Characterization the binding of cytosolic phospholipase A_2 alpha and NOX2 NADPH oxidase in mouse macrophages

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Research Article

Keywords: Cytosolic phospholipase A2α, NOX2 NAPH oxidase, PX-domain, C2 domain

Posted Date: November 11th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1039201/v1

Version of Record: A version of this preprint was published at Molecular Biology Reports on January 29th, 2022. See the published version at https://doi.org/10.1007/s11033-022-07191-w.

Abstract

Background: Previous studies have demonstrated that Cytosolic phospholipase A_2a (cPLA$_2$a) is absolutely required for NOX2 NADPH oxidase activation in human and mouse phagocytes. Moreover, upon stimulation, cPLA$_2$a translocates to the plasma membranes of by binding to the assembled oxidase, forming a complex between its C2 domain and the PX domain of the oxidase cytosolic factor, p47^{phox} in human phagocytes. Intravenous administration of an antisense against cPLA$_2$a that significantly inhibited its expression in mouse peritoneal neutrophil and macrophages also inhibited superoxide production, in contrast to cPLA$_2$a knockout mice that showed normal superoxide production. The aim of the present study was to determine whether there is a binding between cPLA$_2$a-C2 domain and p47^{phox}-PX in mouse macrophages, to further support the role of cPLA$_2$a in oxidase regulation also in mouse phagocytes.

Methods and Results: A significant binding of mouse GST-p47^{phox}-PX domain fusion protein and cPLA$_2$a in stimulated mouse phagocyte membranes was demonstrated by pull down experiments, although lower than that detected by human p47^{phox}-PX domain. Substituting the amino acids Phe98, Asn99 and Gly100 to Cys98 Ser99 and Thr100 in mouse p47^{phox}-PX domain (that are present in human p47^{phox}-PX domain) caused strong binding that was similar to that detected by the human p47^{phox}-PX domain.

Conclusions: the binding between cPLA$_2$a-C2 and p47^{phox}-PX domains exist 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 p47^{phox}-PX domain that facilitate the binding to cPLA$_2$a.

Introduction

The multi-component electron carrier, NOX-2 NADPH oxidase, t transfers electrons from NADPH to molecular oxygen to form superoxides, a precursor of microbicidal oxidants. Its subunits include four cytoplasmic components, p47^{phox}, p67^{phox}, p40^{phox} and Rac2, and a hetero-dimeric trans-membrane glycoprotein flavocytochrome b_{558} composed of gp91^{phox} and p22^{phox} (for reviews [1–3]. The cytosolic components translocate to the plasma membrane upon stimulation and associate with the flavocytochrome b_{558} to form the assembled active oxidase. In resting cells, p47^{phox} is found in an auto-inhibited form thereby preventing its binding to membranes [5]. In stimulated cells, the restrictive conformation of the autoinhibitory region of p47^{phox} is released through phosphorylation of several critical serine residues within its polybasic region [6], and exposing the interactive SH3 domains that direct its translocation to the membranes by binding to specific targets in p22^{phox} [4, 7–9].

cPLA$_2$α that hydrolyzes phospholipids containing arachidonate at the sn-2 position [10], has been implicated as the major enzyme in the formation of eicosanoids. cPLA$_2$α 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 [11]. It was shown that cPLA$_2$ translocates from the cytosol to the nuclear membrane and to the endoplasmic reticulum by an increase of cytoplasmic [Ca^{2+}] in a variety of cells [12] *via* its C2 domain [13] in a calcium binding regions [14].

We have previously demonstrated an essential requirement for cPLA$_2$ in the activation of the assembled phagocyte NADPH oxidase [15], the oxidase-associated H^+ channel [16] and oxidase-associated diaphorase activity [17]. We have demonstrated [18] that cPLA$_2$α 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 granulocyte-like PLB-985 cells. Thus, the ability of cPLA$_2$α to colocalize in two different compartments in the same cells enables it to participate in both eicosanoid production and to regulate NADPH oxidase activation. The activation and translocation of cPLA$_2$α by PMA in mouse macrophages [18, 20] that does not induce an increase in cytoplasmic [Ca^{2+}], together with its translocation to the plasma membrane, suggest the existence of alternative pathways for inducing translocation of cPLA$_2$α that are distinct from the C2 domain phospholipid binding mechanism. The requirement of cPLA$_2$α for oxidase activation is in line with other studies using inhibitors and antisense molecules [21–23] but stands in contrast to observations of normal superoxide production by stimulated phagocytes from cPLA$_2$α-deficient mice [24]. However, the latter may be attributed to compensating isoenzyme expression frequently observed in knockout animal models.

