Characterization of the binding of cytosolic phospholipase A2 alpha and NOX2 NADPH oxidase in mouse macrophages

Yulia Solomonov · Nurit Hadad · Rachel Levy

Received: 1 November 2021 / Accepted: 25 January 2022 / Published online: 29 January 2022
© The Author(s), under exclusive licence to Springer Nature B.V. 2022

Abstract

Background Previous studies have demonstrated that cytosolic phospholipase A2α (cPLA2α) is required for NOX2 NADPH oxidase activation in human and mouse phagocytes. Moreover, upon stimulation, cPLA2α translocates to the plasma membranes by binding to the assembled oxidase, forming a complex between its C2 domain and the PX domain of the cytosolic oxidase factor, p47phox in human phagocytes. Intravenous administration of antisense against cPLA2α that significantly inhibited its expression in mouse peritoneal neutrophils and macrophages also inhibited superoxide production, in contrast to cPLA2α knockout mice that showed normal superoxide production. The present study aimed to determine whether there is a binding between cPLA2α-C2 domain and p47phox-PX in mouse macrophages, to further support the role of cPLA2α in oxidase regulation also in mouse phagocytes.

Methods and results A significant binding of mouse GST-p47phox-PX domain fusion protein and cPLA2α in stimulated mouse phagocyte membranes was demonstrated by pull-down experiments, although lower than that detected by the human p47phox-PX domain. Substituting the amino acids Phe98, Asn99, and Gly100 to Cys98, Ser99, and Thr100 in the mouse p47phox-PX domain (present in the human p47phox-PX domain) caused strong binding that was similar to that detected by the human p47phox-PX domain.

Conclusions The binding between cPLA2α-C2 and p47phox-PX domains exists in mouse macrophages and is not unique to human phagocytes. The binding between the two proteins is lower in the mice, probably due to the absence of amino acids Cys98, Ser99, and Thr100 in the p47phox-PX domain that facilitate the binding to cPLA2α.

Keywords Cytosolic phospholipase A2α · NOX2 NADPH oxidase · PX-domain · C2 domain

Introduction

The multi-component electron carrier, NOX-2 NADPH oxidase, transfers electrons from NADPH to molecular oxygen to form superoxides, a precursor of microbicidal oxidants. Its subunits include four cytoplasmic components, p47phox, p67phox, p40phox and Rac2, and a hetero-dimeric transmembrane glycoprotein flavocytochrome b558 composed of gp91phox and p22phox (for reviews [1–3]). The cytosolic components translocate to the plasma membrane upon stimulation and associate with the flavocytochrome b558 to form the assembled active oxidase. In resting cells, p47phox is found in an auto-inhibited form, thereby preventing its binding to membranes [4]. In stimulated cells, the restrictive conformation of the autoinhibitory region of p47phox is released through phosphorylation of several critical serine residues within its polybasic region [5] and exposing the interactive SH3 domains that direct its translocation to the membranes by binding to specific targets in p22phox [6–9]. In addition, it was reported that the PX domain of p47phox has distinct lipid-binding sites to Phosphatidylinositol 3,4-bisphosphate (PI(3,4)P2) or phosphatidic acid [8–12]. Residues corresponding to His-51, Lys-55, and Arg-70 contribute to forming the atypical binding site of the 47phox-PX domain to PI(3,4)P2. While Lys-55 was shown to be important for recognition and the proper function of phagocyte NADPH oxidase.

cPLA2α that hydrolyzes phospholipids containing arachidonate at the sn-2 position [13] has been implicated as the major enzyme in the formation of eicosanoids. cPLA2α
has two functionally distinct domains: an N-terminal C2 domain necessary for Ca\(^{2+}\)-dependent phospholipid binding and a C-terminal Ca\(^{2+}\)-independent catalytic region [14]. It was shown that \(c\text{PLA}_2\alpha\) translocates from the cytosol to the nuclear membrane and the endoplasmic reticulum by an increase of cytoplasmic [Ca\(^{2+}\)] in a variety of cells [15] via its C2 domain [16] in calcium-binding regions [17]. In \(c\text{PLA}_2\alpha\), the C2-domain is structurally designed to target phosphocholine (PC) -rich membrane regions to increase the catalytic domain’s enzymatic efficiency, which prefers polyunsaturated PC [18]. The function for \(c\text{PLA}_2\alpha\)-C2 domain Tyr 96 was recently reported [19] as a critical specificity determinant for the headgroup of PC, whereas Asn65 tethers with the lipid phosphate moiety and facilitates Ca ions-PC complexation. Further enhancement of C2-domain binding to the membrane, via interaction with Arg59, Arg61, and His 62, was shown when PC-enriched membranes also contained \(c\text{PLA}_2\alpha\) activator, ceramide-1-phosphate [20]. It was recently reported [21] that \(c\text{PLA}_2\alpha\) undergoes C2-domain-dependent oligomerization on membranes independently of its catalytic activity.

