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p47phox, the phagocyte NADPH oxidase/NOX2 organizer: structure, phosphorylation and implication in diseases

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Abbreviations: AIR, auto-inhibitory region; CGD, chronic granulomatous disease; NCF1, neutrophil cytosolic factor 1; NOX, NADPH oxidase; phox, phagocyte oxidase; PRR, proline rich region; PX, phox homology domain; ROS, reactive oxygen species; SH3, *src* homology 3 domain

Abstract

Phagocytes such as neutrophils play a vital role in host defense against microbial pathogens. The anti-microbial function of neutrophils is based on the production of superoxide anion ($O_2^{\bullet-}$), which generates other microbicidal reactive oxygen species (ROS) and release of antimicrobial peptides and proteins. The enzyme responsible for $O_2^{\bullet-}$ production is called the NADPH oxidase or respiratory burst oxidase. This multicomponent enzyme system is composed of two transmembrane proteins (p22phox and gp91phox, also called NOX2, which together form the cytochrome b_{558}) and four cytosolic proteins (p47phox, p67phox, p40phox and a GTPase Rac1 or Rac2), which assemble at membrane sites upon cell activation. NADPH oxidase activation in phagocytes can be induced by a large number of soluble and particulate agents. This process is dependent on the phosphorylation of the cytosolic protein p47phox. p47phox is a 390 amino acids protein with several functional domains: one phox homology (PX) domain, two *src* homology 3 (SH3) domains, an auto-inhibitory region (AIR), a proline rich domain (PRR) and has several phosphorylated sites located between Ser303 and Ser379. In this review, we will describe the structure of p47phox, its phosphorylation and discuss how these events regulate NADPH oxidase activation.

Keywords: CYBB protein, human; NADPH oxidase; neutrophil cytosolic factor 1; neutrophils; phosphorylation; reactive oxygen species; review

Introduction

Phagocytes such as polymorphonuclear neutrophils, eosinophils, monocytes and macrophages constitute one of the most powerful means of host defense against bacteria and fungi (Segal, 2005; Nathan, 2006). Upon infection and inflammation, neutrophils migrate towards the infection site attracted by chemoattractants such as the complement fraction C5a, the N-formyl-methionyl-leucyl-phenylalanine (fMLF) peptide, interleukin 8 (IL-8), platelet activating factor (PAF) or leukotriene B4 (LTB4). At the infection site, neutrophils recognize and engulf the pathogen and activate the release into the vacuole of antibacterial peptides, proteases and reactive oxygen species (ROS) (i.e., superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$) and hypochlorous acid (HOCl)), which together contribute to death and destruction of the bacteria (Babior, 1984; Hampton *et al*., 1998). ROS are produced by phagocytes in a powerful "oxidative burst", characterized by a rapid, cyanide-insensitive increase in oxygen uptake, an increase in glucose consumption and abrupt ROS release. The enzyme responsible for $O_2^{\bullet-}$ production is the multicomponent NADPH oxidase or respiratory burst oxidase (Babior, 1999; El Benna *et al*., 2005). The phagocyte NADPH oxidase and ROS production play a key role in host defense against microbial pathogens as illustrated by a human genetic disorder called chronic granulomatous disease (CGD), which is associated with life-threatening bacterial and fungal infections and is characterized by an absence of ROS production due to a deficiency in one of the components of the NADPH oxidase (Meischl and Roos, 1998; Kannengiesser *et al*., 2008).