The aim of the present study was to explore whether there is a binding between cPLA$_2$α and the assembled NADPH oxidase in mouse macrophages, similar to that reported in human phagocytes [19] and in rat microglia [25]. Such binding between these two proteins may provide an explanation of the mechanism and strengthen our results [26] demonstrating that the absence of cPLA$_2$α by use of oligo antisense against cPLA$_2$α inhibits NOX2 NADPH activity in mouse phagocytes *in vitro* and *in vivo*.

Methods

Neutrophil purification – Neutrophils from healthy volunteers were separated by Ficoll/Hipaque centrifugation, dextran sedimentation, and hypotonic lysis of erythrocytes [15]. 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 macrophage cells were washed from mouse peritoneum with medium RPMI 1640. Cells were cleaned by centrifugation and hypotonic lysis of erythrocytes [26]. 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.

Bacterial Expression and Purification of Recombinant Proteins- PCR was used to subclone the mice $p47^{phox}$ – PX domain in-frame into the expression vector pGEX-4T-2 using primers containing the BamHI or NotI restriction endonucleases sites (underlined): forward- 5'-CGGCGGATCCATGGGGGACAC-3', and reverse – 5'-CATCAGCGGCCGCCACTTTGA AGAAG-3'. The template used was cDNA from mouse macrophages. The GST fusion proteins were overexpressed and purified as described previously [18].

Preparation of cell lysates - $1x10^8$ cells were incubated with 5 mM DFP in PBS for 30 min in 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 centrifugated and resuspend with 1 ml lysis buffer (150 mM NaCl, 1 mM EDTA ,1 mM EGTA, 10 % glycerol, 50 mM HEPES (pH 7.5), 1% Triton X-100, 10 µM $MgCl_2$ containing protease and phosphatase inhibitors: 1mM Benzamidine, 10 mg/ml Aprotinin, 10 mg/ml Leupeptin, 20 mM p-Nitrophenyl Phosphate, 10mM NaF, 5 mM Na_3VO_3 and β-glycerol phosphate 50 mM). The suspension was sonicated 3 times for 20 sec with microsom Heatsystem sonicator and centrifuged for 5 min at 15,600 *g* to remove unbroken cells, nuclei, and granules. To separate the membrane and cytosol fractions the supernatant (cell lysate) was centrifuged for 30 min at 134,000 g [17, 20].

Affinity Binding Assay –GST or GST fusion proteins attached to gluthatione-sepharose beads were added to lysates of stimulated human neutrophils and mouse macrophages and were tumbled end-over-end for overnight at 4°C. The samples were centrifuged, washed six times with phosphate-buffered saline, boiled in SDS sample buffer, 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-$cPLA_2\alpha$ (Cell Signaling Technology, Beverly, MA, USA), rabbit anti GST and Goat anti-$p47^{phox}$ 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-4T-2 expression vector encoding the cDNA of mouse $p47^{phox}$-PX domain was used as a template to generate the desired mutations by the overlap extension polymerase chain reaction [27]. The PCR reactions, using appropriate complementary synthetic oligonucleotides introducing the desired mutation and two additional external primers at the ends of the $p47^{phox}$-PX were performed with Red Load Taq Master / high yield using Thermostable DNA polymerase (LAROVA, Germany).

Primers for mutations: Glu66A + His68N + Thr69P: Forward 5'-GAGGCCGGCGCGAT CAATCCAGAGAACAG-3' Rev 5'-CTGTTCTCTGGATTGATCGCGCCGGCCTC-3' .

Val 73I mutation was done on the amplified fragment using the primers: Forward 5'-CAGA GAACAGAATCATCCCACACCTCCCG-3' Rev 5'-CGGGAGGTGTGGGATGATTCT- GTT CTCTG-3'.