We have previously demonstrated an essential requirement for \(c\text{PLA}_2\alpha\) in the activation of the assembled phagocyte NADPH oxidase using \(c\text{PLA}_2\alpha\) knock out phagocytes like cells that had normal phagocytic activities but did not produce superoxide despite the presence of the assembled oxidase in the membranes after activation, and this activity could be restored by addition of arachidonic acid [22]. In an earlier study, we showed that AA increased the affinity of the assembled NADPH oxidase for NADPH, in purified membranes and in endosomes, separated from stimulated neutrophils [23]. Likewise, specific antisense against \(c\text{PLA}_2\alpha\) that blocked its expression and activity in vitro in various phagocytic cells and in vivo in different mouse models of diseases also inhibited the activity of NOX-2 NADPH oxidase [24–26]. We also showed that \(c\text{PLA}_2\alpha\) activity is required for the oxidase-associated \(H^+\) channel [27] and oxidase-associated diaphorase activity [28]. We have demonstrated [29] that \(c\text{PLA}_2\alpha\) translocates to the plasma membrane by interacting with the assembled oxidase complex in addition to its translocation to nuclear membranes, in peripheral blood neutrophils and phagocyte-like PLB-985 cells. Thus, the ability of \(c\text{PLA}_2\alpha\) to colocalize in two different compartments in the same cells enables it to participate in both eicosanoid production and regulate NOX2-NADPH oxidase activation. The activation and translocation of \(c\text{PLA}_2\alpha\) by PMA in mouse macrophages [29, 30] that does not induce an increase in cytoplasmic [Ca\(^{2+}\)], together with its translocation to the plasma membrane, suggesting the existence of alternative pathways for inducing translocation of \(c\text{PLA}_2\alpha\) that are distinct from the C2 domain phospholipid-binding mechanism. The requirement of \(c\text{PLA}_2\alpha\) for NOX2-NADPH oxidase activation is in line with other studies using inhibitors and antisense molecules [31–34] but stands in contrast to observations of normal superoxide production by stimulated phagocytes from \(c\text{PLA}_2\alpha\)-deficient mice [35]. They reported that \(c\text{PLA}_2\alpha\) inhibition or gene disruption led to complete suppression of neutrophil arachidonate release and eicosanoids biosynthesis but had no effect on neutrophil NADPH oxidase activation, FcγII/III or CD11b surface expression, primary or secondary granule secretion, or phagocytosis of \(Escherichia coli\) in vitro. Surprisingly, although all phagocytic functions were normal, they reported that \(c\text{PLA}_2\alpha\) inhibition or gene disruption diminished neutrophil-mediated \(E. coli\) killing in vitro. Likewise, \(E. coli\) were attenuated in \(c\text{PLA}_2\alpha(–/–)\) mice than wild-type littermates following intratracheal inoculation with live \(E. coli\) in vivo.

The present study aimed to explore whether there is a binding between \(c\text{PLA}_2\alpha\) and the assembled NADPH oxidase in mouse macrophages, similar to that reported in human phagocytes [36] and rat microglia [24]. Such binding between these two proteins may explain the mechanism and strengthen our results [25], demonstrating that the absence of \(c\text{PLA}_2\alpha\) by using oligo antisense against \(c\text{PLA}_2\alpha\) inhibits NOX2-NADPH activity in mice phagocytes in vitro and in vivo.

