Unique targeting of cytosolic phospholipase A\(_2\) to plasma membranes mediated by the NADPH oxidase in phagocytes

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Cytosolic phospholipase A\(_2\) (cPLA\(_2\))–generated arachidonic acid (AA) has been shown to be an essential requirement for the activation of NADPH oxidase, in addition to its being the major enzyme involved in the formation of eicosanoids at the nuclear membranes. The mechanism by which cPLA\(_2\) regulates NADPH oxidase activity is not known, particularly since the NADPH oxidase complex is localized in the plasma membranes of stimulated cells. The present study is the first to demonstrate that upon stimulation cPLA\(_2\) is transiently recruited to the plasma membranes by a functional NADPH oxidase in neutrophils and in granulocyte-like PLB-985 cells. Coimmunoprecipitation experiments and double labeling immunofluorescence analysis demonstrated the unique colocalization of cPLA\(_2\) and the NADPH oxidase in plasma membranes of stimulated cells, in correlation with the kinetic burst of superoxide production. A specific affinity in vitro binding was detected between GST-p47\(_{phox}\) or GST-p67\(_{phox}\) and cPLA\(_2\) in lysates of stimulated cells. The association between these two enzymes provides the molecular basis for AA released by cPLA\(_2\) to activate the assembled NADPH oxidase. The ability of cPLA\(_2\) to regulate two different functions in the same cells (superoxide generation and eicosanoid production) is achieved by a novel dual subcellular localization of cPLA\(_2\) to different targets.

Introduction

The NADPH oxidase is a multicomponent electron transport chain that transfers electrons from NADPH to molecular oxygen to form superoxide, a precursor of microbicidal oxidants. The production of superoxide by NADPH oxidase is one of the most important functions for host defense. However, during altered physiological states reactive oxygen products may promote inflammatory reactions and participate in processes that lead to tissue injury. An understanding of the biochemical processes that regulate NADPH oxidase activity may provide a means to more effectively control the activity of the cells during infection and inflammation. In resting neutrophils, NADPH oxidase is dormant, with unassembled subunits located on the cytosol and the plasma membranes (Babior, 1999; Leto, 1999; Babior et al., 2002). Upon stimulation, the cytosolic components p47\(_{phox}\), p67\(_{phox}\), p40\(_{phox}\), and rac2 translocate to the plasma membranes and associate with the heterodimeric transmembrane glycoprotein, flavocytochrome b\(_{558}\). The cytochrome is comprised of two subunits, gp91\(_{phox}\) and p22\(_{phox}\), that contain heme, flavin, and NADPH-binding sites (Segal and Abo, 1993; Babior, 1999; Leto, 1999; Yu et al., 1999; Segal et al., 2000). The two cytosolic components, p47\(_{phox}\) and p67\(_{phox}\), contain Src homology 3 motifs that direct their translocation to the membranes by binding to specific targets in p22\(_{phox}\) and to each other (Leto, 1999). p47\(_{phox}\) and p40\(_{phox}\) subunits contain phox homology domains that specifically bind to phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3-phosphate (Ellson et al., 2001; Kanai et al., 2001; Sato et al., 2001). These phox homol-

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Abbreviations used in this paper: AA, arachidonic acid; cPLA\(_2\), cytosolic phospholipase A\(_2\); fMLP, formyl-methionyl-leucyl-phenylalanine; OZ, opsonized zymosan; PtdIns(4,5)P\(_2\), phosphatidylinositol 4,5-bisphosphate.
mechanism by which cPLA₂ (Hirabayashi and Shimizu, 2000). To elucidate the nuclear and ER membranes in a variety of cells (for review in the different cell types, as proposed by the authors. In contrast to the findings demonstrating requirement of the cPLA₂ for oxidase activation, resident peritoneal macrophages from cPLA₂-deficient mice exhibited normal stimulated superoxide release (Gijon et al., 2000), which may be attributed to either compensatory effects of isoenzymes frequently observed in models of knockout animals or to the known differences of the PLA₂ isotypes between mice and human/rat (Suzuki et al., 2000). In an earlier study, we showed that AA increases the affinity of the assembled oxidase in purified membranes for NADPH (Rubinek and Levy, 1993). The mechanism by which cPLA₂ regulates the NADPH oxidase activity is not known, particularly since the NADPH oxidase complex is localized in the plasma membrane in stimulated cells, whereas cPLA₂ has been shown to translocate to the nuclear and ER membranes in a variety of cells (for review see Hirabayashi and Shimizu, 2000). To elucidate the mechanism by which cPLA₂ regulates the activation of NADPH oxidase, the present study investigated the cellular localization of cPLA₂ upon stimulation and whether there is any association between cPLA₂ and the NADPH oxidase complex during activation of phagocytic cells.

