Requirement of JNK-Mediated Phosphorylation for Translocation of Group IVA Phospholipase A_2 to Phagosomes in Human Macrophages[1]

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Eicosanoids are a broad family of lipids that play a critical role in host defense against bacterial and fungal infections. The first enzyme in the metabolic pathway for the generation of eicosanoids is group IVA phospholipase A_2, also known as cytosolic phospholipase $A_2\alpha$ (cPLA$_2\alpha$). During phagocytosis, cPLA$_2\alpha$ has been found to translocate to the phagosome, although the molecular mechanism involved in such a translocation has not been elucidated. By using enhanced GFP-tagged proteins we show in this work that a nonphosphorylatable cPLA$_2\alpha$ mutant (S505A) does not translocate to the phagosomes, but a mutant that mimics phosphorylation on Ser^{505} (S505E) does it so readily. During phagocytosis, endogenous cPLA$_2\alpha$ is phosphorylated at Ser^{505}, and inhibitors of JNK, but not of other related kinases such as p38 or the extracellular-regulated kinases 1 and 2, completely block such a phosphorylation. Inhibition of JNK activity also inhibits the translocation of cPLA$_2\alpha$ to phagosomal membranes, as well as arachidonic acid release to the extracellular medium. Moreover, the S505E mutant makes the enzyme refractory to JNK inhibition, translocating normally to phagosomal membranes. Collectively, these data support a key role for JNK-mediated cPLA$_2\alpha$ phosphorylation at Ser^{505} in the sequence of events leading to translocation and activation of the enzyme to phagosomal membranes in human macrophages. ***The Journal of Immunology,*** **2009, 183: 2767–2774.**

Eicosanoids produced by phagocytic cells such us neutrophils and macrophages play a key role in the innate immune system. In addition to their demonstrated pro and antiinflammatory properties, phagocyte-derived eicosanoids have been implicated in the control of infections. In in vitro and in vivo models, the eicosanoids have been shown to modulate the microbicidal activity of phagocytes (1–5).

The first enzyme in the metabolic pathway leading to stimulated eicosanoid production by phagocytic cells is the group IVA phospholipase A_2, also known as cytosolic phospholipase $A_2\alpha$ (cPLA$_2\alpha$)[3] (6–9). cPLA$_2\alpha$ catalyzes the stimulus-dependent release of free arachidonic acid (AA) from its phospholipid storage sites. Free AA will then be available for eicosanoid biosynthesis. cPLA$_2\alpha$ has been found to translocate to the phagosomes after stimulation of the phagocytes, and such a translocation appears to be key for eicosanoid generation and killing of the ingested microbe (10–13).

Because of the key role of cPLA$_2\alpha$ in generating lipid mediators, the enzyme is subjected to a very tight regulation in intact cells (14). The intracellular Ca^{2+} level regulates the ability of cPLA$_2\alpha$ to associate to cellular membranes, which is mediated by a C2 domain present at the N-terminal half of the protein (15). The C2 domain also contains a site for binding to ceramide-1-phosphate, which allosterically activates the enzyme and increases the residence time of the enzyme in membranes (16). The enzyme also possesses a binding site for anionic phospholipids, particularly phosphatidylinositol 4,5-bisphosphate (PIP_2), that may also be involved in anchoring the cPLA$_2\alpha$ to cellular membranes (17–21).

On the other hand, cPLA$_2\alpha$ can be phosphorylated at various sites (e.g., Ser^{505}, Ser^{515}, and Ser^{727}), which may profoundly affect the activity of the enzyme under activation conditions (22). The biological significance of the phosphorylation of Ser^{515} and Ser^{727} is not yet well understood. It has been suggested that phosphorylation at Ser^{727} disrupts the interaction of the enzyme with p11 and annexin A2, resulting in the proper translocation of cPLA$_2\alpha$ to membranes (23), and that phosphorylation at Ser^{515} serves to facilitate the subsequent phosphorylation of cPLA$_2$a at Ser^{505}, which is the one that leads to increased catalytic activity (24, 25).

Phosphorylation of cPLA$_2\alpha$ at Ser^{505} results in an ~2- to 2.5-fold increase in its specific activity (26). Much of the data currently available in the literature suggest that cPLA$_2$ is phosphorylated and activated by either the ERK1/2 (p44/p42) or p38 isoforms of MAPK; the activation mechanism may vary with the cell type and activation regimen (14, 27). In this regard, we have previously shown that phosphorylation activation of the AA-releasing cPLA$_2$ at Ser^{505} occurs during stimulation of mouse macrophage cells by a variety of TLR agonists, and that this phosphorylation is effected by the extracellular-regulated kinases p42 (ERK1) and p44 (ERK2) (28–30). Interestingly, other studies have also suggested

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[3] Abbreviations used in this paper: cPLA$_2\alpha$, cytosolic phospholipase $A_2\alpha$ (group IVA cytosolic phospholipase A_2); AA, arachidonic acid; bis-BODIPY FL C11-PC, 1,2-bis-(4,4-difluoro-5,7-dimethyl-4-bora-3*a*,4*a*-diaza-*s*-indecane-3-undecanoyl)-*sn*-glycero-3-phosphocholine; ECFP, enhanced cyan fluorescent protein; EGFP, enhanced GFP; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLA_2, phospholipase A_2.

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that cPLA$_2\alpha$ phosphorylation on Ser^{505} may also be involved in regulating the affinity of the enzyme for binding to cellular membranes (31). Thus, phosphorylation of cPLA$_2\alpha$ on Ser^{505} may act to increase the phospholipid binding affinity at low calcium concentrations probably by promoting a conformational change that allows a better interaction of the catalytic domain with the membrane phospholipid (31).