The phagocyte NADPH oxidase consists of several proteins that are separated between membranes and cytosol in resting cells (Chanock *et al*., 1994; Babior 1999; El Benna *et al*., 2005). The cytosolic proteins are p47phox (phox: pha-

gocyte oxidase), p67phox, and p40phox, which interact with each other to form a complex and the small G-proteins, Rac1 (in monocytes) or Rac2 (in neutrophils) (Groemping *et al*., 2005). The membrane associated components of the NADPH oxidase are a glycosylated 91-kDa protein (gp91phox or NOX2) and a 22-kDa subunit (p22phox) which together form the flavocytochrome b_{558} (Vignais, 2002). The gp91phox/NOX2 subunit is the electron transfer chain of the active NADPH oxidase because it has binding sites for FAD, NADPH and two hemes. The spatial separation of the NADPH oxidase components ensures that the enzyme is dormant in resting cells. However, in response to stimulation, the cytosolic components migrate almost instantly to the membrane where they assemble with the flavocytochrome b_{558} to form the active enzyme, a process that is tightly regulated by protein-protein interactions and by phosphorylation of p47phox (El Benna *et al*., 1994a; Faust *et al*., 1995; Quinn and Gauss, 2004; Groemping *et al*., 2005).

Characteristic structural features of p47phox

p47phox or NCF1 (for neutrophil cytosolic factor 1), is a protein composed of 390 amino acids with a molecular mass of 44.7 kDa (Lomax *et al*., 1989; Volpp *et al*., 1989). It was estimated to be present in neutrophil cytosol at 100-150 ng/10^6 cells (Leto *et al*., 1991; Jouan *et al*., 1993). The N-terminal amino acid sequence of p47phox has one PX (phox homology) domain (amino acids 4-121), the middle amino acid sequence of p47phox also contains two SH3 domains (amino acids 159-214 (SH3A) and amino acids 229-284 (SH3B)). Its COOH-terminal sequence has a basic charge and is rich in serine and arginine residues and has at least one proline-rich region (PRR) (amino acids 363-368) (Groemping *et al*., 2005) (Figure 1).

In resting state the two p47phox-SH3 domains interact intramolecularly with the C-terminal region of the non phosphorylated protein to keep p47phox in an auto-inhibited state (de Mendez *et al*., 1997; Greomping *et al*., 2003). For this reason the C-terminal region is called the auto-inhibitory region (AIR). The X-ray structure of the auto-inhibited form of p47phox reveals that tandem SH3 domains share an interface which forms a shallow groove that constitutes the peptide binding surface (Groemping *et al*., 2003; Yuzawa *et al*., 2004a). In the auto-inhibited form, this site is occupied by the AIR polybasic region of p47phox and in the activated form it is occupied by a proline rich region of the cytoplasmic domain of p22phox (Yuzawa *et al*., 2004b).

Phosphorylation of p47phox

During human neutrophil stimulation, p47phox is heavily phosphorylated. This phosphoprotein was first identified since it was missing in neutrophils of some CGD-patients (Segal *et al*., 1985). In resting cells, p47phox is not phosphorylated with a highly basic charge (pI > 9), as detected by NEPHGE (non-equilibrium pH gel electrophoresis) two-dimensional gel analysis (Okamura *et al*., 1988). Upon phosphorylation, its pI shifts to acidic range, giving rise to several phosphorylated isoforms corresponding to different phosphorylated states. When cells are stimulated with PMA, the PKC activator, eight to nine phosphorylation states of p47phox are observed in the cytosol and membranes, with the two most acidic forms being located in the membrane (Rotrosen and Leto, 1990; El Benna *et al*., 1994b). This localized and stepwise phosphorylation in the cell indicate a sequential p47phox phosphorylation in the cytosol and membrane. Phosphorylation sites analysis revealed that p47phox is phosphorylated in the carboxy-terminal portion of the protein obtained after cyanogen bromide (CNBr) cleavage (El

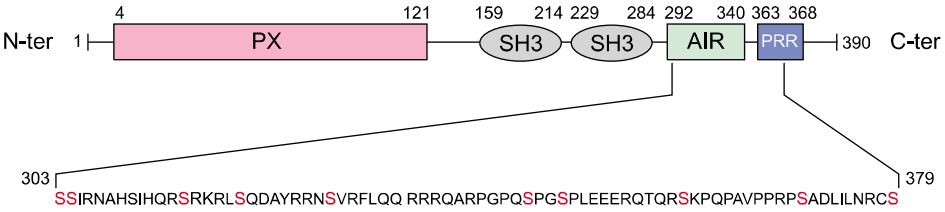

Figure 1. Domain structure of p47phox. p47phox is composed of 390 amino acids with one phox homology (PX) domain (amino acids 4-121), two src homology 3 domains (SH3) domains (amino acids 159-214 (SH3A) and amino acids 229-284 (SH3B)), one autoinhibitory region (AIR) (amino acids 292-340) and a proline-rich region (PRR) (amino acids 363-368). The C-terminal region contains several phosphorylated serines (in red).