**Methods**

**Neutrophil purification**

Forty ml blood with neutrophil count between 3-7X10^6/ ml was drawn from healthy volunteers with their written consent. Neutrophils at 95% purity were obtained by Ficoll/ Hiiapque centrifugation, dextran sedimentation, and hypotonic lysis of erythrocytes [22]. The study was approved by the institutional Human Research Committee of the Soroka University Medical Center (No. 0370-16-SOR).

**Mouse macrophage purification**

4 ml of sterile thioglycolate 4% were injected intraperitoneal to ICR male mice. After 4 days, macrophages were washed from the mouse peritoneum with medium RPMI 1640. Cells were cleaned by centrifugation, and hypotonic lysis of erythrocytes was carried out [25]. The study was performed following approval by the Ben-Gurion University of the Negev committee for ethical care and use of animals in experiments, Authorization No. IL-23-05-2017 and was conducted according to the Israeli Animal Welfare Act following the Guide for Care and Use of Laboratory Animal (National Research Council, 1996).
Bacterial expression and purification of recombinant proteins

PCR was used to subclone the mouse p47phox—PX domain in-frame into the expression vector pGEX-4 T-2 using primers containing the BamHI or NotI restriction endonucleases sites (underlined): forward—5′-CGGCGGATCCATGGG GACAC-3′, and reverse—5′-CATCACGGCGGCCACTTT GA AGAAG-3′. The template used was cDNA from mouse macrophages. The GST fusion proteins were overexpressed and purified as described previously [29].

Preparation of cell lysates

$1 \times 10^8$ cells were incubated with 5 mM diisopropyl fluorophosphate in PBS for 30 min at room temperature. Cells were washed twice with PBS and activated with 50 ng/ml PMA for 3 min in 37 °C. After activation, cell were centrifuged and resuspended with 1 ml lysis buffer (150 mM NaCl, 1 mM EDTA, 10% glycerol, 50 mMHEPES (pH 7.5), 1% Triton X-100, 10 mM MgCl2 containing protease and phosphatase inhibitors: 1 mM Benzamidine, 10 mg/ml Aprotinin, 10 mg/ml Leupeptin, 20 mM p-Nitrophenyl Phosphate, 10 mM NaF, 5 mM Na3VO3 and β-glycerol phosphate 50 mM). The suspension was sonicated three times for 20 s with a Microsom Heat System sonicator and centrifuged for 5 min at 15,600×g to remove unbroken cells, nuclei, and granules.

Affinity binding assay

GST or GST fusion proteins attached to glutathione-sepharose beads were added to lysates of stimulated human neutrophils and mouse macrophages and were tumbled end-over-end overnight at 4 °C. The samples were centrifuged, washed six times with phosphate-buffered saline, boiled in SDS sample buffer at 95 °C for 5 min, and separated SDS-PAGE before immunoblotting.

Immunoblot analysis

After SDS PAGE electrophoresis, proteins were transferred to nitrocellulose membranes. Membranes were incubated in Tris-buffered saline (10 mM Tris, 135 mM NaCl, pH 7.4), with 0.1% Tween 20 (TBS-T) containing 5% nonfat milk for 1.5 h at 20 °C. Blots were incubated with primary antibodies—rabbit anti-cPLA2α (Cell Signaling Technology, Beverly, MA, USA), rabbit anti-GST(Abcam, Waltham, MA, USA), and polyclonal rabbit anti-p47phox that was kindly given to use by Dr. Leto, NIAID, NIH, the USA for overnight at 4 °C and secondary antibodies—peroxidase-conjugated goat anti-rabbit(Amersham Biosciences UK, Buckinghamshire, UK) or rabbit anti-goat (Sigma, Rehovot, Israel) for 1 h at 25 °C and then developed using the enhanced chemiluminescence (ECL) detection system (PerkinElmer, Waltham, MA, USA). Proteins were quantified using densitometry analysis (ImageJ analysis software).

Mutagenesis of expression vectors

pGEX-4 T-2 expression vector encoding the cDNA of mouse p47phox—PX domain was used as a template to generate the desired mutations by the overlap extension polymerase chain reaction [37]. The PCR reactions, using appropriate complementary synthetic oligonucleotides introducing the desired mutation and two additional external primers at the ends of the p47phox—PX, were performed with Red Load Taq Maste high yield using Thermostable DNA polymerase (LAROVA, Germany).