In the present study we have found that cPLA$_2\alpha$ phosphorylation on Ser^{505} appears to be key for translocation of the enzyme to phagosomes in human macrophages. Furthermore, we have uncovered JNK as the enzyme that phosphorylates cPLA$_2\alpha$ during phagocytosis and is responsible for the proper translocation of the enzyme. These studies provide novel insights on the complex regulation of cPLA$_2\alpha$ and hence on the production of eicosanoids during phagocytosis of microorganisms in the human system.

Materials and Methods

Materials

[5,6,8,9,11,12,14,15-^{3}H]AA (200 Ci/mmol) was purchased from Amersham Ibérica. The Abs anti-cPLA$_2\alpha$ and anti-phospho-Ser^{505} cPLA$_2\alpha$, as well as anti-phospho-MAPKs p42, p44, p38, and JNK, were from Cell Signaling Technology. Zymosan labeled with Alexa Fluor 594 and bis-BODIPY FL C11-PC (1,2-bis-(4,4-difluoro-5,7-dimethyl-4-bora-3*a*,4*a*-diaza-*s*-indecane-3-undecanoyl)-*sn*-glycero-3-phosphocholine) were from Molecular Probes. Ficoll-Paque Plus was from GE Healthcare. Gentamicin was purchased from BioWhittaker/Cambrex. Human macrophage nucleofection solution was from Amaxa. Macrophage serum-free medium and RPMI 1640 were purchased from Invitrogen. ECL chemiluminescence substrate was from Amersham Ibérica. All other reagents were from Sigma-Aldrich.

Plasmids

The construct enhanced GFP (EGFP)-cPLA$_2\alpha$ has been described elsewhere (21, 32, 33). For the construction of the enhanced cyan fluorescent protein (ECFP)-S505A-cPLA$_2\alpha$, the EGFP was replaced with ECFP in the plasmid EGFP-cPLA$_2\alpha$ by using the restriction enzymes *Age*I and *Bsr*GI. Then, Ser^{505} was substituted by Ala (S505A) using the QuickChange XL Site-Directed Mutagenesis kit from Stratagene and the oligonucleotides 5′-CAATACATCTTATCCACTGGCGCCTTTGAGTGACTT-3′ (forward) and 5′-GCAAAGTCACTCAAAGGCGCCAGTGGATAAGATGTA-3′ (reverse). For the mutagenesis of Ser^{505} to Glu (S505E) the oligonucleotides used were: 5′-GAATCTCAATACATCTTATCCACTGGAGCCTTTGAGTGACTTTGC-3′ (forward) and 5′-GCAAAGTCACTCAAAGGCTCCAGTGGATAAGATGTATTGAGATTC-3′ (reverse). Mutagenesis was confirmed by sequencing.

Cells

Human macrophages were obtained from buffy coats of healthy volunteer donors obtained from the Centro de Hemoterapia y Hemodonación de Castilla y León (Valladolid, Spain). Briefly, blood cells were diluted 1/1 with PBS, layered over a cushion of Ficoll-Paque, and centrifuged at 750 × *g* during 30 min. The mononuclear cellular layer was then recovered and washed three times with PBS, resuspended in RPMI 1640 supplemented with 2 mM L-glutamine and 40 μg/ml gentamicin, and allowed to adhere to plastic in sterile dishes for 2 h. Nonadherent cells were then removed by extensively washing with PBS. Macrophage differentiation was achieved by incubating the adhered monocytes in RPMI 1640 supplemented with 2 mM L-glutamine, 40 μg/ml gentamicin, and heat-inactivated 5% human serum for 2 wk in the absence of exogenous cytokine mixtures. When needed, human macrophages were transfected by the nucleofection technique (Amaxa), following the kit specifications for human macrophages. Briefly, cells were harvested by treatment with trypsin for 90 min and then by gentle scraping. After washing, the cells were resuspended in 100 μl of human macrophage nucleofector solution plus 5 μg of plasmid. Nucleofection was conducted using the program Y-010, and the cells were resuspended in 400 μl of macrophage serum-free medium (Invitrogen) plus 5% heat-inactivated human serum. Cell viability under all experimental conditions utilized in this study remained always >95%.

Confocal microscopy

Cells were seeded in MatTek dishes and allowed to adhere for 24 h. Medium was then changed by HBSS with 10 mM HEPES and 1.3 mM $CaCl_2$. For some experiments cells were incubated without $CaCl_2$, which was added when needed. Fluorescence was monitored by confocal microscopy using a Bio-Rad Radiance 2100 laser scanning system coupled to a Nikon TE-2000U with a thermostatized chamber (Warner Instruments). The objective was CFI Plan Apochromat 60×, 1.4 numerical aperture, oil immersion. The fluorescence of ECFP was monitored at 457 nm argon excitation using the combination of a long pass barrier filter HQ470LP and a short pass filter HQ520SP. The fluorescence of EGFP was monitored at 488 nm argon excitation using the combination of a long pass filter HQ500LP and a short pass filter HQ560SP. The Alexa Fluor 594 fluorescence was monitored at 543-nm HeNe excitation using a long band pass filter HQ570LP.

