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Co-Compartmentalization of MAP Kinases and Cytosolic Phospholipase A_2 at Cytoplasmic Arachidonate-Rich Lipid Bodies

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Lipid bodies are inducible lipid domains abundantly present in leukocytes engaged in inflammation. They are rich in esterified arachidonate and are also potential sites for eicosanoid-forming enzyme localization. It is therefore of interest to know whether arachidonate-releasing cytosolic phospholipase A_2 ($cPLA_2$) localizes at lipid bodies. Here, we present evidence that $cPLA_2$ and its activating protein kinases, mitogen-activated protein (MAP) kinases, co-localize at lipid bodies. U937 cells express high levels of $cPLA_2$ and contain numerous cytoplasmic lipid bodies. Using double-labeling immunocytochemistry we demonstrated punctate cytoplasmic localizations of both $cPLA_2$ and MAP kinases in U937 cells that were perfectly concordant with fluorescent fatty-acid-labeled lipid bodies. The co-localization of $cPLA_2$ and MAP kinases at lipid bodies was confirmed by subcellular fractionation and immunoblot. Lipid body fractions free of cytosol and other organelles contained significant amounts of [^{14}C]arachidonate-labeled phosphatidylcholine and $cPLA_2$ enzymatic activities. Immunoblotting with specific antibodies identified $cPLA_2$ as well as MAP kinases, including ERK1, ERK2, p85, and p38, in lipid bodies. The co-compartmentalization within arachidonate-rich lipid bodies of $cPLA_2$ and its potentially activating protein kinases suggests that lipid bodies may be structurally distinct intracellular sites active in extracellular ligand-induced arachidonate release and eicosanoid formation. ***(Am J Pathol 1998, 152:759–769)***

Lipid bodies are lipid-rich cytoplasmic inclusions present in macrophages,[1,2] mast cells,[1,2] neutrophils,[2,3] eosinophils,[2,4,5] fibroblasts,[2] endothelial cells,[2] and other cell types.[2] Although leukocytes in normal peripheral blood and macrophages in normal tissues contain very few lipid bodies, lipid bodies are characteristically abundant in cells engaged in inflammation, atherosclerosis, and neoplasia.[2] Lipid body formation can also be induced *in vitro* by exposing leukocytes to inflammatory lipid mediators, including arachidonic acid (AA)[5–8] and platelet-activating factor[9] as well as mitogenic phorbol esters.[5] Using immunogold staining, autoradiography, and subcellular fractionation, lipid bodies are shown to be structurally distinct sites of esterified arachidonate[2,4,6] and cyclooxygenase localization.[10–12] Moreover, increased formation of lipid bodies is associated with enhanced eicosanoid synthesis.[8,9] Therefore, lipid bodies in leukocytes may represent a morphological feature of cellular participation in inflammation and play an active role in heightened eicosanoid formation under pathological conditions. To further evaluate the potential role of lipid bodies in eicosanoid formation, we investigated whether cytosolic phospholipase A_2 ($cPLA_2$) and mitogen-activated protein (MAP) kinases, the enzymes requisite for extracellular ligand-mediated release of arachidonic acid, localize to cytoplasmic lipid bodies.

$cPLA_2$, first purified from macrophages and monocytic U937 cells,[13–15] specifically hydrolyzes AA from the *sn*-2 position of glycerophospholipids[14,16] and thus serves as the rate-limiting enzyme in the formation of eicosanoids and platelet-activating factor. $cPLA_2$ can be activated by a myriad of extracellular stimuli, including lipopolysaccharide,[17] phorbol esters,[18] thrombin,[19,20] interleukin 1,[21] tumor necrosis factor,[22] and epidermal growth factor.[23] One common pathway for $cPLA_2$ activation in response to diverse stimuli may be serine 505 phosphorylation by MAP kinases, also known as extracellular signal-regulated kinase (ERK).[24,25] MAP kinases ERK2 and ERK1 were shown to phosphorylate and activate $cPLA_2$ in cell-free systems[24,25] as well as COS cells,[24] endothelial cells,[26] and macrophages.[27]

MAP kinases have been shown to be present in the nucleus as well as the cytoplasm.[28,29] The localization of MAP kinases in both cytoplasm and nucleus may be essential for signal transduction because some MAP ki-

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nase substrates such as transcription factors c-jun and c-myc are localized in the nucleus.[28,30] Although phosphorylation of $cPLA_2$ by MAP kinases has been demonstrated both in cell-free systems and within cells, it is not clear whether the activating MAP kinases and substrate $cPLA_2$ localize in the same intracellular sites for the rapid activation of $cPLA_2$ requisite for eicosanoid formation.

Here we present evidence on the co-localization of MAP kinases and $cPLA_2$ at cytoplasmic lipid bodies of monocytic U937 cells. The co-compartmentalization of the enzymes requisite for ligand-induced arachidonate mobilization in lipid bodies indicates that cytoplasmic lipid bodies may play an important role in extracellular-signal-induced arachidonate release and eicosanoid formation.

Materials and Methods

Materials

Anti-$cPLA_2$ monoclonal antibody (MAb) and anti-ERK1, anti-ERK3, and anti-p38 polyclonal antibodies (Santa Cruz Biotech, Santa Cruz, CA); anti-pan-ERK, anti-ERK1, anti-ERK2, and anti-annexin VI MAbs (Transduction Laboratories, Lexington, KY); anti-mitochondrial p60 MAb (Calbiochem, San Diego, CA); purified ERK2 (Upstate Biotechnology, Lake Placid, NY); p38 control lysate (New England Biolabs, Beverly, MA); control mouse myeloma immunoglobulins (Organon Teknika Corp., Durham, NC); biotinylated secondary antibodies, Texas-red-conjugated avidin, and Vectastain glucose oxidase avidin-biotinylated enzyme complex (ABC) kit (Vector Laboratories, Burlingame, CA); fluorescent fatty acid 1-pyrenedodecanoic acid (Molecular Probes, Eugene, OR); horseradish-peroxidase-conjugated secondary antibodies and ECL Western blotting kits (Amersham, Arlington Heights, IL); protein assay micro-bicinchoninic acid (BCA) kit (Pierce, Rockford, IL); diolein, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine (Sigma Chemical Co., St. Louis, MO); and [^{14}C]arachidonic acid (^{14}C-AA) and L-α-1-palmitoyl-2-[^{14}C]arachidonyl phosphatidylcholine (DuPont NEN, Boston, MA) were obtained as noted. Rabbit anti-$cPLA_2$ polyclonal antibody and purified $cPLA_2$ were generous gifts of either Dr. James Clark (Genetics Institute) or Dr. Ruth Kramer (Eli Lilly).

