Published in final edited form as:

Free Radic Biol Med. 2017 December ; 113: 1–15. doi:10.1016/j.freeradbiomed.2017.09.007.

Down-regulation of NOX2 activity in phagocytes mediated by ATM-kinase dependent phosphorylation

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Abstract

NADPH oxidases (NOX) have many biological roles, but their regulation to control production of potentially toxic ROS molecules remains unclear. A previously identified insertion sequence of 21 residues (called NIS) influences NOX activity, and its predicted flexibility makes it a good candidate for providing a dynamic switch controlling the NOX active site. We constructed NOX2 chimeras in which NIS had been deleted or exchanged with those from other NOXs (NIS1, 3 and 4). All contained functional heme and were expressed normally at the plasma membrane of differentiated PLB-985 cells. However, NOX2- NIS and NOX2-NIS1 had neither NADPH-oxidase nor reductase activity and exhibited abnormal translocation of p47^{phox} and p67^{phox} to the phagosomal membrane. This suggested a functional role of NIS. Interestingly after activation, NOX2-NIS3 cells exhibited superoxide overproduction compared with wild-type cells.

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Author contributions

As co-corresponding authors, author 1 and author 2 contributed equally to this work.

SB and AP undertook the experiments in Figs. 2–4, and 8. SB did the experiments of Fig. 7. DG performed the confocal microscopy analysis and took photos from the slides prepared by SB and AP. MJS and FF designed the experiments, analyzed the experimental data and wrote the paper. CV performed the directed mutagenesis experiments and cloning to obtain different mutants. AMH and MF conducted the nano-LC-MS/MS analyses with the samples prepared by SB (Fig. 5 and Fig. 6). SMES, HS and PT built a new homology model of the NOX2 dehydrogenase domain taking advantage of the Pr H. Sumimoto crystal structure deposited in the database (Fig. 1).

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Paradoxically, the $V_{\max}$ of purified unstimulated NOX2-NIS3 was only one-third of that of WT-NOX2. We therefore hypothesized that post-translational events regulate NOX2 activity and differ between NOX2-NIS3 and WT-NOX2. We demonstrated that Ser486, a phosphorylation target of ataxia telangiectasia mutated kinase (ATM kinase) located in the NIS of NOX2 (NOX2-NIS), was phosphorylated in purified cytochrome *b*₅₅₈ after stimulation with phorbol 12-myristate-13-acetate (PMA). Moreover, ATM kinase inhibition and a NOX2 Ser486Ala mutation enhanced NOX activity whereas a Ser486Glu mutation inhibited it. Thus, the absence of Ser486 in NIS3 could explain the superoxide overproduction in the NOX2-NIS3 mutant. These results suggest that PMA-stimulated NOX2-NIS phosphorylation by ATM kinase causes a dynamic switch that deactivates NOX2 activity. We hypothesize that this downregulation is defective in NOX2-NIS3 mutant because of the absence of Ser486.

Graphical Abstract

Keywords

NADPH oxidase; neutrophil; NOX; phosphorylation; ataxia telangiectasia mutated (ATM); NOX-specific Insertion Sequence (NIS)

1. Introduction

The family of NADPH oxidases (NOX) catalyzes the reduction of molecular oxygen to form superoxide anion, the starting molecule for reactive oxygen species (ROS) production controlling various physiological and physiopathological processes. The NOX members contain seven members expressed in human tissues, NOX1-5 and Duox1-2 [1]. Catalysis occurs in a core functional unit, shared by all NOX isoforms, that consists of a transmembrane domain with distant homology to the mitochondrial b cytochromes and a cytosolic dehydrogenase domain (DH) related to the Ferredoxin NADP⁺ reductase family (FNR). The core transmembrane domain has six transmembrane helices, two cytosolic loops (named B and D), and three extra-cytosolic loops (A, C, and E), and contains two nonidentical hemes coordinated by four histidine residues located in the third and fifth transmembrane helices.

NOX isoenzymes can be grouped based on their mode of regulation. NOX1-4 form a stable complex with membrane-bound p22^{phox}; NOX1-3 require additional regulatory proteins, whereas NOX4 is constitutively active [2]. NOX5 and the DUOXs have different intra- and intermolecular regulatory interactions. From a functional point of view, the NOX members

are involved in many physiological processes, so their dysfunction provokes a range of pathological processes such as cancer, neurological disorders or cardiovascular disease. In this context, NADPH oxidases are now considered as potential therapeutic targets and major efforts are underway to understand better the regulatory mechanisms of these enzymatic systems [3–5].

The activation and regulatory processes of NADPH oxidases have been extensively studied in phagocytes. In these cells, ROS are toxic mediators involved in the destruction of pathogens. The deficiency of the NOX2-based enzyme complex is responsible for the major genetic form of chronic granulomatous disease (X-CGD), a rare inherited immunodeficiency syndrome (frequency of about 1/200 000) that primarily affects children [6, 7].

The phagocyte NADPH oxidase, NOX2, is dormant in resting cells and becomes activated during phagocytosis to produce superoxide. Indeed, its activity is subtly controlled by a dynamic process involving assembly of several cytosolic proteins (p40^{phox}, p47^{phox}, p67^{phox}, Rac1/2) around the catalytic partner, the heterodimer of p22^{phox}-NOX2 (formerly gp91^{phox}) known as flavocytochrome *b*₅₅₈. This assembly initiates the electron transfer from NADPH to oxygen *via* a molecule of FAD and two hemes, ultimately resulting in the production of oxidizing species involved in the destruction of phagocytosed microorganisms [8]. Phosphorylation is a key regulatory step in the activation of NADPH oxidase. Phosphorylation of cytosolic factors by PKCs, mitogen-activated protein kinase (MAPK) and other kinases, has been widely demonstrated in priming and activation processes [9, 10], while phosphorylation of p22^{phox} correlated with NADPH oxidase activity in activated human neutrophils [11]. Phosphorylation of cytochrome *b*₅₅₈ was first described in 1988 [12]; later experiments showed that PKC promoted binding of cytosolic factors both to NOX2 holoenzyme [13] and to a recombinant DH fragment [10]. However little is known about the molecular mechanisms of NADPH oxidase deactivation or regulation. Various possible hypotheses including phosphorylation/dephosphorylation mechanisms, have been put forward to explain the deactivation of the oxidase complex [14].

At the molecular level, much progress has been made on elucidating the structures of the cytosolic proteins of the complex. X-ray structures of isolated cytosolic protein domains [15–17], molecular envelope and high resolution of some entire cytosolic components, [18–21] and even structural insight in the dynamic changes of the cytosolic proteins upon activation processes [22–24] have been obtained during the last ten years. On the contrary, structural information is still very scarce for the heterodimeric membrane component, cytochrome *b*₅₅₈. Homology modelling therefore offers the best tool available for understanding the structure of NOXs. The first three-dimensional homology model of the DH domain of NOX2 (comprising the NADPH-binding and the FAD-binding subdomains) was built over twenty years ago, based on the significant sequence homology of this region with members of the FNR family [25]. This model provided an extremely useful structural picture of the DH domain, including the identification of an insertion of twenty amino acids in the NADPH binding domain of NOXs compared to the FNR family (which we designate NOX-NIS for NOX-NADPH-domain Insertion Sequence and which corresponds to positions 484–504 in NOX2). Because this insertion appeared to be ideally positioned at the entrance of the NADPH binding site, Taylor et al. proposed that the insertion, modelled as a

α -helix, may control access of NADPH to the binding site (Fig. 1A and 1B). This would explain the need for an activation and/or regulation step in NOX enzymes, in contrast to FNR-type enzymes which are constitutively active. Using site-directed mutagenesis of charged amino acids in the α -helical loop (residues 484–504) and stable transfection in the X⁰CGD- PLB-985 cell model, we highlighted that this region, and more precisely Asp484, H490 and Asp500, were essential for proper assembly of the NADPH oxidase complex related to the electron transfer from NADPH to FAD during the time course of NADPH oxidase activation [26]. However, this activation model has never been clearly validated, leaving some doubt about the precise function of this additional sequence in NOX2 and in the homologues NOX1, NOX3, and NOX4. This ambiguity mainly resides in that all mutants of the NOX2-NIS studied to date are completely devoid of NADPH oxidase activity, which significantly complicates their functional study.