Primers for mutations: Glu66A + His68N + Thr69P: Forward 5′-GAG GCC GGC GCGAT CAATCCAG AGA ACAG-3′ Rev 5′-CTGTTCTCTGATTTGATCCTGCACCGCCTCTC-3′.

Val 73I mutation was done on the amplified fragment using the primers: Forward 5′-CAGA GAA CAG AATC ATC CCA CAC CTC CCG -3′ Rev 5′-GAGGGAGGTGTGGGAT GATTCT- GTT CTCTG-3′.

To generate the second mutation: Phe98C + Asn99S + Gly100T the following primers were used: Forward 5′-CAC TGA ATACT GCAGCACG CTC ATG GGA CTG CC-3′ Rev 5′-GGC AGT CCC ATG AGCGT GCT-GCA GTA TCCAGT-3′. The external primers used for both mutation construct were: Forward 5′-AATG TGC CTG GAT GCG TTC CCA AAA TTA -3′ Rev 5′-ACG CGC CCT GAC GGG CTT GTC TGC -3′.

The mutated products were digested with BamHI and NotI and cloned into a GEX-4 T-2 expression vector, digested with the same enzymes. The vectors were then transformed into Escherichia coli DH-101. The mutated fragments were sequenced using the ABI3100 Genetic Analyzer.

Statistical analysis

The mean differences were analyzed by Student’s t-test.

Results

Since our previous study demonstrated that there is a binding between the C2-domain of cPLA2α and the PX domain of P47phox, these domains were used to study the binding between cPLA2α and P47phox in mouse macrophages. Peritoneal mouse macrophages and human neutrophils were stimulated with 50 ng/ml PMA for 3 min. An affinity binding assay with human GST-cPLA2α-C2 domain, which is identical to the mouse cPLA2α-C2 domain was
performed in both lysates. Addition of GST-cPLA α-C2 domain to mouse macrophage lysate resulted in binding to p47phox, which was much lower than the binding to p47phox in human neutrophil lysate (Fig. 1A). These results may be due to either the low expression of mouse p47phox or the lower affinity of the antibodies to mouse p47phox since the expression of p47phox is also lower in the lysate of mouse macrophages. Thus, we used another strategy and investigated an affinity binding assay, using GST-p47phox-PX protein, to further characterize the binding between p47phox and cPLA α in mouse macrophages. Since p47phox-PX domains in mice and humans, although very similar, are not identical, mouse GST-p47phox-PX construct was engineered, and its efficiency to pull down cPLA α was compared to that of the human GST-p47phox-PX domain. Human GST-p47phox-PX (PX-H) and mouse GST-p47phox-PX (PX-M) or GST were added to either stimulated human neutrophil lysates or stimulated mouse macrophage lysates. As shown in Fig. 1B, affinity binding experiments with mouse GST-p47phox-PX (PX-M) showed significant binding to human or mouse cPLA α but was significantly (p < 0.001) lower in comparison with the binding of human GST-p47phox-PX (PX-H) to either human or mouse cPLA α although the level of cPLA α is similar in both cell lysates.

