Cytosolic Phospholipase A$_2$α Is Targeted to the p47$^{phox}$-PX Domain of the Assembled NADPH Oxidase via a Novel Binding Site in Its C2 Domain

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We have previously demonstrated a physical interaction between cytosolic phospholipase A$_2$α (cPLA$_2$) and the assembled NADPH oxidase on plasma membranes following neutrophil stimulation. The aim of the present study was to define the exact binding sites between these two enzymes. Here we show, based on blot overlay experiments, Förster resonance energy transfer analysis and studies in neutrophils from patients with chronic granulomatous disease deficient in p67$^{phox}$, that cPLA$_2$ specifically binds to p47$^{phox}$ and that p47$^{phox}$ is sufficient to anchor cPLA$_2$ to the assembled oxidase on the plasma membranes upon stimulation. Blot overlay and affinity binding experiments using subfragments of cPLA$_2$ and p47$^{phox}$ demonstrated that the cPLA$_2$-C2 domain and the p47$^{phox}$-PX domain interact to form a complex that is resistant to high salt. Comptuational docking was used to identify hydrophobic peptides within these two domains that inhibited the association between the two enzymes and NADPH oxidase activity in electro-permabilized neutrophils. These results were used in new docking computations that produced an interaction model. Based on this model, cPLA$_2$-C2 domain mutations were designed to explore its interaction p47$^{phox}$ in neutrophil lysates. The triple mutant F35A/M38A/L39A of the cPLA$_2$-C2 domain caused a significant inhibition of the affinity binding to p47$^{phox}$, whereas the single mutant I67A was highly effective. The double mutant M59A/H115A of the p47$^{phox}$-PX domain caused a significant inhibition of the affinity binding to cPLA$_2$. Thus, Ile$^{67}$ of the cPLA$_2$-C2 domain is identified as a critical, centrally positioned residue in a hydrophobic interaction in the p47$^{phox}$-PX domain.

The NADPH oxidase is a multicomponent electron carrier that transfers electrons from NADPH to molecular oxygen to form superoxide, a precursor of microbicidal oxidants. NADPH oxidase subunits include four cytoplasmic components, p47$^{phox}$, p67$^{phox}$, p40$^{phox}$, and Rac2, and a hetero-dimeric transmembrane glycoprotein flavocytochrome b$_{558}$ composed of gp91$^{phox}$ and p22$^{phox}$ (for reviews see Refs. 1, 2). Essential roles for at least four of the phox components is evident from studies on chronic granulomatous disease (CGD)$^2$ patients, who suffer from inherited defects in NADPH oxidase-dependent microbial killing due to defects in the genes encoding these oxidase proteins (3). In resting cells, p47$^{phox}$, p67$^{phox}$, and p40$^{phox}$ exist as a tight cytosolic complex dissociated from the membrane-bound flavocytochrome. Upon stimulation, the cytosolic components translocate to the plasma membrane and associate with the flavocytochrome b$_{558}$ to form the assembled active oxidase. Based on studies of p47$^{phox}$-deficient cells from chronic CGD patients (4), p47$^{phox}$ appears to have a key role in translocation of the cytosolic subunits. p47$^{phox}$ possesses a phox homology (PX) domain, tandem SH3 domains, a series of basic residues, and phosphorylation targets that is also referred to as an autoinhibitory region, and a proline-rich region in the C terminus. In resting cells, p47$^{phox}$ is found in an autoinhibited state due to intramolecular interactions among the PX domain, the tandem Src homology 3 (SH3) domains, and polybasic sequences of p47$^{phox}$, thereby preventing its binding to membranes (5). In stimulating 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). Conformational changes in p47$^{phox}$ following phosphorylation result in unfolding and exposing the novel interactive SH3 domains that direct its translocation to the membranes by binding to specific targets in p22$^{phox}$ and its PX domain that specifically binds phosphatidylinositol 3,4-bisphosphate and phosphatic acid on the plasma membrane (7–9). Neither p40$^{phox}$ nor p67$^{phox}$ is able to translocate to

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$^2$The abbreviations used are: CGD, chronic granulomatous disease; cPLA$_2$, cytosolic phospholipase A$_2$α; SH3, Src homology 3; NCF1, neutrophil cytosolic factor 1; CBR, calcium binding region; MAPK, mitogen-activated protein kinase; MNK1, MAPK interacting kinase; FRET, Förster resonance energy transfer; PX, phox homology; PMA, phorbol 12-myristate 13-acetate; FMLP, formylmethionylleucylphenylalanine; GST, glutathione S-transferase; aa, amino acid(s).
membranes in the absence of p47<sub>phox</sub> as evidenced by the cytoplasmic location of p40<sub>phox</sub> and p67<sub>phox</sub> in stimulated cells from CGD patients who lack a functional p47<sub>phox</sub> (10). However, it appears that p40<sub>phox</sub> functions in retention of p47<sub>phox</sub> and p67<sub>phox</sub> on phagosome membranes through interactions of its PX domain with phosphatidylinositol 3-phosphate, as well as interactions of its PB1 and SH3 domains with p47<sub>phox</sub> and p67<sub>phox</sub>, respectively (11).