Seven years ago, a crystal structure of the NADPH binding domain of NOX2 was released in the Protein Data Bank (PDB 3A1F) by H. Sumimoto and colleagues. This structure, which has not yet been the subject of a publication, strikingly confirmed the structural homology with the FNR family. Despite its presence within this crystallized domain, the NOX-NIS is not visible in the crystal structure suggesting an intrinsic flexibility of this sequence. This, again, is in agreement with its postulated role into a dynamic transition from an inactive to an active enzyme.

In order to gain more information, we first built another homology model of the NOX2 DH domain, taking full advantage of the Sumimoto crystal structure, homologs deposited in the database since the Taylor model, and more recent molecular modelling algorithms (Fig. 1B). While this new model provides additional important details, the question of NOX2-NIS function remained open, partly because the structure of the NOX2-NIS in the model is still speculative. In addition, the conformation of the homologous DH domains is presumed to be in an active conformation and thus does not provide necessary information to understand the inactive conformation of NOX2. To address the importance of the NIS structure we generated NOX2 chimera devoid of the NIS or with NIS sequence exchanged from NOX1, 3 or 4. Finally through a detailed analysis of the prototype NOX2 in the PLB-985 cell line and in human neutrophils, we identified a role of NOX2-NIS in NADPH oxidase down-regulation thanks to a site-specific phosphorylation event.

2. Results

2.1. An updated Homology Model

BLAST and DALI searches reveal the closest crystallized homologs of NOX NADPH domain as flavohemoglobins, flavodoxins, ferredoxins, and b5 reductases that are all members of the FNR family. Because these homologs are constitutively active, their crystal structures are presumed to better reflect the active conformation of the NOX2 DH Domain. However, NOX2 requires protein/protein interactions to switch into an active state, so the crystal structure of its isolated NADPH-binding domain (pdb 3A1F) is expected (although not demonstrated) to be in an inactive conformation. Comparison of the 3A1F structure with several homologous crystal structures (pdb 3EIX, 2GJR, FDR, 1AMO, etc.) reveals no consistent pattern of differences except for the existence of the NOX-NIS, which is invisible

in the 3A1F structure, presumably because of its flexibility. Because the NOX-NIS differentiates NOXs from the other DH homologs of the FNR family, it is a likely major control point for NOX regulation, as proposed by Taylor et al. [25] (Fig. 1B). To improve our picture of the NOX-NIS position and its relationship to the NADPH and FAD binding domains, we made an updated structural model of the NOX2 DH domain, using the 3A1F crystal as the main template for the NADPH domain (Fig. 1C).

The two models agree substantially on the identity and position of secondary structural units. However, in the NADPH-binding subdomain, determined by X-ray (Fig. 1C), some secondary structures are more extended than initially predicted and thus lead to a different positioning of the NOX-NIS. In our model the helical portion of the NOX-NIS is pulled away from the NADPH binding site, while in the Taylor model the helix partly covers it. In addition, the end residues of the NIS are visible in the X-ray structure showing that its orientation in the new model is inverted compared to the old one. The long linker allows for significant mobility of the helix, consistent with the hypothesis proposed by Taylor and colleagues [25] that the interaction of cytosolic factors with the NIS could trigger conformational changes and modify the functional state of NOX2.

2.2. NOX2-NIS mutagenesis description

To test this hypothesis, we deleted or exchanged the NOX-NIS sequence, in a NOX2 context, by site directed mutagenesis. The four mutants constructed in this study are shown in Fig. 2A. Construct NOX2- NIS has a complete deletion of the NOX2-NIS, whereas NOX2-NIS1, NOX2-NIS3 and NOX2-NIS4 were obtained by replacing the NOX2-NIS by those of NOX1, NOX3 and NOX4, resulting in changes more or less important in the protein sequence. We emphasize that NOX3-NIS has the sequence closest to that of NOX2-NIS (66% similarity compared to 30 and 14% for NOX1-NIS and NOX4-NIS respectively). In addition amino acids D484, H490 and D500 (Fig. 1C) described as essential for the interaction with cytosolic factors [26], are perfectly conserved in the chimeras NOX2-NIS1 and NOX2-NIS3. Finally, the mutated protein NOX2-NIS4 has a truncated NIS wherein only residue D484 is preserved.

2.3. Cell surface expression of the wild-type and mutated proteins in the PLB-985 cells

The functional consequences of these mutations were studied in the X-CGD PLB-985 cell line wherein the gene encoding the NOX2 protein was inactivated by homologous recombination [27]. Cells were first transfected with plasmid constructs encoding wild type or mutated proteins and clones presenting a significant expression of NOX2 at the cell surface were selected by flow cytometry as described in the experimental procedures. By flow cytometry and western blot analysis, all chimeras are expressed in the transfected PLB-985 cells at the same level as the wild-type protein (Fig. 2B and C). Moreover, differential spectral analysis of cytochrome *b*₅₅₈ demonstrates that mutagenesis of the NIS of NOX2 does not disturb heme incorporation, either qualitatively or quantitatively (Fig. 2C and Table 1).

2.4. Functional analysis of chimeras

The NADPH oxidase activity of PLB-985 cell lines expressing the NOX2- NIS, NOX2- NIS1 and NOX2-NIS4 chimeras were measured *in cellulo* by chemiluminescence and SOD inhibitable cytochrome *c* reduction test to measure superoxide only. As reported in Table 1, in the absence of stimulation no superoxide production was detected. After activation by PMA or opsonized zymosan (ZOPS), a significant production of ROS and superoxide anions were detected in the cells expressing the WT-NOX2 protein whereas this production was completely abolished in the PLB cells expressing the chimeric proteins NOX2- NIS, NOX2-NIS1 and NOX2-NIS4 (Table 1). These constructs also failed to produce superoxide measured *in vitro* by the SOD sensitive cytochrome *c* reduction assay in which purified membrane fractions from the cell lines were combined with a neutrophil cytosolic fraction containing the p47^{phox}, p67^{phox} and Rac1 proteins. The electron transfer from NADPH to FAD quantified spectrophotometrically by the reduction of INT in the same cell-free system assay (diaphorase activity) was also abolished (Table 1).

Using confocal microscopy, we observed that NADPH oxidase inactivation in the NOX2- NIS and NOX2-NIS1 PLB-985 cells correlates with a defect in the phagosomal membrane translocation of p47^{phox} and p67^{phox} (Fig. 3). As can also be seen in higher magnification images (supplementary Figure 1), after phagocytosis of latex beads p67^{phox} and p47^{phox} do not accumulate in the phagosomal membrane in the NOX2-NIS1 and NOX2- NIS2 mutants but are localized mainly in the cytoplasm around the nucleus, similar to what is observed in KO NOX2 PLB-cells (XCGD cells). Unexpectedly, the lack of activity in NOX2-NIS4 is *not* mirrored by a defect in translocation. In this case, after phagocytosis p47^{phox} and p67^{phox} accumulate and colocalize mainly in the phagosomal membrane around the latex beads where the NADPH oxidase complex is activated, similar to the positive control (NOX2 PLB-985 cells) (Fig. 3 and supplementary Figure 1). Thus despite a possible assembly of the complex, the exchange of the NOX2-NIS sequence by that of NOX4 does not allow the downstream molecular events required for activation to occur. The NOX2-NIS3 chimera is the unique chimera tested that conserves both complex assembly and NADPH oxidase activity.