Culture of U937 Cells

The human monocytic leukemia U937 cell line was obtained from American Type Culture Collection (Rockville, MD). All tissue culture media were from Life Technologies (Gaithersburg, MD). The cells were grown in RPMI 1640 medium containing 10% fetal calf serum, 10 mmol/L L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator. The cells were grown to a density of approximately 1×10^6/ml and serum starved overnight in RPMI 1640 with 0.5% fetal calf serum before being harvested for immunostaining or isolation of lipid bodies.

Electron Microscopy of Lipid-Body-Rich U937 Cells

The electron microscopic analysis of U937 cells was as described previously.[6,31] U937 cells were fixed in a dilute mixture of freshly prepared aldehydes and washed in cold 0.1 mol/L sodium cacodylate buffer. They were centrifuged through molten agar to form cell pellets that were post-fixed in Sym-collidine-buffered osmium tetroxide before dehydration in a graded series of alcohols and embedding in a propylene oxide-Epon sequence. Thin sections were prepared and examined with a Philips 400 electron microscope (Mahwah, NJ).

Double Immunofluorescent/Immunochemical Microscopy

U937 cells were washed twice with HBSS and incubated with 10 μmol/L fluorescent fatty acid 1-pyrenedodecanoic acid for 2 hours at 37°C. Cells were then cytospun and fixed in 3% formaldehyde at room temperature for 10 minutes. Fixed cells were permeabilized with 0.05% saponin solution, and then nonspecific reactive sites were blocked with 1% blocker (Schleicher & Schuell, Keene, NH) or 10% normal goat serum at room temperature for 1 hour. After washing, cells were incubated for 2 hours at room temperature with mouse anti-pan ERK (0.5 μg/ml), rabbit anti-p38 (2 μg/ml), or anti-$cPLA_2$ (5 μg/ml) as primary antibodies and with biotin-conjugated goat anti-mouse (1/500 dilution) or anti-rabbit IgG (1/500 dilution) as secondary antibodies. The immunoreactive MAP kinases, p38, and $cPLA_2$ in cells were then identified by using Texas-red-conjugated avidin (10 μg/ml) or Vectastain glucose oxidase kit following the manufacturer's instruction. In controls, the primary antibodies were substituted with mouse myeloma immunoglobulins or rabbit IgG from nonimmune animals, and the anti-pan-ERK was preabsorbed with purified ERK2 protein (1 μg/ml). Cytoplasmic lipid bodies were identified under excitation at 340 nm whereas MAP kinase immunostaining was visualized under rhodamine excitation at 570 nm. The glucose oxidase immunostaining was examined under light microscopy. The cells were observed and photographed with a 63× objective.

Isolation of Lipid Bodies

Lipid bodies were isolated by subcellular fractionation based on the inherent buoyancy of these lipid-rich structures as detailed before.[6] In brief, U937 cells were collected by centrifugation and washed twice with Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution before being resuspended in 20 mmol/L Tris/HCl buffer containing 0.27 mol/L sucrose, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 μg/ml leupeptin, 0.7 μg/ml pepstatin A, 50 μg/ml $N\alpha$-p-tosyl-L-lysine chloromethyl ketone, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 2 mmol/L dithiothreitol (pH 7.4). The cells were disrupted by a tight-fitting Dounce homogenizer, and the homogenates were centrifuged at $1500 \times g$ for 10 minutes to remove nuclei and undis-

rupted cells. The postnuclear supernatants were then overlaid with Top solution (20 mmol/L Tris/HCl, 1 mmol/L EDTA, 1 mmol/L EGTA, and 2 mmol/L dithiothreitol, pH 7.4) and centrifuged at 150,500 × *g* at 4°C for 60 minutes in a SW41 rotor. Four discrete fractions were recovered separately: a top layer of lipid body fraction (contains most but not all of the lipid bodies), the mid-zone between lipid bodies and cytosol fraction, the cytosol fraction, and a microsome pellet fraction. The pellet fraction was washed and then resuspended in Top solution. Lipid bodies were quickly identified with fluorescent Nile red staining.[6] The protein contents in the four fractions were analyzed by micro-BCA assay using bovine serum albumin as a standard. The activities of lactate dehydrogenase (LDH)[32] and sulfatase C[33] were measured as cytosolic and microsomal markers, respectively.

^{14}C-AA Labeling of Lipid Bodies

Cells were incubated with ^{14}C-AA (2 μCi/10^6 cells, 0.2 μmol/L) for 24 hours in RPMI 1640 supplemented with 0.5% fetal calf serum. The cells were then washed twice in Hanks' balanced salt solution before subcellular fractionation using slightly modified protocols as described above. In brief, the cells were homogenized in disruption buffer containing 0.54 mol/L sucrose, and the homogenates were centrifuged at 1500 × *g* for 10 minutes to pellet the nuclear fraction. The postnuclear supernatants were then overlaid with 1.5 ml of 0.27 mol/L sucrose buffer, 1.5 ml of 0.135 mol/L sucrose buffer, and 1.5 ml of Top solution. After centrifugation at 150,500 × *g* for 60 minutes, 1.5-ml fractions were collected in Eppendorf tubes. The pellet and nuclear fraction were washed and resuspended in Top solution. Aliquots of all 10 fractions were used for the analysis of protein content and radioactivity. To analyze the lipid classes of ^{14}C-AA labeling in lipid bodies, lipids were extracted from lipid body fractions using the Bligh and Dyer procedure[34] and separated by thin-layer chromatography using ethanol/chloroform/ammonia (50/6/6) as the developing system.[35] The radiolabeled lipids were identified by co-migration with unlabeled lipid standards and quantified using Instant Imager (Packard, Meriden, CT).

cPLA$_2$ Enzyme Assay

cPLA$_2$ activity in lipid body and other cellular fractions were assayed as described previously.[14] In brief, 1-palmitoyl-2-[^{14}C]arachidonyl phosphatidylcholine (100 μmol/L, 1 × 10^5 cpm) was dried down and resuspended in reaction buffer containing 2 mmol/L $CaCl_2$ and 70% (v/v) glycerol. The suspension was then sonicated to form mixed micelles of phospholipid and Triton X-100. Aliquots of samples to be assayed were added to the mixed micelles and incubated at 37°C for 30 minutes. The reactions were then quenched and processed as described.[14]