Results

Coimmunoprecipitation of cPLA₂ with NADPH oxidase in membranes of activated neutrophils

The association between cPLA₂ and NADPH oxidase was studied by performing coimmunoprecipitation experiments in resting and stimulated neutrophils with either 1 mg/ml opsonized zymosan (OZ), 50 ng/ml PMA, or 5 × 10⁻⁷ M formyl-methionyl-leucyl-phenylalanine (fMLP). As shown in Fig. 1, addition of antibodies against either p47phox or p67phox resulted in significant immunoprecipitation of the cytosolic components in the membrane fractions of the activated neutrophils. cPLA₂ was coimmunoprecipitated with either of the cytosolic components (p47phox or p67phox) in the membrane fractions of activated neutrophils but not in the membranes of resting neutrophils. In spite of the high levels of p47phox or p67phox immunoprecipitated in the cytosol of resting or activated neutrophils, coimmunoprecipitation of cPLA₂ was not detected. To confirm the binding between cPLA₂ and the NADPH oxidase complex, cPLA₂ was immunoprecipitated, and its association with oxidase components was analyzed. As shown in Fig. 2 A, cPLA₂ was immunoprecipitated in the membrane fractions of neutrophils activated for 1 min with either OZ, fMLP, or PMA. The NADPH oxidase components, p47phox, p67phox, and gp91phox, were coimmunoprecipitated with cPLA₂ in the membrane fractions of activated cells. Traces of cPLA₂ and the oxidase components detected in membranes of resting cells (Figs. 1 and 2) are probably due to some basal stimulation of neutrophils during purification. When cPLA₂ immunoprecipitation was done with preimmune serum, neither cPLA₂ nor the oxidase components were detected (unpublished data), confirming the specificity of the assay.

Figure 1. Coimmunoprecipitation of cPLA₂ with p67phox or p47phox in membranes of stimulated neutrophils. Neutrophils were stimulated with 1 mg/ml OZ, 5 × 10⁻⁷ M fMLP, or 50 ng/ml PMA for 1 min at 37°C. The cell cytosol and solubilized membranes were subjected to immunoprecipitation (IP) with anti-p67phox or anti-p47phox antibodies. The immunoprecipitates were separated by SDS-PAGE electrophoresis, and immunoblotting was performed with anti-cPLA₂ antibodies. The levels of p67phox or p47phox immunoprecipitated were evaluated by immunoblot analysis. The results shown are from one representative experiment out of three that gave identical results.
Pretreatment of neutrophils with 5 μg/ml cytochalasin b, which prevents actin polymerization and disrupts cytoskeleton integrity (Frank, 1990; Tsai et al., 1994; Sheikh and Nash, 1998), did not affect the binding between cPLA2 and the assembled oxidase membranes in stimulated neutrophils (Fig. 2B). Although several studies have suggested that the functional oxidase complex at the plasma membranes is associated with the cytoskeleton (Nauseef et al., 1991; Dusi et al., 1996; Allen et al., 1999), the present findings suggest that assembly of the NADPH oxidase complex and its association with cPLA2 stimulated by these agonists are independent of an intact cytoskeleton in nonadherent neutrophils. Furthermore, in accordance with these results superoxide generation and translocation of the cyto-solic oxidase components to membranes of nonadherent neutrophils pretreated with cytochalasin b was increased when stimulated with fMLP or OZ but was not affected when stimulated with PMA (unpublished data; Dusi et al., 1996; Yan and Novak, 1999; Jiang et al., 2000).

Translocation of cPLA2 to the plasma membranes of activated neutrophils
To determine the exact subcellular localization of cPLA2 after neutrophil activation, resting and stimulated cells were fractionated onto a three-step discontinuous Percoll gradient, resulting in the separation of azurophil granules (α), the specific granules (β1 and β2), and the plasma membranes/secretory vesicles (γ). Fig. 3 demonstrates that both cPLA2 and p47phox were associated with the γ fraction of PMA-stimulated neutrophils as shown earlier for p47phox (el Benna et al., 1994; McAdara Berkowitz et al., 2001). Neutrophil stimulation with either fMLP or OZ produced the same results (unpublished data).