To elucidate the reason for the lower binding by mouse GST-p47phox-PX to cPLA α relatively to that of human GST-p47phox-PX, we looked for the differences in the amino acid sequences in the binding region of the P47phox-PX domains in humans and mice. We have recently reported [36] that Ile67 in the cPLA α-C2 resides in a hydrophobic pocket on the surface of the PX domain and interacts with its residues Pro114, His115 in the α4 helix, and Met59 at the end of the α1 helix. In the mouse p47phox-PX domain, there are changes in some amino acids around the amino acids that participate in the binding (Fig. 2A) and thus may affect the protein folding and the binding. To determine whether these amino acids affect the binding to cPLA α, two constructs of the mouse p47phox-PX domain in which the amino acids were substituted by those present in the human p47phox-PX were engineered. In the first (PX-mut1), Glu66, His68, Thr69, and Val73 were substituted with Ala66, Asn68, Pro69, and Ile73, respectively, and in the second (PX-mut2) Phe98, Asn99, and Gly100 were substituted with Cys98, Ser99, and Thr100, respectively. Figure 2B depicts the interaction between cPLA α-C2 and PX-p47phox domains as presented in our previous study [29] and the two mutations on PX-p47phox. As shown in Fig. 3, GST-p47phox-PX-mut1 (expressing the human residues in mouse PX domain) was much less efficient in binding cPLA α in lysates of stimulated human neutrophils (Fig. 3A) or stimulated mouse macrophages (Fig. 3B) in comparison with wild type mouse GST-p47phox-PX, suggesting that these amino acids present in the human p47phox-PX domain disturb the binding between the domains. GST-p47phox-PX-mut2 was much more effective in binding cPLA α in lysates of stimulated human neutrophils (Fig. 3A) or stimulated mouse macrophages (Fig. 3B) in comparison with wild type mouse GST- p47phox-PX, suggesting that these amino acids (Cys98, Ser99, and Thr100) present in the human p47phox-PX domain contribute to the binding. Moreover, the efficiency of the mouse GST-p47phox-PX-mut2 to bind cPLA α is similar to that of the human GST- p47phox-PX domain in both stimulated human neutrophils lysate (Fig. 4A) and stimulated mouse macrophages lysate (Fig. 4B).

Fig. 1  Binding between cPLA α and p47phox. A Affinity-binding assay between GST-C2 domain and P47phox: GST-C2 domain fusion protein and GST attached to glutathione beads were added to lysates of stimulated human neutrophils and stimulated mouse macrophages and subjected to Western blot analysis for detection of p47phox. The samples were separated on 10% SDS gel and subjected to western blot analysis with anti-p47phox or anti-GST antibodies. The last lanes in the blot indicate the location and expression of p47phox in the gel. Although GST by itself pulled down p47phox in neutrophil lysate it was significantly lower than that pulled down by GST-C2 domain. The results are from a representative experiment out of three. B Affinity-binding assay between GST-PX domain and cPLA α: Human and mouse GST-PX domain fusion proteins and GST attached to glutathione beads were added for 24 h to lysates of stimulated human neutrophils and stimulated mouse macrophages (with 50 ng/ml PMA). The samples were separated on 10% SDS gel and subjected to western blot analysis with anti-cPLA α or anti-GST antibodies. The last lanes in the blot indicate the location and expression of cPLA α and actin. The results are from a representative experiment. *p < 0.01, ***p < 0.001 Shown significance between PX-M and GST and between PX-H and PX-M.
Discussion

The results of the present study show that there is a binding between cPLA₂α and p47^phox mediated by cPLA₂α-C2 domain and cPLA₂α-C2 domain in humans. The binding between the two proteins may provide the mechanism by which arachidonic acid can be released in the oxidase milieu and exert its activation effect. These results are in line with and support our previous results [25], demonstrating that inhibition of cPLA₂α expression in mice by intravenous injections of specific antisense against cPLA₂α, caused inhibition of the superoxide production by peritoneal macrophages. The lower binding of the mouse GST-p47^phox-PX (Fig. 1B) is probably attributed to the expression of Phe98, Asn99, and Gly100 instead of Cys98, Ser99, and Thr100, which are expressed in human p47^phox-PX, since their substitution to the human 98–100 amino acids forming the GST-p47^phox-PX-mut2 had increased the binding to cPLA₂α in both human neutrophil and mouse macrophage lysates (Fig. 3) and was similar to the binding of human GST- p47^phox-PX domain (Fig. 4). Residues 98–100 in the p47^phox-PX domain are not conserved, and there are some differences in other species. Interestingly, when the residues 98–100 are identical in species such as in humans and cows or mice and rats, residues 66, 67, 69, 73 are also identical. Our previous study [29] suggested that Ile67, the first amino acid of the β4 strand of the cPLA₂α-C2 domain, resides in a hydrophobic pocket on the surface of the p47^phox-PX domain.
and interacts with its residues Pro114, His115 in the α4 helix, and Met59 at the end of the α1 helix (as depicted in Fig. 2B). The expression of the aromatic Phe98 and the imido Asn99 in the mouse p47phox-PX domain instead of three polar-uncharged amino acids at the end of α2 helix near the α4-helix containing the amino acids Pro114 and His115 that participate in the binding, probably affected the mouse p47phox-PX structure and reduced its affinity to cPLA2α-C2 domain. It seems that the expression of amino acids Glu66, His68, Thr69, and Val73 in the mouse p47phox-PX, found in the free region, are more effective in forming the binding to cPLA2α-C2 domain than the amino acids Ala66, Asn68, Pro69, and Ile73 expressed in human p47phox-PX since their substitution to the amino acids present in the human p47phox-PX domain (mut1), significantly reduced the affinity binding (Fig. 3). The expression of the negative charged Glu66 in the mouse p47phox-PX domain instead of Ala66, and the expression of hydrophobic Thr69 instead of Pro69, probably caused significant changes in the three-dimensional structure of the protein and affected the location of the α helices. Thus, mouse amino acids 98–100 in the p47phox-PX domain caused a reduction in the binding to cPLA2α-C2 domain while the mouse amino acids 66,67,69,73 increased the binding to cPLA2α yet the whole mouse p47phox-PX domain, although showed binding to cPLA2α-C2 domain, is lower compared with the human p47phox-PX domain (Fig. 1B). The existence of the binding between mouse p47phox-PX and cPLA2α-C2 domains enables the translocation of cPLA2α to the plasma membranes and binding to the assembled oxidase via p47phox, similar to other types of phagocytes. These results align with our previous study
in primary rat microglia [24], demonstrating that cPLA₂α was bound to p47^{phox} in membranes of activated cells and regulated NOX2 NADPH oxidase activity.