Immunoblotting of MAP Kinases and cPLA$_2$

Proteins from cellular fractions were concentrated by precipitation with 20% trichloroacetic acid overnight at 4°C. The precipitates were then washed twice with cold acetone. Protein concentrations were normalized in each fraction after micro-BCA assay. The samples were then prepared in denaturing conditions and separated by electrophoresis in 5 to 20% or 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. After transfer onto nitrocellulose membranes, nonspecific binding sites were blocked with 5% nonfat milk (Bio-Rad, Hercules, CA) in Tris-buffered saline/Tween (TBST; 50 mmol/L Tris/HCl, pH 7.4, 150 mmol/L NaCl, and 0.05% Tween 20) at room temperature for 1 hour. The membranes were then probed with anti-cPLA$_2$ MAb (1 μg/ml), anti-pan ERK (0.1 μg/ml), anti-ERK1 (0.25 μg/ml), anti-p38 (0.25 μg/ml), anti-mitochondrial p60 (1/200), or anti-annexin VI (0.1 μg/ml) in TBST with 1% nonfat dry milk. The proteins of interest were then immunoblotted by incubating the membrane with horseradish-peroxidase-conjugated secondary antibodies (1/5000) in TBST with 1% milk. Detection of antigen-antibody complexes was performed by ECL chemiluminescence (Amersham). When the same membrane was sequentially probed with different antibodies, the blot was stripped in stripping buffer (62.5 mmol/L Tris/HCl, pH 6.8, 2% SDS, 100 mmol/L 2-mercaptoethanol) for 10 minutes at 70°C.

Results

Lipid Bodies in U937 Cells

Monocytic U937 cells contained numerous cytoplasmic lipid bodies that can be easily identified by staining with Oil-red O or osmium tetroxide. As shown in Figure 1, lipid bodies in U937 cells were morphologically similar to lipid bodies in human blood leukocytes[5,6] and lacked a delimiting membrane. Although variable in size, lipid bodies were usually approximately 1 μm in diameter. An electron-dense peripheral rim and some honeycomb-like structures in lipid bodies were observed in some cells. The numbers of lipid bodies in each cell were variable and ranged from 5 to more than 40.

Co-Localization of MAP Kinase and cPLA$_2$ to Lipid Bodies of U937 Cells

We evaluated the intracellular localizations of MAP kinases and cPLA$_2$ by immunofluorescence and immunocytochemistry using specific conditions of cell fixation and permeabilization that prevent dissolution of lipid bodies. To examine whether MAP kinases and cPLA$_2$ co-compartmentalize in cytoplasmic lipid bodies of intact U937 cells, dual-labeling microscopy was used to visualize both lipid bodies and proteins of interest in the lipid bodies in these cells. Cytoplasmic lipid bodies were endogenously labeled with the fluorescent fatty acid 1-pyrenedodecanoic acid and visualized under excitation at 340 nm. Subsequently, cells were fixed and MAP

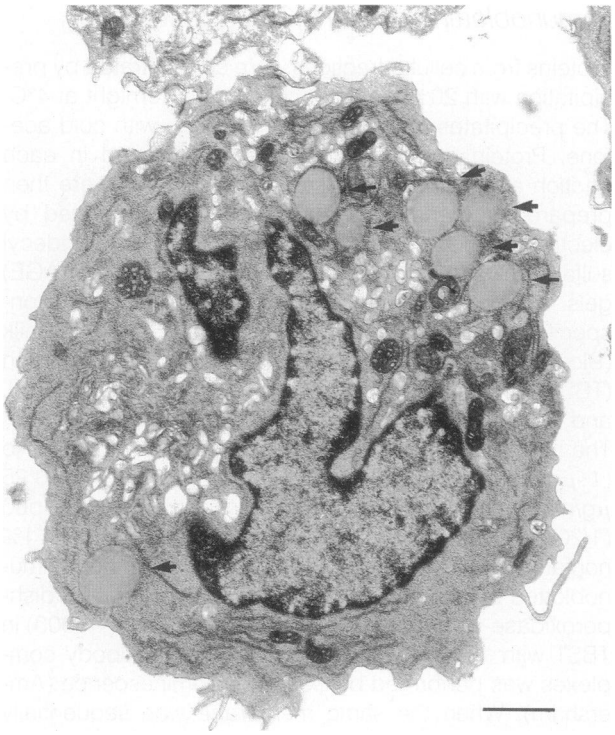

Figure 1. Electron microscopy of the cytoplasmic lipid bodies in U937 cells. The electron microscopy of lipid bodies was performed as described before.[6] As indicated by **arrowheads**, the U937 cells contained numerous cytoplasmic lipid bodies distinctive from any other cellular structures. Bar, 1μm.

kinases were stained by indirect immunofluorescence using an anti-pan-ERK MAb, biotinylated secondary antibodies, and Texas-red-conjugated avidin for visualization under rhodamine excitation.

1-Pyrenedodecanoic acid is a fluorescent fatty acid analogue that enters living cells and incorporates efficiently into both neutral lipids and phospholipids.[36] Incorporation is accompanied by pyrene fluorescence enhancement, and pyrenedodecanoic acid has been shown to label lipid droplets in fibroblasts.[37] As shown in Figure 2, A, C, and E, lipid bodies in U937 cells were easily identifiable by incorporated fluorescent fatty acid as blue-green punctate cytoplasmic inclusions. As the nuclear envelope is rich in both neutral lipids and phospholipids, it also stained with fluorescent fatty acid (Figure 2, A, C, and E).

Cells immunostained with anti-pan-ERK MAb showed focal cytoplasmic MAP kinase staining (Figure 2B) that perfectly matched the punctate fluorescent fatty acid lipid body staining of the same cells (Figure 2A). In addition, there was a prominent nuclear envelope staining with anti-pan-ERK MAb. In control immunostaining, U937 cells were incubated with either ERK2 solid-phase absorbed anti-pan-ERK as primary antibody (Figure 2, C and D) or mouse myeloma immunoglobulin (Figure 2, E and F). In controls, the punctate fluorescent fatty-acid-labeled lipid bodies were present in the U937 cells (Figure 2, C and E), but there was no MAP kinase staining in the same cells with either ERK2 solid-phase absorbed anti-pan-ERK MAb (Figure 2D) or control mouse myeloma immunoglobulin (Figure 2F). Similar results were obtained with glucose oxidase ABC immunocytostaining using anti-pan-ERK and anti-ERK2 MAbs (data not shown), confirming the immunolocalization of MAP kinases to lipid bodies. The localization of MAP kinases to lipid bodies were also demonstrated in human monocytes, eosinophils, and murine 3T3 fibroblasts (data not shown).

