GTPases and reactive oxygen species: switches for killing and signaling

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Summary

In neutrophils and other phagocytic cells, the small GTPase Rac is an essential regulator of a multi-component NADPH oxidase that produces high levels of superoxide, which kills invading pathogens. In many other cell types, Rac and newly discovered relatives of the neutrophil burst oxidase and its subunits have been found associated with production of reactive oxygen species, implicating superoxide production in a wide range of cellular processes not related to host defense. Although the precise role played by Rac in the regulation of these novel oxidases is not known, Rac does control the cellular redox state. Through these pro-oxidant mechanisms, Rac and the novel oxidases modify gene expression, cell proliferation, adhesion and many cell-specific functions.

Key words: Rac, GTPase, ROS, Neutrophil, Oxidase, Nox

Introduction

Neutrophils kill invading microorganisms by producing high concentrations of superoxide through the assembly of a membrane-associated NADPH oxidase, also known as the phagocyte oxidase or respiratory burst oxidase. Historically, this oxidase was thought to be part of a unique and tightly regulated machinery restricted to neutrophils and some other phagocytic cells. However, in recent years, superoxide and other reactive oxygen species (ROS) have been implicated in numerous physiological and pathological processes as signal transduction mediators (reviewed by Droge, 2002). ROS qualify as authentic second messengers because of their regulated production in response to an agonist, the existence of catabolic mechanisms to terminate their signaling, and demonstrable redox-dependent reversible and function-altering modification of target proteins (Cooper et al., 2002).

The small GTPases of the Rho family are key regulators of cell function and are as pleiotropic as ROS in their participation in cellular function. They coordinate extracellular signals received through membrane receptors with cytoskeletal changes and activation of several signaling pathways, participating in processes including phagocytosis, mitogenesis, cell adhesion, gene expression, cell-cycle progression and cell survival (Ridley, 2001; Etienne-Manneville and Hall, 2002).

The small GTPases of the Rho family, like all small GTPases, work as binary switches that exist in GTP-bound or GDP-bound conformations (reviewed by Takai et al., 2001). The switch is activated when an upstream signal activates a GTPase exchange factor (GEF) that, upon binding to the GDP-loaded GTPase, promotes the exchange of GDP for GTP. GTP binding induces a conformational change mainly in two regions – switch 1 and switch 2 – which relay the signal by interacting with and modifying the activity of effector proteins. The Rho GTPases, including RhoA, Rac1 and Cdc42 and many other members, are also thought to interact with some effectors through an additional 13-residue region known as the insert region, which is a distinct feature of this GTPase family (Takai et al., 2001; Wherlock and Mellor, 2002). One of the first Rac effectors to be identified was the phagocyte NADPH oxidase (Abo et al., 1991); to date, the Rho GTPase family has 28 different known effectors, including phosphoinositide (PI) kinases, Ser/Thr kinases and actin-interacting proteins.

The activity cycle is terminated by hydrolysis of GTP by the GTPase. The rate of GTP hydrolysis is accelerated by interaction with GTPase-activating proteins (GAPs). Rho GTPase activity is limited in duration and restricted to a specific subcellular location not only by interaction with specific guanosine nucleotide exchange factors (GEFs) and GAPs but also by additional regulatory mechanisms conferred by the C-terminus. These GTPases are post-translationally modified by isoprenylation, which is necessary for their localization to different membrane compartments. The isoprenoid moiety is masked through formation of complexes with Rho guanine nucleotide dissociation inhibitor (GDI) (Hoffman et al., 2000). Binding to Rho-GDI not only maintains the GTPase in a GDP-bound state and soluble in the cytosol (Regazzi et al., 1992), but also promotes dissociation of GDP-bound GTPases from the membrane. Additional regulation occurs through the preferential localization of the GTPases to different membrane compartments, which is driven by the hypervariable sequence found upstream of the isoprenylation signal (Michaelson et al., 2001). Each of these characteristics plays a crucial role in Rac-mediated control of neutrophil oxidase activity. This article discusses current understanding of how Rac regulates the phagocyte NADPH oxidase and provides insights into the regulation of other ROS-generating enzymes by GTPases.

NADPH oxidase in neutrophils: ROS generation for death

The paradigm for GTPase regulation of ROS production is the assembly and activation of the phagocyte NADPH oxidase. An inability to produce superoxide efficiently because of