Time course binding between cPLA2 and the assembled NADPH oxidase
The binding kinetics between cPLA2 and the assembled NADPH oxidase were studied in peripheral blood neutrophils and in granulocyte-like PLB-985 cells and revealed identical results, as shown for granulocyte-like PLB-985 cells in Fig. 4. Immunoprecipitation of cPLA2 from solubilized membrane fractions could be detected as early as 30 s after stimulation, reaching maximal levels after 1 min of stimulation with either 1 mg/ml OZ or 5 × 10−7 M fMLP and decreasing thereafter (Fig. 4, A and C). Similar results were obtained when the cells were stimulated with 50 ng/ml PMA, with maximal levels after 3 min of stimulation (Fig. 4 B). Since cPLA2 was immunoprecipitated from the membranes, its level dictated the levels of coimmunoprecipitated p47phox and p67phox (Fig. 4, A–C). Thus, the time course appearance of cPLA2, p47phox, and p67phox to the membranes after stimulation was analyzed in order to compare translocation kinetics of these proteins. As shown in Fig. 4, D–F, the oxidase cytosolic components were rapidly and simultaneously translocated to the solubilized membrane fractions after stimulation, preceding the translocation of cPLA2. These results are in line with our previous studies (Levy et al., 1994; Dana et al., 1998; Pessach et al., 2001), demonstrating that the translocation of the oxidase cytosolic components is independent of cPLA2. As shown in Fig. 4, D–F, the kinetics of cPLA2 translocation to the membranes is dependent on the translocation of the kinetics of oxidase cytosolic components and is more rapid when stimulated with OZ or fMLP than with PMA, which is consistent with the development of the burst of superoxides by these agonists (Fig. 4, G–I). Significant translocation of cPLA2 to the membranes of stimulated cells was detected before the production of superoxides, whereas maximal translocation of cPLA2 was detected at 1 min of stimulation with OZ or fMLP and at 3 min of stimulation with PMA (Fig. 4, D–F), which coincided with the onset of superoxide production by these three agonists (Fig. 4, G–I). The detachment of cPLA2 from the membrane fractions precedes that of the cytosolic components p47phox and p67phox (Fig. 4, D–F) and takes place while NADPH oxidase is still being activated to generate superoxide (Fig. 4, G–I).

To determine the molar ratio between the assembled oxidase and cPLA2 in the membranes after stimulation, the lev-
els of cPLA$_2$ and of p47$^{phox}$ (which are representative of the assembled oxidase) in the same membranes from stimulated neutrophils were analyzed by quantitative Western blotting as done by others (Quinn et al., 1993). Representative blots and standard curves obtained by quantitative densitometry of recombinant proteins are shown in Fig. 5. Quantitative analysis revealed that cPLA$_2$ and p47$^{phox}$ translocated to the membranes by the three agonists at a molar ratio of 1:25. Actual measured values for cPLA$_2$ derived from the standard curves were 2.1, 1.6, or 2.3 ng/sample, which were calculated as 25, 20, or 28 pmoles/sample in the membranes from neutrophils stimulated with OZ, fMLP, or PMA, respectively. Actual measured values for p47$^{phox}$ derived from the standard curves were 28, 23, or 31 ng/sample, which were calculated as 604, 504, or 673 pmoles/sample in the membranes from cells stimulated with OZ, fMLP, or PMA, respectively. Despite the different levels of p47$^{phox}$ translocated to the membrane induced by the different agonists, the molar ratio between cPLA$_2$ and p47$^{phox}$ was similar, indicating that the level of p47$^{phox}$ determines the level of cPLA$_2$ that translocates to the membranes. Similar results were obtained in granulocyte-like PLB-985 cells.

**In vivo location of cPLA$_2$ in resting and activated cells**

Based on the in vitro binding between cPLA$_2$ and NADPH oxidase in plasma membranes of stimulated cells, we examined the in vivo location of cPLA$_2$ in resting and activated cells by immunofluorescence microscopy. Granulocyte-like PLB-985 cells are advantageous for the study: first, the expression of gp91$^{phox}$ can be manipulated in these cells (Zhen et al., 1993), thus enabling determination of the role of oxidase for targeting cPLA$_2$ to the plasma membranes, and second, PLB-985 cells differentiated with DMSO exhibit different kinetics for superoxide production (Fig. 4) and for eicosanoid formation (shown at the end of this section), thus facilitating a means to detect the location of cPLA$_2$ in different compartments after stimulation. Double staining immunofluorescence analysis of cPLA$_2$ and the membrane oxidase component gp91$^{phox}$ showed that cPLA$_2$ is found in the cytosol of resting granulocyte-like PLB-985 cells (Fig. 6 A). Upon stimulation with either PMA, OZ, or fMLP, a significant translocation of cPLA$_2$ to the cell periphery was detected where it colocalized with gp91$^{phox}$, confirming the localization of cPLA$_2$ in the plasma membranes of activated phagocytic cells. No increase in the gp91$^{phox}$ immunofluorescence signal was detected after stimulation with either of the agonists (Fig. 6 A), which is in line with the low levels of granules, including gp91$^{phox}$ endomembranes, present in PLB-985 cells differentiated...