2.5. Analysis of the NOX2-NIS3 chimera

The functionality of the NADPH oxidase complex in the NOX2-NIS3 PLB-985 cell line was analysed using the same methodological approach as described above. Unexpectedly, the ROS production in this cell line expressed in total RLU production after PMA or ZOPS activation was 1.5 and 1.3 times higher than the wild type strain respectively (Table 1). When superoxide anions were measured specifically, the activity in the NOX2-NIS3 PLB-985 was 3 to 4 times higher than in the WT-NOX2 cells (Table 1). In addition the modification of oxidase activity observed *in cellulo* was confirmed *in vitro*, where cytochrome *c* and INT reductase activities related to the NOX2-NIS3 membranes were 1.5 to 2 times higher than those measured with membranes expressing the WT-NOX2 protein (Table 1). Replacement of the NIS of NOX2 by that of NOX3 does not disturb p47^{phox} and p67^{phox} translocation to the phagosomal membrane (Fig. 3). We hypothesized that the enhancement of oxidase activity observed in the NOX2-NIS3 chimera could be due to

deregulation of the oxidase activity resulting from the absence of post-translational modifications that can not occur when the NIS2 is replaced by the NIS3.

2.6. Importance of post-translational events for the overproduction of ROS in the NOX2 - NIS3 proteins

In order to confirm our hypothesis, cytochrome *b*₅₅₈ was purified from both resting WT-NOX2 and NOX2-NIS3 PLB-985 cells according to the experimental procedure established by Lord and colleagues [28]. From 1.10^{10} unstimulated WT-NOX2 or mutated NOX2 PLB-985 cells, we obtained 18 ± 2 µg of pure cytochromes *b*₅₅₈ as previously described [29] (Fig. 4A).

As shown in Fig. 4A and B, the flavocytochromes *b*₅₅₈ were pure to homogeneity and share the same spectral features, confirming in both the presence and integrity of the heme moieties. The kinetic parameters of these cytochromes *b*₅₅₈ in the absence of stimulus-induced phosphorylation were determined in a cell-free system composed exclusively of purified proteins (pure flavocytochrome *b*₅₅₈, recombinant p47^{phox}, p67^{phox} and Rac1 proteins). In these conditions, the *V*_{max} of superoxide production catalysed by the NOX2-NIS3 protein was one third that of the wild type NOX2 protein (Fig. 4D). This suggests that the apparent ROS overproduction by this chimera *in vivo* is related to post-translational events that could occur during the activation process and could be different between NOX2-NIS3 and WT-NOX2. Affinity constants for NADPH, p67^{phox} and Rac1 were similar for WT and the NOX2-NIS3 chimera indicating no major structural changes in both resting proteins (Fig. 4D). The *p* values calculated with the Mann–Whitney test were non-significant.

2.7. In cellulo identification of cytochrome *b*₅₅₈ regulatory sites

To assess the importance of phosphorylation events on the regulation of NADPH oxidase activity in the WT-NOX2 protein, we analysed the phosphorylated residues of purified flavocytochrome *b*₅₅₈ with and without PMA activation. Interestingly in NOX2 a unique phosphorylation on serine 486 in the NIS sequence was detected (Fig. 5A). We also detected a phosphorylation on threonine 147 in p22^{phox} (Fig. 6A). For both residues no phosphorylation was observed in unstimulated cytochromes *b*₅₅₈ (Fig. 5B and 6B). In contrast, serine 153 of p22^{phox} was phosphorylated (Fig. 6C) in both resting and activated cytochromes *b*₅₅₈ (Fig. 6D). Ser486 in NOX2 is in the SQ consensus site of ATM kinase (<http://www.cbs.dtu.dk/>) in NOX2 whereas Thr147 and Ser153 in p22^{phox} are in typical PKC phosphorylation sites.

2.8. Involvement of ATM-kinase in the regulation of the NADPH oxidase activity in WT-NOX2 and NOX2-NIS3 mutant

To confirm the involvement of the ATM kinase on the regulation of the NADPH oxidase activity, we measured the phosphorylated form of ATM kinase (phospho-ATM) by autophosphorylation during the time course of PMA activation in human neutrophils (Fig. 7A), and observed an increase as previously reported by Harbort et al. [32]. In addition KU-55933, a specific inhibitor of ATM decreased the phospho-ATM of neutrophils in a concentration dependent way (Fig. 7B). We observed a significant increase of the NADPH

oxidase activity of neutrophils proportional to the amount of KU-55933 added in the medium with a maximal activity at 5 μ M KU-55933 (Fig. 7C and D). This suggests that ATM kinase regulates the ROS production in neutrophils by inhibiting the NADPH oxidase activity. We also measured the response of WT PLB-985 cells to KU-55933 and observed a similar increase in ROS production, although the concentration of inhibitor necessary to elicit maximum ROS production was much lower in than in human neutrophils (0.5 μ M versus 5 μ M, Fig. 7D and E). To confirm the importance of phosphorylation on Ser486 for the modulation of ROS production, the NADPH oxidase activity was measured by chemiluminescence in Ser486Ala-NOX2 and Ser486Glu-NOX2 PLB-985 cells (Fig. 7F). As expected, the loss of the ATM phosphorylation site on NIS2 of the Ser486Ala-NOX2 mutant leads to an increase of NADPH oxidase activity of about 2.5 fold compared to WT-NOX2. This increase is identical to that observed in the NOX2-NIS3 mutant where the ATM phosphorylation site is also absent (Table 1 and Fig. 2A) and in WT PLB-985 cells treated with 0.1 μ M ATM inhibitor KU-55933 (Figure 7E). Also as expected, the NADPH oxidase activity of the Ser486Glu-NOX2 mutant (in which the charge on the glutamate mimics the phosphorylation of Ser486) is significantly diminished.

3. Discussion

From three-dimensional models of the NOX2 dehydrogenase unit using flavohemoglobins, flavodoxins, ferredoxins, and b5 reductases as templates, the NOX-NIS sequence ⁴⁸⁴DESQANHFAVHHDEEKDVITG⁵⁰⁴ which includes a strongly predicted α helix, was proposed to have a regulatory role on NADPH oxidase activation [25]. However until now, the main problem to elucidate the functionality of this enigmatic region was that all the mutants generated from this region were devoid of NADPH oxidase activity.

In this work we developed several methodological approaches i.e. molecular and cellular biology, biochemistry, structural prediction analysis, in order to elucidate the role of this region of NOX2 on the NADPH oxidase activity and regulation. All four NOX-NIS deletion and chimera constructs studied here expressed normally at the plasma membrane in X-CGD PLB-985 cells, and had normal heme content. Based on these results, we concluded that neither deletion nor substitution of the corresponding sequence from NOX1-4 isoforms affect the global structural organization and the targeting of NOX2 protein into the plasma membrane of the PLB-985 cells. Indeed in the crystal structure of the NADPH binding domain of NOX2 deposited by Dr Sumimoto's group, the NOX2-NIS is not visible suggesting this region's high flexibility and thus its low probability of contributing to rigid structural requirements.

If NOX-NIS merely regulates NADPH entry to the cleft containing the NADPH and FAD binding sites, then deletion of this region should give free access to NADPH leading to a constitutive NADPH oxidase activity. However deletion of the sequence (484–504) or replacement by that of NOX1 totally abolished cytosolic factor translocation to the phagosomal membrane, ROS production, and reductase activity. Surprisingly replacement with the NOX4-NIS did *not* disturb this translocation, but *did* disrupt both ROS production and reductase activity. Similar results were obtained with a NOX2/NOX4 chimera expressed in Cos-p22^{phox} cells [30]. The sequences of NOX1-NIS and NOX4-NIS have 7% and 14%

similarity to NOX2-NIS respectively, suggesting that these replacements cause a sizable structural change to the NOX2 DH domain. Taken together, these results demonstrate that NOX2-NIS: 1) is crucial to the NADPH oxidase activity; 2) controls electron transfer from NADPH to FAD; and 3) is important for cytosolic factor translocation to the phagosomal membrane.