To generate the second mutation: Phe98C + Asn99S + Gly100T the following primers were used: Forward 5'-CACTGAATACTGCAGCACGCTCATGGGACTGCC-3' Rev 5'GGCAGTCCCATGAGCGTGCTGCAGT ATTCAGTG-3'. The external primers used for both mutation construct were: Forward 5'-AATGTGCCTG GATGCGTTCCCAAAATTA-3' Rev 5'-ACGCGCCCTGACGGGCTTGTCTGC-3'.

The mutated products were digested with BamHI and NotI and cloned into pGEX-4T-2 expression vector, digested with the same enzymes. The vectors was 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 C2-domain of $cPLA_2\alpha$ and PX domain of $P47^{phox}$, these domains were used to study the binding between $cPLA_2\alpha$ and $P47^{phox}$ in mouse macrophages. The peritoneal macrophages were stimulated with 50 ng/ml PMA for 10 min and membranes were separated. An affinity binding assay with human GST-$cPLA_2\alpha$-C2 domain which is identical to the mouse $cPLA_2\alpha$-C2 domain performed in membranes of stimulated peritoneal macrophages resulted with efficient binding to $p47^{phox}$ in stimulated human neutrophil membranes, but with much lower binding to $p47^{phox}$ in stimulated mouse macrophage membranes (Fig. 1A). These results are probably due to the low expression of mouse $p47^{phox}$ as shown in the lysates. We than investigated an affinity binding assay, using GST-$p47^{phox}$–PX protein, to further characterize the binding between $p47^{phox}$ and $cPLA_2\alpha$ in mouse macrophages. Since $p47^{phox}$-PX domains in mouse and human, although very similar are not identical, mouse GST-$p47^{phox}$–PX construct was engineered and its efficiency to pull down $cPLA_2\alpha$ from stimulated mouse macrophage lysates and human neutrophil lysates was analyzed. Human GST-$p47^{phox}$-PX (PX-H), and mouse GST-$p47^{phox}$-PX (PX-M) or GST, were added to either stimulated human neutrophil lysates or stimulated mouse macrophage lysates. As shown in Figure 1B, affinity binding experiments with mouse GST-$p47^{phox}$-PX (PX-M) showed a significant binding to human or mouse $cPLA_2\alpha$ but was significantly ($p<0.001$) lower in comparison with the binding of human GST-$p47^{phox}$ –PX (PX-H) to either human or mouse $cPLA_2\alpha$.

In order to elucidate the reason for the lower binding by mouse GST-$p47^{phox}$-PX to $cPLA_2\alpha$ relatively to that of human GST-$p47^{phox}$-PX, we looked for the differences in the amino acid sequences in the binding region of the $P47^{phox}$-PX domains in human and in mouse. We have recently reported [19] that Ile67 in the $cPLA_2\alpha$-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 in the end of the α1 helix. In the mouse $p47^{phox}$-PX domain there are changes in some amino acids around the amino acids that participate in the binding

(Fig. 2) and thus can affect the protein folding and the binding. To determine whether these amino acids affect the binding to cPLA$_2$α, two constructs of the mouse p47^{phox}-PX domain in which the amino acids were substituted by those present in the human p47^{phox}-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. As shown in Fig. 3, GST-p47^{phox}-PX-mut1 was much less efficient in binding cPLA$_2$α in lysates of stimulated human neutrophils (Fig. 3A) or stimulated mouse macrophages (Fig. 3B) in comparison with wild type mouse GST-p47^{phox}-PX, suggesting that these amino acids present in the human P47^{phox}-PX domain disturb the binding between the domains. GST-p47^{phox}-PX-mut2 was much more effective in binding cPLA$_2$α in lysates of stimulated human neutrophils (Fig. 3A) or stimulated mouse macrophages (Fig. 3B) in comparison with wild type mouse GST- p47^{phox}-PX, suggesting that these amino acids (Cys98, Ser99 and Thr100) present in the human p47^{phox} -PX contribute to the binding. Moreover, the efficiency of the mouse GST-p47 ^{phox}-PX-mut2 to bind cPLA$_2$α is similar to that of human GST- p47^{phox}-PX domain in both stimulated human neutrophil lysate (Fig. 4A) and in stimulated mouse macrophages lysate (Fig. 4B).