**Figure 4.** The time course association and translocation of cPLA$_2$ and the cytosolic components of NADPH oxidase in membranes of stimulated granulocyte-like PLB-985 cells. PLB-985 cells were differentiated with 1.25% DMSO for 4 d. The cells were stimulated with 1 mg/ml OZ (A), 50 ng/ml PMA (B), or 5 x 10$^{-7}$ M fMLP (C) for the indicated times at 37°C. The cell membranes were subjected to immunoprecipitation (IP) with anti-cPLA$_2$ antibodies, followed by immunoblot of p67$^{phox}$ and p47$^{phox}$. The bottom lane in each experiment shows the levels of cPLA$_2$ detected in the immunoprecipitates. Time course translocation of cPLA$_2$, p67$^{phox}$, and p47$^{phox}$ to cell membranes after stimulation with OZ (D), PMA (E), or fMLP (F) was detected by immunoblot analysis. 2 x 10$^6$ cell membrane equivalent were applied per lane. Superoxide production stimulated with either OZ (G), PMA (H), or fMLP (I) as detected by cytochrome C reduction. The results are from one representative experiment out of three presenting identical results.

**Figure 5.** The molar ratio between cPLA$_2$ and NADPH oxidase in the membranes of stimulated cells. Samples of recombinant cPLA$_2$ or p47$^{phox}$ and membranes (bottom inset) of neutrophils stimulated with 1 mg/ml OZ, 5 x 10$^{-7}$ M fMLP for 1 min or 50 ng/ml PMA for 3 min (top inset) were analyzed by SDS-PAGE electrophoresis. 2 x 10$^6$ cell membrane equivalents were applied per lane. The Western blots were analyzed using quantitative densitometry, and the relative optical density of each band was plotted against the amount of protein added per lane. The correlation coefficient for the cPLA$_2$ standard curve is 0.99 and for the p47$^{phox}$ standard curve is 0.92. The quantities of cPLA$_2$ and p47$^{phox}$ in membranes of stimulated cells were determined from the standard curves. The results are from one representative experiment out of three presenting identical results.
ated with DMSO but in contrast to PLB-985 cells differentiated with 1,25(OH)2D3 (unpublished data) or to PLB-985 cells differentiated with DMF (Pedruzzi et al., 2002). The kinetics of cPLA2 translocation stimulated with fMLP is depicted in Fig. 6 B. Maximal levels of cPLA2 were detected in the cell periphery 1 min after stimulation. 5 min after stimulation, a significant fraction of cPLA2 reappeared in the cytosol. This subcellular localization of cPLA2 stimulated with fMLP is similar to that detected by immunoblotting analysis (Fig. 4 D). The immediate translocation of cPLA2 to the membranes correlates with the rapid onset of superoxide upon stimulation (Fig. 4). Translocation of cPLA2 to the nucleus was studied by double labeling of cPLA2 and of lamin B as a marker for nuclear membranes (Olins et al., 2001). As shown in Fig. 6 C, activation of the cells with fMLP for 15 min induced translocation of cPLA2 to the nuclear membranes where it colocalized with lamin B. To determine the kinetics of cPLA2 translocation to the nuclear membranes, the nuclei of cells stimulated for different time duration were separated and analyzed for the presence of cPLA2 by immunoblot analyses. Translocation of cPLA2 to the nuclear membranes could be detected only 15 min after stimulation, in correlation with the time course of PGE2 secretion as shown in Fig. 6 D, which depicts stimulation of granulocyte-like PLB-985 cells after stimulation with 5 × 10−7 M fMLP. 2 × 10⁶ cell equivalent of nuclear fraction was applied in each lane. Equal amounts of the nuclear fractions were confirmed by detection of the levels of lamin B in each sample by immunoblot analysis.

