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Role of the small GTPase Rac in p22^{phox}-dependent NADPH oxidases

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Abstract

The superoxide-producing phagocyte NADPH oxidase gp91^{phox}/Nox2 and the non-phagocytic oxidases Nox1 and Nox3 each form a complex in the membrane with p22^{phox}, which provides both stabilization and a docking site for organizer proteins. The p22^{phox}-complexed Nox2 and Nox1 are dormant on their own, and their activation requires soluble supportive proteins such as a Nox organizer (p47^{phox} or Noxo1) and a Nox activator (p67^{phox} or Noxa1). The small GTPase Rac directly binds to the activators, and thus plays an essential role in the Nox2-based oxidase containing p47^{phox} and p67^{phox} or a positive role in Nox1 activity supported by Noxo1 and Noxa1. Although Nox3 complexed with p22^{phox} constitutively produce superoxide, the production can be enhanced by supportive proteins. Here we compare the roles of Rac in these p22^{phox}-dependent oxidases using the organizer and activator in different combinations. Expression of constitutively active Rac1(Q61L) is essential for activation of the Nox2- or Nox1-based oxidase containing the organizer p47^{phox} and either p67^{phox} or Noxa1. When these oxidases use Noxo1 as an organizer instead of p47^{phox}, they produce a small but significant amount of superoxide without expression of Rac1(Q61L), although the production is enhanced by Rac1(Q61L). Thus p47^{phox} is likely related to strict dependence on Rac. The Nox3-based oxidase has a similar tendency in the change of the dependence: Rac plays a positive role in Nox3 activation in the presence of p47^{phox} and either p67^{phox} or Noxa1, whereas Rac fails to upregulate Nox3 activity when p47^{phox} is replaced with Noxo1. We also demonstrate that, in the Nox3-based oxidase containing solely p67^{phox} as supportive protein, expression of Rac1(Q61L) enhances not only superoxide production but also membrane translocation of p67^{phox}. Since the enhancements are not observed with a mutant p67^{phox} defective in binding to Rac, this GTPase appear to directly recruit p67^{phox} to the membrane.
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Keywords: Rac; gp91^{phox}; Nox1; Nox3; p67^{phox}

1. Introduction

Reactive oxygen species (ROS) are produced for various biological events including host defense, hormone synthesis, and signal transduction [1–7]. Deliberate production of ROS is at least partly catalyzed by the Nox-family NADPH oxidases, which are integrated into the membrane; the family encompasses seven members identified in humans: five Nox proteins (Nox1, Nox2, Nox3, Nox4, and Nox5) and two dual oxidases (Duox1, and Duox2) [1–7]. Among them, Nox1, Nox2 (originally known as gp91^{phox}), and Nox3 are genetically closest to each other. The founder member Nox2 is predominantly expressed in phagocytes; the phagocyte oxidase, dormant in resting cells, is activated during phagocytosis to produce superoxide, a precursor of a powerful microbicidal ROS. The significance of this oxidase system in the killing of ingested microbes is evidenced by the recurrent, life-threatening infections that occur in patients with chronic granulomatous disease (CGD), whose phagocytes genetically lack the superoxide-producing activity [8]. Nox1 is expressed in a variety of cells including colon epithelial cells and vascular smooth muscle cells, and has recently been shown to

Abbreviations: ROS, Reactive oxygen species; Nox, NADPH oxidase; CGD, chronic granulomatous disease; PMA, phorbol 12-myristate 13-acetate; TPR, tetratricopeptide repeat; Noxo1, Nox organizer 1; Noxa1, Nox activator 1.

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participate in angiotensin II-mediated hypertension [9–11]. Although Nox3 was initially identified as an oxidase abundant in the human fetal kidney, it is currently known to be expressed also in the inner ear and involved in the morphogenesis of otoconia, which is essential for perception of balance and gravity [12].

In phagocytes, Nox2 forms a mutually stabilizing complex in the membrane with p22^{phox}, which provides a docking site for soluble regulatory proteins [1–7]. Activation of Nox2 requires the small GTPase Rac and two supportive proteins: the Nox organizer p47^{phox} and the Nox activator p67^{phox}. In the cytoplasm of resting cells, p47^{phox} is complexed with p67^{phox} via the tail-to-tail interaction [13–15], whereas Rac associates with the inhibitor protein Rho-GDI [16]. During phagocytosis or upon cell stimulation with soluble agents such as phorbol 12-myristate 13-acetate (PMA), the p47^{phox}–p67^{phox} complex and Rac are recruited to the membrane, where they assemble with the membrane components Nox2 and p22^{phox} to form an active oxidase complex. p47^{phox} associates in a direct manner with p22^{phox}, which is essential for oxidase activation, and thus tethers p67^{phox} to p22^{phox} [17–21]; and GTP-bound Rac interacts with p67^{phox} via directly binding to the N-terminal domain comprising four tetratricopeptide repeat (TPR) motifs [22–24]. This interaction is required for oxidase activation as well, because a mutant p67^{phox} carrying the substitution of Glu for Arg-102 in the third TPR neither binds to Rac nor supports superoxide production [22].