The most surprising results were obtained with the NOX2-NIS3 chimera. This change induced an increase of ROS production with a normal translocation of cytosolic factors to the phagosomal membrane in transfected cells after PMA and ZOPS stimulation and also in a cell free system. In contrast, using non-activated purified proteins in a totally cell free system, the chimera demonstrates a lower activity but no important changes to the affinity constants for NADPH, p67^{phox} and *Rac1*. From this we supposed that the structural change introduced by the NOX3-NIS led to differential effects of post-transcriptional modifications (e.g., phosphorylation) on WT vs. NOX2-NIS3 chimera. Thus phosphopeptides of purified flavocytochrome *b*₅₅₈ from resting or PMA activated PLB-985 cells were analyzed by MS-MS. Serine 486 and threonine 147 in the NOX2-NIS and p22^{phox} respectively were phosphorylated only after PMA activation, whereas serine 153 in p22^{phox} was phosphorylated in both situations. Although serine 153 phosphorylation in p22^{phox} is new information, it does not seem related to the activation of NOX2. In contrast, site-directed mutagenesis has already shown that phosphorylation of p22^{phox} on threonine 147 enhances NADPH oxidase activity by promoting p47^{phox} binding [13]. Thus threonine 147 is important for maintaining the PMA-stimulated activity that depends on phosphorylation by PKC.

We demonstrated for the first time a direct phosphorylation of NOX2 on Ser486 after PMA activation in WT PLB-985 cells. Ser486 is in a SQ consensus phosphorylation site for ATM kinase. ATM kinase is a serine/threonine protein kinase and is an important sensor of DNA damage. In response to genotoxic insults ATM kinase is activated by phosphorylation and then initiates and coordinates the DNA damage response [31]. Moreover, it has been previously demonstrated that ATM kinase is directly activated by oxidative stress [33]. Recently Harbort et al. highlighted that deficiencies in the DNA damage response seen in CGD cases could explain pathologic inflammations found in this disease [32]. They provided a clue that might connect oxidative stress with NOX regulation. They demonstrated that in neutrophils ATM kinase is activated by the respiratory burst and that this activation modulates neutrophil functions to slow down the inflammation.

With our work we provide evidence connecting this observation to molecular mechanism occurring at the level of NOX2. We verified that ATM kinase is phosphorylated after PMA stimulated NOX2 activation in intact human neutrophils and that this phosphorylation is inhibited by KU-55933, a specific ATM kinase inhibitor. Our functional study of the WT-NOX2-Ser486Ala and the WT-NOX2-Ser486Glu expressed in PLB-985 cells demonstrates that serine 486 in NOX2 is essential for an ATM-dependent down-regulation of the phagocytic NADPH oxidase activity after PMA stimulation. This event can induce a deactivation process of the NADPH oxidase complex probably necessary to protect against DNA damage within the phagocytes. To reinforce these results we showed that KU-55933 treatment of human neutrophils before PMA activation increases NADPH oxidase activity in

a dose dependent manner. The efficiency of KU-55933 depends on the cell type because WT PLB-985 cells appear more sensitive to this inhibitor than human neutrophils. This is probably due to the differing level of enzyme expression between the two cell types.

The NIS3 sequence, closest to that of NIS2 (66% similarity with NOX2-NIS), contains all the important charged residues D484 and D500 previously demonstrated as involved in the assembly of the complex [26], but does not have the SQ consensus phosphorylation site for ATM kinase (and Ser486). In consequence ATM kinase cannot phosphorylate the NIS3 in NOX2-NIS3 cells which can explain the higher NADPH oxidase activity observed after PMA stimulation. This underlines further the role of NOX2 phosphorylation, underestimated up to now, in the overall activation/regulation process. Indeed, activation of PKs engenders *in fine* a multitude of phosphorylation events targeting proteins of the NADPH oxidase complex, allowing the assembly and the regulation of production of superoxide anions. We propose a modulation of the NADPH oxidase activity level triggered by the activated ATM kinase following its autophosphorylation (Fig. 8).

Despite the crystal structure of the NOX2 NADPH binding domain and the newer homology model that includes the NIS (Fig. 1C), the structural changes in response to phosphorylation and/or protein/protein interactions are still unknown. The much longer NIS of NOX5 has been demonstrated to contain the EF-hand binding site that confers calcium control to NOX5 [35], highlighting the importance of protein/protein interactions in this area to control protein activity. This region is still invisible in the very recent crystal structure of a NOX5 homolog [36], confirming its probable mobility. As noted above, the NIS in general probably controls NADPH accessibility to its binding site, because many NIS substitutions in the NOX2 background affect the initial electron transfer [26, 37]. Based on its position, we envision this accessibility effect to result from motion of the NIS. This idea is supported by the fact that the NOX2-NIS3 chimera is more active than WT NOX2, indicating that NIS position could be regulated by the ATM kinase phosphorylation. Such differences could change the intrinsic structure or flexibility of the NIS, thus affecting interactions with nearby structural elements within the NOX2 protein; or modifying the interactions with NOX2's cytosolic partners; or both. Evidently the changes introduced in NOX2 by the NIS1 are incompatible with the activation of NOX2 in response to stimulating agents, while the changes introduced by the NIS3 leads to an overproduction of ROS. The lack of activity of the NIS deletion construct or the replacement by the NIS4 (closer in extent to that of homologous constitutively active DH domains) indicates a minimum length necessary for a linker in this region to support the NADPH oxidase activation process. In conclusion oxidative stress must be mastered by the cells in order to avoid DNA damage and destruction of surrounding cells and tissues. This control is available for both for phagocytic cells and also for non-phagocytic cells such as vascular cells or neurons. Thus the NIS of NOXs could be an interesting target to modulate the NADPH oxidase activity for therapeutic applications.

4. Experimental procedures

4.1. Chemicals and reagents

Phorbol 12-myristate 13-acetate (PMA), dimethylformamide (DMF), cytochrome *c* (horse heart, type VI), diisopropylfluorophosphate (DFP), horseradish peroxidase (HRPO), N-alpha-tosyl-L-lysyl-chloromethyl ketone (TLCK), latex beads polystyrene (3µm), IonoNitroTetrazolium *p*-Iodonitrotetrazolium Violet, INT, 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride (INT), luminol, Triton X-100, sodium dithionite, flavine adenine dinucleotide (FAD), desoxyribonuclease I, ribonuclease B, superoxide dismutase, phosphatidyl choline type XVI-E and mammalian proteases inhibitors cocktail P8340 were obtained from Sigma Chemical Co. (St Louis, MO, USA). Guanosine 5'-O-(3-thiotriphosphate) (GTPγS), reduced nicotinamide adenine dinucleotide (NADPH), leupeptin, and pepstatin were from Roche (Meylan, France). Foetal bovine serum, RPMI 1640, geneticin G418, Alexa Fluor 488 goat-F(ab')₂ fragment anti-rabbit IgG1 (H+L), Alexa Fluor 546 donkey-F(ab')₂ fragment anti-goat IgG1 (H+L) were from Invitrogen (Cergy Pontoise, France). Monoclonal antibody specific for NOX2 7D5 was purchased from MBL Medical and Biological Laboratories (Naka- Nagoya, Japan). For confocal analysis polyclonal anti-rabbit p47^{phox} raised against residues 371–390 in the C terminal region and goat polyclonal antibodies against p67^{phox} C-19 were from Santa Cruz Biotechnology, Inc (Heildeberg, Germany). For western blot the antibodies used against ATM and phospho-ATM (S1981) were purchased from Thermo Fischer Scientific (Courtaboeuf, France), the monoclonal antibodies 54.1 and 44.1, raised against p22^{phox} and gp91^{phox} were purchased from Santa Cruz Biotechnology, Inc (Heildeberg, Germany), and the monoclonal antibodies against p47^{phox} was purchased from BD Biosciences (Le Pont-de-Claix, France). Endofree plasmid Maxi Kit and QIAprep Spin Miniprep Kit were purchased from Qiagen (Courtaboeuf, France). Octyl glucoside and paraformaldehyde were purchased from Pierce Thermo Scientific (Rockford, USA). The Sephaglas kit and molecular weight markers (Page Ruler®) were obtained from Amersham (Uppsala, Sweden) and Fermentas (Burlington, Canada) respectively. Nitrocellulose sheets for Western Blotting were purchased from Bio-Rad (Marne-la-Coquette, France). Phosphatidic acid was purchased from Coger (Paris, France).