Discussion

The results of the present study show that there is a binding between cPLA$_2$α and p47^{phox} mediated by cPLA$_2$α-C2 domain and p47^{phox}-PX in mouse macrophages, similar to that of human phagocytes although with a lower affinity. 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 and support our previous results [26] demonstrating that inhibition of cPLA$_2$α expression in mice by the use of specific antisense against cPLA$_2$α, caused inhibition superoxide production by peritoneal macrophages. The lower binding of mouse GST- p47^{phox}-PX is probably attributed to 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 acid forming the GST-p47^{phox} –PX-mut2 had increased the binding to cPLA$_2$α 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). Our previous study suggested that Ile67, the first amino acid of the β4 strand of the cPLA$_2$α-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 in the end of the α1- helix. The expression of three polar-uncharged amino acids, instead of the aromatic Phe98 and the imido Asn99 at the end of α2– helix approximate the α4-helix containing the amino acids Pro114 and His115 that participate in the binding probably affected the mouse p47^{phox}-PX structure and its affinity binding to cPLA$_2$α-C2 domain. It seems that the expression of amino acids Glu66, His68, Thr69 and Val73 in the mouse p47^{phox}-PX, found in the free region, are more effective in forming the binding to cPLA$_2$α-C2 domain than the amino acids Ala66, Asn68, Pro69 and Ile73 expressed in human p47^{phox}-PX since their substitution to the amino acids

present in the human p47^{phox}-PX domain, significantly reduced the affinity binding (Fig. 3). The expression of the negative charged Glu66 in the mouse p47^{phox}-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, some of the different amino acids in the mouse p47^{phox}-PX domain caused a reduction in the affinity binding to cPLA$_2$α and some caused an increase, but the whole mouse p47^{phox}-PX domain showed lower affinity binding to cPLA$_2$α in comparison with the human p47^{phox}-PX domain. However, there was a binding of mouse p47^{phox}-PX domain with cPLA$_2$α (Fig. 3) and of GST-C2 domain with membranes p47^{phox} (Fig. 1A) as shown by affinity binding assays in stimulated mouse macrophages membranes, suggesting that similar to other types of phagocytes cPLA$_2$α translocates to the plasma membranes and binds to the assembled oxidase *via* p47^{phox}. These results are in line with our previous study in primary rat microglia [25] demonstrating that cPLA$_2$α was bound to p47^{phox} in membranes of activated cells and regulated NOX2 NADPH oxidase activity.

In agreement with our results, it was reported [28] that during phagocytosis of zymosan by mouse peritoneal macrophages, cPLA$_2$α translocates in a Ca^{2+}-independent manner to the forming phagosomes with kinetics similar to acquisition of the plasma membrane and prior to phago-lysosome 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- cPLA$_2$α. The translocation of cPLA$_2$α to the zymosan forming phagosomes, was also demonstrated and colocalization with 5-lipoxygenase, 5-lipoxygenase-activating protein, and leukotriene C4 synthase was found in mouse peritoneal macrophages [29].

In conclusion, the binding between the cPLA$_2$α-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 human p47^{phox}-PX domain facilitate the binding to cPLA$_2$α and their absence in the mouse p47^{phox}-PX domain reduced the binding. The binding between both enzymes suggests that mouse NOX2-NADPH oxidase is also regulated by cPLA$_2$α and may demonstrate the molecular mechanism by which cPLA$_2$α is able to activate the assembled oxidase.

Declarations

Acknowledgements

This research was supported by a donation from Miss Dorothy Polayes, USA

entitled: the role of cPLA$_2$a in oxidative stress and the pathogenesis of inflammation.

Funding

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Contribution

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.

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Ethic 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 in Molecular Biology Reports.