The recent identification of the novel MAP kinase p38[38] and its role in the activation of $cPLA_2$ in thrombin-stimulated platelets[39] also prompted us to examine the possible association of this kinase with lipid bodies. As the indirect immunofluorescent method described above was not sensitive enough to label p38 and $cPLA_2$ in U937 cells, the localization of these two proteins were evaluated using glucose oxidase ABC immunostaining protocols in cells fixed and permeabilized after incorporation of fluorescent fatty acid. As shown in Figure 3, we found similar focal p38 (Figure 3B) and $cPLA_2$ staining (Figure 3D) that coincided with punctate, blue fluorescing lipid bodies (Figure 3, A and C, respectively). The localization of p38 and $cPLA_2$ to lipid bodies was specific as there was no immunostaining in U937 cells when control nonimmune IgG was used as primary antibody (Figure 3F), although fluorescing punctate lipid bodies were present in these cells (Figure 3E). It should be noted that the images of two cells in Figure 3A were out of focus because the attention was paid to the other three cells during photography. The lipid bodies in U937 cells were visible sometimes under regular light microscopy as seen in Figure 3F due to their specific light scatter intensity.[40] However, there is a significant difference between the purple immunostaining in Figure 3, B or D, and the lipid body shadows in Figure 3F.

Isolation of Lipid-Body-Enriched Fractions by Subcellular Fractionation

To confirm our immunocytochemical findings, we examined the localization of MAP kinases and $cPLA_2$ in lipid body and other cellular compartments by subcellular fractionation. As shown in Figure 4A, the buoyant lipid body fraction contained approximately 8% of cellular proteins but negligible LDH and sulfatase C specific activities. The mid-zone and cytosolic fractions contained most of the cytosolic LDH activity whereas the pellet contained more than 70% of the microsomal marker enzyme arylsulfatase c. These data indicate that the lipid-body-enriched fraction was essentially free of cytosolic and microsomal contamination.

[^{14}C]Arachidonate Labeling of Lipid Bodies

Cells were cultured in the presence of ^{14}C-AA and processed as described in Materials and Methods. Eight equal fractions from the sucrose gradient, pellet, and the nuclear fraction were collected and assayed for protein content and total [^{14}C]arachidonate-labeled lipid. The top two fractions from the sucrose gradient contained most of the cytoplasmic lipid bodies and were highly enriched with arachidonate-labeled lipids (Figure 4B).