Benna *et al*., 1994a). This C-terminal fragment contains multiple phosphorylated sites encompassing serines 303 to 379 (El Benna *et al*., 1994a; 1996b).

Regarding the role of the p47phox phosphorylated sites in NADPH oxidase activation, results obtained from combined mutation of all phosphorylated serines (Ser303, Ser304, Ser315, Ser320, Ser328, Ser345, Ser348, Ser359, Ser370 and Ser379) demonstrated that p47phox phosphorylation is absolutely required for NADPH oxidase activation in EBV-transformed lymphocytes B (Faust *et al*., 1995). Individual mutation of each serine showed that only the mutation of serine 379 to alanine completely inhibited NADPH oxidase activation (Faust *et al*., 1995), while mutation of serine 303 alone or 304 or 328 or 359 or 370 to alanine inhibited almost 50% NADPH oxidase activation, and mutation of serine 315 or 320 or 348 to alanine had no effect. Double mutations revealed that two pairs of phosphorylated serines (serines (303 + 304) and serines (359 + 370)) are necessary for NADPH oxidase activation (Inanami *et al*., 1998; Johnson *et al*., 1998). The importance of these phosphorylated sites of p47phox was also shown in COS-phox cells stimulated with PMA (Price *et al*., 2002) or a physiological stimulus such as fMLF (Cheng *et al*., 2007).

In *in vitro* conditions, p47phox is phosphorylated on selective sites by different type of protein kinases such as PKC α, β, δ, and ζ (Dang *et al*., 2001a; Fontayne *et al*., 2002), PKA (El Benna *et al*., 1996a; Kramer *et al*., 1988), MAPKinase ERK2 and p38MAPKinase (El Benna *et al*., 1996b), protein casein kinase 2 (CKII) (Park *et al*., 2001), AKT (Chen *et al*., 2003; Hoyal *et al*., 2003), IRAK-4 (Pacquelet *et al*., 2007), p21- activated kinase (PAK) (Martyn *et al*., 2005), a phosphatidic acid-activated kinase (Waite *et al*., 1997) and src kinase (Chowdhury *et al*., 2005).

In agreement with the above selective phosphorylation of p47phox, it seems that *in vivo*, depending on the nature of the agonist encountered by neutrophils, a combination of kinases could participate to the phosphorylation of p47phox. Various protein kinases have been implicated in the phosphorylation of p47phox and regulation of NADPH oxidase in intact cells. Thus PKCζ (Dang *et al*., 2001a), PKCβ (Dekker *et al*., 2000; Korchak *et al*., 2001), PKCδ (Bey *et al*., 2004; Brown *et al*., 2003; Cheng *et al*., 2007), PAK (Martyn *et al*., 2005), ERK1/2 (Dewas *et al*., 2000) and AKT (Chen *et al*., 2003) were shown to play a stimulatory role in fMLF- or PMA-induced NADPH oxidase activation. Proinflammatory cytokines such as GM-CSF and TNFα which do not activate NADPH oxidase but prime its activation in response to a secondary stimulus such as fMLF (El Benna *et al*., 2008), induce partial phosphorylation of p47phox on Ser345 by ERK1/2 or p38MAPK and promote NADPH oxidase assembly (Dang *et al*., 1999, 2006; Dewas *et al*., 2003). While phosphorylation of p47phox by PKC, PAK and AKT in neutrophils has a positive stimulatory effect on NADPH oxidase activation and pre-phosphorylation by p38MAPkinase and ERK1/2 results in the enhancement of this effect, some data have suggested that the phosphorylation of p47phox by PKA or CKII could have a negative inhibitory effect (Bengis-Garber *et al*., 1996; Park *et al*., 2001).

