtained from three independent transfections. Protein levels of Rac, p67phox and p47phox were analysed by immunoblotting.

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this leads to the failure of Pak activation but affects neither Rac binding to p67phox nor activation of gp91phox/Nox2 [17,37]. As shown in Figure 3(C), Rac1–ΔIns1(Y40C/Q61L) fully supported superoxide production by gp91phox/Nox2, indicating that Rac1–ΔIns1 directly activates gp91phox/Nox2. This is supported by the finding that Rac1–ΔIns1(Q61L) with a substitution of a lysine residue for Ala27 failed to activate gp91phox/Nox2 (Figure 3C). This replacement is known to have no effect on Rac binding to Pak, but it does abrogate the ability of Rac to bind to p67phox, and hence the subsequent oxidase activation [17,38].

The Ala27 in the switch I region of Rac is naturally replaced by lysine in Cdc42 (Figure 1A), and this protein is also incapable of activating gp91phox/Nox2 (Figure 3D). In addition, Gly30 is substituted with serine in this GTPase. Interestingly, the double replacement by the Rac-derived residues (i.e. a K27A/S30G substitution) enables Cdc42 to fully interact with p67phox in a GTP-dependent manner [11]. As shown in Figure 3(D), this double mutant Cdc42 activated gp91phox/Nox2 to the same extent as Rac1. The activation was dependent on GTP, since the T17N substitution, which renders Cdc42 in an inactive GDP-bound state, resulted in a loss of the activity (Figure 3D). It should be noted that, of the 13 amino acids in the insert region, only six residues are conserved between Cdc42 and Rac (Figure 1B). This is consistent with this finding that the insert region of Rac1 and Rac2 is dispensable for oxidase activation.

Comparison of the ability of the insert-region-deleted Rac proteins to induce gp91phox/Nox2 in a whole-cell activation system

We next compared the activities of the Rac proteins, with the various insert region mutations, with that of Rac1–ΔIns1(Q61L) using a whole-cell system for activation of gp91phox/Nox2 (Figure 4A). As shown in Figure 4(A), Rac1–ΔIns3(Q61L) was as effective as the Rac1–ΔIns1(Q61L) protein in supporting superoxide production by gp91phox/Nox2. Like Rac1–ΔIns1(Q61L), Rac1–ΔIns2(Q61L) and Rac1–ΔIns4(Q61L) activated gp91phox/Nox2 to a higher extent than the insert-region-retaining Rac1 (Figure 4A). Similarly, Rac2–ΔIns3(Q61L) and Rac2–ΔIns4(Q61L) fully supported superoxide production by gp91phox/Nox2 (Figure 4B). These findings further support the conclusion that the insert region is not required for gp91phox/Nox2 activation in vivo.

Compared with the other mutant proteins, we detected there were smaller amounts of Rac2–ΔIns2(Q61L) protein, even though the cells were transfected with the same doses of the expression plasmid vector (Figure 4B). This result indicated that this mutant protein is unstable. To estimate the activity of Rac2–ΔIns2(Q61L), we compared it with that of the insert-region-retaining Rac2, at similar protein levels. As shown in Figure 4(C), Rac2–ΔIns2(Q61L) showed a comparable ability to activate gp91phox/Nox2. Thus the ΔIns2 insert region truncation in Rac2 does not appear to lead to a loss of the activity, despite the instability of the resultant protein.

We further tested the role of the insert region of Rac in gp91phox/Nox2 activation when cells were treated with arachidonic acid, a physiological stimulant, instead of PMA. As shown in Figure 4(D), all the insert-region-deleted Rac1(Q61L) proteins produced a greater amount of superoxide in response to arachidonic acid than the Rac1(Q61L) protein.

The role for the insert region of Rac1 in localization to phagosomes

The phagocyte NADPH oxidase is activated in both phagosomal and plasma membranes [5–8]. Rac1, the predominant isoform of the Rac GTPases in macrophages, is known to localize to both phagosomes and plasma membranes, when expressed as a GFP-fusion protein in macrophage-like RAW264.7 cells [39]. To determine the role of the insert region in Rac localization...
Role for the insert region of Rac in Nox activation