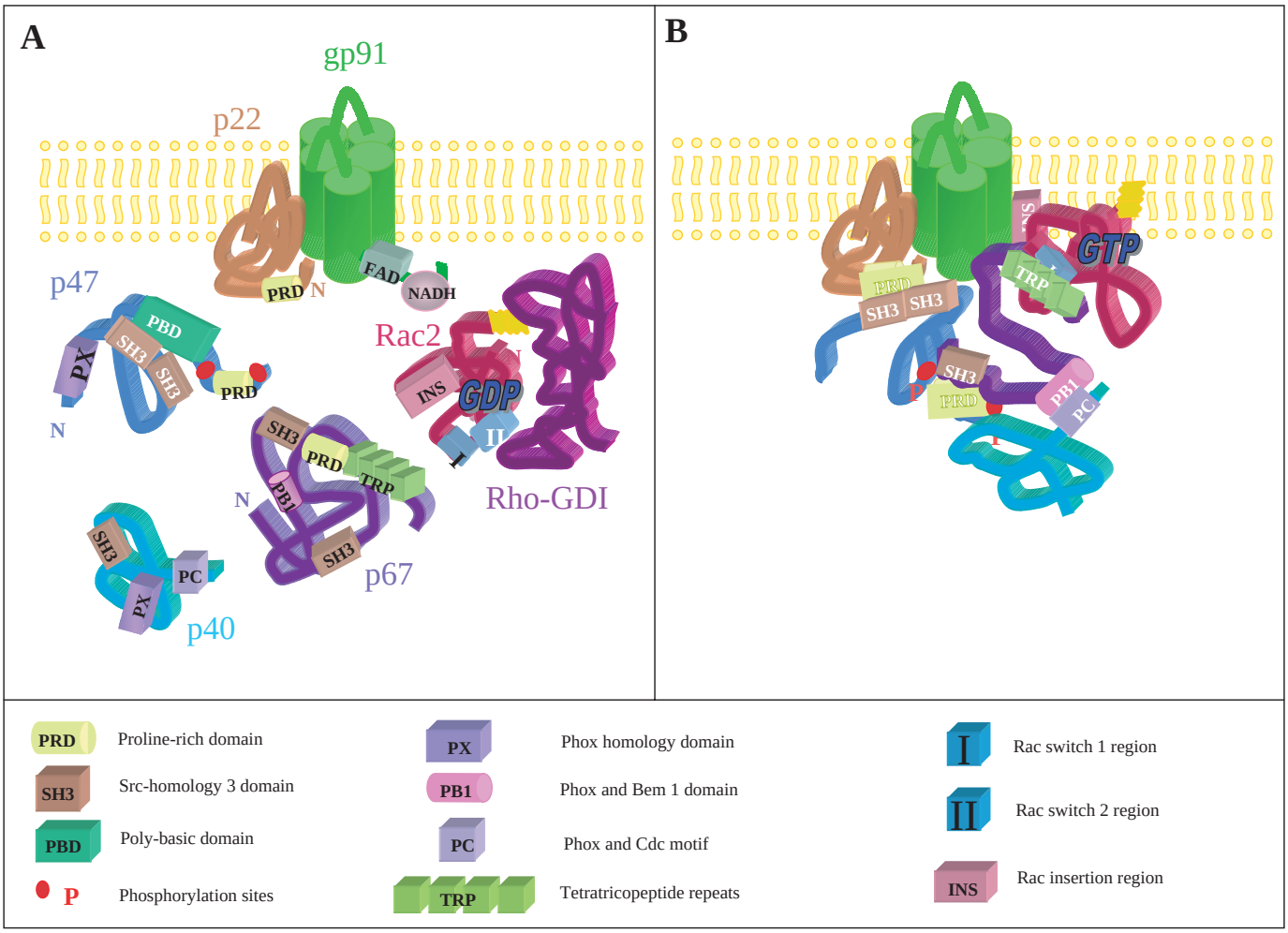

Fig. 1. Multiple domain interactions drive assembly and function of the NADPH oxidase. The diagram depicts the protein domains and motifs in all the oxidases subunits involved in assembly. The domain localization is approximate and does not exclude additional possible interactions between the subunits. For clarity, the lettering color changes from black to white when the domain is involved in a functional interaction in each state. In the resting state (A), the tandem SH3 domains of $p47^{phox}$ form a groove that binds the poly-basic region in the C-terminal domain. Following signaling from soluble ligands and from phagocytosis, assembly is triggered and the domain interactions change (B). Phosphorylation in the C-terminal domain of $p47^{phox}$ and phosphoinositides binding to PX domains drive the interaction of the tandem SH3 domains with the PRD domain of $p22^{phox}$ and the PRD interaction with an SH3 domain of $p67^{phox}$. Concurrently, Rac translocates to the membrane and interacts with $p67^{phox}$ and possibly $gp91^{phox}$ in a GTP-dependent fashion.

mutations disrupting oxidase function causes the inherited, life-threatening chronic granulomatous disease (CGD). Analysis of the genetic loci associated with this disease led to the identification of the subunits of the NADPH oxidase: $gp91^{phox}$, $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$. Biochemical studies revealed the participation of additional components: the small GTPase Rac and $p40^{phox}$. Subsequently, successful in vitro reconstitution uncovered the hierarchical series of interactions between the different components necessary for assembly and activation of the oxidase (Fig. 1).

Assembly and activation of the phagocyte NADPH oxidase

The assembly mechanism of the multi-component phagocyte NADPH oxidase ensures tightly regulated production of high superoxide levels (reviewed by Babior et al., 2002). The NADPH oxidase catalytic core is a membrane-associated cytochrome b558, which exists as an inactive heterodimer formed by $p22^{phox}$ and $gp91^{phox}$ in the resting neutrophil. The $gp91^{phox}$ subunit is a flavocytochrome containing an NADPH-binding site, a non-covalently bound flavin adenine dinucleotide (FAD) molecule and two non-identical heme groups coordinated by two pairs of histidine residues (Biberstine-Kinkade et al., 2001). The FAD-binding feature of this enzyme is the site for diphenylene iodonium (DPI) inhibition of superoxide production (O'Donnell et al., 1993). The $p22^{phox}$ subunit has a regulatory role: it is essential for oxidase activation and recruitment of additional cytosolic factors (Dinauer et al., 1991).

Upon stimulation, four cytosolic proteins – $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and the GTPase Rac – translocate to the plasma membrane to assemble a fully active oxidase complex, which uses electrons from NADPH to reduce oxygen to form

superoxide. The $p47^{phox}$ subunit drives assembly, triggering the translocation of and interaction between the different subunits of the complex through discrete domains (Fig. 1). In the resting state, $p47^{phox}$ exists in an autoinhibited closed conformation formed through intramolecular binding of the two SH3 domains in the N-terminal region to a poly-basic sequence in the C-terminus (Leto et al., 1994; Shiose and Sumimoto, 2000). Phosphorylation of this region by protein kinase C (el Benna et al., 1994; Park and Babior, 1997), Akt (Chen et al., 2003) and/or mitogen-activated protein (MAP) kinases (Dewas et al., 2000) inhibits this interaction, permitting binding of the exposed N-terminal SH3 domains to the proline-rich domain (PRD) of $p22^{phox}$ (Leto et al., 1994) and hence to cytochrome b558 (DeLeo et al., 1995; Dang et al., 2002). Besides phosphorylation, assembly and activation can also be driven by several non-physiological stimuli, such as SDS or C-terminal truncation of $p47^{phox}$ (Ago et al., 1999; Shiose and Sumimoto, 2000). Activating conformational changes also permit the interaction of the Phox homology (PX) domain of $p47^{phox}$ with 3′-phosphorylated phosphoinositides, the products of activated PI 3-kinase (Ago et al., 2003). The $p47^{phox}$ subunit binds through its C-terminal PRD to the C-terminal SH3 domain of $p67^{phox}$ (Leto et al., 1994), initiating translocation to the membrane and binding of the other subunits. The failure of $p47^{phox}$-deficient individuals to translocate $p40^{phox}$ and $p67^{phox}$ underscores the essential role of this subunit in oxidase assembly (Heyworth et al., 1991). However, $p47^{phox}$ is not intrinsically required for superoxide production, since full oxidase activity can be reconstituted in vitro without $p47^{phox}$ by increasing the concentrations of Rac2 and $p67^{phox}$ (Freeman and Lambeth, 1996; Koshkin et al., 1996; Diebold and Bokoch, 2001) or by generating a $p67^{phox}$-Rac2 chimera (Gorzalczany et al., 2002).

