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Identification of Phosphorylation Sites of Human 85-kDa Cytosolic Phospholipase A_2 Expressed in Insect Cells and Present in Human Monocytes*

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The phosphorylation sites on the human, 85-kDa cytosolic phospholipase A_2 ($cPLA_2$) were identified using recombinant $cPLA_2$ expressed in *Spodoptera frugiperda* (Sf9) cells. Analysis by high performance liquid chromatography of tryptic digests of ^{32}P-labeled recombinant $cPLA_2$ showed four major peaks of radiolabeled phosphopeptides. The phosphorylated residues were identified as Ser-437, Ser-454, Ser-505, and Ser-727 using mass spectrometry and automated Edman sequencing. Sf9 cells infected with recombinant virus expressing $cPLA_2$ exhibited a time-dependent release of arachidonic acid in response to the calcium ionophore A23187 or the protein phosphatase inhibitor okadaic acid, which was not observed in Sf9 cells infected with wild-type virus. Stimulation of Sf9 cells with A23187 and okadaic acid also increased the level of phosphorylation of $cPLA_2$. Okadaic acid, but not A23187, induced a gel shift of $cPLA_2$ and increased the level of phosphorylation of Ser-727 by 4.5-fold, whereas the level of phosphorylation of the other sites increased by 60% or less in response to both agonists. To determine whether the same sites on $cPLA_2$ were phosphorylated in mammalian cells, human monocytes were studied. Okadaic acid stimulation of monocytes induced a gel shift of $cPLA_2$, increased the release of arachidonic acid, and increased the level of phosphorylation of $cPLA_2$ on serine residues. Comparison of two-dimensional peptide maps of tryptic digests of ^{32}P-labeled recombinant $cPLA_2$ and human monocyte $cPLA_2$ demonstrated that the same peptides on $cPLA_2$ were phosphorylated in mammalian cells as in insect cells. These results show that the Sf9-baculovirus expression system is useful for investigation of the phosphorylation sites on $cPLA_2$. The results also suggest that phosphorylation of the $cPLA_2$ by protein kinases other than mitogen-activated protein kinase may be important for the regulation of arachidonic acid release.

Arachidonic acid is the precursor for a variety of proinflammatory lipid mediators including the leukotrienes and prostaglandins. The production of these potent agents is largely controlled by the availability of free arachidonic acid. The release of arachidonic acid from membrane phospholipid is a regulated process that is catalyzed by phospholipase A_2 and occurs in many cell types in response to a variety of physiological and pharmacological agonists. Current evidence implicates the 85-kDa, cytosolic PLA_2 ($cPLA_2$)[1] as an important enzyme in mediating agonist-induced arachidonic acid release and eicosanoid production (1, 2). $cPLA_2$, which shows specificity for arachidonic acid-containing substrates (3–5), is posttranslationally regulated both by phosphorylation and by the level of intracellular calcium. Calcium is not required for catalytic activity of $cPLA_2$ but is required for binding of the $cPLA_2$ to phospholipid vesicles (6–8). Calcium-dependent membrane binding of $cPLA_2$ occurs at calcium concentrations of 300 nM or greater and is mediated by a calcium-dependent lipid binding domain present at the amino terminus of $cPLA_2$ (6, 9, 10). Stimulation of mast cells by calcium ionophore or by IgE/antigen has recently been shown to induce translocation of the $cPLA_2$ from the cytosol to the nuclear membrane (11).

Treatment of a variety of cell types with diverse physiological agonists such as growth factors, thrombin, colony-stimulating factor-1, interleukin-1, lipopolysaccharide (LPS), bacteria, vasopressin, and zymosan induce phosphorylation of $cPLA_2$ resulting in an increase in its activity (1, 12–17). In stimulated macrophages, platelets, fibroblasts, and Chinese hamster ovary cells, phosphorylation of $cPLA_2$ occurs exclusively on serine residues (1, 13, 18). $cPLA_2$ is a substrate for protein kinase C, cyclic AMP dependent kinase (protein kinase A), and mitogen-activated protein (MAP) kinase *in vitro*, but only phosphorylation by MAP kinase induces a consistent increase in $cPLA_2$ activity and decreases its electrophoretic mobility (gel shift) (19–21). $cPLA_2$ has one consensus site for MAP kinase at Ser-505 (21). Evidence for a role for MAP kinase in $cPLA_2$ phosphorylation and activation has been provided in transfection studies showing that agonist-stimulated arachidonic acid

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[1] The abbreviations used are: $cPLA_2$, cytosolic phospholipase A_2; LPS, lipopolysaccharide; MAP kinase, mitogen-activated protein kinase; Sf9 cell, *S. frugiperda* cell; PMA, phorbol myristate acetate; HSA, human serum albumin; PBS, phosphate-buffered saline; wild-type virus, wild-type *A. californica* multiply enveloped nuclear polyhedrosis virus; recombinant virus, recombinant *A. californica* multiply enveloped nuclear polyhedrosis virus expressing $cPLA_2$; FBS, fetal bovine serum; Me_2SO, dimethyl sulfoxide; HPLC, high performance liquid chromatography; micro-LC/MS, micro liquid chromatography electrospray ionization mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; u, atomic mass units.

release is diminished in Chinese hamster ovary cells overexpressing $cPLA_2$ that has been mutated at Ser-505 compared with arachidonic acid release that is seen in cells overexpressing the wild-type enzyme (21). Although a role for MAP kinase phosphorylation of $cPLA_2$ is implicated, analysis of two-dimensional phosphopeptide maps of ^{32}P-labeled $cPLA_2$ from stimulated macrophages suggests that this enzyme is phosphorylated on multiple sites and that several of these sites show increased phosphorylation after cell stimulation (13). Recent studies have also provided evidence for MAP kinase-independent pathways leading to $cPLA_2$ phosphorylation and activation (18, 22, 23).