p47phox the phagocyte NADPH oxydase (NOX2) organizer (NOXO2) undergoes conformational changes upon cell activation

NADPH oxidase is dormant in resting cells but becomes active when cells are stimulated. In resting cells, almost 100% of p47phox is located in the cytosol alone or in a complex containing equimolar of p67phox and p40phox (Park *et al*., 1994; Lapouge *et al*., 2002). As described above, in resting non phosphorylated state, the two p47phox-SH3 domains, interact intramolecularly with the p47phox C-terminal sequence to keep the protein in an auto-inhibitory state. p47phox also interacts with p67phox and p40phox via their respective SH3 domains and PRR sequences (Wientjes *et al*., 1996; Grizot *et al*., 2001) or non PRR sequences (Kami *et al*., 2002).

During activation, approximately 10-20% of these proteins migrate to the plasma membrane (Clark *et al*., 1990; El Benna *et al*., 1994b) and the 80-90% of p47phox remain in the cytosol. p47phox binds to cytochrome b_{558} (gp91phox/NOX2 and p22phox) during activation since translocation of p47phox to the plasma membranes is impaired in neutrophils from gp91phox or p22phox deficient CGD patients (Heyworth *et al*., 1991). While cytochrome b_{558} is the central docking site for the cytosolic components that translocate to the plasma membrane, p47phox is the subunit responsible for transporting the whole cytosolic complex (p47phox-p67phox-p40phox) to the docking site during NADPH oxidase activation. It mediates the early binding step to gp91phox/NOX2 and to p22phox and subsequently allows p67phox to interact with and to activate gp91phox/NOX2 (De Leo *et al*., 1996; Dang *et al*., 2001b; Nisimoto *et al*., 1999). It is considered as the organizer subunit since it coordinates

the interaction of the different NADPH oxidase subunits allowing the formation of an active complex.

When p47phox is phosphorylated, the interaction of the SH3 domains switches from the AIR to the p22phox-polyproline rich-sequence (Ago *et al*., 1999; Huang *et al*., 1999; Greomping *et al*., 2003). It implies that phosphorylation induces conformational changes of p47phox. Several reports suggest that p47phox phosphorylation induces conformational changes of the protein (Park and Babior, 1997; Swain *et al*., 1997; Park and Park, 1998). In resting state, p47phox has a constrained conformation due to the tight interaction between SH3 domains and the AIR (Yuzawa *et al*., 2004a). Phosphorylation of p47phox in its carboxy-terminal tail relaxes this interaction, allowing the binding of the cryptic SH3 domains to the proline-rich region of p22phox (Figure 2). Phosphorylation of only three sites of p47phox (Ser303, 304 and 328) is required to induce this conformational change to a state accessible for p22phox thereby bringing p67phox and p40phox in proximity to gp91phox/NOX2 initiating assembly of the enzyme (Shiose and Sumimoto, 2000).

p47phox has a PX domain (a sequence of about 125 amino acids) which binds to phosphatidylinositol 3,4-biphosphate and phosphatidic acid (Kanai *et al*., 2001; Karathanassis *et al*., 2002; Stahelin *et al*., 2003). Upon phosphorylation of the C-terminal region of p47phox, the PX domain is also released which allows its binding to phosphatidylinositol 3,4-biphosphate and phosphatidic acid (Karathanassis *et al*., 2002; Ago *et al*., 2003). This binding is important for membrane localization of the complex and may help to assemble the NADPH oxidase complex at precise sites of the ingested pathogens (Zhan *et al*., 2002; Stahelin *et al*., 2003). During activation p47phox also binds to the cytoskeleton and membrane cytoskeleton (Nauseef *et al*., 1991; Woodman *et al*, 1991; El Benna *et al*., 1994b, 1999). The p47phox PX domain binds also to moesin (Wientjes *et al*., 2001) and this PX/moesin-interaction could mediate association of p47phox to actin cytoskeleton (Zhan *et al*., 2004).