The $p67^{phox}$ subunit is essential for superoxide production, and interacts directly with $gp91^{phox}$ (Dang et al., 2001). The N-terminal domain contains tetratricopeptide (TRP) repeats (1-4) that interact with Rac (Nisimoto et al., 1997), which results in increased affinity of $p67^{phox}$ for $gp91^{phox}$.

The $p40^{phox}$ subunit forms a constitutive complex with $p47^{phox}$ and $p67^{phox}$, and is translocated to the membrane when the oxidase is activated (Wientjes et al., 1993). The SH3 domain of $p40^{phox}$ interacts with the PRD of $p47^{phox}$ (Sathyamoorthy et al., 1997), and the PC domain interacts with the PB1 domain of $p67^{phox}$ (Ito et al., 2001). The functional relevance of these interactions has been difficult to address because $p40^{phox}$ is dispensable for reconstituted oxidase activity in vitro, and no mutations in this subunit have so far been found to be associated with CGD. However, $p40^{phox}$ increases the affinity of $p47^{phox}$ for the cytochrome in vitro (Cross, 2000) and oxidase reconstitution experiments in non-phagocytic cells show that $p40^{phox}$ expression stimulates membrane translocation of $p47^{phox}$-$p67^{phox}$ and superoxide production in a stimuli-selective and cell-context-dependent fashion (Kuribayashi et al., 2002).

Role of Rac in phagocyte NADPH oxidase activation

Rac2 is essential for reconstituted oxidase activity in vitro and in vivo, as revealed by CGD in patients bearing mutations in this GTPase (Ambruso et al., 2000; Williams et al., 2000) (recently reviewed by Dinauer, 2003). However, Rac is not necessary for the translocation of the other cytosolic subunits because, in systems that lack Rac, these subunits are independently recruited to the membrane (Roberts et al., 1999). Similarly, in systems that lack $gp91^{phox}$, $p47^{phox}$ and $p67^{phox}$, Rac still translocates (Heyworth et al., 1994). Tyrosine kinase inhibitors selectively abolish chemoattractant-induced Rac2 translocation independently of $p67^{phox}$ and $p47^{phox}$ (Dorseuil et al., 1995), which supports the idea that parallel pathways drive Rac2 and the other cytosolic subunits to the membrane. In resting neutrophils, Rac is geranylgeranylated (Kinsella et al., 1991) and localizes to the cytosol, forming a complex with Rho-GDI (Abo et al., 1991). Several GEFs are present in neutrophils and could potentially localize Rac to the membrane, including P-Rex1, which is activated by 3′-phosphorylated phosphoinositides and heterotrimeric G proteins (Welch et al., 2002), and Vav, which is activated by tyrosine phosphorylation (Zheng et al., 1996).

Once at the membrane, Rac binds directly to $p67^{phox}$ and probably to $gp91^{phox}$. The residues N26 and G30 in the switch 1 region of Rac interact with loops connecting the TRP motifs in the $p67^{phox}$ N-terminal TRP (Diekmann et al., 1994; Lapouge et al., 2000), whereas the insert region (amino acids 120-137) is thought to bind $gp91^{phox}$ (Freeman et al., 1996; Diebold and Bokoch, 2001). The detailed mechanism for Rac-mediated regulation of oxidase activity is controversial, because it has been proposed that Rac modulates the electron transfer process by binding to $p67^{phox}$ (Freeman and Lambeth, 1996; Gorzalczany et al., 2002) or by directly regulating electron transfer in $gp91^{phox}$ (Diebold and Bokoch, 2001).

The molecular basis for Rac2 specificity

Another striking aspect of Rac-dependent NADPH oxidase activation is the selectivity for Rac2, which is the prominent Rac isoform expressed in hematopoietic cells, rather than for Rac1. The molecular basis for this selectivity might be relevant to the mechanisms for Rac-mediated ROS generation in non-myeloid tissues, where Rac1 is the prevalent isoform. $Rac2^{-/-}$ individuals display a CGD phenotype (Ambruso et al., 2000; Williams et al., 2000), even though Rac1 is present in neutrophils, is activated by *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) (Li et al., 2002) and can functionally substitute for Rac2 in reconstituted systems [e.g. using the purified oxidase) (Abo et al., 1991); or transfected cell lines) (Price et al., 2002)]. This lack of complementation in vivo suggests a specific role for Rac2 in at least two possible circumstances: Rac2 could be required for oxidase function either in response to specific signal transduction pathways or could reside at a distinct subcellular location. In both scenarios (summarized below), an isoform-selective GEF could regulate the distinct Rac2 functions during neutrophil activation.

Given that Rac1 can substitute for Rac2 in vitro, the non-complementable Rac2 function in vivo could lie upstream of oxidase assembly. This would explain why $Rac2^{-/-}$ mice display a partial phenotype, in which superoxide production in response to fMLP, phorbol 12-myristate 13-acetate (PMA) and Ig-dependent phagocytosis is blocked but is not blocked in response to complement-dependent phagocytosis (Kim and Dinauer, 2001). $Rac2^{-/-}$ individuals not only show a CGD phenotype but also display defects in leukocyte adhesion and actin organization (Williams et al., 2000). This phenotype is