4.2. Site-directed mutagenesis and expression of recombinant gp91^{phox} in X-CGD PLB-985 cell line

Gp91^{phox} site-directed mutagenesis was performed with the Quick Change™ Mutagenesis Kit (Stratagene) according to manufacturer's instructions, using two sets of complementary oligonucleotides harboring the desired mutation. DNA sequences were verified by DNA sequencing on both strands using the Big Dye Kit (Cogenics, Grenoble, France). The wild type or mutated gp91^{phox} cDNA were then subcloned into the mammalian expression vector pEF-PGKneo as previously described [36]. The transfection of X-CGD PLB-985 cell line (lacking NOX2 expression and NADPH oxidase activity) [27] with the pEF-PGKneo constructs was performed by electroporation and clone selection was done by limiting dilution in 1.5 mg/ml of geneticin.

4.3. Cell culture and granulocyte differentiation

X-CGD and transfected PLB-985 cell lines were grown in RPMI-1640 supplemented with 10% fetal bovine serum (v/v), 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-Glutamine at 37°C in a humidified 5% CO₂ atmosphere. Geneticin (0.5 mg/ml) was added to maintain the selection pressure of the plasmid in the transfected cells. For granulocytic differentiation, PLB-985 cells (2×10⁵ cells/ml) were exposed to 0.5% dimethylformamide (v/v) for 6 days [38].

4.4. Assessment of gp91^{phox} or NOX2 protein expression by flow cytometry

NOX2 expression in transgenic PLB-985 cells was assessed by using monoclonal antibodies directed against an external epitope of the protein [39] as previously described [26]. Results are expressed as mean fluorescence intensity values in arbitrary units (au). All experiments were done in triplicate.

4.5. Cytosol and membrane fraction preparation from neutrophils and transfected PLB-985 cell lines

Neutrophils and differentiated PLB-985 cells were harvested by centrifugation (1000 g for 10 min at room temperature) and treated with 3 mM diisopropyl-fluorophosphate for 15 min on ice. In experiments of phosphorylation cells stimulated were resuspended in 1×10⁸ cells/ml in phosphate-buffered saline containing 0.9 mM Ca²⁺ and 0.5 mM Mg²⁺ and treated by 5µM cytochalasin B for 15 min at 37°C and stimulated by PMA (20 ng/µl) during 10 min at 37°C. After centrifugation, cells were resuspended in at 2×10⁸ cells/ml in a buffer A (10 mM Hepes (pH 7.4), 10 mM NaCl, 100 mM KCl, 12 mM EGTA, 3.5mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 2 µM leupeptin, 2 µM pepstatin and for phosphorylation's experiments also with 1% phosphatase inhibitor cocktail 3 (v/v). Cells were disrupted by sonication and the homogenate was centrifuged at 1000 g for 15 min at 4°C. The supernatant was withdrawn and centrifuged at 200000 g for 1 hour at 4°C. The high speed supernatant was referred to as the cytosol, and the pellet consisting of crude membranes was resuspended in buffer B (100 mM Hepes (pH 7.4), 10 mM NaCl, 100 mM KCl, 1 mM EDTA, 0.1mM DTT, 1 mM phenylmethylsulfonyl fluoride, 2 µM leupeptin, 2 µM pepstatin and for phosphorylation's experiments also with 1% phosphatase inhibitor cocktail 3 (v/v) [26].

4.6. Immunoaffinity purification of the Cytochrome b₅₅₈ from PLB-985 membrane fractions

PLB-985 membrane fractions were incubated with 1 M NaCl for 30 min at 4°C in Dounce homogenizer. Following centrifugation at 200 000 g for 1 hour at 4°C, the pellet was resuspended in buffer C (10 mM Hepes (pH 7.4), 10 mM NaCl, 100 mM KCl, 1 mM EDTA, 0.1mM DTT, 2% octyl-glucoside (w/v), 1 µL/ml mammalian protease inhibitor cocktail, 1 mM phenylmethylsulfonyl fluoride, 2 µM leupeptin, 2 µM pepstatin, 100 µg/ml deoxyribonuclease I and 100 µg/ml ribonuclease B and for phosphorylation experiments also with 1% phosphatase inhibitor cocktail 3 (v/v) for 45 min at 4°C under constant agitation. Following centrifugation at 200 000 g for 45 min at 4°C, detergent extracts were added to mAbs CS9-Sepharose beads previously equilibrated with buffer D (10 mM Hepes (pH 7.4), 10 mM NaCl, 100 mM KCl, 1 mM EDTA, 0.1mM DTT, 1.2% octyl-glucoside (w/v), 1 µl/ml

mammalian protease inhibitor cocktail, 1 mM phenylmethylsulfonyl fluoride, 2 μ M leupeptin, 2 μ M pepstatin, 100 μ g/ml deoxyribonuclease I and 100 μ g/ml ribonuclease B and for phosphorylation's experiments also with 1% phosphatase inhibitor cocktail 3 (v/v) and the resulting mixture was rotated for 1 hour at 4°C [28], [29]. After centrifugation, the resin was washed four times with 5 ml of ice-cold buffer D. For elution of cytochrome *b*₅₅₈, the affinity matrix was incubated with 600 μ L buffer D and 200 μ M Ac-AEARKKKPSEEEAA-NH₂ for 15 min at room temperature. The protein content of the elution fraction was assessed by cytochrome *b*₅₅₈ differential spectral, immunoblot analysis, silver-stained SDS PAGE or Nano-LC-MS/MS analyses.

4.7. Relipidation of purified cytochrome *b*₅₅₈

Relipidation was performed in the presence of 99% L- α -phosphatidylcholine (Type XVI-E, Sigma Aldrich) (v/v) and 18:1 phosphatidic acid (DOPA, Avanti Polar Lipids). Lipids preparation was achieved accordingly to [40]. Phospholipids [100 μ g in buffer C (50 mM K₂HPO₄ (pH 7.3), 150 mM NaCl, 2 mM MgCl₂, 2 mM EDTA, 15% glycerol (v/v))] containing 1.2% OG (w/v) were mixed with approximately 400 pmol of purified cytochrome *b*₅₅₈. The mixture was diluted six fold with the buffer C without detergent and was either incubated for 30 min on ice, prior to testing oxidase activity in the cell-free assay, or stored at -80°C until use.

4.8. Expression and purification of recombinant p47^{phox}, p67^{phox} and Rac1 proteins

P47^{phox}, p67^{phox} and Rac1 were expressed in *Escherichia coli* as a glutathione S-transferase fusion proteins using pGEX-2T vector and purified as previously described [29].

4.9. Protein digestion

After purification, proteins precipitation was performed with a TCA/acetone procedure. Extracts were solubilized in Laemmli buffer and proteins were stacked in the top of a 4–12% NuPAGE gel (Invitrogen) before R-250 Coomassie blue staining. The gel band was manually excised and cut into pieces before being washed by six successive incubations in 25 mM NH₄HCO₃ for 15 min each, followed by six incubations in 25 mM NH₄HCO₃ containing 50% acetonitrile (v/v). Gel pieces were then dehydrated with 100% acetonitrile (v/v) and incubated with 10 mM DTT in 25 mM NH₄HCO₃ for 45 min at 53 °C and with 55 mM iodoacetamide in 25 mM NH₄HCO₃ for 35 min in the dark. Alkylation was stopped by the addition of 10 mM DTT in 25 mM NH₄HCO₃ (10-min incubation). Gel pieces were then washed again by incubation in 25 mM NH₄HCO₃ followed by dehydration with 100% acetonitrile (v/v). Modified trypsin (sequencing grade, Promega, Madison, WI) in 25 mM NH₄HCO₃ was added to the dehydrated gel pieces for incubation at 37 °C overnight. Peptides were extracted from gel pieces in three sequential extraction steps (each 15 min) in 30 μ L of 50% acetonitrile (v/v), 30 μ L of 5% formic acid (v/v), and finally 30 μ L of 100% acetonitrile (v/v). Pooled supernatants were splitted into 2 parts: 10% for proteome analysis and 90% for phosphopeptides enrichment. Both were dried under vacuum.