Cytosolic phospholipase A<sub>2</sub>α (cPLA<sub>2</sub>α), which hydrolyzes phospholipids containing arachidonate at the sn-2 position (12), has been implicated as the major enzyme in the formation of eicosanoids. cPLA<sub>2</sub>α has two functionally distinct domains: an N-terminal C2 domain necessary for Ca<sup>2+</sup>-dependent phospholipid binding, and a C-terminal Ca<sup>2+</sup>-independent catalytic region (13). It was shown that cPLA<sub>2</sub>α translocates from the cytosol to the nuclear membrane and to the endoplasmic reticulum by an increase in cytoplasmic [Ca<sup>2+</sup>] in a variety of cells (14). The C2 domain of cPLA<sub>2</sub>α has been co-crystallized with calcium (15), and the structure revealed that it binds two calcium ions at one end of the domain between three loops, called calcium binding regions (CBRs): CBR1, CBR2, and CBR3 (16). The calcium ions neutralize the negative electrostatic potential surrounding the hydrophobic residues located at the tips of CBRs 1 and 3, thereby facilitating their interactions with the hydrophobic portions of the lipid head groups of PC-enriched membranes (16). Phosphorylation of cPLA<sub>2</sub>α is important for regulating release of arachidonic acid in cells, but this process is not clearly understood. The catalytic domain of cPLA<sub>2</sub>α contains several functionally important phosphorylation sites (for review see Ref. 17), Ser<sup>505</sup>, Ser<sup>572</sup>, and Ser<sup>515</sup>, which are phosphorylated by mitogen-activated protein kinases (MAPKs), mitogen-activated protein kinase interacting kinase (MNK1), or a related MAPK-activated protein kinase, and calmodulin kinase II, respectively. It is suggested that, depending on the cell type and agonist used for activation, phosphorylation may function to regulate cPLA<sub>2</sub>α catalytic activity and membrane binding.

We have previously demonstrated an essential requirement for cPLA<sub>2</sub>α in activation of the assembled phagocyte NADPH oxidase (18), the oxidase-associated H<sup>+</sup> channel (19), and oxidase-associated diaphorase activity (20). The absolute requirement of cPLA<sub>2</sub>α for oxidase activation is in line with other studies (21–23) utilizing inhibitors and isoenzymes. In contrast, phagocytes from cPLA<sub>2</sub>α-deficient mice showed a normal stimulated superoxide release (24) that could be attributed to the effect of other compensating isoenzymes, a response frequently observed in knockout animal models. Our most recent study (25) demonstrated that in peripheral blood neutrophils and granulocyte-like PLB-985 cells, cPLA<sub>2</sub>α translocates to the plasma membrane by interacting with the assembled oxidase complex in addition to its translocation to nuclear membranes. Thus, the ability of cPLA<sub>2</sub>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<sub>2</sub>α by PMA (25, 26), which does not induce an increase in cytoplasmic [Ca<sup>2+</sup>], together with its translocation to the plasma membrane suggest the existence of alternative pathways for inducing translocation of cPLA<sub>2</sub>α that are distinct from the C2 domain phospholipid-binding mechanism. In agreement with our results, it was recently reported (27) that during phagocytosis of zymosan, cPLA<sub>2</sub>α translocates in a Ca<sup>2+</sup>-independent manner to the forming phagosomes in kinetics similar to acquisition of the plasma membrane and prior to phagolysosome fusion. The aim of the present study was to explore the nature of the interaction between cPLA<sub>2</sub>α and the assembled NADPH oxidase, which anchors cPLA<sub>2</sub>α to the plasma membrane upon stimulation of neutrophils or granulocyte-like PLB cells.

**EXPERIMENTAL PROCEDURES**

**Neutrophil Purification**—Neutrophils from healthy volunteers or from CGD patients were separated by ficoll/Hypaque centrifugation, dextran sedimentation, and hypotonic lysis of erythrocytes (18). p47<sub>phox</sub>-deficient CGD patients and p67<sub>phox</sub>-deficient CGD patients lacking expression of these cytosolic oxidase proteins due to mutations in the genes for neutrophil cytosolic factor 1 on chromosome 7q11.23 and in the gene for neutrophil cytosolic factor 2, located on 1q25, respectively, were enrolled in the study. The study was approved by the institutional Human Research Committee of the Soroka University Medical Center.

**Cell Culture and Differentiation**—PLB-985 leukemic cell lines and gp91<sub>phox</sub>-deficient PLB-985 cells lacking normal expression of normal gp91<sub>phox</sub> (X-CGD), provided by M. C. Dinauer (James Whitcomb Riley Hospital for Children, Indianapolis, IN), were grown in stationary suspension culture in RPMI 1640 and differentiated toward the granulocyte phenotype with 10<sup>−7</sup> M retinoic acid as described earlier (18).

**Retroviral Transduction of gp91<sub>phox</sub>-deficient PLB-985 Cells**—Retroviral gp91<sub>phox</sub> was expressed in gp91<sub>phox</sub>-deficient PLB-985 cells as done in our previous study (20).

**Cell Stimulation**—Cells (5 × 10<sup>6</sup> cells/ml) in Hanks’ balanced salt solution buffer were incubated with 1 mg/ml opsonized zymosan prepared as described before (28), 50 ng/ml PMA or 5 × 10<sup>−7</sup> M fMLP for 3 min at 37 °C.

**Superoxide Anion Measurements**—The production of superoxide anion (O<sub>2</sub><sup>−</sup>) by intact cells was measured as the superoxide dismutase inhibitable reduction of ferricytochrome c (18).

**Isolation of Membrane and Cytosol Fractions**—Isolation of membrane and cytosol fractions was performed exactly as described earlier (29).

**Coimmunoprecipitation**—Immunoprecipitation was performed as described earlier (25). The detection of cPLA<sub>2</sub>α or the NADPH oxidase components after SDS-PAGE electrophoresis was analyzed as described previously (26).

**Bacterial Expression and Purification of Recombinant Proteins**—PCR was used to subclone the different cPLA<sub>2</sub>α regions: C2, Lid, or catalytic domain B, in-frame into the expression vector pGEX-4T-2 using primes containing the EcoRI or Xhol restriction endonucleases sites (underlined): 5′-TATAGAATTCGTCATTATATAAGATCCCTAC-3′, 5′-TATCGAGATTATACCTCAGAGACATTCTTCTAG-3′, 5′-TATAGAATTCGATTATTATATGGAACAGC-3′, 5′-TATCGAGATTACTCGTCCACCATGAA-3′, 5′-TATAGAATTCGAAATTTATGGGAACAGC-3′, and 5′-TATAGAATTCGAAATTTATGGGAACAGC-3′, respectively. The tem-
plate used was pMT2 plasmid containing the cloned gene from the cDNA library. GST catalytic domain A construct has been described earlier (26). The GST fusion proteins were overexpressed and purified as described previously (30). To isolate the p47phox N-terminal protein, the GST protein was cleaved from GST-p47phox N-terminal protein by the addition of 5 μg of factor Xa (New England Biolabs).