Implication of p47phox in diseases

Chronic Granulomatous Disease (CGD)

The importance of p47phox and ROS production by NADPH oxidase in host defenses is illustrated by a life-threatening genetic disorder called chronic granulomatous disease (CGD), in which the NADPH oxidase enzyme is dysfunctional in phagocytes, leading to life-threatening bacterial and fungal infections (Dinauer, 1993; Roos *et al*., 1996). CGD is an inherited immune deficiency in which human phagocytes are unable to produce ROS. Recurrent, often life-threatening bacterial and fungal infections usually start during childhood. Common infectious syndromes include pneumonia and lung abscesses, skin and soft tissue infections, lymphadenopathy, suppurative lymphadenitis, osteomyelitis and hepatic abscesses. The most common pathogens encountered in CGD patients are gram-positive bacteria (*Staphylococcus aureus*), gram-negative bacteria (*Salmonella, Pseudomonas cepacia, Serratia marcescens*…) and fungi (*Aspergillus, Candida albicans*). Aspergillus species can cause intractable pneumonia and sometimes septicemia in CGD patients, and are a frequent cause of death (Meischl and Roos, 1998; Kannengiesser *et al*., 2008). CGD results from mutations in the NADPH oxidase component genes, namely the CYBB gene (Xp21)

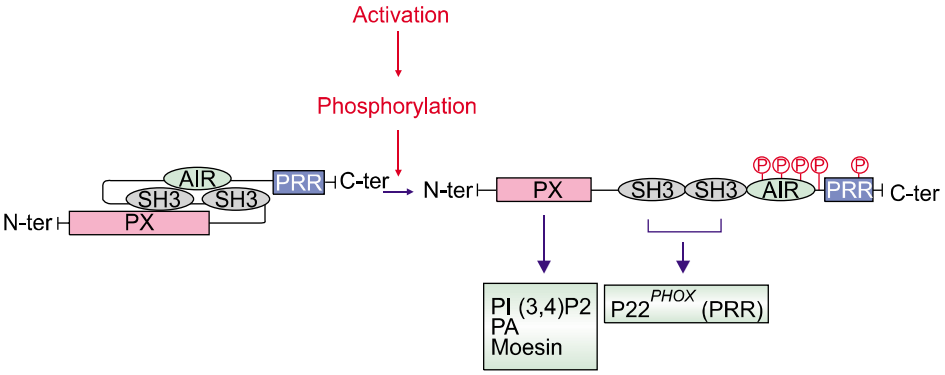

Figure 2. Phosphorylation of p47phox induces conformational changes and changes domains interactions. In resting state the two p47phox-SH3 domains interact with the C-terminal region AIR to keep the protein in an auto-inhibited state. Upon activation, p47phox is phosphorylated, this phosphorylation induces conformational changes allowing the binding of the cryptic SH3 domains to the proline-rich region (PRR) of p22phox and PX domain to phosphatidylinositol 3,4-biphosphate (PI3,4P), phosphatidic acid (PA) and moesin.

that encodes the gp91phox subunit, and the CYBA gene (16q24), the NCF1 gene (7q11) and the NCF2 gene (1q25) which encode p22phox, p47phox, p67phox, respectively. The most frequent form of CGD (approximately 70% of all cases) is the X-linked gp91phox-deficient form, followed by the autosomal form deficient in p47phox (approximately 25%) (Meischl and Roos, 1998; Kannengiesser *et al*., 2008). Less frequent forms are autosomal CGD deficient in p67phox ($<$ 5%) and p22phox ($<$ 5%) (Roos *et al*., 1996). Most of the p47phox deficient patients have a GT deletion at the start of exon two, resulting in a frameshift and lack of expression of the protein (Gorlach *et al*., 1997).