4.10. Phosphopeptides enrichment

Phosphopeptides were enriched from samples with TiO₂ beads (GL Science) in batch mode with a modified protocol from [41]. Briefly samples were mixed during one hour with 0.6mg beads in loading buffer [1M Glycolic acid in 80% acetonitrile (v/v), 5% TFA (v/v)]. Beads were washed 3 times with loading buffer, 80% acetonitrile with 1% TFA (v/v) and finally 10% acetonitrile (v/v) with 0.1% TFA (v/v). Phosphopeptides were eluted with 10% ammonia solution (v/v) during 10 min. After acidification with formic acid, peptides were desalted using C18 ultra-micro tip columns (Harvard Apparatus) and dried under vacuum.

4.11. Nano-LC-MS/MS analyses

The dried peptides were resuspended in 5% acetonitrile (v/v) and 0.1% trifluoroacetic acid (v/v) and analyzed by online nano-LC-MS/MS (RSLC, Dionex and Q-Exactive Plus; Thermo Fisher Scientific). Peptides were sampled on a 300 μ m $\times$ 5-mm PepMap C18 precolumn and separated on a 75 μ m $\times$ 250-mm 3 μ m C18 column (PepMap, Dionex). The nano-LC method consisted in a 120-min gradient at a flow rate of 300 nL/min, ranging from 5% to 11% acetonitrile (v/v) in 0.1% formic acid (v/v) in 22 min, then a second step from 11% to 25% in 90 min and a final step from 25% to 33% in 8 min. MS and MS/MS data were acquired using Xcalibur (Thermo Fisher Scientific). Spray voltage were set at 1.6 kV. Survey full-scan MS spectra (m/z = 400–1,600) were acquired in the Orbitrap with a resolution of 70,000 after accumulation of 10^6 ions (maximum filling time, 200 ms). For proteome analysis, the 10 most intense ions from the preview survey scan were fragmented by Higher energy collision dissociation (resolution 17500, collision energy, 30%) after accumulation of 10^5 ions (maximum filling time, 50 ms). For phosphopeptides analysis accumulation for MSMS was set to 10^6 ions (maximum filling time, 250 ms).

4.12. Mass spectrometry bioinformatics data analyses

Data were automatically processed using Mascot Daemon software (version 2.5, Matrix Science). Peak lists were generated with Mascot Distiller version 2.5.1.0 software (Matrix Science) from the LC-MS/MS raw data. Searches against Uniprot database (human taxonomy) and against contaminants database (138809 sequences in total for these 2 databases) and the corresponding reversed databases were performed using Mascot (version 2.5.1). ESI-FTICR was chosen as the instrument, Trypsin/P as the enzyme and 2 missed cleavage allowed. Precursor and fragment mass error tolerances were set respectively at 10 ppm and 25 mDa. Peptide modifications allowed during the search were as follows: carbamidomethylation (C, fixed) acetylation (protein N-term, variable), oxidation (M, variable) and phosphorylation (S, T, Y, variable). The Proline software (version 1.0) was used to filter the results: conservation of rank 2 PSM, PSM false discovery rate < 1% (as calculated on peptide scores by using the reverse database strategy), and minimum of one specific peptide per identified protein group [41].

4.13. Measurement of NADPH oxidase activity in intact cells

ROS production was measured by chemiluminescence as previously described [26]. Briefly, 5×10^5 human neutrophils or granulocytic differentiated PLB-985 cells in phosphate-buffered saline containing 0.9 mM Ca²⁺ and 0.5 mM Mg²⁺ were mixed with 20 mM glucose, 20 μ M

luminol and 10 units/ml of horseradish peroxidase. The NADPH oxidase reaction was initiated by adding PMA (80 ng/ml) or when indicated, opsonized zymosan (ZOPS) (0.64 mg/ml). Relative light units (RLU) were recorded at 37° Cover a time course of 60 min in a luminoscan luminometer (LabSystems, Helsinki, Finland). In some experiments, human neutrophils or PLB-985 cells (5×10^5) were pre-incubated with the ATM inhibitor KU-55933 (15 to 500 nM) for 15 min at 37°C before stimulation.

4.14. Measurement of NADPH oxidase and iodinitrotetrazolium reductase activities in a broken cell system

NADPH oxidase activity was measured *in vitro* using transfected PLB-985 plasma membranes (100 µg) and cytosol (300 µg) from human neutrophils in a reaction mixture containing 20 µM GTP-γS, 5 mM MgCl₂ and an optimal amount of arachidonic acid in a final volume of 100 µl. After incubation for 10 min at 25°C, the NADPH oxidase reaction was initiated in the presence of cytochrome *c* (100 µM) and NADPH (150 µM). The specificity of superoxide production was checked by adding 50 µg/ml superoxide dismutase to stop the kinetic reduction. The diaphorase activity was measured under the same *in vitro* conditions, except that cytochrome *c* was replaced by 50 µM of iodinitrotetrazolium [43]. In some experiments, plasma membranes of PLB-985 cells and cytosol of neutrophils were replaced by purified cytochrome *b*₅₅₈ (5 nM) and purified recombinant proteins p67^{phox}, p47^{phox} and Rac1 preloaded with GTP-γ-S (1 µM). We added 10 µM FAD to the reaction medium when purified cytochrome *b*₅₅₈ was used [44].

4.15. Analysis of in cellulo p47^{phox} and p67^{phox} translocation to the phagosomal membrane

Differentiated PLB-985 cells (5×10^5) were deposited on glass slides and activated with 10 µl of PMA-treated latex beads [100 µL of PMA 40 µg/ml were mixed with latex beads (3 µm) in 100 µL sterile PBS] or ZOPS 0.64 mg/ml for 15 min at 37 °C. Cells were fixed in 4% paraformaldehyde (v/v) for 10 min and permeabilized by 0.25% Triton X-100 (v/v). Fixed cells were incubated with primary antibodies [rabbit polyclonal anti-p47^{phox} and goat polyclonal anti-p67^{phox} (Santa Cruz)]. Alexa Fluor 488 F(ab')₂ fragments of goat anti-rabbit IgG (H+L) and Alexa Fluor 546 F(ab')₂ fragments of donkey anti-goat IgG (H+L) were used as second antibodies (Molecular Probes). Cellular nuclei were visualized by Hoechst 33258 staining. [45]. Cells were examined with a confocal laser-scanning microscope and analyzed by Leica confocal software.

4.16. Analytical methods

SDS-PAGE was performed in slab gels containing 12% acrylamide (w/v) and polypeptides were transferred electrophoretically onto nitrocellulose sheets [46]. The immunodetection of the NOX subunits [47], ATM, phospho-ATM (S1981), and p47^{phox} was performed using specific antibodies listed in chemicals reagents. Secondary antibodies were polyclonal anti-mouse- or anti-rabbit-IgG labeled with horseradish peroxidase and the presence of immune complexes was detected by chemiluminescence using an ECL kit (Amersham Biosciences).

Expression of wild or mutated NOX2 in PLB-985 cells were followed by cytochrome *b*₅₅₈ differential (reduced minus oxidized) spectral analysis recorded at room temperature with a

DU 640 Beckman spectrophotometer. A molecular absorption coefficient of $\epsilon_{426 \text{ nm}} = 106 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for the Soret band was used for quantification [48].

Protein concentration was determined by the Bradford method [49] using Bio-Rad protein-assay reagent and bovine serum albumin as a standard.