AA release

Cells were labeled with 0.5 μCi/ml [^{3}H]AA for 20 h. Afterward, the cells were extensively washed with PBS containing with 0.5 mg/ml fatty acid-free BSA to remove unincorporated fatty acid. Cells were then stimulated for 1 h in RPMI 1640 medium containing 0.5 mg/ml fatty acid-free BSA to blunt fatty acid reacylation (34), and supernatants were removed. When inhibitors were used, the cells were pretreated with them for 30 min before stimulation. Cell monolayers were overlaid with ice-cold phosphate buffer containing 0.05% Triton X-100 and scraped. Radioactivity was quantified by liquid scintillation counting, and AA release was referred to total radioactivity for each condition.

Immunoblot

Cells were lysed and 50–100 μg of total protein was separated by standard 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). Primary Abs were used as recommended by the manufacturer, and HRP linked to anti-rabbit donkey IgG or anti-mouse sheep IgG was used as a secondary Ab (1/5000 in PBS (pH 7.4) with 0.5% defatted dry milk and 0.1% Tween 20). Specific proteins were visualized using ECL chemiluminescent substrate (Amersham) and a digital image acquisition system (VersaDoc; Bio-Rad).

Preparation of opsonized zymosan

Zymosan was prepared as described elsewhere (35, 36). Briefly, zymosan particles were suspended in PBS, boiled for 60 min, and washed three times. The particles were opsonized by incubation with heat-inactivated complement human serum for 20 min at 37°C, in a ratio of 1 ml of serum per 3 mg of particles. Zymosan was then washed three times with PBS and sonicated in RPMI 1640 or HBSS during 15 min. This opsonization gives responses that are primarily mediated by IgG receptors. No endogenous phospholipase A$_2$ activity was detected in the zymosan batches used in this study, as assessed by in vitro activity assay (37, 38).

Synchronized phagocytosis

These experiments were conducted as described elsewhere with minor modifications (39). Macrophages were seeded over glass coverslips, allowed to adhere, and then washed with RPMI 1640 supplemented with 40 μg/ml gentamicin. Cells were then kept at 4°C for 5 min, and opsonized zymosan was then added. After 15 min of incubation, coverslips were transferred to plates with RPMI 1640 at 37°C, and the phagocytosis was allowed to proceed for different periods of time. Reactions were stopped by fixation with 3% paraformaldehyde and 3% sacarose for 15 min if they were to be analyzed by microscopy. After three washes with PBS, the coverslips were mounted in glass slides with antifade medium. Samples were then analyzed by confocal microscopy.

Frustrated phagocytosis

This procedure was conducted as described elsewhere (40). Briefly, human macrophages were dislodged from the culture plates by trypsin treatment and were resuspended in RPMI 1640 containing 10 mM HEPES and 2 mM EDTA. Cells were gently stirred for 2–3 h to allow for the reexpression of membrane receptors. Cells were then washed, resuspended in RPMI 1640 containing 10 mM HEPES and 2 mM $MgCl_2$, and plated over MatTek dishes treated or not with 10 mg/ml pure IgG. After 30 min at 37°C, the cells were monitored by confocal microscopy. Some images were obtained in the *x*,*z*-axis to have a better view of the cell membranes attached to the glass. In some experiments, the cells were labeled with 5 μM bis-BODIPY FL C$_{11}$-PC for 30 min, washed twice, and processed for frustrated phagocytosis. Fluorescence was monitored by confocal microscopy using 488-nm argon excitation and the combination of a HQ500 long band pass filter and HQ560 short band pass filter.