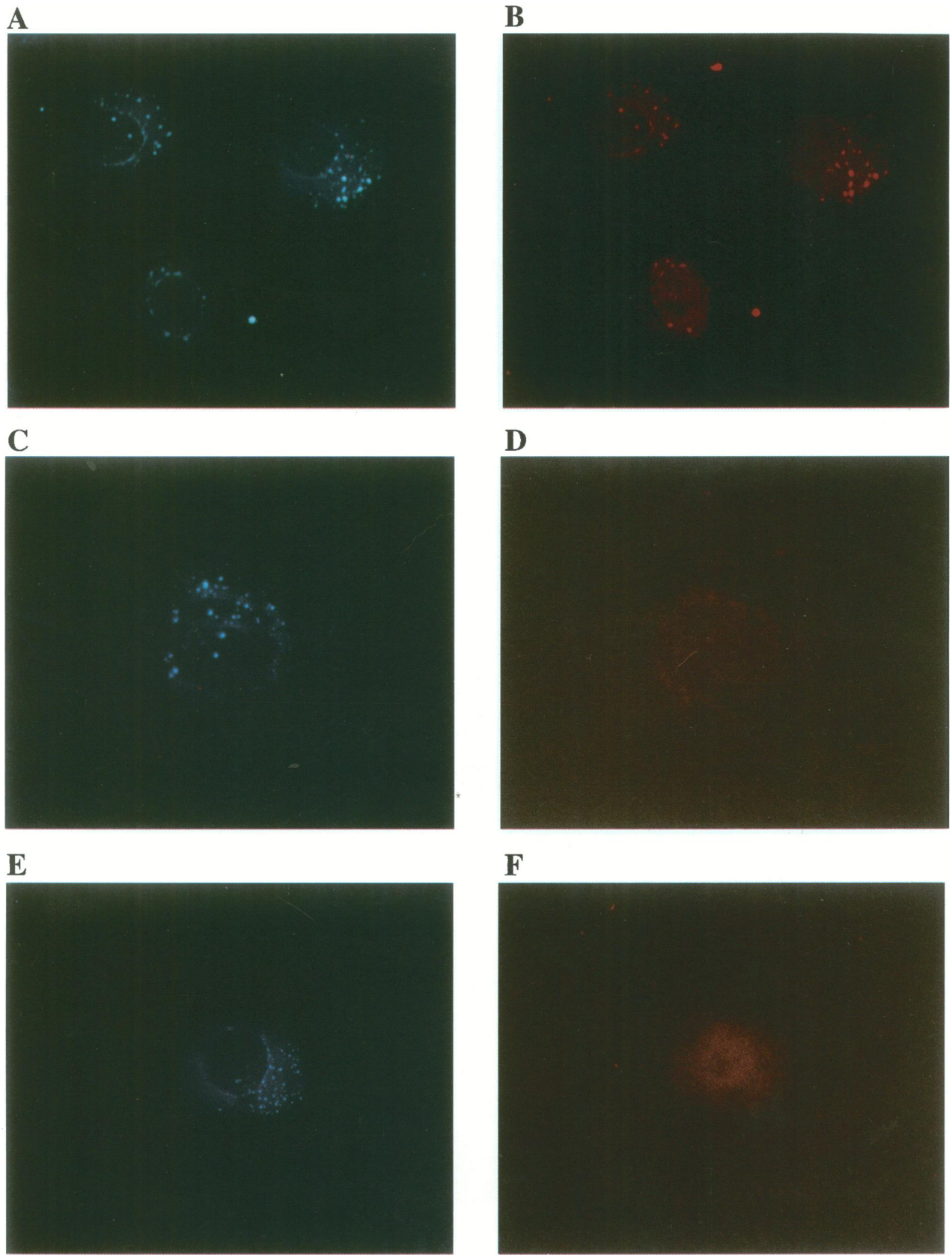

Figure 2. The localization of MAP kinases to lipid bodies of U937 cells by dual-labeling fluorescent microscopy. The lipid bodies in U937 cells were labeled with fluorescent fatty acid 1-pyrenedodecanoic acid and visualized under excitation at 340 nm (**A**, **C**, and **E**). MAP kinases were stained by indirect immunofluorescence using Texas-red-conjugated immunocomplex for visualization under rhodamine excitation at 570 nm (**B**, **D**, and **F**). In immunostaining with anti-pan MAP kinase MAb there was perfect co-localization of punctate staining for MAP kinases (**B**) with lipid bodies (**A**). In contrast, there was no MAP kinase staining in control cells immunostained with either ERK2 solid phase absorbed anti-pan MAP kinase (**D**) or nonimmune mouse myeloma immunoglobulin (**F**).

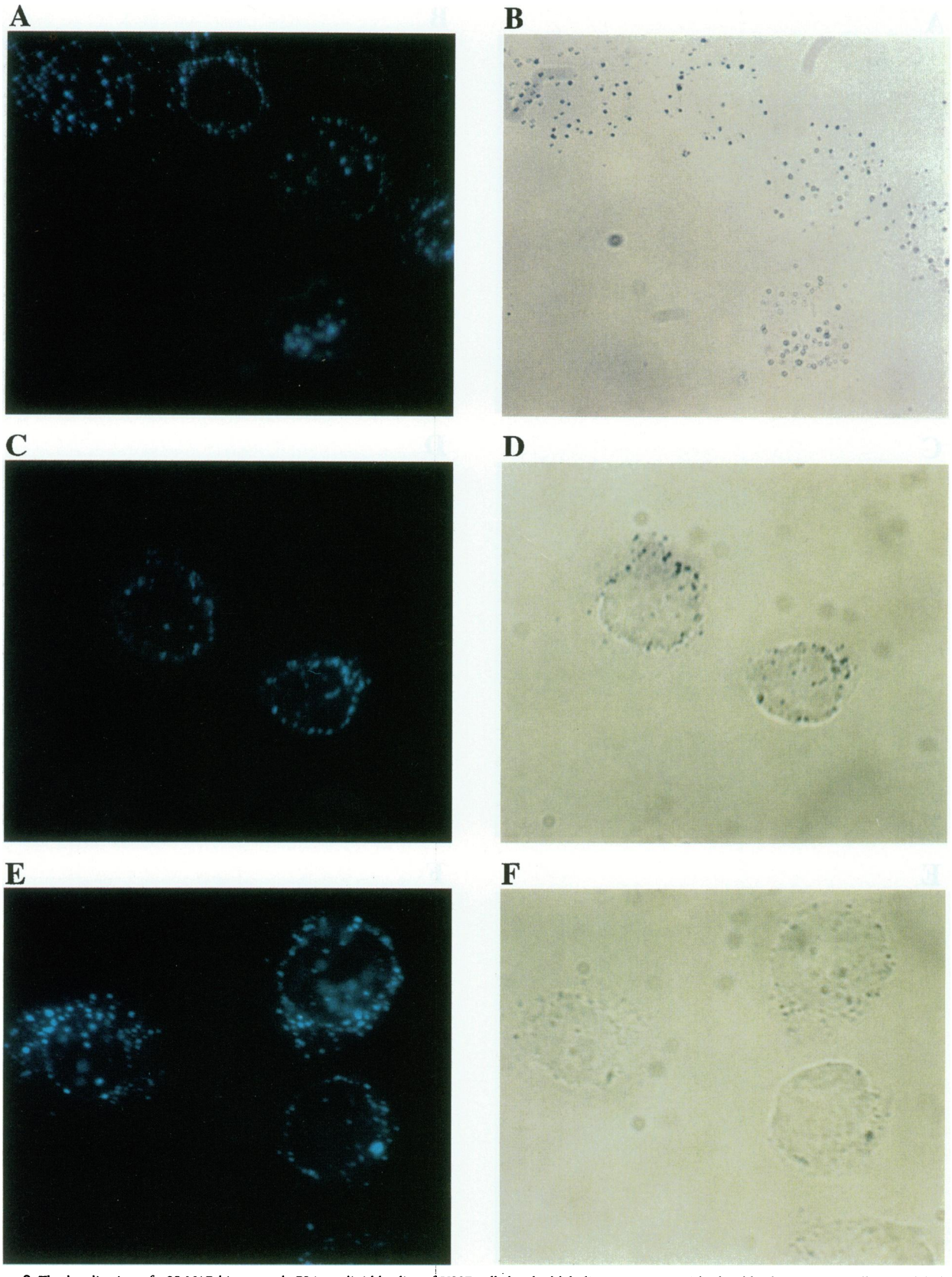

Figure 3. The localization of p38 MAP kinase and $cPLA_2$ to lipid bodies of U937 cells by dual-labeling microscopy. The lipid bodies in U937 cells were labeled with fluorescent fatty acid 1-pyrenedodecanoic acid and visualized under excitation at 340 nm (**A**, **C**, and **E**). P38 (**B**) or $cPLA_2$ (**D**) was stained using specific anti-p38 or anti-$cPLA_2$ antibodies and glucose oxidase ABC kit. The same IgG isotype from nonimmune animals was used as control (**F**). The punctate staining of p38 (**B**) and $cPLA_2$ (**D**) in U937 cells matched lipid body labeling in the same cells (**A** and **C**, respectively). The pictures are representative of four independent experiments.