379  Figure 7  The role of the insert region of Rac1 and Rac2 in activation of Nox3

+  (A and B) Myc–Rac1(Q61L) or Myc–Rac1–ΔIns1(Q61L) were co-expressed in HEK-293 cells with Nox3 and (A) Myc–p67phox or (B) Myc–Noxa1. (C and D) Myc–Rac2(Q61L) or Myc–Rac2–ΔIns1(Q61L) were co-expressed in HEK-293 cells with Nox3 and (C) Myc–p67phox or (D) Myc–Noxa1. Chemiluminescence change was continuously monitored at 37°C with Diogenes. Each graph represents the means ± S.D. of the chemiluminescence values measured for 10 min and obtained from three independent transfections. Protein levels of Rac, p67phox and Noxa1 were analysed by immunoblotting. The vertical lines in the panels indicate the grouping of images from different parts of the same gel.

during phagocytosis, we expressed GFP-fused Rac1–ΔIns1 in RAW264.7 cells. As shown in Figure 5, Rac1–ΔIns1 was recruited not only to the plasma membrane but also to phagosomes upon ingestion of IgG-coated beads. Thus the insert region is dispensable for targeting of Rac to phagosomes, an event that plays a crucial role in activation of the phagocyte oxidase.

The role for the insert region of Rac1 and Rac2 in Nox1 activation

It is known that Rac1 is involved in activation of the non-phagocytic oxidase Nox1. In the presence of Noxo1 and Noxa1, Nox1 produces superoxide and the rate of superoxide formation is increased approx. 2-fold by ectopic expression of an active Rac protein [15–17]. Rac1–ΔIns1(Q61L) enhanced superoxide production by Nox1 to the same extent as Rac1(Q61L) in HEK-293 (Figure 6A) and HeLa (results not shown), cells, with both co-expressing Noxo1 and Noxa1. Rac2 was also capable of facilitating Nox1 activation, and this occurred independently of the insert region (Figure 6B). These findings indicate that the insert region of Rac1 and Rac2 does not contribute to activation of Nox1.

The role of the insert region of Rac1 and Rac2 in Nox3 activation

Although Nox3, another non-phagocytic oxidase, constitutively produces superoxide even in the absence of oxidase regulatory proteins, such as p47phox, p67phox, Noxo1 and Noxa1 [18], superoxide production is significantly enhanced by either p67phox or Noxa1 in a Rac-dependent manner [15,21]. The removal of the insert region did not attenuate the effect of Rac1 on the enhancement by p67phox (Figure 7A) or by Noxa1 (Figure 7B). Rac2 also facilitated Nox3 activation in a manner independent of the insert region (Figures 7C and 7D). Thus Nox3 activation does not require the insert region of Rac1 and Rac2.

The role of the insert region of Rac3 in activation of Nox1–3

We finally tested whether Rac3, another member of the Rac GTPase family in mammals, has the ability to activate the Nox family oxidases, and, if so, whether the insert region plays a role. As shown in Figure 8, Rac3 was capable of participating in activation of gp91phox/Nox2, Nox1 and Nox3, to the same extent as Rac1. The deletion of the insert region did not affect the abilities of Rac3, again indicating that this region is dispensable.

DISCUSSION

In the present study we show that the insert region of the three Rac GTPases is dispensable for activation of the phagocyte oxidase gp91phox/Nox2 and the non-phagocytic oxidases Nox1 and Nox3. The dispensability explains the reason why the insert region of Rac is equivalent to that of Cdc42 in activation of gp91phox/Nox2 at the cellular level (Figure 3D), despite having less than 50% identity.
in amino acid sequence. Earlier studies have described normal cell-free activation of the phagocyte oxidase by Rac-Cdc42 [38] and Rac-RhoA [40] chimeras, in which the Rac insert region is replaced by the corresponding regions in Cdc42 and RhoA. Notably, of the 13 amino acid residues, only three are conserved between the insert regions of Rac1 and RhoA (Figure 1B).

The present study demonstrates that Rac1-ΔIns2 fully activates gp91phox/Nox2 both in vivo and in vitro (Figures 2B and 4A). A previous in vitro study [25] reported that Rac1-ΔIns2 was only approximately half as effective as the wild-type protein in activating the phagocyte NADPH oxidase. However, it was also reported in a whole-cell system that Rac1-ΔIns2 exhibited two thirds of the activity of the insert-region-retaining Rac1, at a lower protein expression level, and that this mutant protein did elicit comparable oxidase activation at a higher expression level [27]. Although Rac2-ΔIns3 supports oxidase activation to the same extent as Rac1(Q61L) in a whole-cell system in the present study (Figure 4B), it was previously reported that superoxide production induced by Rac2-ΔIns3 is approx. 3-fold less than that for the wild-type protein in a cell-free activation system for gp91phox/Nox2 [28]. The reason for the discrepancy between the present and previous studies is unknown.