The importance of p47phox in host defense was also demonstrated in a p47phox knockout mice (Jackson *et al*., 1995). These mice developed lethal infections and granulomatous inflammation similar to those encountered in human CGD patients. This animal model mirrors human CGD and confirms a critical role for the phagocyte NADPH oxidase in mammalian host defense.

Atherosclerosis

ROS are important contributors to the pathophysiology of human vascular diseases such as atherosclerosis and hypertension. The expression of NADPH oxidase subunits in human coronary arteries is correlated with the severity of atherosclerosis (Lavigne *et al*, 2001; Clempus and Griendling, 2006). NADPH oxidase and its subunit p47phox are indeed expressed in human and mice smooth muscle cells which produce ROS (Lavigne *et al*, 2001). It was hypothesized that NADPH oxidase is involved in growth factor-induced smooth muscle cell proliferation and atherosclerosis (Brandes *et al*., 2002). Chen *et al*. (2004) have observed decreased neointimal formation in gp91phox/NOX2-deficient mice in response to arterial injury. Since p47phox regulates the activation of NOX2, excessive NOX2 activation via p47phox dysregulation could be involved in this process. Barry-Lane *et al*. (2001) showed that p47phox is required for atherosclerosis lesion progression in $ApoE^{-/-}$ mice. Indeed, atherogenic mice deficient in p47phox ($ApoE^{-/-}$/$p47phox^{-/-}$) had less lesion area than atherogenic mice ($ApoE^{-/-}$).

Rheumatoid Arthritis

Although ROS production by NADPH oxidase plays a key role in host defenses against microbial pathogens, excessive ROS release can also damage bystander host tissues, thereby amplifying inflammatory reactions (Babior 1984, 2000; El Benna *et al*., 2008). Increasing evidence suggests that NADPH oxidase is involved in inflammatory diseases such as rheumatoid arthritis. Rheumatoid arthritis (RA) is a systemic inflammatory disorder most commonly targeting the joints. The pathophysiology of RA involves dysregulated cytokine production and neutrophil accumulation in synovial fluid (Firestein, 2004). Synovial fluid from arthritic patients contains a large number of neutrophils and macrophages suggesting that they may contribute to tissue injury. NADPH oxidase activity and phosphorylation of p47phox is highly increased in neutrophils from arthritic patients (Dang *et al*., 2006). This up-regulation could be due to the action of proinflammatory cytokines such as TNFα, which is found in high concentrations in the synovial fluids of these patients. Using $p47phox^{-/-}$ mice, Van Lent *et al*. (2005) showed that NADPH-oxidase-driven ROS production determines chondrocytes death. These cells are important cells in the joints death and partly regulates metalloprotease- mediated cartilage matrix degradation during interferon gamma stimulated immune complex arthritis.

Hepatic fibrosis

Liver fibrosis is the common consequence of chronic liver injury (Albanis and Friedman, 2001). ROS produced by different cells in the liver play an important role in this process (Parola and Robino, 2001). Human hepatic stellate cells (HSCs), a fibrogenic cell type, express p47phox and produce ROS. Angiotensin II a pro-oxidant and fibrogenic cytokine, which induces phosphorylation of p47phox and ROS production. In a experimental liver fibrosis model, $p47phox^{-/-}$ mice showed attenuated liver injury and fibrosis compared to wild type mice (Bataller *et al*., 2003).

Conclusion

The phagocyte NADPH oxidase plays a key role in host defense against microbial pathogens by generating superoxide anion and other ROS molecules. Excessive ROS release can also damage surrounding host tissues involved in inflammatory reaction. A tight regulation of the NADPH oxidase complex is therefore necessary to avoid tissue injuries. p47phox with its different domains and several phosphorylated sites is a main regulator of NADPH oxidase activation by organizing the complex. Thus p47phox is the phagocyte NADPH oxidase (NOX2) organizer.

Better understanding of the different mechanisms and transduction pathways involved in p47phox phosphorylation and interaction will be necessary to design new therapeutic agents to downregulate ROS hyperproduction in inflammatory diseases without decreasing the normal response to bacterial-derived stimuli.

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