Overlay Assay—Recombinant p47phox and p67phox were a kind gift from Prof. Edgar Pick (Tel-Aviv University, Israel). The different cPLA2 fusion proteins or cell lysates of 5 × 10⁷ (25) were separated on SDS-PAGE gel without β-mercaptoethanol and boiling. Protein renaturation was performed by incubation in 25% isopropanol solution for 30 min before immunoblotting (20).

Affinity Binding Assay—GST or GST fusion proteins were added to lysates of resting or stimulated neutrophils or to recombinant p47phox N-terminal in phosphate-buffered saline, and were tumbled end-over-end for 1 h at room temperature. The samples were washed six times with phosphate-buffered saline, boiled in SDS sample buffer, and separated SDS-PAGE before immunoblotting.

FRET Analysis—Glass-adherent neutrophils or granulocyte-like PLB cells were fixed with 3.7% formaldehyde in phosphate-buffered saline, either resting or after stimulation for 3 min with 50 ng/ml PMA. The cells were co-immunostained with anti-cPLA2 mouse monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and either p67phox or p47phox polyclonal rabbit antibodies (31) and with Cy3-conjugated (anti-rabbit IgG) and Cy5-conjugated (anti-goat IgG) secondary antibodies, respectively (Jackson ImmunoResearch Laboratories). Primary and secondary antibodies were diluted 1:500/1:4000 for neutrophils and 1:400/1:500 for granulocyte-like PLB cells, respectively. Cells were imaged with a Zeiss LSM 510 laser scanning confocal microscope using the 543 nm Green HeNe laser line and a 560–615 nm band-pass filter for Cy3 and the 633 nm Red HeNe laser line and a 650 long pass filter for Cy5. Under these conditions, bleed-through between the Cy3 and Cy5 channels was insignificant. Cy3-sensitized emission of Cy5 was measured directly in the Cy5 channel while exciting Cy3 with the 543 nm line. A relative FRET index was calculated by normalizing the intensity of Cy3-sensitized Cy5 emission by the direct Cy3 emission intensity. FRET was further validated by donor recovery after acceptor photobleaching (32), by comparing Cy3 emission intensity before and after selective photobleaching of Cy5 with the 633 nm laser line. Mean fluorescence intensities were measured by calculating the average gray level of a circular region of interest encompassing each analyzed cell, correcting each channel for the background (offset), the average autofluorescence (determined in unstained samples), and the low bleed through between channels (calculated using singly stained samples).

Neutrophil Permeabilization and Superoxide Production—Neutrophils were electroporated exactly as described earlier (20) based on a previous study (34). The effect of the synthetic peptides on binding and NADPH oxidase activity was studied as described (35). The cell suspension was supplemented with 800 μM synthetic peptides (Sigma) incubated on ice for 30 min, centrifuged, and resuspended in the same supplemented buffer containing 200 μM peptide and 150 mM cytochrome c, stimulated for immediate measurement of superoxide production at 22 °C.

Molecular Modeling—p47phox-PX with bound phosphatidylinositol 3-phosphate was docked to the C2 domain of cPLA2 (36) using the protein-protein docking program MolFit (37–40). The initial step consisted of MolFit docking of the p47phox-PX in complex with phosphatidylinositol 3-phosphate and cPLA2-C2 domain (36) (PDB code 1rlw), employing translational interval of 1.05 Å and rotational interval of 12°. The docking models were filtered, selecting models in which the active site in the catalytic domain of intact cPLA2 (15) pointed toward the membrane, as deduced from the position of phosphatidylinositol 3-phosphate. In the second docking stage, the interactions of inhibitory peptides identified experimentally in this study were up-weighted. A similar filtering procedure for the position of the active site of cPLA2 was employed as in the first docking stage, and the accepted models were clustered. The final models were energy-minimized. Details of the procedure are given as supplemental text.

Mutagenesis of Expression Vectors—pGEX-4T-2 expression vector encoding the cDNA of cPLA2-C2 domain or pGEX-3X expression vector encoding the cDNA of p47phox-PX domain were used as a template, to generate the desired mutations introduced according to the molecular modeling, and generated by the overlap extension PCR (41). The PCR reactions, using appropriate complementary synthetic oligonucleotides...
cPLA$_2$-C2 and p47$^{\text{phox}}$-PX Interaction

FIGURE 2. Translocation of cPLA$_2$ to the plasma membranes in p67$^{\text{phox}}$-deficient neutrophils and in a model of differentiated PLB-958 cells lacking p67$^{\text{phox}}$. A, translocation p47$^{\text{phox}}$ and cPLA$_2$ to plasma membrane in stimulated p67$^{\text{phox}}$-deficient neutrophils expressing cPLA$_2$, and p47$^{\text{phox}}$, but not p67$^{\text{phox}}$. Stimulation was induced by FMLP (5 $\times$ 10$^{-7}$ M) for 3 min at 37 °C. Similar results were obtained when cells were stimulated by PMA (not shown).