In agreement with our results, it was reported [38] that during phagocytosis of zymosan by mouse peritoneal macrophages, cPLA₂α translocates in a Ca^{2+}-independent manner to form phagosomes with kinetics similar to its translocation to the plasma membrane and before phagosomal fusion. F4/80, a cell surface macrophage protein highly expressed on resident peritoneal macrophages, was used as a marker to monitor plasma membrane internalization during uptake of zymosan. They showed that F4/80 fluorescence was found around the zymosan particle and on extensions of the plasma membrane adjacent to the forming phagosome, and its localization entirely overlapped with GFP-cPLA₂α. The translocation of cPLA₂α to the zymosan forming phagosome was also demonstrated: colocalization with 5-lipooxygenase, 5-lipooxygenase-activating protein, and leukotriene C4 synthase was found in mouse peritoneal macrophages [39].

In conclusion, the binding between the cPLA₂α-C2 and p47^{phox}.PX domains is not unique to human phagocytes; it was demonstrated in mouse macrophages, although to a lower extent. Specific amino acids 98–100 in the human p47^{phox}.PX domain facilitate the binding to cPLA₂α, and their absence in the mouse p47^{phox}.PX domain reduced the binding. But amino acids 66–69 and 73 are more efficient in mice than in the human p47^{phox}.PX domain for the binding formation. The binding between both enzymes suggests that mouse NOX2-NADPH oxidase is also regulated by cPLA₂α and may provide the molecular mechanism by which cPLA₂α can activate the assembled oxidase.

Acknowledgements This research was supported by a donation from Miss Dorothy Polayes, USA entitled: the role of cPLA₂α in oxidative stress and the pathogenesis of inflammation.

Author contributions YS designed and performed the experiments and analyzed data. NH helped in methodology. RL guided the study and wrote the manuscript. All authors read the manuscript and approved its publication.

Funding This research was supported by a donation from Miss Dorothy Polayes, USA entitled: the role of cPLA₂α in oxidative stress and the pathogenesis of inflammation.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The study was approved by the institutional Human Research Committee of the Soroka University Medical Center (No. 0370-16-SOR) and by the Ben-Gurion University of the Negev committee for ethical care and use of animals in experiments, Authorization No. IL-23-05-2017.

Consent for publication All authors approved the manuscript and agreed to publish it in Molecular Biology Reports.