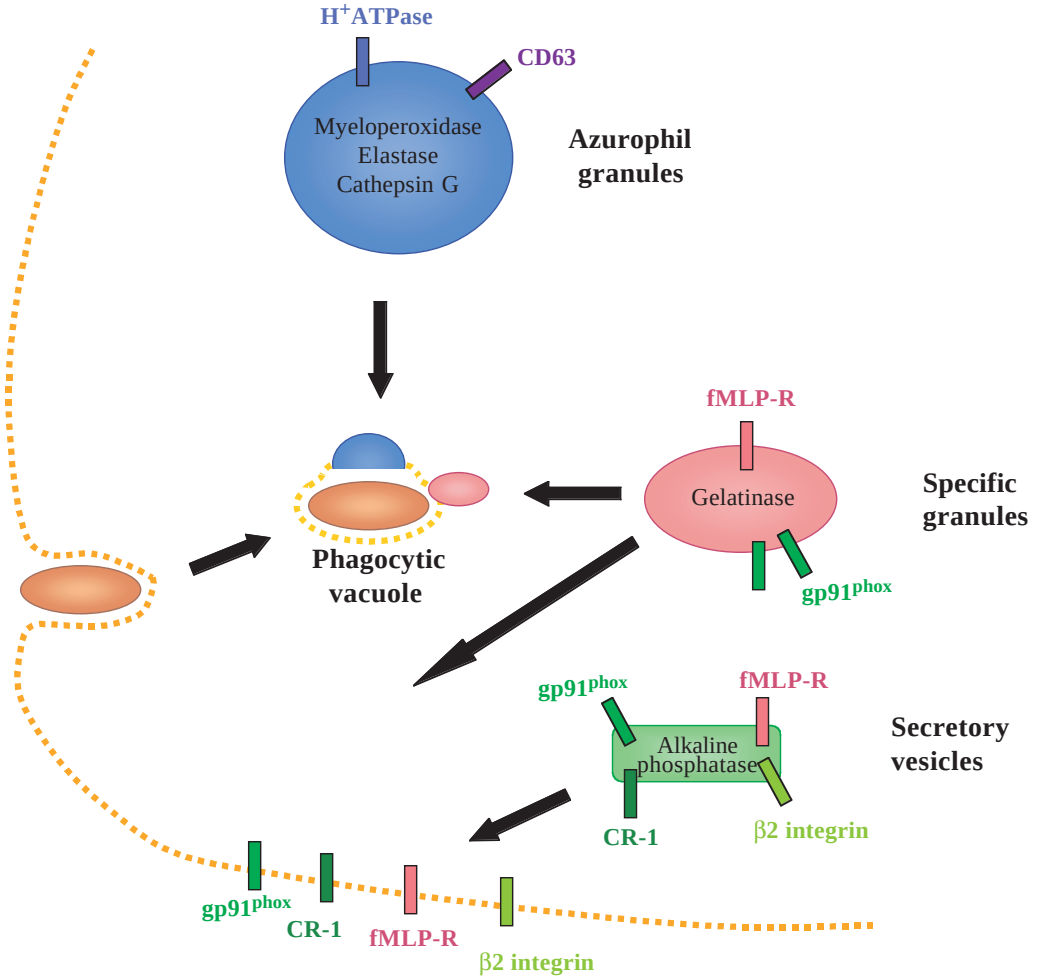

Fig. 2. NADPH oxidase localization in neutrophils. Diagram based on published information (Borregaard and Cowland, 1997). Approximately 10% of the oxidase is located at the plasma membrane and 90% is located in two separate compartments: the secretory vesicles and specific granules. Secretory vesicles contain several proteins [including complement receptor (CR-1), integrin β2, *N*-formyl-methionyl-leucyl-phenylalanine receptor (fMLP-R)] and are rapidly translocated to the plasma membrane upon regulated exocitosis. Another fraction of gp91^{phox} is included in specific granules, which can either fuse with the phagosome or eventually with the plasma membrane.

consistent with the participation of Rac2 in other upstream events related to oxidase activation, such as chemotaxis (Roberts et al., 1999), granule mobilization (Zhong et al., 2003) and phagocytosis (Caron and Hall, 1998).

Alternatively, Rac2 could act at a specific subcellular location. Although the sequence identity shared by Rac 1 and Rac2 is high, the main difference lies in the hypervariable C-terminal tail, which is thought to confer distinct localization of small GTPases (Michaelson et al., 2001) and efficient geranylation (Tao et al., 2002). In contrast to Rac2, Rac1 has a poly-basic sequence in the hypervariable region before the prenylation motif, which is necessary and sufficient to direct preferential localization to the plasma membrane (Hancock et al., 1990). The Rac2 hypervariable region lacks negative residues, localizing the protein mainly to intracellular membranes, and this partitioning is augmented by Rho-GDI co-expression (Michaelson et al., 2001). Deletion of this motif confirms the functional importance of the hypervariable region, rendering the mutant unable to complement Rac2-specific functions because of deficient prenylation and de-localization (Tao et al., 2002).

The cellular context of ROS production in neutrophils

Studies of the NADPH oxidase in intact cells have revealed the complexity of its regulation. In resting neutrophils, the oxidase has a dual localization: a small fraction (10%) is at the plasma membrane and 90% is located in two separate intracellular compartments: the secretory vesicles and specific granules (Borregaard et al., 1983; Sengelov et al., 1992) (Fig. 2). Both plasma membrane and intracellular locations are functionally relevant. In mouse neutrophils, fMLP receptor activation leads to superoxide release into the extracellular space, which is proposed to be responsible for the tissue-damaging effects of neutrophil activation (Bylund et al., 2003), probably through rapid mobilization of secretory granules (Borregaard et al., 1987; Sengelov et al., 1992). The oxidase included in specific granules is incorporated into the phagosome, but can also be mobilized to the plasma membrane. The intracellular pools of oxidase are functional, because superoxide production has been directly detected in non-phagosomal compartments (Johansson et al., 1995; Kobayashi et al., 1998). This intracellular pool of membrane-associated oxidase is regulated by PMA-activated protein kinase Cδ and 3′-phosphorylated phosphoinositides generated by PI 3-kinase (Brown et al., 2003).