B, no translocation of p47$^{\text{phox}}$ or cPLA$_2$ to plasma membrane in stimulated p47$^{\text{phox}}$-deficient neutrophils expressing cPLA$_2$, and p67$^{\text{phox}}$ but not p47$^{\text{phox}}$. Cell stimulation as in A. C, translocation p47$^{\text{phox}}$, p67$^{\text{phox}}$, and cPLA$_2$, to plasma membrane in stimulated neutrophils expressing cPLA$_2$, and both p47$^{\text{phox}}$ and p67$^{\text{phox}}$. Cell stimulation as in A. D, coimmunoprecipitation of p47$^{\text{phox}}$ with cPLA$_2$ in p67$^{\text{phox}}$-deficient neutrophil membranes and in healthy controls membranes. The cell-solubilized membranes were subjected to immunoprecipitation with anti-cPLA$_2$, antibodies, separated by SDS-PAGE, and immunoblotted with anti-cPLA$_2$, anti-p67$^{\text{phox}}$, and anti-p47$^{\text{phox}}$, antibodies. E, p47$^{\text{phox}}$ and p67$^{\text{phox}}$ protein expression in cytosols from PLB-958 cells differentiated for 2 and 6 days with 10$^{-7}$ M retinoic acid. F, immunoblot analysis of cytosolic oxidase components and of cPLA$_2$, was performed to detect their translocation to the membranes of differentiated PLB cells after stimulation by 50 ng/ml PMA or 5 $\times$ 10$^{-7}$ M fMLP. G, coimmunoprecipitation of p47$^{\text{phox}}$ with cPLA$_2$, in membranes of differentiated PLB cells for 2 or 6 days. Solubilized membranes of unstimulated and stimulated differentiated PLB-958 cells (as in F) were subjected to immunoprecipitation with anti-cPLA$_2$, antibodies, applied to SDS-PAGE, and followed by immunoblotting with anti-p67$^{\text{phox}}$, anti-p47$^{\text{phox}}$, or anti-cPLA$_2$, antibodies. For both cell types the results are from a representative experiment out of three. 2 x 10$^6$ cell cytosol equivalents or 2 x 10$^7$ cell membrane equivalents were applied per lane. When cPLA$_2$, immunoprecipitation was performed with preimmune serum, cPLA$_2$ and oxidase components were not detected (not shown).

FIGURE 3. Recombinant protein p47$^{\text{phox}}$ binds cPLA$_2$. Lysate equivalents of 1 x 10$^6$ cells from unstimulated (−) or FMLP-stimulated (+) neutrophils (as in Fig. 2) were separated on 7% SDS gel, renatured, transblotted to nitrocellulose, and incubated with 5 μg/ml recombinant p47$^{\text{phox}}$ (A) or 5 μg/ml recombinant p67$^{\text{phox}}$ (B) followed by detection with antibodies against the recombinant protein (overlap), Western blot analysis (W.B.) with anti-cPLA$_2$, (right gels), anti-p47$^{\text{phox}}$, or anti-p67$^{\text{phox}}$, (middle gels) antibodies was performed to indicate the location of cPLA$_2$, p47$^{\text{phox}}$, and p67$^{\text{phox}}$ and to demonstrate the specificity of the antibodies excluding nonspecific detection of other proteins. The results are from a representative experiment out of three with identical results. Similar results were obtained when cells were stimulated by PMA (not shown).

introducing the desired mutation and two additional primers at the ends of the cPLA$_2$-C2 fragment and of p47$^{\text{phox}}$-PX, were performed with Red Load Taq Master/high yield using Thermostable DNA polymerase (Larova, Germany) with appropriate complementary synthetic oligonucleotides that introduced the desired mutation and two additional primers at the ends of the cPLA$_2$-C2 fragment. The mutated products were digested with EcoRI and XhoI or with BamH1 and EcoRI and cloned into pGEX-4T-2 or pGEX-3X expression vectors, respectively, 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. The first three mutations in cPLA$_2$-C2 (F35A plus M38A plus L39A) were mutated in combination to generate triple mutation. The E67A mutation was mutated alone or in addition to the three mutations shown above. M59A and H115A in the p47$^{\text{phox}}$-PX were mutated alone or together.

RESULTS AND DISCUSSION

Flavocytochrome b$_{558}$ Alone Is Not Sufficient to Anchor cPLA$_2$ to Plasma Membranes after Cell Stimulation—To define the exact binding domains between cPLA$_2$ and the assembled NADPH oxidase we first explored the oxidase subunits responsible for recruitment of cPLA$_2$ to the plasma membrane. To study whether cPLA$_2$ binds the cytochrome b$_{558}$, cPLA$_2$ translocation to the plasma membranes was examined in undifferentiated X-CGD PLB cells transduced with full-length gp91$^{\text{phox}}$ (XCGD-gp91) as reported in our previous studies (20, 25). As shown in Fig. 1A, membranes were prepared from XCGD-gp91 PLB cells transduced with M59A alone. During differentiation the cells acquire the NADPH oxidase subunits as reported previously (18). In the presence of the cytosolic oxidase sub-
units in differentiated XCGD-gp91<sup>phox</sup> PLB cells, stimulation did cause translocation of cPLA<sub>2</sub> to the plasma membranes, consistent with assembly of the oxidase, as determined by the translocation of p47<sup>phox</sup>. In addition, the specific protein kinase C inhibitor GF-109203X attenuated PMA-stimulated translocation of both p47<sup>phox</sup> and cPLA<sub>2</sub> in a similar dose-dependent
manner (Fig. 1B), consistent with our previous study reporting that GF-109203X causes the same dose response inhibition of p47<sup>phox</sup> phosphorylation, oxidase assembly, and activity (26). Taken together, these results suggest that cPLA<sub>2</sub> does not bind directly to flavocytochrome b<sub>558</sub> but, rather, binds to the assembled oxidase complex.