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Figures

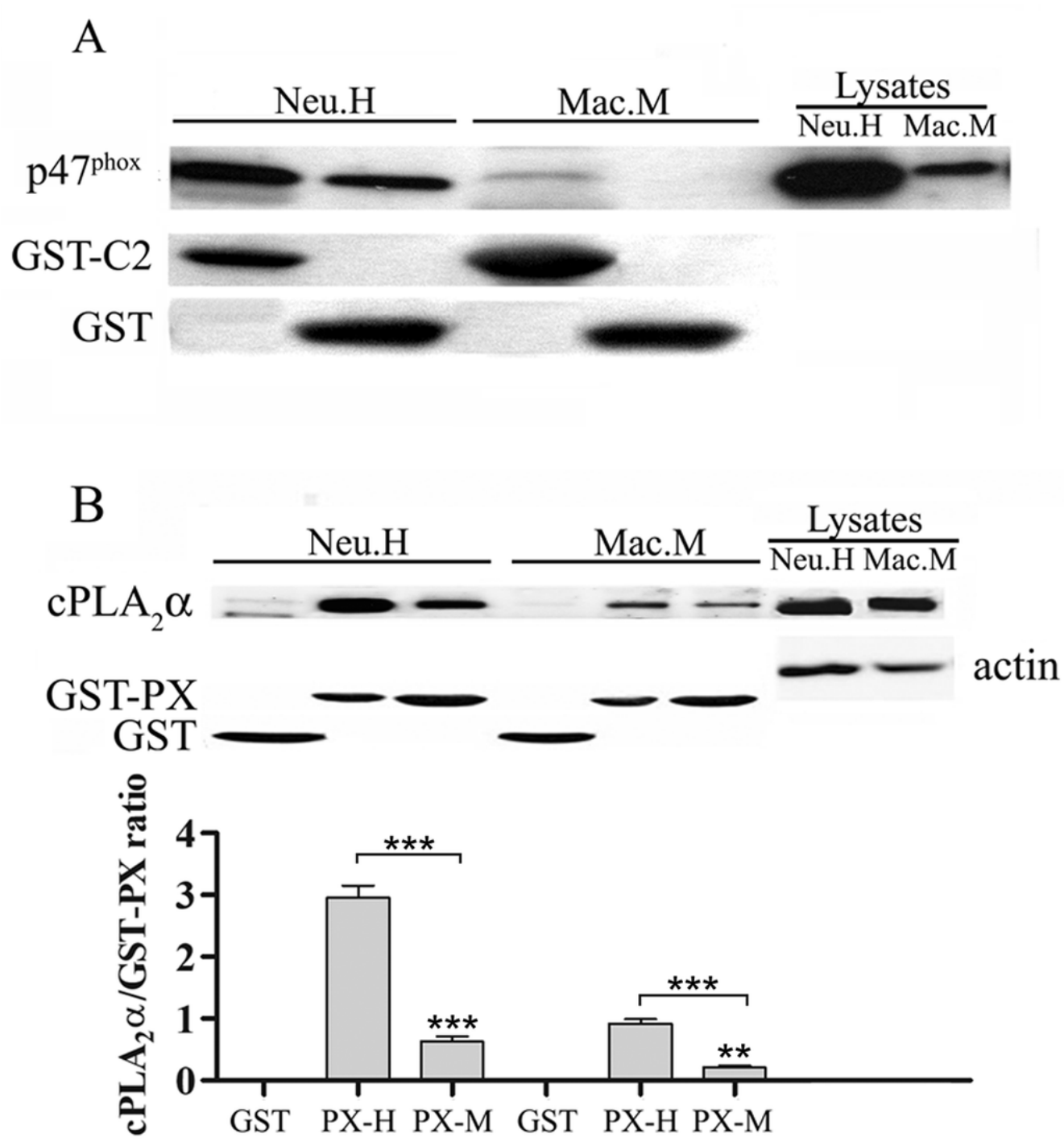

Figure 1

Binding between cPLA2phox and p47phox. A. Affinity-binding assay between GST-C2 domain and membranes P47phox: GST-C2 domain fusion protein and GST attached to glutathione beads were added to membranes 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. The results are from a representative experiment out of three. B. Affinity-binding assay between GST-PX domain and cPLA2α, Human and mouse GST-PX domain fusion proteins and GST attached to glutathione beads were added to lysates of stimulated human neutrophils and stimulated mouse macrophages (with 50 ng/ml PMA) for 24 hours. The samples were separated on 10% SDS gel and subjected to western blot analysis with anti-cPLA2α or anti-GST antibodies. The last lanes in the blot indicate the location and expression of cPLA2α 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.