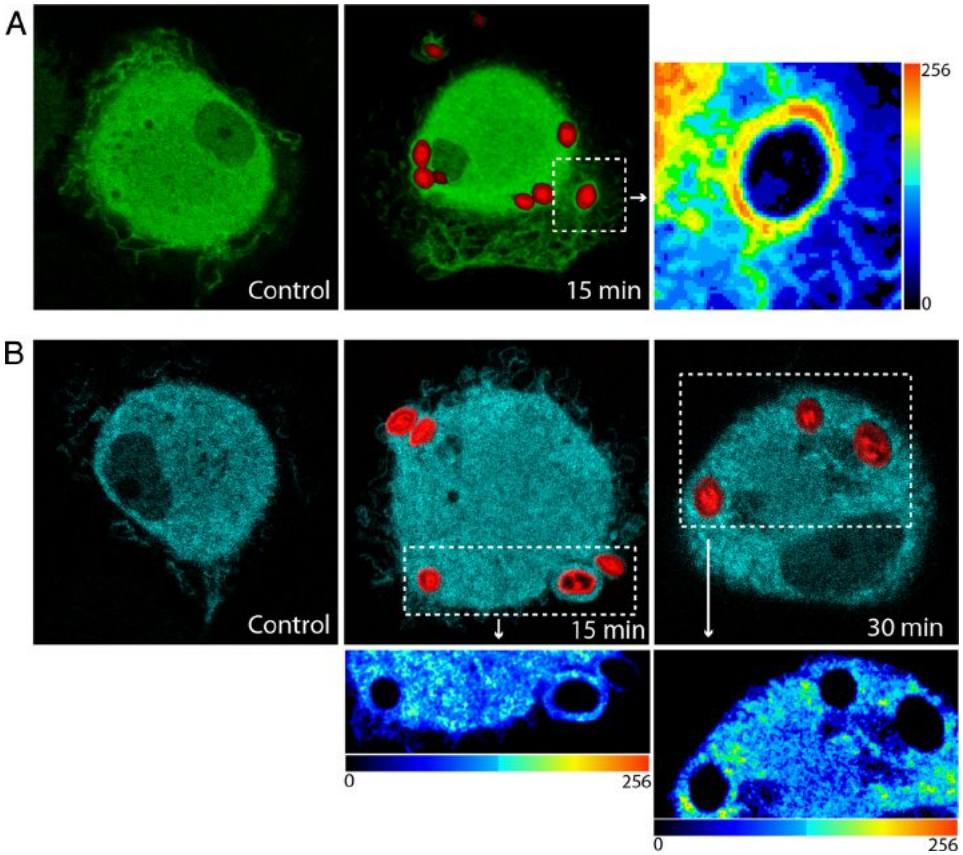

FIGURE 1. Analysis of the translocation of EGFP-cPLA$_2\alpha$ and ECFP-S505A-cPLA$_2\alpha$ to phagosomes in human macrophages. Cells transfected with the construct EGFP-cPLA$_2\alpha$ (*A*) or ECFP-S505A-cPLA$_2\alpha$ (*B*) were subjected to synchronized phagocytosis with opsonized Alexa Fluor 594-zymosan, fixed at different time points, as indicated, and analyzed by confocal microscopy (*A* and *B*). Some phagosomes were selected (dotted squares) and enlarged, showing the intensity of the fluorescence in pseudocolor. Selected cells are representative of most cells in the samples analyzed.

Results

Phosphorylation of cPLA$_2\alpha$ is necessary for translocation to the phagosome in human macrophages

In addition to internal membranes such as those of the Golgi, endoplasmic reticulum, or perinuclear membranes, cPLA$_2\alpha$ also translocates to phagosomes during the phagocytic process (11). However, the molecular mechanism underlying this translocation remains unknown. To address the role, if any, of cPLA$_2\alpha$ phosphorylation at Ser^{505} in translocation of the enzyme to the phagosome, we utilized human macrophages transfected with a mutant, ECFP-S505-cPLA$_2\alpha$, where Ser at position 505 is replaced by Ala, thereby preventing phosphorylation at that position. The cells were treated with opsonized zymosan as a phagocytic stimulus for different periods of time, and the movement of cPLA$_2\alpha$ was studied by confocal microscopy. Fig. 1 shows that, while the wild-type EGFP-cPLA$_2\alpha$ readily translocated to phagosomes, the mutant ECFP-S505A-cPLA$_2\alpha$ failed to do so at any time tested up to 120 min.

To confirm the above findings under different conditions, an experiment of frustrated phagocytosis was performed, utilizing glass plates coated with IgG (40–45). In this kind of experiment, the IgG-coated glass would represent the phagocytosable particle, and the macrophage membranes closer to the glass would behave as a nascent phagosome (40–45). Utilizing this procedure, the macrophages exposed to IgG-coated glass surfaces responded by translocating the wild-type EGFP-cPLA$_2\alpha$ to the membranes more proximal to the glass surface, that is, the "forming phagosome" (Fig. 2*A*). In contrast, the mutant ECFP-S505A-cPLA$_2\alpha$ did not move (Fig. 2*B*). Collectively, these results uncover the requirement for cPLA$_2$ phosphorylation on Ser^{505} for translocation to the phagosomal membrane.

cPLA$_2\alpha$ phosphorylation during phagocytosis

To identify the kinase implicated in the phosphorylation of cPLA$_2\alpha$ on Ser^{505} during phagocytosis, the phosphorylation/activation state of various members of MAP kinase family were examined in human macrophages after stimulation with opsonized zymosan as a phagocytic stimulus. As shown in Fig. 3, phosphorylation of cPLA$_2\alpha$ was detected in a time-dependent manner. Because the Ab used for cPLA$_2\alpha$ in these experiments is specific for phospho-Ser^{505}, the increases observed in cPLA$_2\alpha$ phosphorylation actually reflect increases in the phosphorylation of the Ser^{505}, and not in other residues. Of the MAP kinase family members examined, only the phosphorylation of JNK p54 was clearly observed with a time course that corresponded well with that of cPLA$_2\alpha$ (Fig. 3). Minor time-dependent increases in the phosphorylation state of ERK1 (p42), ERK2 (p44), and JNK p46 were also detected, and the phosphorylation state of p38 did not appreciably change (Fig. 3).

In the next series of experiments, well-established kinase inhibitors were utilized to identify the kinase involved in cPLA$_2\alpha$ phosphorylation on Ser^{505}. The methoxyflavone derivative PD98059 was used to selectively inhibit the ERKs (46), the pyridinyl-imidazole SB203580 was used to selectively inhibit p38 (47), and the anthrapyrazolone inhibitor SP600125 was used to selectively block JNK (48). Of these, only SP600125 quantitatively blocked the phosphorylation of cPLA$_2\alpha$ (Fig. 3), suggesting that JNK is the kinase that phosphorylates cPLA$_2\alpha$ in stimulated human macrophages. This is a remarkable and unexpected finding, since many of the data available in the literature implicate p38 and/or the ERKs but not JNK in this process (14, 27). In accordance with the above data, we also found that SP600125 strongly

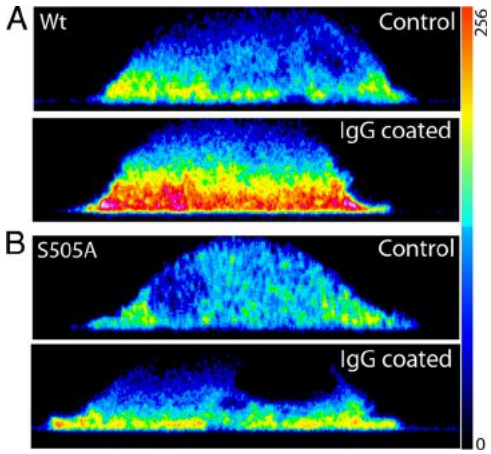

FIGURE 2. cPLA$_2\alpha$ translocation during frustrated phagocytosis in human macrophages. Cells transfected with the construct EGFP-cPLA$_2\alpha$ (*A*) or ECFP-S505A-cPLA$_2\alpha$ (*B*) were plated over noncoated glass (Control) or IgG-coated glass (IgG coated) and allowed to adhere for 30 min. Fluorescence was analyzed by confocal microscopy. Images of the *x*,*y*-axis of the cells were taken, and the intensity of the fluorescence is shown in pseudocolor. The cells in the images are representative of most cells in the same preparation.