The role of NADPH oxidase in targeting cPLA2 to the plasma membranes

To determine the role of NADPH oxidase in directing cPLA2 to the plasma membranes, we studied the subcellular location of cPLA2 in granulocyte-like gp91phox-deficient PLB-985 cells in which no oxidase assembly occurs (Bibertine-Kinkade et al., 1999). As shown by immunoblot analysis, cPLA2 could not be detected in membranes of granulocyte-like gp91phox-deficient PLB-985 cells stimulated for 1 min with OZ, PMA, or fMLP, whereas it was clearly detected in membrane fractions of stimulated granulocyte-like PLB-985 cells (Fig. 7 A). These results were confirmed by
confocal laser scanning microscopy, demonstrating that granulocyte-like PLB-985 and granulocyte-like gp91phox-deficient PLB-985 cells were stimulated as in the legend to Fig. 1. The cell membranes were separated on 10% SDS-PAGE and subjected to Western blot analysis against cPLA2. The results are from one representative experiment out of three that gave identical results. (B) Resting granulocyte-like and granulocyte-like gp91phox-deficient PLB-985 cells were double stained as in the legend to Fig. 6 A. cPLA2 is found in the cytosol of both type of cells, whereas gp91phox was detected only in granulocyte-like PLB-985 cells. (C) Granulocyte-like gp91phox-deficient PLB-985 cells and the cell line expressing retroviral gp91phox differentiated toward the granulocytic lineage before and after stimulation (as in the legend to Fig. 1) were double stained (as in the legend to Fig. 6 A). cPLA2 is found in the cytosol before and after activation in gp91phox-deficient PLB-985 cells but translocates to cell periphery after stimulation following the expression of retroviral gp91phox protein in these cells.

In vitro interaction between cytosolic oxidase components and cPLA2

To determine affinity binding between cPLA2 and the oxidase components, GST-p47phox and GST-p67phox fusion proteins were used. As shown in Fig. 8, cPLA2 was pulled down from lysates of stimulated, but not resting, neutrophils by either GST-p47phox or GST-p67phox fusion protein but not from lysates of resting cells, suggesting that the binding occurs only with the phosphorylated form of cPLA2. In addition, p67phox was also pulled down by GST-p47phox fusion protein, and p47phox was pulled down by GST-p67phox fusion protein. These experiments demonstrate the in vitro affinity binding between cPLA2 and the oxidase cytosolic components but do not determine whether cPLA2 is associated independently with both p47phox and p67phox or whether it is bound to one cytosolic component and takes up the other, since p47phox and p67phox are found in a complex at the membranes of activated cells. Similar results were obtained in granulocyte-like PLB-985 cells (unpublished data). Although cPLA2 was specifically bound to each of the oxidase cytosolic components in vitro using recombinant GST fusion proteins, such binding between cPLA2 and the soluble p47phox and p67phox in the cytosol did not occur in physiological conditions (Fig. 1). Rather cPLA2 binds the cytosolic oxidase components when found in the assembled NADPH oxidase after translocation to the plasma membrane, in accordance with our previous study demonstrating that the assembly of the oxidase is independent of cPLA2 (Dana et al., 1998). The behavior of the recombinant GST-fused cytosolic proteins may reflect the effects of the fusion on the conformation and exposure of sites interacting with cPLA2 that become exposed in the native proteins only when forming membrane complexes after cell stimulation.