4.17. Sequence Alignment and Homology Modeling

A large and diverse set of NOX DH domain sequences was aligned with PromalS3D [50], constraining the alignment with crystal structures of b5 reductases (1UMK, 2EIX), ferredoxin nadp reductases (1GJR, 1FNB), flavodoxin reductase (1FDR), and nitric oxide dioxygenase or flavohemoglobin (1GVH). Default parameters were used except that PromalS was used in the initial alignment. Additional mammalian NOX sequences were identified by BLAST, chosen on the basis of diverse levels of similarity to the human probe, and added to the original alignment using MAFFT [51]. Mammalian NOX subfamilies were abstracted from the original alignment and sequence logos of the NIS region were created using WebLogo3 [52]. Crystal structure templates were identified by BLAST searches using the DH domain of NOX2 as a probe and by DALI searches using the crystal structure of the NADPH binding domain of NOX2 (PDB ID 3AIF) as a probe. Templates were chosen based on sequence and structural similarity. An initial model was built in InsightII (Accelrys), which allows significant manual choice and manipulation of templates; this model was energy minimized using InsightII's standard molecular dynamics routines. Another model was built at the Robetta site [53], which used 3AIF and flavohemoglobin as its primary templates. Robetta includes extensive minimization and quality reports. Homology models and crystal structure templates were compared in Chimera [54] via its Matchmaker function with default values except iteration was performed until no pair exceeded $2.0 \text{ \AA}$, circular permutation was allowed and the superposition/alignment was iterated until convergence. RMSD values are reported along with the number of corresponding positions that were structurally aligned by this method. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311).

4.18. Statistics

All results are expressed as mean $\pm$ SD. Significant differences were identified using the Student test; $p < 0.05$ was considered significant.

Acknowledgments

The authors are grateful to Pr. Mary C. Dinauer for the generous gift of X-CGD PLB-985 cells. We are very grateful to Cécile Martel and Michelle Mollin for their enthusiasm at work at the CDiReC. This article is dedicated to the memory of Didier Grunwald, our friend who left us tragically.

Funding

MJS is grateful for support from the University Joseph Fourier, Faculty of Medicine; the Ministry of Education and Research, MENRT; the Regional Clinical Research Department, DRCI, Grenoble University Hospital, the CGD Research Trust grant award reference J4G/09/09 and from the National Institutes of Health Grant N01-AI-30070 from the Immunodeficiency Network and the Primary Immunodeficiency Disease Consortium. FF thanks for support from ANR 2010-1536-01 "prototype NOX" and the Institut Universitaire de France.

Abbreviations

NOX	NADPH oxidase
ROS	reactive oxygen species
CGD	chronic granulomatous disease
PMA	Phorbol 12-myristate 13-acetate
fMLP	formyl-methionylleucyl-phenylalanine
DMF	dimethylformamide
DFP	diisopropylfluorophosphate
DPI	diphenylene iodonium
INT	iodonitrotetrazolium
SOD	superoxide dismutase
NOX	NADPH oxidase
O₂^{•-}	superoxide
ATM	ataxia-telangectasia mutated

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Highlights

NIS sequence is a flexible structure supporting a functional role in NOX enzymes

NIS sequence of NOX2 regulates its NADPH oxidase activity in phagocytes

ATM kinase is activated by ROS production in phagocytes after PMA stimulation

Ser486 phosphorylation in NIS-NOX2 by ATM inhibits the NADPH oxidase activity

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Fig. 1. Representation of the new three-dimensional homology model of the cytosolic dehydrogenase domain of NOX2

A) Structure-based sequence alignment including individual sequences of crystallized dehydrogenase domain homologs of the FNR family (identified with PDB ids), and sequence logos of mammalian NOX subfamilies. Blue bar indicates a conserved beta strand; red bars indicate conserved alpha helices. Black Bracket indicates the approximate extent of the NOX Insertion Sequence (NIS). * indicates the location of Ser486 and the ATM phosphorylation site SQ in the NIS is underlined. Logos were obtained from alignments of 20 mammalian NOX1 sequences, 28 NOX2, 30 NOX3, and 28 NOX4 as described in Experimental Procedures. B) NOX2 DH domain homology model from Taylor et al [25], represented in blue ribbons, except the NOX2NIS highlighted in gold. Approximate locations of FAD (yellow sticks) and NADPH (gray sticks). C) Our improved NOX2 DH model is represented as ribbons, dark green for the NADPH binding domain -corresponding to the 3A1F structure- and pale green for the FAD binding domain. NOX2-NIS is again in gold color. Side chains of critical residues for cytosolic factor interactions (D484, H490, D500) and for phosphorylation by ATM kinase (Ser486) are shown in stick representation. Molecular graphic images were produced using PyMOL software. (2-column fitting image).

Fig. 2. Cell surface expression of the wild type and mutated NOX2 proteins in transfected X-CGD PLB-985 cells

A) Mutagenesis of the α helix_(484–504) of NOX2 (NOX2-NIS) - The wild type amino acid sequence of the NOX2-NIS is on the top line. The mutations introduced in this sequence are shown in shaded boxes. The black squares indicate the amino acid residues that are identical to the wild type sequence whereas the dashes correspond to the NOX2 amino acids deleted in the mutant sequences. Amino acids D484, H490 and D500 described as essential for assembly with the cytosolic factors are underlined. **B)** Flow cytometry analysis of NOX2 expression –Differentiated transfected X-CGD PLB-985 cells ($5 \cdot 10^5$) were incubated with the NOX2 monoclonal antibody 7D5, combined with a PE-conjugated anti-mouse IgG, as described in “the experimental procedures”. Mouse IgG1 isotype was used as an irrelevant monoclonal antibody. **C)** Immunoblot analysis of both subunits of cytochrome b_{558} , NOX2 and p22^{phox}, was performed in 1 % Triton X-100 (v/v) soluble extract (80 μ g) from PLB-985 mutant cells, subjected to SDS-PAGE, blotted onto a nitrocellulose sheet and revealed with monoclonal antibodies 54.1 and 44.1, respectively. **D)** Cytochrome b_{558}

differential spectra performed with the same detergent extracts as described in *C*, were recorded at room temperature with a DU 640 Beckman spectrophotometer. MW, molecular weight markers (kDa) (1.5-column fitting image).

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Fig. 3. Cytosolic factors translocation to the phagosomal membrane in transfected PLB-985 cells stimulated by latex beads

P47^{phox} and *p67^{phox}* translocation to the phagosomal membranes were evaluated by confocal microscopy analysis in 5×10^5 wild type or mutated PLB-985 cells stimulated by PMA-treated latex beads for 15 min at 37 °C as described in “the experimental procedures”. NOX2 cells represent the positive control and XCGD cells represent the negative control of *p47^{phox}* and *p67^{phox}* translocation to the phagosomal membranes. The + indicates normal cytosolic factor translocation while – indicates absence of cytosolic factor translocation to the phagosomal membrane. Scale bar is 10 μ m. These results are representative of observations obtained in at least four independent experiments. (2-column fitting image).

Fig. 4. Purification of cytochromes *b*₅₅₈ from transfected PLB-985 cells

A) Silver-stained SDS PAGE of purified WT-NOX2 and NOX2-NIS3 cytochromes *b*₅₅₈ (30 picomol) and immunoblot analysis of both subunits of purified cytochromes *b*₅₅₈ performed with 1.5 picomol of purified cytochromes *b*₅₅₈ using 54.1 and 44.1 monoclonal antibodies directed against NOX2 and p22^{phox} respectively. The intermediate band revealed by the 44.1 antibody is probably a dimer of p22^{phox}. B) Differential spectrum analysis of purified cytochromes *b*₅₅₈. The reduced-minus-oxidized difference spectra were recorded at room temperature with a DU 640 Beckman spectrophotometer. C) Purification factors and yields of the purification process of WT and NOX2-NIS3 cytochrome *b*₅₅₈. D) Kinetic parameters of the purified WT-NOX2 and NOX2-NIS3 cytochromes *b*₅₅₈. NADPH oxidase activity was reconstituted in a cell-free system assay using purified WT or NOX2-NIS3 cytochromes *b*₅₅₈ (5 nM), recombinant cytosolic proteins p47^{phox}, p67^{phox} and Rac1 (1 μM each), in the presence of NADPH (150 μM). Kinetic parameters were determined by varying the concentration of one parameter keeping the others at a saturating level. The data represent average ± SD of at least three independent experiments. The statistical significances of the differences were evaluated by the non-parametric Mann-Whitney test. *p<0.05. MW, molecular weight markers (kDa). (1.5-column fitting image).