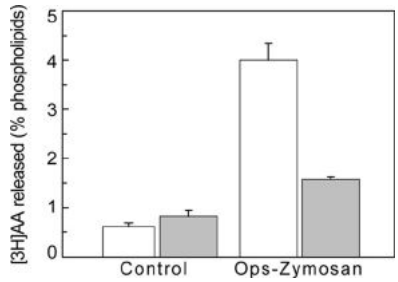

FIGURE 4. Effect of the JNK inhibitor SP600125 on AA release. Human macrophages labeled with [^{3}H]AA were treated with vehicle (Control) or opsonized zymosan for 60 min (Ops-Zymosan) in the absence (open bars) or presence (gray bars) of 10 μM SP600125. [^{3}H]AA release was analyzed as described under *Materials and Methods*. The data are representative of at least three different experiments done in triplicates and data are given as mean values ± SD.

inhibited the extracellular release of AA induced by opsonized zymosan in the human macrophages (Fig. 4).

JNK regulates the translocation of cPLA$_2\alpha$ to phagosomal membranes

Human macrophages transfected with the construct EGFP-cPLA$_2\alpha$ were subjected to synchronized phagocytosis in the presence of the above-mentioned MAP kinase inhibitors. As shown in Fig. 5, in the cells pretreated with the JNK inhibitor SP600125, the EGFP-cPLA$_2\alpha$ construct did not move to phagosomal membranes after the cells were exposed to opsonized zymosan. Fig. 5 also shows that neither PD98059 nor the SB203580 blunted the translocation of EGFP-cPLA$_2\alpha$ to the phagosomes, thus highlighting the specificity of action of SP600125.

The effect of the MAP kinase inhibitors was also investigated in frustrated phagocytosis experiments. As shown in Fig. 6, translocation of EGFP-cPLA$_2\alpha$ to the membranes closer to the IgG-coated glass (forming phagosome) was not inhibited by PD98059 or SB203580, but was very strongly diminished in the presence of the JNK inhibitor SP600125. Collectively, these experiments demonstrate that JNK regulates the translocation of cPLA$_2\alpha$ to the phagosome.

To verify that the cPLA$_2\alpha$ translocates to the phagosome in a functionally active form, we utilized cells loaded with fluorogenic phospholipase substrate bis-BODIPY FL C11-PC (49) and subjected them to a frustrated phagocytosis assay. A dramatic increase in fluorescence was observed in the proximity of the IgG-coated glass (Fig. 7), indicating that an A-type phospholipase is acting at that place (where the "phagosome" is being initiated). That such a phospholipase is actually cPLA$_2\alpha$ was verified by conducted experiments in the presence of the specific cPLA$_2\alpha$ inhibitor pyrrophenone (50). Pyrrophenone, at doses as low as 1 μM, strongly diminished the fluorescence increase in the cells exposed to IgG-coated glass, indicating that such a fluorescence increase is due to cPLA$_2\alpha$ activation. The increase in fluorescence observed in cells attached to the IgG-coated glass was also completely inhibited by the JNK inhibitor SP600125 (Fig. 7). Collectively, these data indicate that JNK-mediated cPLA$_2\alpha$ phosphorylation during phagocytosis allows the proper translocation of cPLA$_2\alpha$ to the phagocytic cup and attendant phospholipid hydrolysis to yield free AA.

Mutation of Ser^{505} to glutamic acid mimics cPLA$_2\alpha$

To further characterize the role of Ser^{505} phosphorylation in cPLA$_2\alpha$ translocation to the phagosome, a mutant was constructed where Ser^{505} was changed to glutamic acid, to mimic phosphorylation at that residue (31) (ECFP-S505E-cPLA$_2\alpha$). Human macrophages were transfected with the mutant ECFP-S505E-cPLA$_2\alpha$

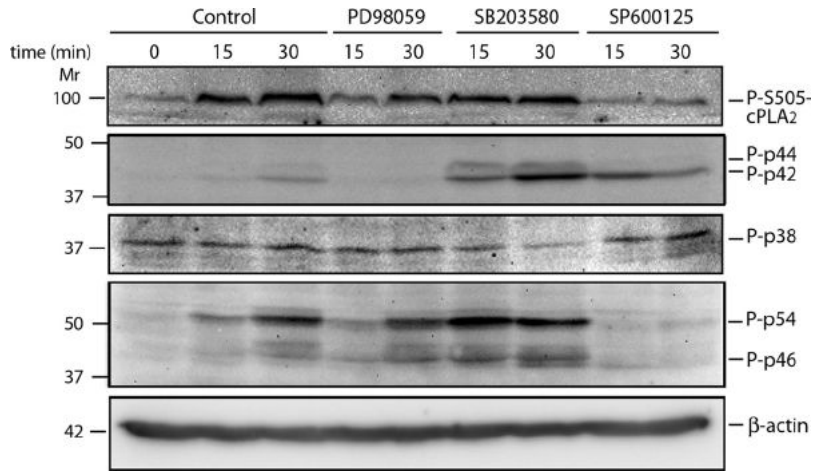

FIGURE 3. Analysis of the kinases implicated in cPLA$_2\alpha$ phosphorylation during phagocytosis of opsonized zymosan. Human macrophages were stimulated with opsonized zymosan at different periods of time, as indicated. Some of the samples were preincubated with specific kinase inhibitors, 10 μM PD98059, 10 μM SB203580, and 10 μM SP600125, as indicated. Phosphorylation of cPLA$_2\alpha$ (S505), ERKs p42–p44, p38, and JNKs p46–p54 was analyzed by immunoblot using specific anti-phospho Abs. The results are representative of three different experiments.