References

1. Groemping Y et al (2003) Molecular basis of phosphorylation-induced activation of the NADPH oxidase. Cell 113(3):343–355
2. Groemping Y, Rittinger K (2005) Activation and assembly of the NADPH oxidase: a structural perspective. Biochem J 386(Pt 3):401–416
3. Leto TL (1999) The respiratory burst oxidase. Inflammation: basic principles and clinical correlates. Lippincott, Williams, and Wilkins Press, Philadelphia, pp 769–786
4. Ueyama T et al (2008) Sequential binding of cytosolic phox complex to phagosomes through regulated adaptor proteins: evaluation using the novel monomeric Kusabira-green system and live imaging of phagocytosis. J Immunol 181(1):629–640
5. Ago T et al (1999) Mechanism for phosphorylation-induced activation of the phagocyte NADPH oxidase protein p47(phox). Triple replacement of serines 303, 304, and 328 with aspartates disrupts the SH3 domain-mediated intramolecular interaction in p47(phox), thereby activating the oxidase. J Biol Chem 274(47):33644–33653
6. Heyworth PG et al (1991) Neutrophil nicotinamide adenine dinucleotide phosphate oxidase assembly. Translocation of p47-phox and p67-phox requires interaction between p47-phox and cytochrome b558. J Clin Invest 87(1):352–356
7. Sato TK, Overduin M, Emr SD (2001) Location, location, location: membrane targeting directed by PX domains. Science 294(5548):1881–1885
8. Kanai F et al (2001) The PX domains of p47phox and p40phox bind to lipid products of PI(3,4)K. Nat Cell Biol 3(7):675–678
9. Karathanassis D et al (2002) Binding of the PX domain of p47(phox) to phosphatidylinositol 3,4-bisphosphate and phosphatic acid is masked by an intramolecular interaction. Embo J 21(19):5057–5068
10. Stampoulis P et al (2012) Atypical membrane-embedded phosphatidylinositol 3,4-bisphosphate (PI(3,4,5)-P2) binding site on p47(phox) Phox homology (PX) domain revealed by NMR. J Biol Chem 287(21):17848–17859
11. Chandra M et al (2019) Classification of the human phox homology (PX) domains based on their phosphoinositide binding specificities. Nat Commun 10(1):1528
12. Kervin TA, Wiseman BC, Overduin M (2021) Phosphoinositide recognition sites are blocked by metabolite attachment. Front Cell Dev Biol 9:690461
13. Clark JD, Milona N, Knopf JL (1990) Purification of a 110-kilodalton cytosolic phospholipase A2 from the human monocytic cell line U937. Proc Natl Acad Sci USA 87(19):7708–7712
14. Nalefski EA et al (1994) Delineation of two functionally distinct domains of cytosolic phospholipase A2, a regulatory Ca(2+)-dependent lipid-binding domain and a Ca(2+)-independent catalytic domain. J Biol Chem 269(27):18239–18249
15. Glover S et al (1995) Translocation of the 85-kDa phospholipase A2 from cytosol to the nuclear envelope in rat basophilic leukemia cells stimulated with calcium ionophore or IgE/antigen. J Biol Chem 270(25):15359–15367
16. Dessen A et al (1999) Crystal structure of human cytosolic phospholipase A2 reveals a novel topology and catalytic mechanism. Cell 97(3):349–360
17. Essen LO et al (1997) A ternary metal binding site in the C2 domain of phosphoinositide-specific phospholipase C-delta1. Biochemistry 36(10):2753–2762
18. Malmberg NJ, Van Buskirk DR, Falke JJ (2003) Membrane-docking loops of the cPLA2 C2 domain: detailed structural analysis of the protein-membrane interface via site-directed spin-labeling. Biochemistry 42(45):13227–13240
19. Hirano Y et al (2019) Structural basis of phosphatidylcholine recognition by the C2-domain of cytosolic phospholipase A2alpha. Elife. https://doi.org/10.7554/eLife.44760
20. Pettus BJ et al (2004) Ceramide 1-phosphate is a direct activator of cytosolic phospholipase A2. J Biol Chem 279(12):11320–11326
21. Ward KE et al (2020) The cytosolic phospholipase A2alpha N-terminal C2 domain binds and oligomerizes on membranes with positive curvature. Biomolecules 10(4):647
22. Dana R et al (1998) Essential requirement of cytosolic phospholipase A2 for activation of the phagocyte NADPH oxidase. J Biol Chem 273(1):441–445