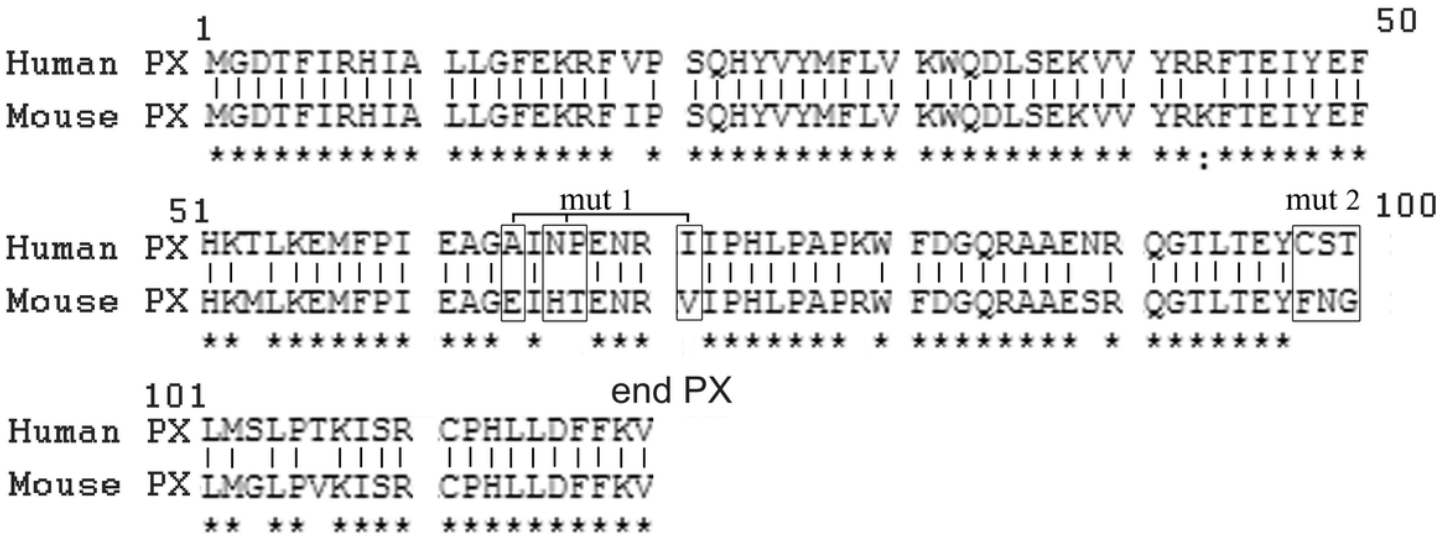

Figure 2

Comparison between human and mouse p47phox-PX domains. Amino acid that are different are not marked with stars and amino acids that participate in the binding between p47phox-PX domain and cPLA2α C2-domain in human are sign with squares.