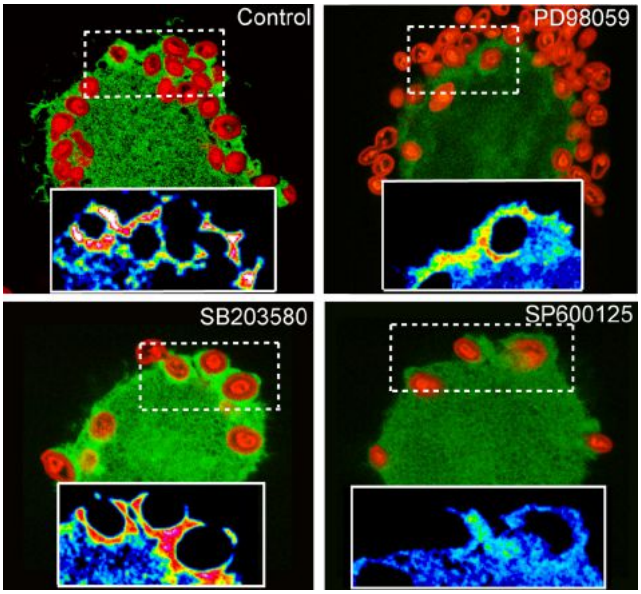

FIGURE 5. Effect of kinase inhibitors on the translocation of $cPLA_2\alpha$ to phagosomes. Human macrophages were transfected with the construct EGFP-$cPLA_2\alpha$, preincubated with 10 μM of the indicated inhibitors for 30 min, and subjected to synchronized phagocytosis experiments with opsonized Alexa Fluor 594-zymosan for 30 min, fixed, and analyzed by confocal microscopy. Some phagosomes have been selected (dotted squares) and enlarged, showing the intensity of the fluorescence in pseudocolor. Selected cells are representative of most cells in the samples analyzed.

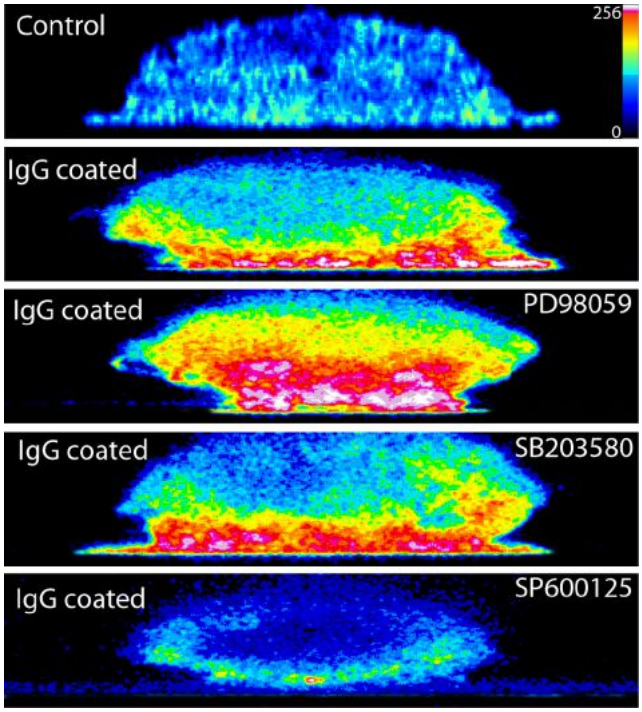

FIGURE 6. Effect of kinase inhibitors on the translocation of $cPLA_2\alpha$ during frustrated phagocytosis. EGFP-$cPLA_2\alpha$-transfected human macrophages were subjected to frustrated phagocytosis over noncoated glass (Control) or IgG-coated glass (IgG coated) in the absence or presence of 10 μM PD98059, SB203580, and SP600125, as indicated. Cells were analyzed by confocal microscopy, and images of the *x*,*z*-axis of the cells were taken. The intensity of the fluorescence is shown in pseudocolor. Representative cells of more than 30 analyzed are shown.

and subjected to frustrated phagocytosis. As shown in Fig. 8, the behavior of ECFP-S505E-$PLA_2\alpha$ was similar to that of the wild-type enzyme, translocating to the membranes closer to the coated IgG glass. Interestingly, the inhibitor SP600125 did not blunt the ability of the mutated enzyme to translocate (membranes closer to the IgG coated glass, Fig. 8*B*). This was to be expected, since the mutant mimics the phosphorylated enzyme, and thus JNK activity would not be necessary to achieve $cPLA_2\alpha$ phosphorylation under these settings. Of note, in some experiments, the ECFP-S505E-$PLA_2\alpha$ mutant was already partially found in the membranes closer to the glass, even in the absence of IgG coating (Fig. 8*B*), an effect that was not appreciated with the nonphosphorylated construct EGFP-$cPLA_2\alpha$. This behavior of the ECFP-S505E-$PLA_2\alpha$ mutant provides additional support to the idea that phosphorylation of the enzyme at Ser^{505} is important for translocation, because signals that would not induce the movement of a wild-type $cPLA_2\alpha$, that is, those occurring for the attachment of the cell to glass, are sufficient to translocate an "already phosphorylated enzyme", namely the ECFP-S505E-$cPLA_2\alpha$ mutant.