Discussion

The results of the present study are the first to demonstrate that in granulocytic cells, which contain abundant levels of NADPH oxidase, stimulation induces an immediate and transient recruitment of cPLA2 to the plasma membranes, in addition to its already known translocation to the nuclear
membranes (Marshall et al., 2000). The translocation of cPLA₂ to the membrane fractions of stimulated neutrophils has been shown earlier by us and by others (Durston et al., 1994; Hazan et al., 1997; Marshall et al., 2000). In addition, several studies performed in a variety of cells including platelets, fibroblasts, and keratinocytes (Kast et al., 1993; Schalkwijk et al., 1995; McNicol and Shibou, 1998; Kitanani et al., 2000) have reported translocation of cPLA₂ to membrane fractions. However, the present study demonstrates that cPLA₂ is localized in the plasma membranes upon stimulation. The absence of cPLA₂ translocation to the plasma membranes in granulocyte-like gp91phox-deficient PLB-985 cells and the restoration of cPLA₂ translocation, after the expression of retroviral gp91phox protein, indicate that NADPH oxidase is responsible for anchoring cPLA₂ to the plasma membranes. The translocation of cPLA₂ to the membranes occurred after assembly of the oxidase and coincided with the onset of superoxide production (Fig. 4). The affinity binding between GST-p47phox or GST-p67phox with cPLA₂ only in lysates of stimulated cells (Fig. 8) suggests a direct binding with cPLA₂ in its phosphorylated form. In earlier studies (Dana et al., 1998), we have shown that AA by itself and not its metabolites is required for activation of the assembled NADPH oxidase enzyme. The colocalization of both enzymes (the assembled NADPH oxidase and cPLA₂) in the same compartment and their direct binding during the onset of superoxide production provides a means by which AA released by cPLA₂ is able to activate the assembled NADPH oxidase. The precise mechanism by which AA regulates the NADPH oxidase in whole cells is not yet known. Several studies suggest that AA induces structural changes in NADPH oxidase components that may promote productive interaction between the different oxidase subunits, thereby enabling full oxidase activation or directly affecting the function of flavocytochrome b (Foubert et al., 2002). The 1:25 molar ratio between cPLA₂ to the assembled oxidase in the membranes of stimulated cells (Fig. 5) indicates that one copy of cPLA₂ is able to provide sufficient levels of AA for several copies of the NADPH oxidase found in its environment. Our current observations are most consistent with a model in which cPLA₂-generated AA might be a cofactor acting in the intact phagocytic cell to enhance the affinity of the assembled NADPH oxidase for NADPH (Rubiniek and Levy, 1993) probably by induction of structural changes. cPLA₂ dissociates from the membranes before the oxidase cytosolic components (Fig. 4, D-F) and while the oxidase is still functioning (Fig. 4, G-I), suggesting that cPLA₂ is required for the activation of NADPH oxidase but not for maintaining its activity.

Recent studies have suggested that cPLA₂ has three functionally distinct domains: an NH₂-terminal C2 domain necessary for Ca²⁺-dependent phospholipid binding, a COOH-terminal Ca²⁺-independent catalytic region (Nalefski et al., 1994), and a putative pleckstrin homology domain within this region that is responsible for the interaction with phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂; Mosior et al., 1998). Subcellular localization of the various C2 domains upon cytoplasmic [Ca²⁺] elevation has been shown to correlate with their phospholipid binding specificity. It has also been shown that the cPLA₂-C2 domain has specificity to the phosphatidylycholine-rich nuclear envelope and ER (Gijon et al., 1997; Perisic et al., 1999; Hurley and Misra, 2000) and that aromatic and hydrophobic residues in the calcium binding loop of the cPLA₂-C2 domain are important for its lipid specificity (Stahelin et al., 2003b). In contrast, the PKCα-C2 domain has specificity to the phosphatidylserine-rich plasma membranes (Oancea and Meyer, 1998; Corbalan-Garcia et al., 1999) and ASn₁₈₉ plays a key role in this specificity (Stahelin et al., 2003b). Several studies have shown that cPLA₂ translocates from the cytosol to the nuclear membrane and to the ER by calcium ionophores or agonists such as histamine or IgE/antigen, which increase cytoplasmic [Ca²⁺] in a variety of cells (Peters-Golden and McNish, 1993; Glover et al., 1995; Schievella et al., 1995; Sierra-Honigmann et al., 1996). However, PMA, which does not induce an increase in cytoplasmic [Ca²⁺], caused activation and translocation of cPLA₂ (Hazan et al., 1997; Qiu et al., 1998), suggesting the existence of alternative pathways that induce translocation of cPLA₂ not involving elevation of cytoplasmic [Ca²⁺], which is necessary for the C2 domain phospholipid binding. The results of our present study suggest that the cPLA₂-C2 domain does not participate in the translocation of cPLA₂ to the plasma membranes, since cPLA₂ translocation is inconsistent with its C2 domain phospholipid binding specificity. In addition, cPLA₂ translocation to the cell periphery did not occur in the absence of a functional NADPH oxidase (Fig. 7), and it can be induced with PMA (Fig. 3 and Fig. 6 A), which does not cause elevation of cytoplasmic [Ca²⁺]. However, we cannot rule out the possibility that the binding of cPLA₂ to the assembled oxidase is mediated through the cPLA₂-C2 domain. Similar to our observation that the C2 domain–containing cPLA₂ binds NADPH oxidase, a recent study (McAdara Berkowitz et al., 2001) has shown that C2 domain–containing protein JFC₁, which is restricted to the plasma membranes/secretory vesicles, binds p67phox without affecting the interaction between p47phox and p67phox. High levels of PtdIns(4,5)P₂ have been detected in neutrophil plasma membranes upon stimulation (Botelho et al., 2000; Martin, 2001), and PtdIns(4,5)P₂ has been shown to display a particularly dramatic effect on the activity of cPLA₂ (Mosior et al., 1998). However, it seems that the cPLA₂ pleckstrin homology domain by itself does not play a critical role in targeting cPLA₂ to the plasma membranes, since in the absence of the functional oxidase cPLA₂ did not translocate to this compartment, as shown in gp91phox-deficient granulocytic PLB-985 cells (Fig. 7). Thus, the assembled NADPH oxidase appears to be the major determinant in directing cPLA₂ to the plasma membranes, although the interaction sites among cPLA₂, NADPH oxidase, and the plasma membranes are not yet defined.