Fig. 5. LC-MS/MS analysis of peptide NNAGFLSYNIYLTGWDESpQANHFAVHHDEEKDVITGLK from NOX2 phosphorylated on Ser486

Briefly 1×10^8 PLB-985 cells/ml treated by $5 \mu\text{M}$ cytochalasin B for 15 min at 37°C were stimulated by PMA ($0.5 \mu\text{g}$) during 10 min at 37°C . Cytochrome b_{558} was purified as described in “the experimental procedures”. **A)** HCD spectrum from the precursor ion 883.406Th in the 5+ charge state obtained at 97.2 min. This spectrum was scored 108 by Mascot and confidence on phosphosite specific assignment was 100%. Fragment ions matching with the theoretical spectrum are annotated. This peptide was identified only in activated cells. **B)** Extracted ion chromatogram of m/z 883.406Th both in resting and PMA activated differentiated WT-NOX2 PLB-985 cells. (2-column fitting image).

Fig. 6. MS/MS analysis of peptides ERPQIGGTphIK and QPPSphNPPPRPPAEAR from p22^{phox} phosphorylated on Thr147 and Ser153 respectively

Briefly 1×10^8 PLB-985 cells/ml treated by $5 \mu\text{M}$ cytochalasin B for 15 min at 37°C were stimulated by PMA ($0.5 \mu\text{g}$) during 10 min at 37°C . Then cytochrome *b*₅₅₈ purification was done as described in “the experimental procedures”. **A)** HCD spectrum from the precursor ion 589.80Th in the 2+ charge state obtained at 18 min. This spectrum was scored 37 by Mascot. Fragment ions matching with the theoretical spectrum are annotated. This peptide was identified only in activated cells. **B)** Extracted ion chromatogram of m/z 589.80Th both in resting and PMA activated differentiated WT-NOX2 PLB-985 cells. **C)** HCD spectrum from the precursor ion 564.27Th in the 3+ charge state obtained at 16.2min. This spectrum

was scored 26 by Mascot. Fragment ions matching with the theoretical spectrum are annotated. Multiple spectra were acquired both in resting and activated samples. *D)* Extracted ion chromatogram of m/z 564.27Th both in resting and PMA activated differentiated WT-NOX2 PLB-985 cells. (2-column fitting image).

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Fig. 7. Effect of ATM inhibition on the NADPH oxidase activity in human phagocytes
A) Western blot analysis of ATM phosphorylation (Ser1981) in 5.10^6 human neutrophils during the time course of PMA activation (20 ng/ml) for 15 or 30 min. Soluble extracts (100 μ g of protein) in RIPA buffer (Thermo Fischer Scientific, Courtaboeuf, France) were subjected to SDS-PAGE, blotted onto a nitrocellulose sheet and visualized with polyclonal antibodies against ATM, phospho-ATM and monoclonal antibodies against p47^{phox}. Blot is representative of 4 independent experiments. **B)** Western blot analysis of the inhibition of ATM phosphorylation in human neutrophils pre-incubated for 15 min with KU-55933 (0.5 and 5 μ M) and then activated with PMA (20 ng/ml) for 15 or 30 min. Blot is representative of 3 independent experiments. **C)** Kinetics of ROS production by human neutrophils (5.10^5) pre-incubated with the ATM inhibitor KU-55933 (0.5 μ M to 10 μ M) and activated by PMA (80 ng/ml) for 60 min. The NADPH oxidase activity is expressed as the peak value of relative luminescence units (RLU) measured in 15 min from human neutrophils (**D**) or differentiated WT-NOX2 PLB-985 cells. (**E**). The data represent average $\pm$ SD of at least 3

independent experiments. *F*) ROS production expressed as the peak values of RLU measured in 15 min was measured by luminol-amplified chemiluminescence for 60 min in $5 \cdot 10^5$ differentiated WT-NOX2, Ser486Ala-NOX2 and Ser486Glu-NOX2 PLB-985 cells after PMA stimulation. Values represent the mean $\pm$ SD of at least 3 independent experiments. The statistical significances of the differences of NADPH oxidase activity in presence of KU-55933 vs. the control absence of KU-55933 were evaluated by the Student test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.0001$. (1.5-column fitting image).

Fig. 8. Regulation of ROS production in neutrophil by ATM activation

The NADPH oxidase complex is activated after PMA stimulation leading to the phosphorylation of cytosolic factors and p22^{phox} subunits by PKC (on Thr147) and other kinases for optimal assembly of the complex. The activated NADPH oxidase complex can produce ROS in large quantities (step 1). In order to protect the cell from DNA damage, ROS can activate ATM kinase by autophosphorylation (step 2) which in turn phosphorylates Ser486 in the NIS of NOX2 (step 3). The consequence is a conformational change in NOX2 leading to a decrease in ROS production. (1.5-column fitting image).

Table 1

In cellulo and *in vitro* NADPH oxidase activity in transfected X-CGD PLB-985 cell lines

Cytochrome *b*₅₅₈ was quantified using Soret band absorption of 1 % Triton X-100 (v/v) soluble extracts from X-CGD and transfected PLB-985 cells, considering that cytochrome *b*₅₅₈ contain two hemes. ROS production was measured by luminol-amplified chemiluminescence in 5.10⁵ differentiated PLB-985 cells after PMA or opsonized zymosan (ZOPS) stimulation. RLU represents the sum of relative luminescence units measured in 60 min. *In vitro* NADPH oxidase activity was reconstituted in a broken cell system with purified plasma membranes (100 µg) from transfected X-CGD PLB-985 cells in presence of human neutrophil cytosol (300 µg) and activated with an optimal concentration of arachidonic acid as described in the experimental procedures. INT reductase activity was determined in the same conditions as described above, except that cytochrome *c* was replaced by INT. Activities were also expressed as percentage of activity compared with the WT-NOX2 PLB-985 cell line. Values in this table represent the mean ± SD of triplicate determinations.

n=3	INTACT CELLS										CELL-FREE-SYSTEM			
	ROS production (total RLU)					O ₂ ⁻ production nmol/min/10 ⁶ cells					NADPH oxidase activity (nmol/min/mg prot)		Diaphorase activity (nmol/min/mg prot)	
	PLB-985 cells	cytochrome <i>b</i> ₅₅₈ (pmol/mg/prot)	PMA	% of control	ZOPS	% of control	PMA	% of control	ZOPS	% of control		% of control		% of control
WT-NOX2	26.3 ± 2.9	261 ± 16	100	95.7 ± 9.7	100	0.40 ± 0.06	100	0.04 ± 0.01	100	90.7 ± 9.8	100	101 ± 10	100	
X-CGD	0	0.6 ± 0.03	0	1.01 ± 0.08	1	0	0	0	0	20.0 ± 28	22	2.8 ± 0.8	3	
NOX2- NIS	19.5 ± 2.5	0.10 ± 0.01	0	0.85 ± 0.01	0	0	0	0	0	21.0 ± 4.4	23	3.7 ± 1.5	4	
NOX2-NIS1	18.5 ± 2.8	0.17 ± 0.05	0	0.10 ± 0.02	1	0	0	0	0	25.3 ± 4.9	30	8.0 ± 1.0	0	
NOX2-NIS3	23.5 ± 3.1	390 ± 41	150	125 ± 10	131	1.57 ± 0.25	392	0.11 ± 0.02	275	187 ± 6	206	159 ± 13	157	
NOX2-NIS4	25.2 ± 3.8	0.15 ± 0.05	0	0.86 ± 0.05	0	0	0	0	0	13.7 ± 0.6	15	5.5 ± 0.9	0	