Discussion

$cPLA_2\alpha$ plays a key role in innate immunity by mediating the production of eicosanoids with microbicidal capacity (1–5). During phagocytosis, $cPLA_2\alpha$ is known to translocate to the phagosome (11); however, the mechanisms governing such a translocation have not been elucidated. Understanding these mechanisms would help identify novel molecular targets with obvious therapeutic potential in the treatment of infectious diseases. In this regard we have demonstrated in the present work that phosphorylation of $cPLA_2\alpha$ on Ser^{505} by JNK is an important step for the proper binding of the enzyme to phagosomal membranes in an active manner in human macrophages.

The involvement of $cPLA_2\alpha$ in the generation of microbicidal lipids is supported by different lines of evidence. Free fatty acids such as AA are known to synergize with oxygen radicals or reactive nitrogen intermediates to promote bacterial killing (51–53). Mice lacking $cPLA_2\alpha$ manifest a reduced clearance of bacteria in vivo (12), which is also the case for 5-lipoxygenase-deficient mice (5). It has been suggested that leukotrienes enhance microbicidal activity in macrophages by activating the NADPH oxidase (2, 3, 54). Thus, an argument has been made in favor of a therapeutic use of receptor agonists capable of activating $cPLA_2\alpha$ such as ATP in combination with other antimicrobial drugs to efficaciously treat complex infections (13, 55–57). In the context of this work, it could be hypothesized that drugs that enhance JNK activity could, in combination with other antimicrobial agents, constitute a possible therapy to treat complex infections.

The biological significance of the phosphorylation of $cPLA_2\alpha$ on Ser^{505} is not fully understood. It is well known that phosphorylation of $cPLA_2\alpha$ at Ser^{505} increases the specific activity of the enzyme (14, 26, 27). However, such an increase is rather modest, that is, only ~1.5–2.0-fold (14, 26, 27), which suggests that Ser^{505} phosphorylation may serve other physiological roles (14, 27). In this regard, Das et al. (31) have proposed that phosphorylation of $cPLA_2\alpha$ on Ser^{505} facilitates membrane penetration of residues Ile^{399}, Leu^{400}, and Leu^{552}, with all of them being present in the catalytic domain of the enzyme, thereby allowing hydrophobic interactions to sustain membrane binding of $cPLA_2\alpha$ in response to transient calcium increases. The behavior of $cPLA_2\alpha$ significantly varies depending on whether its C2 domain is fully active (i.e., at Ca^{2+} concentrations near or >1 μM). Under these conditions,

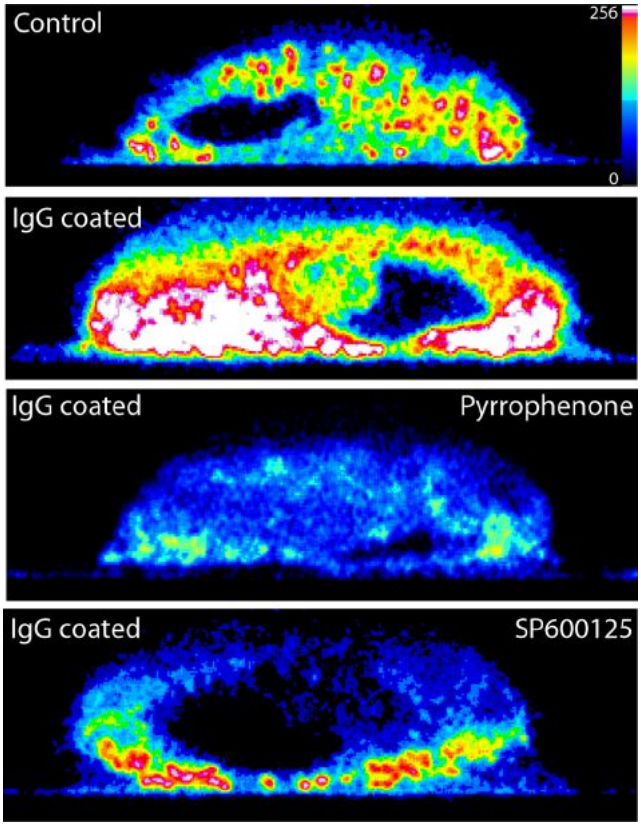

FIGURE 7. Effect of the JNK inhibitor SP600125 on cPLA$_2\alpha$ "in situ" activity during phagocytosis. Human macrophages labeled with bis-BODIPY FL C11-PC and pretreated with vehicle, 10 μM SP600125, or 1 μM pyrrophenone as indicated were plated on noncoated glass (Control) or on IgG-coated glass (IgG coated) for 30 min, and fluorescence was analyzed by confocal microscopy. Images of the *x*,*z*-axis of the cells were taken, and the intensity of the fluorescence was analyzed in pseudocolor. Representative cells of more than 30 analyzed are shown.

cPLA$_2\alpha$ translocates to membrane without any other apparent requirement (27). However, at lower Ca^{2+} concentrations, other cofactors may be involved in helping the enzyme associate with membranes. The data by Das et al. (31) suggest that Ser^{505} phosphorylation may be one of these. From a pathophysiological point of view, this is a relevant observation, because occupancy of surface receptors on cells generally promotes low and transient increases in intracellular Ca^{2+} concentrations that lead to sustained cPLA$_2\alpha$ activation responses. This is also the case in our system of zymosan-stimulated human macrophages, where the rises in the intracellular Ca^{2+} concentrations peak at ~250 nM (J. Casas and M. A. Balboa, unpublished data), leading to cPLA$_2\alpha$ translocation to the phagosome in a manner that depends on Ser^{505} phosphorylation (this study).