Eicosanoid generation has been shown to be regulated in part by perinuclear envelope localization or translocation of individual enzymes of leukotriene and prostaglandin biosynthesis (Ueno et al., 2001). The perinuclear translocation of cPLA₂, shown in a variety of cells, is in agreement with its role in leukotriene and prostaglandin formation. Similarly, our present study demonstrates a correlation between the kinetics of cPLA₂ translocation to nuclear membranes and PGE₂ production in stimulated granulocyte-like PLB-985 cells (Fig. 6, C and D). The differences in the kinetics of su-
peroxide production and PGE₂ formation in PLB-985 cells differentiated with DMSO enabled us to efficiently follow the distribution of cPLA₂ during stimulation, from early translocation to the cell periphery to later translocation to the nuclear envelope. Thus, the mechanism which permits the participation of cPLA₂ in two different processes in the same cell (regulation of NADPH oxidase and eicosanoid production) is controlled by localization of the enzyme in different subcellular compartments.

In conclusion, the use of combined biochemical and microscopical approaches enhanced our ability to gain better insight into cellular processes and to clearly demonstrate that upon activation of peripheral blood neutrophils and granulocyte-like PLB-985 cells, both of which contain abundant cPLA₂ in the cell periphery of stimulated granulocyte-like PLB-985 cells, both of which contain abundant cPLA₂, the enzyme translocates to the plasma membranes where it binds the assembled oxidase complex and releases AA, which promotes oxidase activity. The absence of cPLA₂ in the cell periphery of stimulated granulocyte-like gp91phox-deficient PLB-985 cells and its translocalization after the expression of retroviral gp91phox protein in these cells indicate that cPLA₂ is anchored to the plasma membranes by the assembled oxidase. The physical association between these two enzymes enables the regulation of NADPH oxidase by cPLA₂-generating AA. The novel dual subcellular localization of cPLA₂ in different compartments, first in the plasma membranes and then in the nucleus, provides a molecular mechanism for the participation of cPLA₂ in different processes in the same cells. The exact binding sites between these two enzymes and the target for AA action are currently under investigation.

Materials and methods
Neutrophil purification
Neutrophils were separated by Ficoll/Hypaque centrifugation, dextran sedimentation, and hypotonic lysis of erythrocytes (Dana et al., 1998).

Cell culture and differentiation
PLB-985 and gp91phox-deficient PLB-985 cells lacking the expression of normal gp91phox (provided by M.C. Dinauer, James Whitcomb Riley Hospital for Children, Indianapolis, IN) were grown in stationary suspension culture in RPMI-1640 as described earlier (Dana et al., 1998). The optimal concentration of 1.2% of DMSO was added to 2 × 10⁵ PLB-985 cells/ml at their logarithm growth phase to induce differentiation toward the granulocyte phenotype. Mac-1 antigen determination was detected by indirect immunofluorescence as described previously (Hazav et al., 1989).

Isolation of membrane and cytosol fractions
Neutrophils were suspended at 10⁵ cells/ml in phosphate buffer saline and treated with 5 mM disopropylfluorophosphate for 30 min at room temperature before stimulation. Membrane and cytosol fractions were prepared as described previously (Levy et al., 1990).