Nox1, also forming a heterodimer with p22^{phox} [25,26], is inactive without supportive proteins; it can be activated by p67^{phox} and p47^{phox} but to a much lesser extent than Nox2 [26–29]. Full activation of Nox1 requires the p67^{phox} homologue Noxa1 (Nox activator 1) and the p47^{phox} homologue Noxo1 (Nox organizer 1) [26–29], in which Noxo1 tethers Noxa1 to p22^{phox}, analogous to p47^{phox} binding to p67^{phox} and p22^{phox} [26,30,31]. In the presence of Noxa1 and Noxo1, PMA enhances superoxide production by Nox1, although Nox1 generates superoxide without cell stimulants [26–29]. Recent studies have shown that Rac participates in activation of the Nox1-based oxidase containing Noxo1 and Noxa1 [30–34]. In this system, Rac functions by binding to Noxa1, because superoxide production by Nox1 is severely impaired when the wild-type Noxa1 is replaced with a mutant one carrying the substitution of Glu for Arg-103 in the third TPR [30–32], a protein defective in binding to Rac [26]. Whereas the activity of Nox2 supported by both p47^{phox} and p67^{phox} in HeLa or HEK293 cells requires further expression of constitutively active Rac1(Q61L) [29,30], Nox1 with coexpressed Noxo1 and Noxa1 produces substantial amounts of superoxide, which is enhanced only slightly, if any, by Rac1(Q61L) [30–32]. Nevertheless, the involvement of Rac in Nox1 is supported by several lines of evidence including the observation that Nox1 activation is impaired by RNA interference-mediated knockdown of Rac1 [30–32]. Thus there might be a difference in the demand for Rac between Nox1 and Nox2: endogenous Rac is sufficient for Nox1, whereas ectopic expression of active Rac is required for Nox2 in the whole cell reconstitution system.

Like Nox1 and Nox2, Nox3 forms a complex with p22^{phox}, which is essential for Nox3 activity [35]. On the other hand, although Nox4 associates with p22^{phox}, Nox4 activity is likely independent of the association [25,36,37]; Nox5 and Duox oxidases do not seem to form a complex with p22^{phox} [5,37]. Thus the first three oxidases Nox1, Nox2, and Nox3 are functionally close to each other in that their activity is dependent on p22^{phox}. In addition, only the three p22^{phox}-dependent oxidases can be regulated by the Nox organizers and activators, which are not involved in regulation of Nox4, Nox5, Duox1, and Duox2 [5,25,36,37]. This is consistent with the fact that p22^{phox} functions as a anchoring site for the organizer proteins. In contrast to Nox2 and Nox1, however, Nox3 does not exhibit strict requirements for both "activator" and "organizer" components: Nox3, complexed with p22^{phox}, produces a substantial amounts of superoxide without supportive proteins [35]; and the activity of Nox3 can be enhanced by p47^{phox}, p67^{phox}, or Noxo1 alone, or by the combination of p47^{phox} and p67^{phox} [31,35,38,39]. In addition, compared with Nox2 and Nox1, the role of Rac in Nox3 regulation has been somewhat enigmatic: this GTPase does not seem to strongly affect Nox3 activity supported by Noxo1 and Noxa1 [35], whereas it likely up-regulates Nox3-based oxidases reconstituted by p67^{phox} or Noxa1 alone [31]. In the latter cases, however, it has been unknown how the activator proteins are targeted to the membrane in the absence of the organizer proteins. In contrast to these p22^{phox}-dependent oxidases, Rac does not appear to participate in activation of Nox4 [36], Nox5 [40], or Duox [41].

To further understand the roles of Rac in Nox1, Nox2, and Nox3, here we have reconstituted these p22^{phox}-dependent oxidases in various cell types using the organizer and activator in different combinations. Expression of Rac1(Q61L) is absolutely required for activation of the Nox2- or Nox1-based oxidase containing the organizer p47^{phox} and either p67^{phox} or Noxa1. When Noxo1 replaces p47^{phox}, these oxidases produce a significant amount of superoxide without expression of Rac1(Q61L), although the production is enhanced by Rac1(Q61L). Thus p47^{phox} is likely related to strict dependence on Rac; a similar difference between p47^{phox} and Noxo1 may occur in Nox3-based oxidases. We also show that Rac facilitates the p67^{phox}-supported Nox3 activity and membrane translocation of this activator protein.

2. Materials and methods

2.1. Plasmid construction

The human cDNAs encoding Nox1, Nox2, Nox3, p22^{phox}, p47^{phox}, p67^{phox}, Noxo1, Noxa1, Rac1, and RhoGDI were prepared as previously described [30,35]. Mutations leading to the indicated amino acid substitutions were introduced by polymerase chain reaction-mediated site-directed mutagenesis. The cDNAs for Nox1 and Nox2, and Nox3 were ligated to the mammalian expression vector pcDNA3.0 (Invitrogen). The cDNA encoding Rac1(Q61L), p22^{phox}, Noxo1, p47^{phox},

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