23. Rubinek T, Levy R (1993) Arachidonic acid increases the activity of the assembled NADPH oxidase in cytoplasmic membranes and endosomes. Biochim Biophys Acta 1176(1–2):51–58
24. Szaingurten-Solodkin I, Hadad N, Levy R (2009) Regulatory role of cytosolic phospholipase A2alpha in NADPH oxidase activity and in inducible nitric oxide synthase induction by aggregated Abeta1–42 in microglia. Glia 57(16):1727–1740
25. Raichel L, Slava Berger LK, Hadad N, Karter M, Williams RO, Feldmann M, Levy R (2008) Reduction of the elevated cPLA2 expression by olioantisenses—a new anti-inflammatory therapy in a mouse model of collagen induced arthritis. Eur J Immunol 38(1):1–11
26. Malada-Edelstein YF, Hadad N, Levy R (2017) Regulatory role of cytosolic phospholipase A2 alpha in the induction of CD40 in microglia. J Neuroinflammation 14(1):33
27. Lowenthal A, Levy R (1999) Essential requirement of cytosolic phospholipase A(2) for activation of the H(+) channel in phagocyte-like cells. J Biol Chem 274:21603–21608
28. Pessach I et al (2001) Essential requirement of cytosolic phospholipase A(2) for stimulation of NADPH oxidase-associated diaphorase activity in granulocyte-like cells. J Biol Chem 276(36):33495–33503
29. Shmelzer Z et al (2003) Unique targeting of cytosolic phospholipase A2 to plasma membranes mediated by the NADPH oxidase in phagocytes. J Cell Biol 162(4):683–692
30. Hazan I et al (1997) Cytosolic phospholipase A2 and its mode of activation in human neutrophils by opsonized zymosan. Correlation between 42/44 kDa mitogen-activated protein kinase, cytosolic phospholipase A2 and NADPH oxidase. Biochem J 326(3):867–876
31. Li Q, Cathcart MK (1997) Selective inhibition of cytosolic phospholipase A2 in activated human monocytes. Regulation of superoxide anion production and low density lipoprotein oxidation. J Biol Chem 272(4):2404–2411
32. Bae YS et al (2000) Independent functioning of cytosolic phospholipase A2 and phospholipase D1 in Trp-Lys-Tyr-Met-Val-D-Met-induced superoxide generation in human monocytes. J Immunol 164(8):4089–4096
33. O’Dowd YM et al (2004) Inhibition of formyl-methionyl-leucyl-phenylalanine-stimulated respiratory burst in human neutrophils by adrenaline: inhibition of phospholipase A2 activity but not p47phox phosphorylation and translocation. Biochem Pharmacol 67(1):183–190
34. Zhao X et al (2002) Cytosolic phospholipase A2 (cPLA2) regulation of human monocyte NADPH oxidase activity. cPLA2 affects translocation but not phosphorylation of p67phox AND p47phox. J Biol Chem 277(28):25385–25392
35. Rubin BB et al (2005) Cytosolic phospholipase A2-alpha is necessary for platelet-activating factor biosynthesis, efficient neutrophil-mediated bacterial killing, and the innate immune response to pulmonary infection: cPLA2-alpha does not regulate neutrophil NADPH oxidase activity. Groups IV, V, and X phospholipases A2s in human neutrophils: role in eicosanoid production and gram-negative bacterial phospholipid hydrolysis. Extended lamivudine retreatment for chronic hepatitis B: maintenance of viral suppression after discontinuation of therapy. J Biol Chem 280(9):7519–7529
36. Shmelzer Z et al (2008) Cytosolic phospholipase A2alpha is targeted to the p47phox-PX domain of the assembled NADPH oxidase via a novel binding site in its C2 domain. J Biol Chem 283(46):31898–31908
37. Ho SN et al (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 77(1):51–59
38. Girotti M et al (2004) Cytosolic phospholipase A2 translocates to forming phagosomes during phagocytosis of zymosan in macrophages. J Biol Chem 279(18):19113–19121
39. Balestrieri B et al (2006) Group V secretory phospholipase A2 translocates to the phagosome after zymosan stimulation of mouse peritoneal macrophages and regulates phagocytosis. J Biol Chem 281(10):6691–6698

Publisher’s Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.