Immunoprecipitation
Coat antisera raised against recombinant p47phox or p67phox (Leto et al., 1991) or rabbit antisera raised against cPLA₂ (Hazan et al., 1997) was added to 3 × 10⁴ membranes or cytosol cell equivalents in 500 μl solubilization buffer (150 mM NaCl, 5 mM EDTA, 10 mM Tris, pH 7.5, 1% sodium deoxycholate, and 1% NP-40), and they were incubated on ice overnight. The extracts were brought to a volume of 1 ml in solubilization buffer containing 30 μl of 50% slurry of recombinant protein G-sepharose. The samples were tumbled end-over-end for 1 h and washed twice with 1 ml solubilization buffer containing 20% (wt/vol) sucrose and 0.15% (wt/vol) BSA and twice with 1 ml solubilization buffer containing 20% sucrose. The samples were boiled in SDS sample buffer and electrophoresed on a 10 or 7% SDS-PAGE. For detection of protein translocation, cell membranes (2 × 10⁶ cell equivalent) were separated by SDS-PAGE electrophoresis. The resolved proteins were electrophotographically transferred to nitrocellulose, and the detection of cPLA₂ or the oxidase components was analyzed as described previously (Hazan et al., 1997).

Subcellular fractionation
Subcellular fractionation was performed as described by others (Keijser et al., 1999). Neutrophils (5 × 10⁷) treated with disopropylfluorophosphate were suspended in relaxation buffer (as described earlier; Levy et al., 1990) and disrupted by nitrogen cavitation at 400 pounds per square inch. Nuclei and unbroken cells were pelleted by centrifugation at 500 g for 10 min at 4°C. The supernatant was decanted and loaded onto a precooled discontinuous density gradient Percoll, and 10% concentrated relaxation buffer and distilled water were mixed to give solutions of densities of 1.05, 1.10, and 1.12 g/ml. The gradients were centrifuged at 32,800 g for 35 min at 4°C using a fixed angle Beckman Coulter JA20 rotor. Four visible bands were collected, and the markers for azurophil granules (α), specific granules (β), and plasma membranes (γ) were analyzed as described previously (Kaufman et al., 1996).

Immunofluorescence microscopy
Preparation of labeled cells was done as described by others (Bingham et al., 1999) with some modifications. Cells were adhered on coverslips for 30 min at 37°C. The cells were stimulated with various agonists for the desired duration and fixed with 3% (wt/vol) formaldehyde. The first antibodies against cPLA₂, gp91phox, and lamin B (Santa Cruz Biotechnology) dissolved in PBS containing 0.2% saponin were added for 1 h at room temperature. Cy2- or Cy3-conjugated antibodies (Jackson Immunoresearch Laboratories) were used as secondary antibodies. The fluorescence was visualized using a four channel Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss Microlmaging, Inc.). The LSM 510 software was used for imaging cPLA₂, gp91phox, and lamin B localization.

Retroviral transduction of gp91phox-deficient PLB-985 cells
Retroviral gp91phox was expressed in gp91phox-deficient PLB-985 cells (Zhen et al., 1993) as done in our previous study (Pessac et al., 2001).

Affinity-binding assay
The GST fusion proteins were affinity purified on glutathione-sepharose as described previously (Leto et al., 1994). 50 μl (1 μg) of GST-p47phox or GST-p67phox bound to glutathione-sepharose beads was added to lysates of 5 × 10⁵ resting or stimulated neutrophils prepared as described before (Hazan-Haley et al., 2000) and was tumbled end-over-end for 2 h at 4°C. Bound proteins were washed three times in 15 vol of ice cold 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, 0.15 mM PMSF, and 10 mM Pipes (pH 7.5), eluted with 2% SDS, and analyzed by SDS-PAGE followed by immunoblotting with the appropriate antibodies.

Superoxide anion measurements
The production of superoxide anion (O₂⁻) by intact cells was measured as the superoxide dismutase inhibitable reduction of ferricytochrome c (Dana et al., 1998).

Preparation of nuclei
Nuclei were separated from granulocyte-like PLB cells (2 × 10⁷ cells) before and after stimulation as described by others in neutrophils (Surette et al., 1998). Stimulated cells were pelleted and resuspended in 600 μl of ice-cold NP-40 lysis buffer (0.1% NP-40, 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF). The cells were vortexed for 15 s, kept on ice for 5 min, and centrifuged at 300 g for 10 min at 4°C. The resulting pellets (the nuclei containing fractions) were then immediately solubilized in electrophoresis sample buffer and processed for SDS-PAGE and immunoblot determination of cPLA₂ and the nuclear lamin B. Nuclear integrity was verified directly by light microscopy, which also revealed that intact cells were rarely observed in nuclei-containing fraction (<2%).

PGE₂ determination
PGE₂ levels were determined in the supernatant of stimulated cells by RIA using commercial kits (NEN Life Science Products). The samples were immediately stored at −70°C and analyzed during 1 wk of the experiments.

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