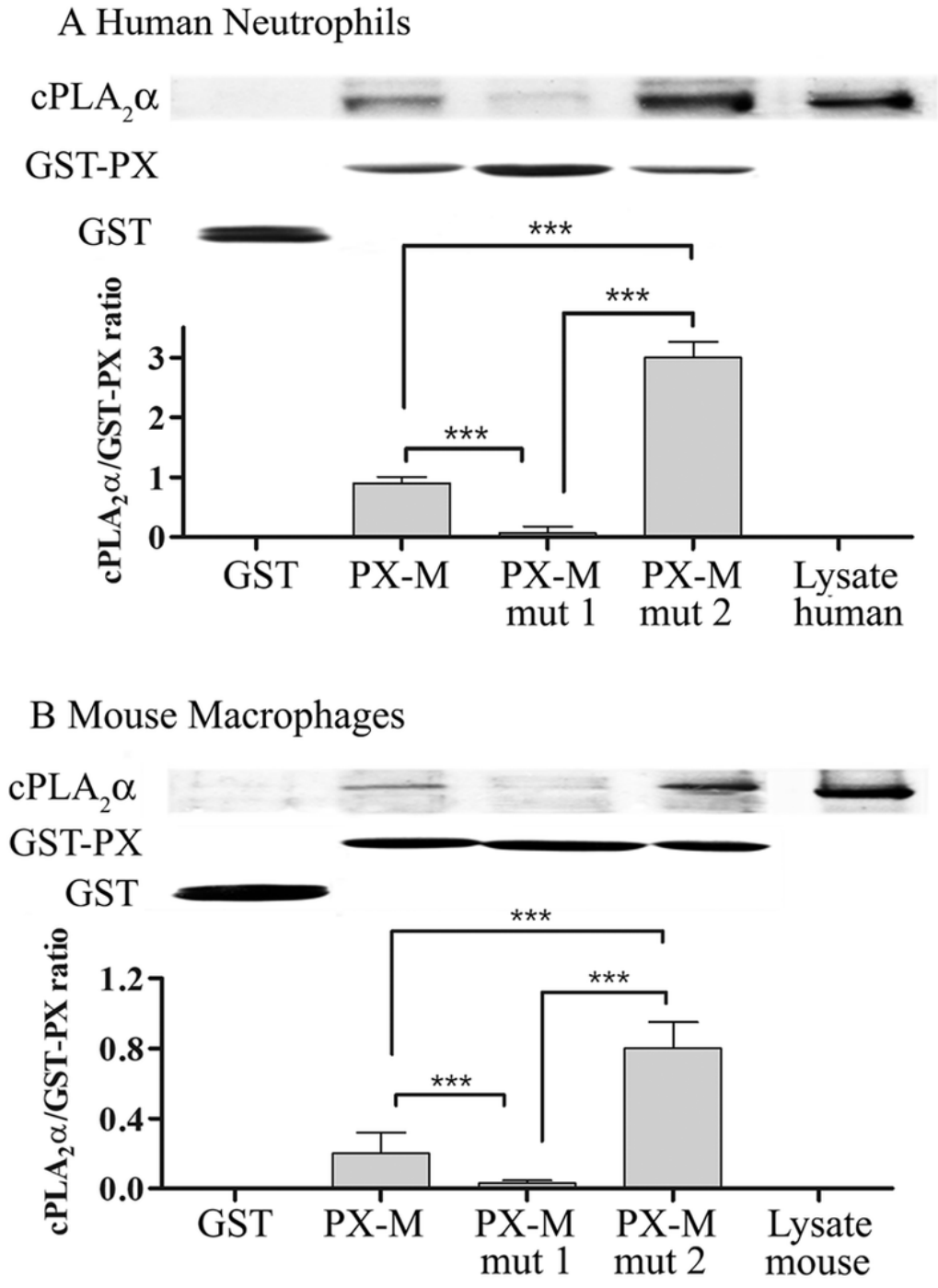

Figure 3

Binding between mouse p47phox-PX domain and mouse p47phox-PX domains mutants to cPLA2α. Affinity-binding assay - mouse GST p47phox-PX domain (PX-M), mouse mutant 1 GST p47phox-PX domain (PX-Mut1), mouse mutant 2 GST p47phox-PX domain (PX-Mut2) and GST alone attached to glutathione beads were added to stimulated human neutrophil lysates (A) or stimulated mouse macrophage lysates (B). The samples were separated on 10% SDS gel and subjected to western blot

analysis with anti-cPLA2α or anti-GST antibodies. The last lane in each blot indicates the location of cPLA2α. The results are from a representative experiment. The bar graphs present the means+SEM of the density ratio of cPLA2α to the different GST-PX bands of three experiments. ***P<0.001.