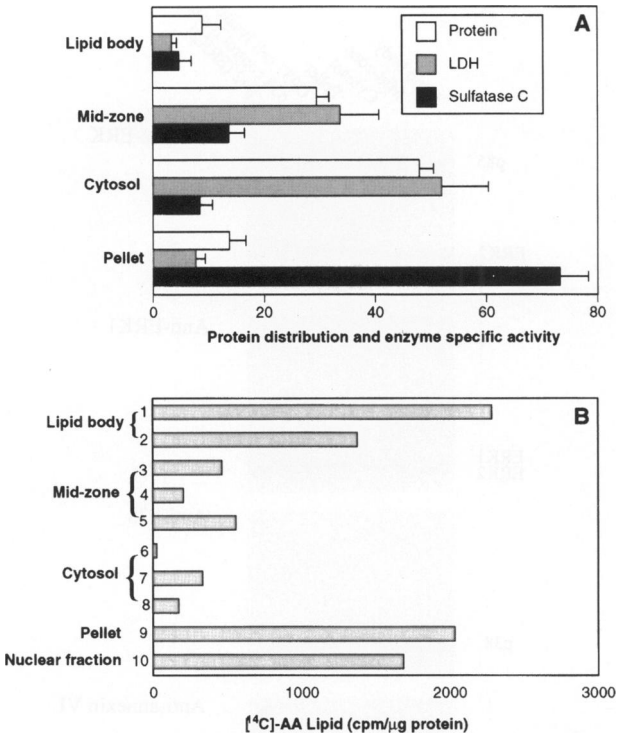

Figure 4. Characterization of lipid bodies and other cellular fractions. **A:** The lipid body and other cellular fractions were isolated as described in Materials and Methods. Four discrete fractions were recovered separately: a top layer of lipid bodies (contains most but not all lipid bodies), the mid-zone between lipid bodies and cytosol fraction, the cytosol fraction, and the microsome pellet. The protein contents in each fraction are shown as a percentage of total proteins recovered whereas the LDH and sulfatase C were presented as specific activities. The low LDH and sulfatase C activities in lipid body fraction therefore indicate that this fraction was essentially free of the contamination by cytosol and microsome. The data presented are means ± SEM of four independent experiments. **B:** Eight 1.5-ml fractions were collected after ultricentrifugation, and the pellet as well as nuclear fraction were resuspended in 1.5 ml of buffer. The top two fractions, which contained most of the lipid bodies, were highly enriched with [^{14}C]arachidonate lipids. The data are representative of three independent experiments.

They contained approximately 20% of total [^{14}C]-AA-lipid. An appreciable amount of labeling was also seen in the mid-zone fraction number, 5 which contained glycosylphosphatidylinositol-linked membrane protein CD36 (data not shown). All of the other arachidonate-labeled lipids were highly localized to the microsomal pellet and nuclear fractions.

Distribution of [^{14}C]Arachidonate Labeling in the Glycerolipid Classes of Lipid Bodies

The distribution of ^{14}C-AA labeling in different classes of glycerolipids in lipid bodies was further analyzed by thin-layer chromatography and autoradiography. As shown in Figure 5, although neutral lipids were the major class of ^{14}C-AA-labeled glycerolipids in lipid bodies, more than 30% of ^{14}C-AA labeling in lipid bodies were phospholipids. More significantly, phosphatidylcholine, a major substrate for cPLA$_2$, is also a major class of phospholipid labeled by ^{14}C-AA in lipid bodies. ^{14}C-AA-labeled phosphatidylcholine accounted for 16% of total ^{14}C-AA lipids in lipid bodies. Approximately 11% of ^{14}C-AA labeling

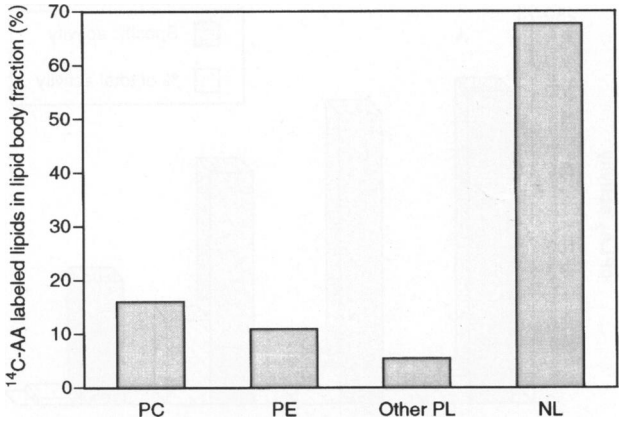

Figure 5. Distribution of ^{14}C-AA labeling in different classes of glycerolipids of lipid bodies isolated from U937 cells. Cells were incubated with ^{14}C-AA for 24 hours as described in Materials and Methods. Lipid bodies were isolated, and their lipids were extracted using the Bligh procedure[34] and separated by thin-layer chromatography. Neutral lipids and major classes of phospholipids labeled by ^{14}C-AA were quantified and expressed as a percentage of the total ^{14}C-AA labeling in lipid bodies. Data are representative of three different experiments.

was in phosphatidylethanolamine. Other phospholipids, including phosphatidylinositol and phosphatidylserine, constitute less than 6% of total ^{14}C-AA labeling in lipid bodies. These results indicate that lipid bodies contain significant amounts of phospholipids that can serve as the substrate for cPLA$_2$.

Association of cPLA$_2$ with Isolated Lipid Bodies

The association of cPLA$_2$ protein with lipid bodies in U937 cells (Figure 2B) was further evaluated by enzymatic assay and protein immunoblot. The cPLA$_2$ activity in subcellular fractions was analyzed by measuring the release of radiolabeled arachidonic acid from the *sn*-2 position of 1-palmitoyl-2-[^{14}C]arachidonyl phosphatidylcholine. Significant amounts of cPLA$_2$ enzymatic activities were detected in the lipid body fraction as well as in the interface and cytosol fractions (Figure 6A). It should be pointed out that, although cPLA$_2$ specific activity is very high in the lipid body fraction, the cytosol contains more than 50% of total cPLA$_2$ activity due to the higher protein concentration in this fraction.

We verified the presence of cPLA$_2$ in lipid body fractions by immunoblotting the protein with specific monoclonal (Figure 6B) and polyclonal (data not shown) antibodies. Both types of cPLA$_2$ antibodies recognized a single major band of protein in the lipid body fraction (Figure 6B). This immunoreactive protein had an apparent mass of 110 kd in SDS-polyacrylamide gel and comigrated with purified cPLA$_2$ protein. In accord with the immunocytochemical staining and the cPLA$_2$ enzymatic activity assay results, immunoblotting corroborated the prominent association of cPLA$_2$ with lipid bodies isolated from U937 cells.