cPLA<sub>2</sub> Is Bound to p47<sup>phox</sup> in the Assembled Oxidase—To determine which cytosolic component in the assembled oxidase is bound to cPLA<sub>2</sub> after stimulation, neutrophils from p67<sup>phox</sup>-deficient CGD patients were studied, as shown by immunoblot of their cytosol (Fig. 2A). Activation of these cells resulted in translocation of p47<sup>phox</sup> and cPLA<sub>2</sub> to the membrane fractions (Fig. 2A) similar to the translocation of these proteins in neutrophils from healthy donors, who express all oxidase components (Fig. 2C). In contrast, when neutrophils from p47<sup>phox</sup>-deficient CGD patients were stimulated, there was no translocation of either p67<sup>phox</sup> or cPLA<sub>2</sub> (Fig. 2B). The lack of p67<sup>phox</sup> translocation is expected, because it is dependent on the translocation of p47<sup>phox</sup>, which is missing in these cells. Addition of antibodies against cPLA<sub>2</sub> to the membrane fractions of activated neutrophils from p67<sup>phox</sup>-deficient CGD patients caused a significant immunoprecipitation of cPLA<sub>2</sub> and coinmunoprecipitation of p47<sup>phox</sup> similar to that observed in stimulated neutrophils from healthy controls (Fig. 2D). Similar results were obtained in PLB-985 cells differentiated with retinoic acid, which, similar to HL-60 cells (29), serve as a model for p67<sup>phox</sup>-deficient cells, because the induction of p47<sup>phox</sup> precedes that of p67<sup>phox</sup>. As shown in Fig. 2C, PLB-985 cells differentiated for 2 days with retinoic acid express only p47<sup>phox</sup> and not p67<sup>phox</sup>, and were not capable of producing superoxide following stimulation by PMA (0.2 ± 0.07 nmol/10<sup>6</sup>/min), while after 6 days of differentiation, with the appearance of p67<sup>phox</sup> protein (Fig. 2E), they produced significant stimulated superoxide (11.5 ± 1.7 nmol/10<sup>6</sup>/min). The translocation of cPLA<sub>2</sub> to the plasma membranes was studied in differentiated PLB-985 cells for 2 and 6 days, which express similar levels of p47<sup>phox</sup> but differ in their expression of p67<sup>phox</sup>. As shown in Fig. 2F, cPLA<sub>2</sub> translocated to the plasma membranes after stimulation with fMLP or PMA, which correlated with p47<sup>phox</sup> translocation, regardless of the presence of p67<sup>phox</sup>. Immunoprecipitation of cPLA<sub>2</sub> from membrane fractions of stimulated cells resulted in coinmunoprecipitation of p47<sup>phox</sup> in cells lacking or expressing p67<sup>phox</sup> protein (Fig. 2G). These results show a direct binding between p47<sup>phox</sup> and cPLA<sub>2</sub> in stimulated neutrophils and granulocyte-like PLB cells and that p47<sup>phox</sup> is sufficient for the translocation of cPLA<sub>2</sub> to the plasma membranes, whereas p67<sup>phox</sup> is not involved in this process. The absence of cPLA<sub>2</sub> translocation in the absence of the assembly of the oxidase suggests that cPLA<sub>2</sub> does not bind the oxidase membrane components, consistent with the results in Fig. 1B.

Although p67<sup>phox</sup> is not required to anchor cPLA<sub>2</sub> to the assembled oxidase, there is a possibility of interaction between these two proteins within the assembled oxidase after the translocation of cPLA<sub>2</sub>. Overlay experiments were performed to determine whether cPLA<sub>2</sub> binds only p47<sup>phox</sup> or also binds p67<sup>phox</sup>, because in the absence of p47<sup>phox</sup> subunit, in neutrophils of p47<sup>phox</sup>-deficient CGD patients, there is no translocation of p67<sup>phox</sup> to the membranes. As shown by the overlay experiments (detailed in the legends) presented in Fig. 3A, recombinant p47<sup>phox</sup> was bound to p67<sup>phox</sup> in lysates of both resting and stimulated cells (as expected) and was bound to cPLA<sub>2</sub> only in the lysates of activated cells. In contrast, recombinant p67<sup>phox</sup> did not bind cPLA<sub>2</sub> in resting or stimulated cells, although it could bind p47<sup>phox</sup> (Fig. 3B), indicating that there is no direct binding between cPLA<sub>2</sub> and p67<sup>phox</sup>. The binding of recombinant-autoinhibited p47<sup>phox</sup> to cPLA<sub>2</sub> in lysates of stimulated cells suggests that p47<sup>phox</sup> does not need to be phosphorylated to bind cPLA<sub>2</sub>, whereas cPLA<sub>2</sub> has to be phosphorylated for binding p67<sup>phox</sup>, which is in accordance with our previous study (25).

The binding of cPLA<sub>2</sub> to p47<sup>phox</sup> but not to p67<sup>phox</sup> was further supported by FRET measurements. Because efficient FRET occurs only at a distance of a few nanometers, its detection indicates a molecular range interaction between the labeled proteins (32, 42). FRET between Cy3-labeled cPLA<sub>2</sub> and Cy5-labeled p47<sup>phox</sup> or p67<sup>phox</sup> was performed, and FRET indices were consistent with binding between cPLA<sub>2</sub> and p47<sup>phox</sup>, but not p67<sup>phox</sup>, both in neutrophils and in granulocyte-like cells after stimulation (Fig. 4, A, B, E, and F), in correspondence with...
the binding of cPLA₂ and p47phox, but not p67phox (Fig. 3). FRET was not detectable in unstimulated cells further supporting our previous finding (25) that though these proteins are both located in the cytosol, they do not interact prior to stimulation. Moreover, there is only partial translocation of p47phox (~10%) (43) and of cPLA₂ (25) to the plasma membrane. FRET is detected only in the cell periphery and not in the cytosol, consistent with our suggestion (25) that the binding of cPLA₂ occurs after the assembly of the oxidase. To further validate our FRET measurements, we tested for donor recovery after acceptor photobleaching (32). Indeed, photobleaching of Cy5-labeled p47phox resulted in a 28% increase in Cy3-labeled cPLA₂ fluorescence in both types of stimulated cells, but not in unstimulated cells (Fig. 4, A, B, C, D, H, and K). Thus we show here by two different methods (Figs. 3 and 4) that cPLA₂ is anchored to the assembled oxidase upon stimulation through its interaction with p47phox but not with p67phox.