In the reports showing that the Rac1 insert region is not involved in a cell-free activation of the phagocyte oxidase, alanine residues are used to replace amino acids 123–133 [29–31]. This mutation is equivalent to that in Rac1-ΔIns4, which has an ability to fully activate the oxidase both in vivo and in vitro (Figures 2B and 4B). On the other hand, Rac1-ΔIns2 and Rac2-ΔIns3 lack amino acids 124–135, which might induce a subtle but significant conformational change that affects protein function and/or stability under certain conditions. In this context it should be noted, although the insert region of Rac1 is distant from the switch I region (Figure 1C), there is a dynamic coupling between the regions [41]. In addition, the ΔIns2 truncation of amino acids

Figure 8  The role of the insert region of Rac3 in activation of NADPH oxidases

Myc–Rac3(Q61L), Myc–Rac3-ΔIns1(Q61L) or Myc–Rac1(Q61L) were co-expressed in HEK-293 cells with (A) gp91phox /Nox2, Myc–p67phox and FLAG–p47phox, (B) Nox1, Myc–Noxa1 and FLAG–Noxo1 or (C) Nox3 and Myc–p67phox or Myc–Noxa1. After pre-incubation for 5 min, cells were incubated at 37 °C with or without 200 ng/ml PMA. Chemiluminescence change was continuously monitored with Diogenes. Each graph represents the means ± S.D. of the chemiluminescence values measured for 10 min and obtained from three independent transfections. Protein levels of Rac, p67phox, p47phox, Noxa1 and Noxo1 were analysed by immunoblotting.

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124–135 results in a marked destabilization of Rac2 (Figure 4B), although the same mutation did not largely affect the stability of Rac1. It seems possible that, depending on experimental conditions, Rac1–ΔIns2 and Rac2–ΔIns3 may become somewhat unstable, which leads to a reduced activation of gp91phox/Nox2 as observed in previous studies [25,27,28]. Under the present cell-free and whole-cell conditions, all of the four mutant Rac proteins lacking the insert region (ΔIns1–ΔIns4) are capable of inducing a full activation of the NADPH oxidase (Figures 2 and 4).

Rac exists in a heterodimer with RhoGDI (Rho GDP dissociation inhibitor) in the cytoplasm of phagocytes, and interacts upon cell stimulation with p67phox at phagosomal and plasma membranes to activate the phagocyte oxidase [5–8]. The insert region contributes to neither the Rac–RhoGDI dimer [23,24] nor the Rac–p67phox interactions [11], suggesting that the insert region is not involved in membrane translocation of Rac. Nevertheless, it was still possible that the insert region had such a role given that the insert region of Rac1 was reported to bind to membrane phosphoinositides and was therefore considered to participate in membrane targeting [42]. The present study demonstrates that a mutant Rac1 lacking the insert region is normally recruited to phagosomal and plasma membranes (Figure 5). Thus the Rac insert region is not required for membrane recruitment as well as not being required for subsequent activation of the phagocyte oxidase.

The insert region of other members of the Rho subfamily, such as RhoA and Cdc42, has been implicated in a variety of biological effects. RhoA induces Rho kinase activation and cellular transformation in a manner dependent on the insert region [34], the insert region of Cdc42 indirectly participates, but in activation of phospholipase D1 [33] and in the social amoeba Dictyostelium, the insert region of RacG is essential for phagocytosis and chemotaxis [43]. However, little is known about direct targets for the insert region of Rac1 lacking the insert region is normally recruited to the Rac–p67phox region contributes to neither the Rac–RhoGDI dimer [23,24] nor the Rac–RhoGDI complex and the structural basis for the regulation of Rho proteins by RhoGDI complex [28,45]. The present findings, however, suggest that this interaction makes a minor contribution to oxidase activation.

We also show in the present study that Rac3, a third member of the Rac GTPases in mammals, is capable of activating the three NADPH oxidases, Nox1, gp91phox/Nox2 and Nox3 (Figure 8). Rac3 is predominantly expressed in the brain [2–4]. As the gp91phox/Nox2- and Nox1-based oxidase also exist in the brain [46–48], it is possible that oxidase activation by Rac3 occurs in vivo. Rac3 is also present in leukemia and cancer cells [4,49]. In these cells, Rac3-mediated activation of NADPH oxidases, which leads to ROS production, could be important because ROS, via induced DNA damage, are known to be associated with cancer [2,7].

**AUTHOR CONTRIBUTION**

Kei Miyano and Hirotumi Koga designed and performed experiments, analysed data and wrote the manuscript. Reiko Minakami designed and performed experiments and analysed data. Hideki Sumimoto designed the study, analysed data and wrote the manuscript.

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