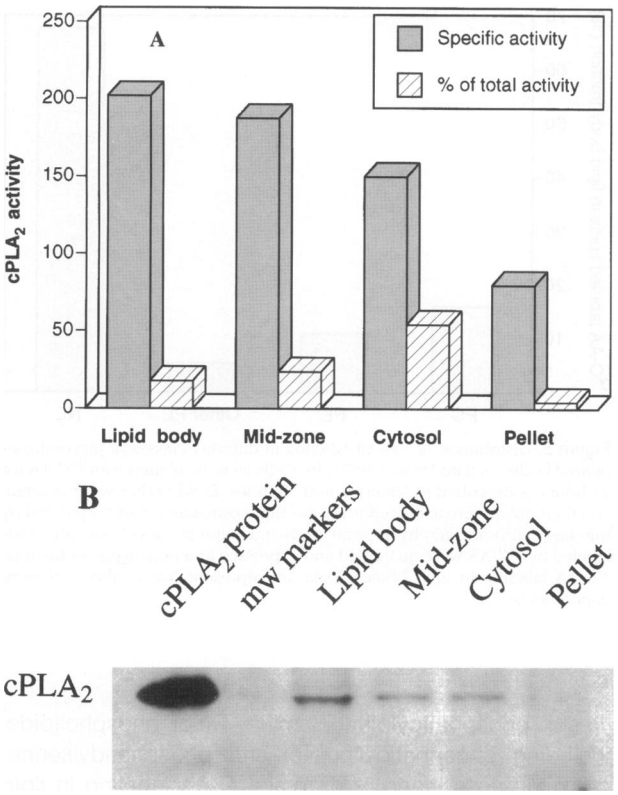

Figure 6. Localization of $cPLA_2$ in isolated lipid body fractions of U937 cells. **A:** $cPLA_2$ enzymatic activity in lipid body and other cellular fractions. The cellular fractions were incubated with 1-palmitoyl-2-[1-^{14}C]arachidonyl phosphatidylcholine at 37°C for 30 minutes. $cPLA_2$ activity was analyzed by measuring the release of ^{14}C-AA. The results are presented as specific activity (pmol/minutes/mg protein) and percentage of total activity. The data are representative of five similar independent experiments. **B:** Proteins (20 μg) from lipid body and other cellular fractions were electrophoresed on 5 to 20% SDS-PAGE gels and immunoblotted with anti-$cPLA_2$ MAb. Polyclonal antibodies recognized the same protein band at 110 kd that co-migrated with purified $cPLA_2$ protein. The blot is representative of three independent experiments.

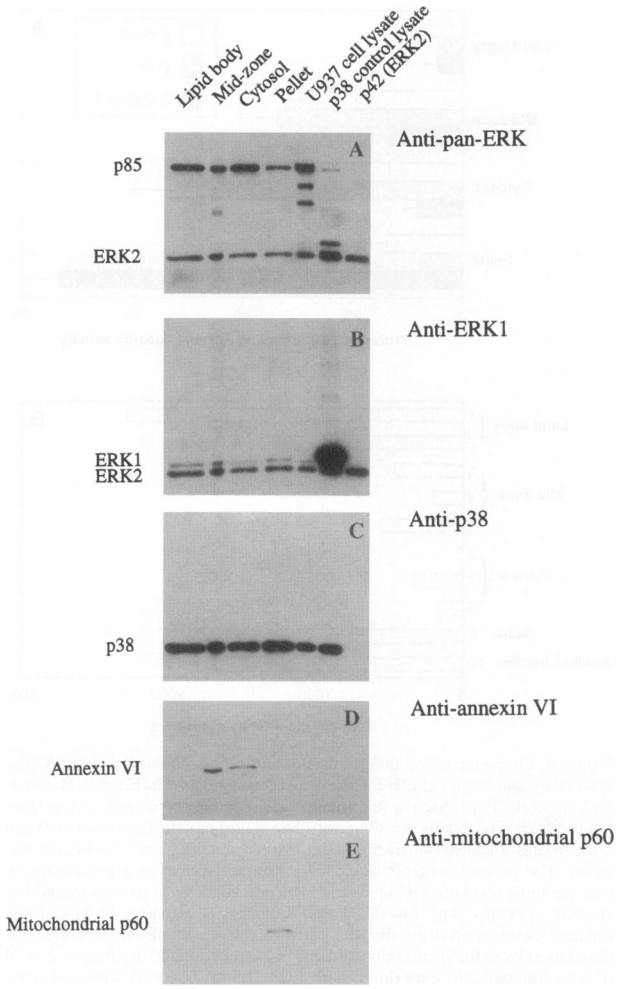

Figure 7. Association of MAP kinases with lipid bodies of U937 cells. Proteins (20 μg) extracted from different cellular fractions, U937 cell lysate, p38 control lysate, and purified ERK2 were separated by 12% SDS-PAGE gels and blotted sequentially with anti-pan-ERK (**A**), anti-ERK1 (**B**), anti-p38 MAP kinase (**C**), anti-annexin VI (**D**), and anti-mitochondrial p60 (**E**). MAP kinase ERK2 and p38 control lysates were applied as positive controls for the evaluation of the antibody specificity. The data are representative of at least three independent experiments.

Association of MAP Kinases with Isolated Lipid Bodies

The localization of MAP kinases to lipid bodies was also further evaluated. To examine the isoforms of the MAP kinases associated with isolated lipid bodies, proteins extracted from different cellular fractions of U937 cells were separated by SDS-PAGE and blotted with anti-pan-ERK, anti-ERK1, and anti-p38 antibodies. In unfractionated U937 cell lysates, four immunoreactive protein bands were detected by the anti-pan-ERK MAb (Figure 6A). ERK2 in U937 cells was identified by the identical migration rate with purified ERK2 protein. One of the other detected bands likely represents MAP kinase p85. Some batches of anti-pan-ERK MAb also recognized p44 (ERK1; data not shown). In addition to p42 and p85, the anti-pan-ERK MAb also recognized p45 MAP kinase in p38 control lysates prepared from c-6 glioma cells.

The lipid body fractions, despite their paucity of total protein, contained significant amounts of ERK2 and p85, which were also present in other subcellular fractions (Figure 7A). Using a polyclonal antibody against ERK1 (p44), significant amounts of ERK1 as well as ERK2 were found to be present in lipid body fractions (Figure 7B). In conjunction with results shown in Figure 2, the substantial association of $cPLA_2$ activators ERK1 and ERK2 with lipid bodies may serve to ensure rapid phosphorylation of $cPLA_2$ on lipid bodies in response to extracellular stimuli, such as cytokines and growth factors. Consistent with findings by others,[41] our results localized ERK3 only to the nuclear fraction (data not shown). The nuclear localization of ERK3 and other MAP kinases was in accord with our findings of MAP kinase immunostaining on the nuclear envelope (Figure 2).