In Vitro Interaction between the Cytosolic p47phox Component N-Terminal Domain and the cPLA₂-C2 Domain—To determine the p47phox binding domains for cPLA₂, three GST fusion constructs of p47phox were expressed (Fig. 5A): GST-p47phox N-terminal (1–138 aa) (GST-C2), GST-cPLA₂-catalytic domain A (123–260 aa) (GST-CDA), GST-cPLA₂-Lid region (370–550 aa) (GST-Lid), and GST-cPLA₂-catalytic domain B (530–749 aa) (GST-catalytic domain B). These four fusion proteins were separated on SDS-PAGE, renatured, and transblotted to nitrocellulose. Their expression and location were determined by Western blot analysis with anti-GST antibodies. The location of the different GST fusion domains of cPLA₂ was determined using anti-GST antibodies (left gel). p47phox N-terminal was bound only to the cPLA₂-C2 domain. A, affinity-binding GST-cPLA₂-C2 and p47phox N-terminal domains GST-cPLA₂-C2 or GST recombinant proteins attached to glutathione-Sepharose beads were added to recombinant p47phox N-terminal (GST-C2+NT or GST+NT, respectively). The samples were separated on 10% SDS gel and subjected to Western blot analysis with anti-p47 antibodies (right gel). In addition, in each gel GST-cPLA₂ and p47phox N-terminal proteins were loaded to verify their location (GST-C2 and NT, respectively). The left gel was immunoblotted with anti-GST antibodies to indicate the location and amount of GST-cPLA₂-C2 and GST used. The right gel was immunoblotted with anti-p47phox antibodies, and its location was verified by comparison with the migration of the p47phox N-terminal (NT). In the right lane, in which only GST-C2 was loaded, p47phox antibodies did not react, excluding nonspecific identification of other proteins. The results are from a representative experiment out of three. B, affinity binding assay between GST-cPLA₂-C2 and whole p47phox protein from lysates of neutrophils. The GST-cPLA₂-C2 domain (GST-C2) or GST alone attached to glutathione-Sepharose beads were added to lysates of unstimulated neutrophils (~) or neutrophils stimulated by FMLP (+). The samples were separated on 10% SDS gel and subjected to Western blot analysis with anti-p47phox (upper gel) or anti-GST antibodies (lower gel). The results are from a representative experiment out of three. Similar results were obtained when cells were stimulated by PMA (not shown).

FIGURE 6. Binding of the recombinant protein p47phox N-terminal to the C2 region of cPLA₂. A, schematic presentation of the cPLA₂ domain as GST fusion proteins. The ball symbolizes GST. Amino acid residue numbers are shown in parentheses. B, overlay p47phox N-terminal binds to cPLA₂-C2. 20 ng of each of the recombinant GST fusion cPLA₂ domains were separated on 10% SDS gels. The right blot was incubated with 5 μg/ml recombinant p47phox N-terminal followed by immunoblotting with anti-p47phox antibodies. The location of the different GST fusion domains of cPLA₂ was determined using anti-GST antibodies (left gel). p47phox N-terminal was bound only to the cPLA₂-C2 domain. C, affinity-binding GST-cPLA₂-C2 and p47phox N-terminal domains GST-cPLA₂-C2 or GST recombinant proteins attached to glutathione-Sepharose beads were added to recombinant p47phox N-terminal (GST-C2+NT or GST+NT, respectively). The samples were separated on 10% SDS gel and subjected to Western blot analysis with anti-p47phox (right gel). In addition, in each gel GST-cPLA₂-C2 and p47phox N-terminal proteins were loaded to verify their location (GST-C2 and NT, respectively). The left gel was immunoblotted with anti-GST antibodies to indicate the location and amount of GST-cPLA₂-C2 and GST used. The right gel was immunoblotted with anti-p47phox antibodies, and its location was verified by comparison with the migration of the p47phox N-terminal (NT). In the right lane, in which only GST-C2 was loaded, p47phox antibodies did not react, excluding nonspecific identification of other proteins. The results are from a representative experiment out of three. D, affinity binding assay between GST-cPLA₂-C2 and whole p47phox protein from lysates of neutrophils. The GST-cPLA₂-C2 domain (GST-C2) or GST alone attached to glutathione-Sepharose beads were added to lysates of unstimulated neutrophils (~) or neutrophils stimulated by FMLP (+). The samples were separated on 10% SDS gel and subjected to Western blot analysis with anti-p47phox (upper gel) or anti-GST antibodies (lower gel). The results are from a representative experiment out of three. Similar results were obtained when cells were stimulated by PMA (not shown).
Addition of GST-cPLA2-C2 to lysates of unstimulated or stimulated neutrophils ((Fig. 6D, lower gel) pulled down the cytosolic oxidase component p47^phox (Fig. 6D, upper gel), indicating that cPLA2-C2, similar to the whole cPLA2, can bind both forms of p47^phox, the autoinhibited unphosphorylated form, as shown for the recombinant p47^phox (Fig. 3A), and the phosphorylated-open form in stimulated cells. The affinity binding between recombinant p47^phox and cPLA2 was detected only in lysates of stimulated neutrophils (Fig. 3), and FRET between p47^phox and cPLA2 occurred only after cell stimulation (Fig. 4), indicating that cPLA2 has to be in its phosphorylated form to bind p47^phox. In contrast, binding of GST-cPLA2-C2 to p47^phox N-terminal (Fig. 6) appears to be facilitated by the absence of the rest of cPLA2, bypassing the need for phosphorylation-dependent conformational changes required for binding of the complete cPLA2.