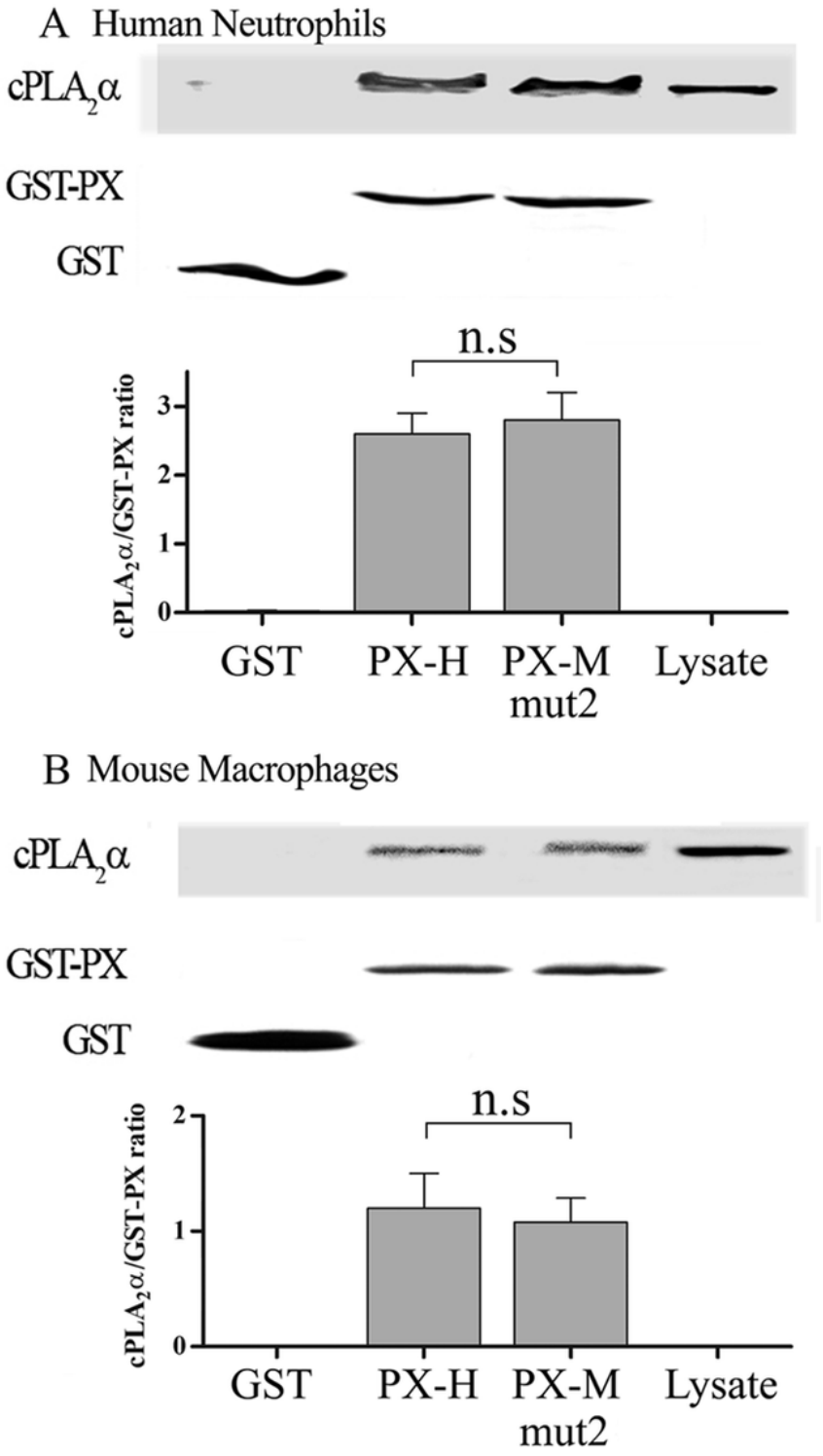

Figure 4

Comparison between the binding of human p47phox-PX domain and mouse mutant 2 p47phox-PX domain to cPLA2α. Affinity-binding assay - human GST p47phox-PX domain (PX-H) or mouse mutant 2

GST p47phox-PX domain (PX-Mut2) and GST alone attached to glutathione beads were added to stimulated human neutrophil lysates (A) or mouse macrophage lysates (B). The samples were separated on 10% SDS gel and subjected to western blot analysis with anti-cPLA2α or anti-GST antibodies. The last lane in each blot indicates the location of cPLA2α. The results are from a representative experiment. The bar graphs represent the means+SEM of the density ratio of cPLA2α. to the different GST-PX bands of three experiments.