Besides restricting the toxicity of superoxide, the activation of NADPH oxidase in intracellular compartments has been proposed to be necessary for protease activity, another component of the microbicidal machinery in the phagosome. The functional interdependence of superoxide and proteases is

suggested by the diminished *Staphylococcus aureus* digestion associated with reduced superoxide generation in CGD (Segal et al., 1981) and to studies using DPI-mediated oxidase inhibition (Reeves et al., 2002). Recently, a mechanistic explanation was provided for this dependence (Reeves et al., 2002). They showed that the vectorial generation of superoxide directed into the phago-lysosome produces a high negative charge across the vacuolar membrane, causing $[K^+]$ to enter the vacuole and an accompanying rise in pH and hypertonicity. The alkaline and high $[K^+]$ generated provide proper conditions for elastase and cathepsin G activity, these being essential effectors of superoxide in microorganism killing. Importantly, this mechanism restricts the hazardous activity involved in microorganism killing not only by physically confining it to the vacuole but also by ensuring it is only activated therein.

Although the NADPH oxidase has a mostly intracellular localization in neutrophils, it is located exclusively on the plasma membrane in other cell types, such as macrophages and non-myeloid cells. When reconstituted in COS cells, the oxidase localizes to the plasma membrane and displays agonist-regulated activity in response to PMA or arachidonic acid, although with reduced efficiency compared with neutrophils (Price et al., 2002). Intriguingly, in this case, Rac1 is necessary and sufficient to induce membrane translocation of $p47^{phox}$ with $p67^{phox}$, and of $p47^{phox}$ independently of $p67^{phox}$, and thus drives oxidase assembly and activation through multiple pathways. Mutations in residues 27 and 30 of the Rac effector region make Rac unable to reconstitute the neutrophil oxidase in vitro (Kwong et al., 1995) and also completely eliminate ROS production in COS cells. However, other mutations that abolish the binding of CRIB-domain-containing effectors (e.g. PAK), or that lie in the insert region, partially reduce ROS production (Price et al., 2002). Thus, these experiments demonstrate that NADPH oxidase is active in non-myeloid cells given the proper components, and implicate Rac1 at several steps in the signal transduction cascade beyond the direct activation of the oxidase.

NADPH oxidases in other tissues: ROS generation for signaling

Searches for homologous mechanisms of superoxide production in non-hematopoietic cells, relying on identification of relatives of the different oxidase subunits, sensitivity to DPI and Rac-mediated regulation, have identified several related oxidases in non-myeloid tissues. The subunit $gp91^{phox}$ and its relatives have been named the Nox family (for NADPH oxidase), where $gp91^{phox}$ is Nox2 (reviewed by Lambeth et al., 2000). Although they share variable degrees of sequence identity (20-60%), family members have the same domain organization, including heme-binding histidines, FAD/NADPH-binding domains and membrane-spanning domains. Their expression correlates with increased superoxide production, and they are DPI sensitive. However, little is known about their functions.

Several tissues express Nox1, including colon, uterus, prostate and vascular smooth muscle (Lassegue et al., 2001; Suh et al., 1999). Overexpression of Nox1 in NIH-3T3 cells induces low levels of constitutive superoxide production, leading to cell transformation and an increase in tumorigenic potential when the cells are transplanted into nude mice (Arnold et al., 2001). Expression of the Nox3 isoform is restricted to fetal kidney (Cheng et al., 2001). Nox4 is expressed in the cortex of adult and fetal kidney, arterial vascular smooth muscle cells and in osteoclasts (Geiszt et al., 2000; Shiose et al., 2001; Sorescu et al., 2002; Yang et al., 2001). In osteoclasts, Nox4 cooperates with $gp91^{phox}$ in bone resorption (Yang et al., 2001). When expressed in HEK293 cells, Nox4 increases NADPH-dependent superoxide production but is insensitive to arachidonic acid and Rac2-GTP (Shiose et al., 2001) and is not regulated by the novel subunits Nox-organizing protein 1 (NOXO1) and Nox-activating protein 1 (NOXA1) (J. D. Lambeth, personal communication). Interestingly, in contrast to Nox1, Nox4 inhibits cell growth (Shiose et al., 2001) and induces senescence (Geiszt et al., 2000) in NIH-3T3 fibroblasts. Nox5 is a novel homolog highly expressed in spleen and testis (Banfi et al., 2001; Cheng et al., 2001). It has an additional domain, harboring three EF-hand motifs in the N-terminal tail. This domain is thought to confer responsiveness to calcium. When Nox5 is expressed in HEK293 cells, only a fraction of the total superoxide produced is scavenged by exogenously added superoxide dismutase, which suggests a dual distribution of Nox5 between the plasma membrane and an intracellular compartment (Banfi et al., 2001).

The activity of the novel Nox proteins also depends on regulatory subunits. Whereas $p47^{phox}$ and $p67^{phox}$ reconstitute PMA-regulated superoxide production in Nox1-expressing HEK293 cells (Banfi et al., 2003), few non-myeloid tissues except endothelial cells, vascular smooth muscle cells and adventitia express these subunits. Recently, NOXO1 and NOXA1 were identified as relatives of $p47^{phox}$ and $p67^{phox}$. Their expression patterns correlate only partially with Nox1 expression (Banfi et al., 2003; Geiszt et al., 2003; Takeya et al., 2003), suggesting that they might also regulate other oxidases. These new subunits share a relatively low level of sequence identity with $p47^{phox}$ and $p67^{phox}$ (20-30%). However, they have a similar domain structure and stimulate superoxide production when expressed in heterologous systems with Nox1 or Nox2, and in combination with their phagocytic counterparts $p47^{phox}$ and $p67^{phox}$, although with different efficiency (Geiszt et al., 2003; Takeya et al., 2003). NOXO1 retains the functional domains of $p47^{phox}$ that interact with the other subunits, such as the SH3, PRD and PX domains. However, it lacks the poly-basic domain and the phosphorylation sequences involved in autoinhibition and phosphorylation-dependent activation, thus rendering complex formation apparently insensitive to agonist control. Interestingly, agonist regulation is cell-type-dependent: NOXO1 expression reconstitutes agonist-regulated $gp91^{phox}$ activity in K562 myeloid cells and Nox1 activity in NIH-3T3 cells, but agonist-insensitive activity in HEK293, COS7 and Caco cells (Banfi et al., 2003; Geiszt et al., 2003; Takeya et al., 2003). This indicates that additional factors are involved in controlling oxidase activity. The $p67^{phox}$ relative NOXA1 lacks the N-terminal SH3 domain and the binding function of the PB1 domain, but retains the C-terminal SH3 domain, the TRP domain and the activation domain essential in $p67^{phox}$ for oxidase activation. Thus, it could still interact with Rac, Nox1, $gp91^{phox}$/Nox2, $p47^{phox}$ and NOXO1 but not with $p40^{phox}$ (Takeya et al., 2003). The observations discussed above

involved reconstitution in heterologous systems and overexpression of proteins and, thus, might not be physiologically relevant in every case. However, they demonstrate the potential functional compatibility of the different relatives, which could provide additional regulatory mechanisms through varying the subunits of the oxidase.