Determining the Binding Sites between cPLA2-C2 and the p47^phox N-terminal—Findings based on the reported structure of p47^phox in the assembled oxidase (2) and our results demonstrating that cPLA2 translocates and binds to the NADPH oxidase only after its assembly (Figs. 1B and 4 and Ref. 25) and that the binding is located in the p47^phox-PX domain (Fig. 5) suggest that the binding site is located on an exposed surface of the p47^phox-PX domain. After stimulation, the structure of p47^phox is opened due to its phosphorylation, which allows binding to the C-terminal domains of gp91^phox (44) and p22^phox through its SH3 domains (6, 45) and to phosphatidylinositol 3,4-bisphosphate and phosphatic acid on the plasma membrane through two basic pockets within its PX domain (6, 9). Thus, other exposed PX domain surfaces appear to function as a docking site for cPLA2 on the assembled NADPH oxidase, without interfering with the interaction of p47^phox with the plasma membrane, with the flavocytochrome b558 or with p67^phox. This notion is supported by the observed binding between the recombinant p47^phox protein and cPLA2 (Fig. 3) and by affinity binding between p47^phox in lysates of unstimulated neutrophils and the GST-cPLA2-C2 construct (Fig. 6D).

In both cases, p47^phox is found in its autoinhibited, folded conformation (1, 6, 30, 46, 47) in which the sole region of the p47^phox N-terminal domain available for binding cPLA2 is its outer surface. This is because the inner surface that binds the membranes via its phosphatidylinositol 3,4-bisphosphate lipid binding pockets is normally masked by intramolecular interactions with the C-terminal domains in the autoinhibited form of p47^phox (2, 9).

A computer-based docking search to predict the binding interface between p47^phox and cPLA2 was conducted as described under “Experimental Procedures.” In the first docking stage, interactions involving p47^phox-PX residues implicated in membrane binding were disregarded, because cPLA2 binds p47^phox after oxidase assembly. In addition, models that did not position the active site of the catalytic domain of cPLA2 near the cellular membranes (48) were discarded. The search produced several clusters of models with similar geometric electrostatic hydrophobic complementarity scores. A few linear peptides that were buried at the p47^phox-PX and cPLA2-C2 interfaces in these models were selected to determine the binding sites by competitive binding experiments. Electroporated neutrophils were shown to be permeable to small peptides used to inhibit NADPH oxidase assembly and activation (35). We used this methodology to determine the effects of the different peptides on binding of cPLA2 to the assembled NADPH oxidase and on the production of superoxide by the oxidase after cell stimulation. Of all the peptides analyzed (Table 1), only two were found to be inhibitory: peptide 33-GAFGDML-39, derived from the p47^phox-PX domain. These two peptides significantly inhibited cPLA2 translocation to the plasma membrane, with the flavocytochrome b558 or with p67^phox. This notion is supported by the observed binding between the recombinant p47^phox protein and cPLA2 (Fig. 3) and by affinity binding between p47^phox in lysates of unstimulated neutrophils and the GST-cPLA2-C2 construct (Fig. 6D).

| Source | Sequence | Superoxide production (% of control) | Binding Inhibition |
|-------|----------|-------------------------------------|-------------------|
| p47-PX | 3-MDGTFIR-9 | 130 ± 35 | - |
| | 10-HIALLGF-16 | 95 ± 9 | - |
| | 58-EMPPIEAGA-66 | 35 ± 12 | + |
| | 71-NRIPHLPAPK-81 | 110 ± 20 | - |
| | 106-LPTKISRC-113 | 118 ± 15 | - |
| | 132-QTKKPET-138 | 110 ± 15 | - |
| cPLA2-C2 | 33-GAFGDML-39 | 24 ± 12 | + |
| | 32-KGAFGDMLD-40 | 121 ± 16 | - |
| | 84-ANYVMDETL-102 | 120 ± 19 | - |

Shown are the sequences and topographical locations of the PX and C2 domain peptides used in the assay. Neutrophils were electroporated in the presence of 0.8 mM concentrations of each peptide (as described under “Experimental Procedures”) before stimulating with PMA (50 ng/ml). Superoxide production is expressed as % of control ± S.E. of seven different experiments performed in duplicates. Similar results were obtained when the cells were stimulated with the inhibition of binding between cPLA2 and NADPH oxidase shown in an example in Fig. 7 (A and B) is summarized in the right column (Binding Inhibition).
The two peptides (GAFGDM separators) and EMFPIEAGA were also the only ones that inhibited stimulated superoxide production in electroporated neutrophils (Table 1), emphasizing the requirement for cPLA$_2$ binding to NADPH oxidase activation. Extension of the inhibitory peptides 33–39 to 32–40 aa resulting with addition of one charged amino acid on each side (Lys and Asp) caused a loss of the inhibitory effect, supporting the results (Fig. 5C) demonstrating that the interaction is primarily of a hydrophobic nature. All the other p47phox peptides examined had no effect (Table 1), suggesting their corresponding protein domains do not play a significant role in the protein interaction. The PX domain of p47phox has a flat, compact shape that consists of three β strands, four α helices, and an exposed proline-rich segment held by the two helices α1 and α2 that has been shown to bind SH3 domains in other proteins (33, 49). However, the peptide competition experiments suggest that the exposed proline-rich region of the p47phox PX domain, 71 NRIIPHLPAKP 81 aa, is not involved in binding to cPLA$_2$. Further investigation of this interaction will require more detailed structural and functional studies.