A striking feature of this work is the finding that JNK mediates the phosphorylation of cPLA$_2\alpha$ on Ser^{505} in zymosan-stimulated human macrophages. With the exception of a recent paper that appeared while our manuscript was in preparation (58), we are not aware of other studies implicating JNK in the translocation activation of cPLA$_2\alpha$. Most of the data currently available in the literature implicate either the ERKs and/or p38 (14, 27). Note, however, that most of these previous studies used nonphysiological stimuli such as phorbol esters or calcium ionophores and/or were conducted utilizing cell lines. In a more physiological setting, it has been described that inhibition of the ERK pathway, although decreasing the AA release, does not directly affect the phosphorylation and translocation of cPLA$_2\alpha$ (59). The involvement of p38 in the phosphorylation of cPLA$_2\alpha$ has been described in thrombin-stimulated platelets and opsonized zymosan-treated neutrophils, but its role in AA release remains controversial (22, 60, 61). Note as well that some differences in the involvement of various MAP kinase family members in cPLA$_2\alpha$ phosphorylation may be species-specific.

JNK activation occurs after ischemia/reperfusion in many tissues, including lung, kidney, liver, brain, and heart (62). The JNK inhibitor SP600125 has been used to decrease ischemia/reperfusion injury, especially in lung, kidney, and liver. cPLA$_2\alpha$ activation has been shown to occur during these processes, and the absence of cPLA$_2\alpha$ protects against postischemic brain injury, as shown in mouse knockout models (63). Additionally, SP600125 has been found to blunt allergen-induced increase in bronchial responsiveness in a murine model of chronic airway inflammation. Again, animals deficient in cPLA$_2\alpha$ exhibit no airway hyperresponsiveness after Ag challenge (64). Taking these observations together with the results described in our work, it is tempting to speculate that at least part of the effects of SP600125 described above may be due to the inhibition of cPLA$_2\alpha$ phosphorylation, translocation, and activation under those circumstances.

As indicated above, while this manuscript was in preparation, a study was published linking JNK to cPLA$_2\alpha$ phosphorylation also under pathophysiologically relevant conditions (58). In that work, CTLs were found to mediate tissue destruction in celiac disease by activating cPLA$_2\alpha$ through NKG2D receptors, thus liberating AA to promote degranulation and cytolysis, and finally tissue inflammation and target killing. The authors identified that both ERKs and JNK in an independent additive manner are the kinases responsible for the phosphorylation and activation of cPLA$_2\alpha$. In this regard, we also detected ERK activation in our zymosan-stimulated human macrophage system, as well as mild inhibition of

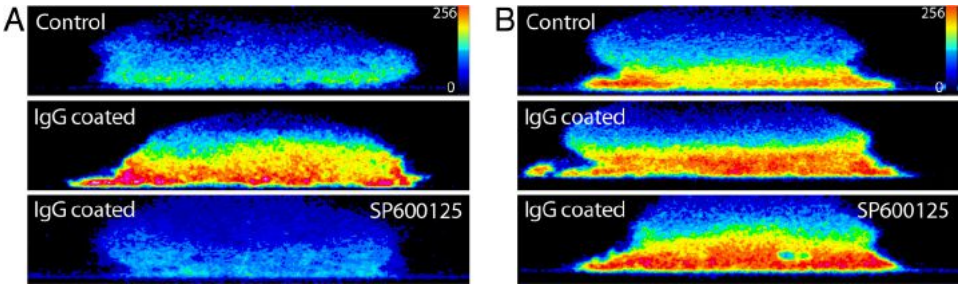

FIGURE 8. Translocation of the ECFP-S505E-cPLA$_2\alpha$ mutant during frustrated phagocytosis. Human macrophages transfected with either EGFP-cPLA$_2\alpha$ (*A*) or the ECFP-S505E-cPLA$_2\alpha$ mutant (*B*) were plated over noncoated glass (Control) or IgG-coated glass (IgG coated) in the absence or presence of the inhibitor SP600125 (as indicated), allowed to adhere for 30 min, and fluorescence was analyzed by confocal microscopy. Images of the *x*,*z*-axis of the cells were taken, and the intensity of the fluorescence is shown in pseudocolor. Representative cells of more than 30 analyzed are shown.

$cPLA_2\alpha$ phosphorylation by an ERK inhibitor. However, in our system the effect of JNK inhibition on $cPLA_2\alpha$ phosphorylation, translocation, and AA release is much stronger, producing an almost complete blockage of these events. An important observation that follows from taking these results together is the complexity of $cPLA_2\alpha$ as a possible pharmacological target to treat disease. In celiac disease, where activation of $cPLA_2\alpha$ greatly contributes to the pathology of the disease, inhibition of $cPLA_2\alpha$, either directly or indirectly, would theoretically be beneficial for the patient (58). On the other hand, during infections of invading microorganisms, the enhancement of $cPLA_2\alpha$ activity or its activating kinase JNK would be theoretically beneficial because it would increase the antimicrobial capacity of the phagocytic cell.

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Disclosures

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