Novel functions of the neutrophil NADPH oxidase homologs

The existence of superoxide-producing oxidases in non-phagocytic tissues suggests that superoxide has a role beyond microbial clearance. Their expression patterns are consistent with a role in general cellular homeostasis, including cell proliferation, senescence and/or apoptosis, as well as tissue-specific functions in bone resorption, oxygen sensing in the kidney and control of vascular tone in blood vessels.

Superoxide controls vascular tone in blood vessels by inactivating nitric oxide produced by endothelial cells to induce smooth muscle relaxation. All three cell types forming the vascular wall – endothelial cells, smooth muscle and adventitial fibroblasts – produce superoxide and express several members of the Nox family. In the vascular cell wall, the expression of $gp91^{phox}$/Nox2, Nox1 and Nox4 account for constitutive low levels and agonist-induced superoxide production (reviewed by Lassegue and Clempus, 2003). Endothelial cells express $gp91^{phox}$/Nox2 with all the phagocytic subunits (Li and Shah, 2002), Nox1 and Nox 4 (Sorescu et al., 2002). Interestingly, in this cell type, the oxidase is found in an intracellular compartment, is pre-assembled and has a low basal constitutive activity. Angiotensin II increases superoxide production through phosphorylation of $p47^{phox}$ (Li and Shah, 2003). Vascular smooth muscle cells in culture express Nox1, $gp91^{phox}$/Nox2 and Nox4, along with $p22^{phox}$, $p67^{phox}$ and $p47^{phox}$ (Lassegue et al., 2001; Touyz et al., 2002). After stimulation with PMA, angiotensin II and platelet-derived growth factor (PDGF), $p47^{phox}$ generates oxidase activity necessary for proliferation and contraction (Lavigne et al., 2001). Adventitial fibroblasts also exhibit constitutive and angiotensin II-modulated oxidase activity and express $p67^{phox}$, $p47^{phox}$, $gp91^{phox}$ and $p22^{phox}$ (Pagano et al., 1997). Whether this tissue expresses NOXO1 and NOXA1 is currently not known. The role of $p47^{phox}$-dependent $gp91^{phox}$ activation in angiotensin-II-induced increases in systolic blood pressure has been examined in vivo. Initial studies showed that interfering with oxidase assembly by introducing a peptide that disrupts the binding of $p47^{phox}$ to $gp91^{phox}$ (and potentially to Nox1 and Nox4) conjugated to the cell-permeable peptide of HIV viral coat (HIV-tat) successfully counteracts systolic blood pressure increases when co-administered with angiotensin II to animals (Rey et al., 2001). The selectivity of this approach should be further developed once we know more about the assembly mechanisms of the different oxidases.

The oxidase is also involved in the progression of vascular diseases. In the hypertensive state, increased superoxide production in the vascular wall is thought to mediate smooth muscle cell hypertrophia and proliferation (reviewed in Landmesser et al., 2002; Zalba et al., 2001). Increased superoxide production and oxidase expression is associated with the development of atherosclerotic lesions and disease progression (Landmesser et al., 2002; Sorescu et al., 2002; Patel et al., 2000; Sorescu et al., 2001).

Role of Rac and oxidants in non-myeloid tissues

Although many independent studies connect Rac to the control of superoxide production in non-phagocytic cells, little is known about its regulation of the newly discovered oxidases, and thus this represents an important question to be addressed. The specific expression patterns of the subunits of the novel oxidases suggest that they have cell-type-specific roles in hormone synthesis, oxygen sensing and bone resorption, which could all be Rac regulated.

Studies with Rac mutants have implicated Rac-dependent ROS generation in non-myeloid cells during signal transduction by growth factors and control of adhesion and proliferation (Fig. 3). Growth factor binding [epidermal growth factor (EGF) or platelet-derived growth factor (PDGF)] promotes ROS production as a necessary intermediary for signal transduction to proceed (Sundaresan et al., 1995; Bae et al., 1997). Growth factor receptors activate Rac to produce DPI-sensitive ROS through a PI 3-kinase-dependent mechanism (Bae et al., 2000). This appears to be the mechanism used in vascular smooth muscle cells to produce ROS in response to angiotensin II. This vasoconstrictor induces a bi-phasic ROS response: a first early ROS peak dependent on protein kinase C activation, and a second, steady rise dependent on Rac activation through Src, EGF receptor (EGFR) transactivation and PI 3-kinase (Seshiah et al., 2002). Growth-factor-triggered ROS generation through Rac regulates receptor tyrosine kinases by controlling tyrosine phosphatases, which are known targets of oxidants. Tyrosine phosphatases have an oxidation-susceptible cysteine residue in the active site that is readily modified by H_2O_2, and this abrogates catalytic activity (Meng et al., 2002). This short-term inactivation of phosphatases increases receptor tyrosine kinase activity and is necessary for signal transduction (Lee et al., 1998).