The association of p38 with lipid bodies was also demonstrated by immunoblot. As shown in Figure 7C, the specific anti-p38 antibody recognized a single protein band of approximately 38 kd, which was identical with p38 from the positive control lysate.

The localization of specific MAP kinases and $cPLA_2$ to lipid bodies after subcellular fractionation was further supported by additional studies. The lipid body fraction

was not only free of cytosolic and microsomal contamination (Figure 4A) but also distinct from endocytic pathway vesicles because the endosomal marker protein annexin VI[42] was shown to localize exclusively to mid-zone and cytosol fractions (Figure 7D). There was also no contamination of mitochondria and endoplasmic reticulum in our lipid body fractions as the mitochondrial marker protein p60 (Figure 7E) and endoplasmic reticulum marker IP3 receptors (data not shown) were shown to be absent from isolated lipid bodies and to be predominantly localized in the pellet fractions (Figure 7, D and E). Thus, lipid bodies isolated by subcellular fractionation, despite their low content of total protein, contained ERK1, ERK2, p38, and p85 MAP kinases as well as cPLA$_2$. Taken together, our findings indicate that a significant pool of cPLA$_2$ and its potentially activating MAP kinases localize at arachidonate-rich lipid bodies. The functional role of these lipid-body-associated enzymes in inflammatory reaction is under further investigation.

Discussion

A number of findings support roles for MAP kinases ERK1, ERK2, and p38 in the activation of cPLA$_2$ and the extracellular ligand-induced formation of inflammatory eicosanoids.[24,26,27,39,43] In this report, we demonstrate the co-compartmentalization of several MAP kinases and cPLA$_2$ in cytoplasmic lipid bodies of U937 cells. This finding for the first time places cPLA$_2$ and its upstream activating kinases together in association with cytoplasmic arachidonate-rich lipid domains. The co-compartmentalization of enzymes required to initiate liberation of arachidonate within lipid bodies may be important in the extracellular signal-initiated formation of paracrine eicosanoid mediators of inflammation.

Previous studies reported diffuse localizations of MAP kinases and cPLA$_2$ in the cytoplasm.[29,44,45] The fact that the localization of these enzymes to lipid bodies was not appreciated may result from differences in immunostaining protocols and cell types used in related studies. First, lipid bodies are not recognized, or even preserved, on routine light and fluorescent microscopy when alcohol- or acetone-based fixation and permeabilization solutions are used. Both methanol and acetone solubilize these cytoplasmic inclusions.[6] Second, in some studies, cells overexpressing MAP kinases[28,46] and cPLA$_2$[45] were used for immunofluorescent staining, and the intracellular localization of overexpressed proteins may differ from that of constitutively expressed proteins. Finally, the localizations of MAP kinase and cPLA$_2$ may be cell-type dependent. To date, no study on the localization of MAP kinases and cPLA$_2$ in lipid-body-rich U937 cells has been reported. The localization of these proteins to lipid bodies is not restricted, however, to U937 cells as similar lipid body localization has been noted in the RAW murine macrophage cell line and rat basophil leukemia cell line as well as human eosinophils and monocytes (unpublished data).

Although the enzymatic pathways for the formation of a variety of eicosanoids are well understood, the cPLA$_2$ activation site and cellular sources of arachidonic acid remain less clear. cPLA$_2$ has a calcium-dependent phospholipid-binding motif on its amino acid sequence and may be activated by submicromolar amounts of Ca^{2+}.[47,48] It was shown that cPLA$_2$ translocated from cytosol to endoplasmic reticulum and/or nuclear envelope in response to stimulation with either agonist or calcium ionophore in rat basophilic leukemic cells[44] and CHO cells overexpressing cPLA$_2$.[45] In light of other findings on the localization of the enzyme prostaglandin endoperoxide H synthase (cyclo-oxygenase) in nuclear and endoplasmic reticulum membranes,[49–51] and the enzyme 5-lipoxygenase and 5-lipoxygenase-activating protein in nuclear membranes,[52–56] the nuclear envelope and/or endoplasmic reticulum membrane may be an important site for the liberation of arachidonic acid for specific eicosanoid formation. Such sites proximate to the nucleus may be suited to the role of eicosanoids as autocrine mediators of transcription and nuclear activation.

The formation of leukotriene B_4 by nuclei-free neutrophilic cytoplasts,[57] however, strongly suggests alternative intracellular sites for eicosanoid formation. Here, our findings provide new evidence to implicate lipid bodies as an alternative intracellular site for cPLA$_2$ activation and eicosanoid formation. First, lipid bodies are inducible cytoplasmic domains and are present in diverse types of cells,[2,5] in particular, leukocytes engaged in inflammatory, immunological, atherosclerotic, and neoplastic processes.[2,4,6,7] Second, lipid bodies are intracellular depots of esterified arachidonate[2,6,7] as well as cytoplasmic sites for cyclo-oxygenase[11,12] and 5-lipoxygenase[58] localization. Finally, increased formation of lipid bodies has been correlated with the enhanced generation of prostaglandin E_2 and leukotriene B_4 by neutrophils[8] and prostaglandin E_2 and leukotriene C_4 by eosinophils.[9] Conversely, suppression of lipid body formation blocked enhanced eicosanoid formation.[8,9] In conjunction with these lines of evidence, the localization of MAP kinases ERK1, ERK2, and p38 and cPLA$_2$ with phosphatidylcholine in lipid bodies strongly suggests that cytoplasmic lipid bodies may be intracellular sites of eicosanoid formation distinct from those in the nuclear envelope and plasma membrane.

In conclusion, we have demonstrated the co-localization of MAP kinases and cPLA$_2$ at cytoplasmic lipid bodies. Such discrete subcellular association of both upstream kinases and cPLA$_2$ with an esterified arachidonate reservoir may represent a distinct and efficient mechanism for extracellular-ligand-induced eicosanoid formation. Our results therefore suggest that lipid bodies may be a cytoplasmic locus of eicosanoid synthesis and play a pivotal role in inflammation.

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