### Figures

**Figure 7. Mapping the association between cPLA$_2$ and p47phox-PX Interaction**

- **A**. Inhibition of cPLA$_2$ translocation to the plasma membranes by specific peptides. Neutrophils were electro-permeabilized in the presence of 0.8 mM concentrations of each peptide (as described under “Experimental Procedures”) before stimulation with FMLP (5 × 10$^{-7}$ M) or PMA (50 ng/mL). cPLA$_2$ translocation to the plasma membranes of activated neutrophils was attenuated in the presence of 33 GAFGDM (α, inhibitory peptide), but not in the presence of 33 ANYVMDETL (β, non-inhibitory peptide). The assembly of the NADPH oxidase, as indicated by p47phox translocation to the plasma membranes, was not affected by the presence of the peptides. The Na$^+$/K$^-$ATPase expression indicates the amounts of membranes loaded in each lane. The results are from a representative experiment out of three independent experiments.

- **B**. Affinity binding assay between mutated GST-p47phox-PX and cPLA$_2$ protein from lysates of stimulated neutrophils (with 50 ng/ml PMA). The inhibition peptides 33–39 to 32–40 aa result in expression of one charged amino acid on each side (Lys– or Asp–) demonstrating that the interaction is primarily of a hydrophobic nature. All the other p47phox peptides examined had no effect (Table 1), suggesting their corresponding protein domains do not play a significant role in the protein interaction. The PX domain of p47phox has a flat, compact shape that consists of three β strands, four α helices, and an exposed proline-rich segment held by the two helices α1 and α2 that has been shown to bind SH3 domains in other proteins (33, 49). However, the peptide competition experiments suggest that the exposed proline-rich region of the p47phox PX domain, 71 NRIIPHLPAKP 81 aa, is not involved in binding to cPLA$_2$. Further investigation of this interaction will require more detailed structural and functional studies.

### Table 1

| Peptide | WT (%) | M/A (%) | FMLI/A (%) | FLM/A (%) |
|---------|--------|---------|------------|-----------|
| 33 GAFGDM | 100 | 98 | 97 | 96 |
| 33 ANYVMDETL | 100 | 100 | 100 | 100 |
| 33 EMFPIEAGA | 100 | 98 | 97 | 96 |
| 33 NRIIPHLPAKP | 100 | 100 | 100 | 100 |

The mean ± S.E. of the analysis of the seven experiments is presented.
thermore, the region containing 3MDGTFIR9 aa and 10HIALLG16 aa, covering most of the β strand (Ile6–Lys16), does not mediate binding to cPLA2. Neither do most of the α4 helix nor the C terminus of the PX domain, because peptides 106LPTKISR113 and 132QTKPET138 aa had no effect on binding or superoxide production.

The experimental results presented in Fig. 7 (A and B) and Table 1 were then used in the second docking stage in which interactions involving either peptide 58EMFPIEAGA66 of PX or peptide 33GAFGDML39 of C2 were favored (see “Experimental Procedures”). This led to the formation of a single cluster of six models in which these two peptides were at the interface, whereas the other tested peptides were not. Notably, the biased docking procedure produced more models in which these two peptides contributed to the interface, but it did not require that they interact with each other. Hence, the direct interaction between these peptides (Fig. 7E) is an independent result, not enforced by the weighting scheme employed in the computations. The six models in the final cluster, although similar, form two subgroups that differ in the details of the interaction in that in one subgroup residues Phe35, Met38, and Leu39 of cPLA2 are centrally positioned in the interface and in the other subgroup the centrally positioned residue is Ile67. The interface in both subgroups is mostly hydrophobic, in correspondence with the experimental results (Fig. 5C). To examine these models and to define the relative role of the cPLA2-C2 amino acid residues participating in the binding to p47phox-PX, mutated GST constructs were engineered, and their efficiency for pulling down p47phox from neutrophil lysates was analyzed. As shown in Fig. 7C, substitution of Phe35, Met38, and Leu39 to Ala resulted in a slight decrease in binding affinity to p47phox (of 30 ± 7%) compared with the wild type, whereas substitution of Ile67 to Ala was very efficient in inhibiting binding to p47phox, reaching ∼93 ± 3% inhibition. Substitution of all four amino acids to Ala caused a 96 ± 2% inhibition of binding to p47phox, which was not significantly different from the effect of Ile67 substitution alone. The mutagenesis results clearly favor one of the two docking models, in which Ile67 is centrally positioned in the interface. Thus, Ile67, the first amino acid of the β strand, resides in a hydrophobic pocket on the surface of the PX domain and interacts with its residues Pro114 and His115 in the α4 helix, and Met59 in the end of the α1 helix (the brown patch in Fig. 7E). Substitution of His115 to Ala in the GST construct of p47phox-PX domain did not affect the binding affinity to cPLA2 in stimulated neutrophil lysate compared with the wild type, whereas substitution of Met59 to Ala cause significant inhibition of the binding to cPLA2 (32 ± 8%). Substitution of both His115 and Met59 to Ala was very efficient in inhibiting the binding to cPLA2 in stimulated neutrophil lysate reaching about 60% inhibition. Pro114 was not mutated as it would have changed the PX domain conformation. The two inhibitory peptides (Fig. 7, A and B, and Table 1), highlighted in green and yellow in Fig. 7E, are at the edge of the interface. Thus, it is likely that they interfere with the hydrophobic interaction between cPLA2-C2 and p47phox-PX, preventing the formation of the complex. In addition, the PX domain peptide 58EMFPIEAGA66 probably directly inhibits the binding, because it contains Met59, which participates in the binding with cPLA2-C2 Ile67 (Fig. 7D).

In conclusion, we have clearly demonstrated that the binding between cPLA2 to the assembled oxidase upon activation of neutrophils or granulocyte-like PLB-985 cells is mediated by the p47phox cytosolic subunit. Our study shows that the cPLA2-C2 domain, which is responsible for anchoring cPLA2 to the membrane, also contains other binding sites that engage cPLA2 with the assembled oxidase and promote its activity.

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