Rac-dependent ROS production is necessary for cell adhesion and spreading, when GTPases coordinate remodeling of adhesion complexes with organization of actin. During cell adhesion, Rac-induced ROS downregulate Rho activity to disrupt stress fiber formation, whereas Rac promotes organization of actin into cortical structures required for cell spreading (Nimnual et al., 2003). Thus, ROS coordinate Rho and Rac activity during cell spreading to regulate distinct cytoskeleton-remodeling pathways. In response to Rac, oxidants produced by a DPI-sensitive mechanism inactivate low-molecular-weight phosphotyrosine phosphatase (LMW-PTPase), relieving p190RhoGAP inhibition. Tyrosine-phosphorylated p190RhoGAP stimulates Rho GTPase activity, thus preventing Rho-dependent organization of actin stress fibers at early steps of cell spreading (Arthur and Burridge, 2001). Such a mechanism is probably responsible for the downregulation of Rho after RGD-peptide-dependent integrin stimulation and for the inhibition of cell spreading observed in catalase-overexpressing cells (Nimnual et al., 2003). Although the identity of the oxidase remains unknown, DPI sensitivity and the requirement for the Rac insert region suggest the participation of a Nox-family member. Interestingly, LMW-PTPase also dephosphorylates p120FAK, inhibiting cell spreading (Rigacci et al., 2002); thus the control of FAK

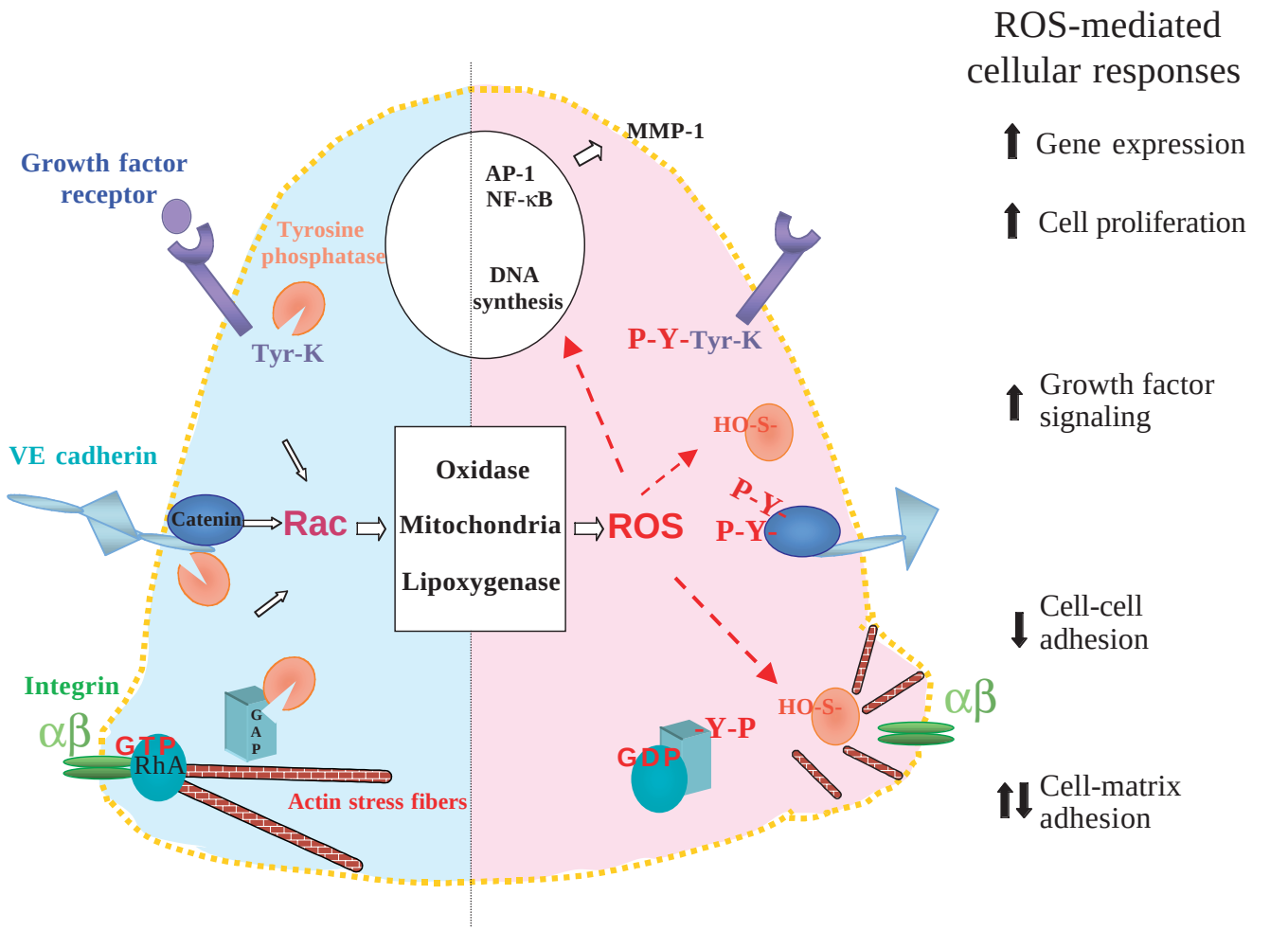

Fig. 3. Rac changes the redox state of the cell through the engagement of multiple superoxide sources to modulate signal transduction. Downstream of several cell-surface receptors, Rac is activated to shift the intracellular redox state by triggering superoxide generation from several alternative sources. Tyrosine phosphatases are one of the known targets for superoxide, which are inactivated upon oxidation of a catalytic cysteine residue, thus increasing phosphotyrosine content of several effector proteins in Rac-mediated signal transduction pathways.

phosphorylation is another regulatory target for ROS produced during cell spreading (Chiarugi et al., 2003).

Rac-dependent ROS are also involved in disruption of cell-cell contacts. Transient ROS generation blocks endothelial cell-cell adhesion by disrupting homotypic VE-cadherin interactions involving complexes containing highly phosphorylated α-catenin (van Wetering et al., 2002). Strong endothelial cell-cell adhesion correlates with low levels of tyrosine-phosphorylated catenins and elevated LMW-PTPase activity (Taddei et al., 2002). Therefore, it is likely that Rac-dependent superoxide generation in endothelial cells disrupts cell-cell adhesion by transient LMW-PTPase inhibition (Caselli et al., 1998), leading to increased phosphorylation of catenins. The source of the ROS in this case remains unknown.

Rac-dependent superoxide generation is necessary for Ras to achieve its full transforming potential. ROS production downstream of oncogenic Ras cooperates with signal transduction activated by Raf kinase (Irani et al., 1997; Joneson and Bar-Sagi, 1998; Karnoub et al., 2001). Efforts to elucidate the mechanism of Rac-mediated ROS production in this case, using mutations in the effector-binding residues or in the insert region, give conflicting results depending on the cell type and the read-out used. Activated Rac expression in COS cells induces DPI-sensitive superoxide production that depends on the insert region but is independent of residues 37 and 40 in the switch I region (Joneson and Bar-Sagi, 1998). These results suggest a role for an NADPH oxidase but contrast with the requirement for these residues in reconstitution of Rac-dependent Nox2 activity in COS cells (Price et al., 2002). This implicates a different member of the family and a different mechanism for Rac regulation of the oxidase. In contrast to COS cells, in NIH-3T3 cells the insert region is dispensable for induction of ROS by Rac and DNA synthesis downstream of oncogenic Ras (Karnoub et al., 2001). DPI sensitivity and Rac effector domain mutants support a role for an oxidase but by a mechanism independent of the insert region (Karnoub et al., 2001). These discrepancies probably in part reflect the existence of alternative mechanisms for Rac-dependent ROS production but might relate to the immortalized phenotype of the cell lines used and to the extent of phenotypic changes induced by persistent or transient expression of Ras. This difference is apparent in REF52 fibroblasts, where transient

Ras expression induces mitogenesis by a Rac-dependent mechanism (Joneson and Bar-Sagi, 1998) but stable Ras expression leads to cell-cycle arrest and senescence. This latter outcome reproduces the effect of Ras expression in non-immortalized cell lines, where the forced expression of this oncogene induces growth arrest and cell senescence through superoxide production in mitochondria (Lee et al., 1999).

These mechanistic variations are reminiscent of the effects of Nox activity reconstitution in different cell types and the participation of Rac in several stages of the activation mechanisms in COS cells in which the oxidase has been reconstituted. They also reveal that other ROS sources are under the control of GTPases.

Other sources and mechanisms of ROS generation

Rac also mediates ROS generation by alternative, DPI-insensitive mechanisms. During integrin-driven cell spreading on fibronectin, Rac induces a rise in ROS, which is only partially inhibited by DPI; nordihydroguaiaretic-acid-sensitive 5-lipoxygenase (LOX) is the additional ROS source (Chiarugi et al., 2003). LOX produces superoxide in vitro (O'Donnell and Azzi, 1996) and can regulate redox-sensitive NF-κB activation (Bonizzi et al., 1999). Vascular endothelial growth factor (VEGF) receptor engagement in endothelial cells activates LOX downstream of Rac and generates ROS by a DPI-independent mechanism (Colavitti et al., 2002). After integrin engagement by soluble antibodies in primary fibroblasts, Rac triggers ROS production by yet another mechanism. In this case, Rac activation after integrin crosslinking precedes, and is required for, DPI-insensitive ROS generation and changes in membrane potential in mitochondria (Werner and Werb, 2002). These signaling steps are part of a signal transduction cascade leading to NF-κB activation and metalloprotease induction. In this case, mitochondria constitute a further source for ROS: superoxide production is coupled to the rate of electron transport through the respiratory chain and is modulated during signal transduction, apoptosis, senescence and gene expression (Quillet-Mary et al., 1997; Chandel et al., 1998; Lee et al., 1999; Nemoto et al., 2000). The other GTPases of the Rho family – RhoA and Cdc42 – control ROS generation, probably by alternative mechanisms and sources, because they are unable to activate the NADPH oxidase. Expression of constitutively activated RhoA in primary fibroblasts leads to an increase in H_2O_2 production (Werner and Werb, 2002) by an unknown mechanism. Cdc42 mediates a redox-controlled pro-angiogenic response to ethanol in an immortalized endothelial cell line. Ethanol activates Cdc42 to generate a long-lasting rise in intracellular H_2O_2, and both events are necessary to induce endothelial cell migration and an angiogenic response in vitro (Qian et al., 2003). However, whether these mechanisms involve Rac as a switch to activate an oxidase, or other sources of ROS production, has not been evaluated.

Conclusion

Our understanding of NADPH oxidase assembly and activation in neutrophils has helped us to identify similar mechanisms for superoxide generation in other tissues, and relatives of the NADPH oxidase subunits have been discovered. However, how much functional resemblance exists between neutrophils and other systems is still unclear. A new level of complexity is introduced by the co-existence of several members of the family in some cell types, where they share common activating stimuli and probably subunits. Differential regulation is evident among the newly discovered members of the Nox family, which have novel regulatory domains (such as Nox5) and different subunits (such as NOXO1).

Rac, as a general regulator of superoxide production in non-myeloid tissues, constitutes an additional potential regulator of these novel oxidases, given that NOXA1 interacts with Rac, and Rac activity is necessary and sufficient for reconstitution of $gp91^{phox}$ activity in COS cells. However, whether this GTPase regulates these novel oxidases by mechanisms similar to those operating in the neutrophil has not been explored directly. The fact that the Rac and Rho GTPases function as temporal and morphological coordinators for signal transduction further underscores the relevance of these studies.

Investigation of ROS production by different sources could have significant implications for our understanding of signal transduction not only because different sources should be subjected to distinct regulation but also because a differential localization could target distinct processes. A good example is the NADPH oxidase itself, where localized superoxide generation plays a determinant role in vacuolar function (Reeves et al., 2002). An example in non-phagocytic cells involves EGFR signaling, where ROS-dependent phosphatase inactivation is necessary for EGF-induced lateral signal propagation at the plasma membrane (Reynolds et al., 2003). When EGF stimulation triggers the endogenous mechanism for ROS production, tyrosine phosphorylation of receptors is selectively modified at the plasma membrane but not in endocytic compartments. By contrast, when ROS are provided from the extracellular milieu by adding H_2O_2, EGFR phosphorylation is modified in both compartments.

The temporal regulation and engagement of different ROS sources provides a way to modify the function of a signal transduction cascade in progress, either at different steps or branches. In these circumstances, early ROS production by a membrane-associated source could regulate signaling events occurring at the plasma membrane, for example enabling growth factor receptor signaling or focal adhesion formation, whereas late ROS production by a different mechanism could modulate intracellular kinases and redox-sensitive transcription factors. Thus, the same second messenger could generate different responses, such as cell growth versus senescence, by modification of different target proteins.

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