Identification of the phosphorylation sites on $cPLA_2$ is important for understanding its mechanisms of regulation. To identify the phosphorylation sites, we have taken advantage of the baculovirus expression system in which we have previously demonstrated that $cPLA_2$ is expressed as a phosphoprotein and from which large amounts of $cPLA_2$ can be obtained (24). It has been observed that the sites that are phosphorylated on proteins expressed in baculovirus-infected insect cells and mammalian cells are often the same (25, 26). A recent report has demonstrated that $cPLA_2$ is phosphorylated on Ser-505 when expressed in insect cells (27). In this report we demonstrate that *Spodoptera frugiperda* (Sf9) cells expressing $cPLA_2$ can be induced to release arachidonic acid in response to the agonists calcium ionophore A23187 and the protein phosphatase inhibitor okadaic acid and that this functional response in okadaic acid-treated cells correlates with increased phosphorylation of a novel site on $cPLA_2$. Evidence is also presented demonstrating that the sites phosphorylated on $cPLA_2$ in infected Sf9 cells also occur on $cPLA_2$ in human monocytes.

EXPERIMENTAL PROCEDURES

Materials

Grace's supplemented phosphate-free medium and RPMI 1640 phosphate-free medium were obtained from Life Technologies, Inc. RPMI 1640 medium and calcium- and magnesium-free Hanks' balanced salt solution were from Whittaker Bioproducts (Walkersville, MD). Sequencing grade, modified trypsin was from Promega. [^{32}P]Orthophosphoric acid (8,500–9,000 Ci/mmol) and [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (100 Ci/mmol) were from DuPont NEN. LPS was from List Biological Labs (Campbell, CA). Okadaic acid and phorbol myristate acetate (PMA) were from LC Services Co. (Woburn, MA). Zymosan, phenylmethylsulfonyl fluoride, leupeptin, aprotinin, and A23187 were from Sigma. Human serum albumin (HSA) was obtained from Intergen Co. (Purchase, NY). Prior to use as a stimulus the zymosan was suspended in calcium- and magnesium-free phosphate-buffered saline (PBS) and boiled for 10 min, followed by centrifugation. After resuspension in PBS, the boiling procedure was repeated two more times. For preparation of opsonized zymosan, the zymosan was incubated with a 1:1 dilution of human serum in 0.9% saline solution for 30 min at 37 °C and washed once with PBS before final resuspension in PBS.

Cell Culture

Sf9 cells were grown and infected with either wild-type *Autographa californica* multiply enveloped nuclear polyhedrosis virus (wild-type virus) or recombinant *A. californica* multiply enveloped nuclear polyhedrosis virus expressing $cPLA_2$ (recombinant virus) as described previously (24). Cell manipulations were performed at 65–70 h postinfection unless otherwise indicated. Human mononuclear cells were purified from healthy donors on plasma/Percoll gradients (28). The mononuclear cells were washed in calcium- and magnesium-free Hanks' balanced salt solution and then plated at 5×10^6 cells/well in 12-well plates and incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. After 1 h adherent cells were vigorously rinsed three times with Hanks' balanced salt solution to remove nonadherent lymphocytes, and then 1 ml of RPMI 1640, supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin sulfate, 100 units/ml penicillin G, and 0.29 mg/ml glutamine was added. Approximately 15% of the cells initially seeded adhered to the culture dish as determined by the number of nuclei/well using a Coulter counter.

[^{3}H]Arachidonic Acid Release

Sf9 cells plated at 0.5×10^6 cells/well in 12-well plates in 1.0 ml of Grace's supplemented medium containing 10% FBS were labeled with [^{3}H]arachidonic acid (0.5 μCi/ml) for 18 h. The cells were rinsed three times with Grace's supplemented medium containing 0.1% HSA to remove unincorporated arachidonic acid and then stimulated in Grace's supplemented medium containing 0.1% HSA with okadaic acid (1 μM) or A23187 (0.5 μg/ml). After stimulation, the medium was removed and centrifuged at $1,400 \times g$ for 10 min. The cells were scraped into 1 ml of calcium- and magnesium-free PBS containing 0.1% Triton X-100. The amount of radioactivity in the cells and media was measured by liquid scintillation spectrophotometry.

Human monocytes were allowed to adhere for 4 h and then labeled with [^{3}H]arachidonic acid (0.25 μCi/ml) in RPMI 1640 containing 10% FBS, 100 μg/ml streptomycin sulfate, 100 units/ml penicillin G, and 0.29 mg/ml glutamine. After 16 h the cells were rinsed three times with RPMI 1640 containing 0.25% HSA. Cells were stimulated in 1 ml of RPMI 1640 containing 0.25% HSA with PMA (500 nM), A23187 (0.5 μg/ml), okadaic acid (1 μM), opsonized zymosan (60 particles/cell) or LPS (1 μg/ml). After stimulation, the medium was removed and centrifuged at $1,400 \times g$ for 10 min. The cells were scraped into 1 ml of calcium- and magnesium-free PBS containing 0.1% Triton X-100. The amount of radioactivity in the cells and media was measured by liquid scintillation spectrophotometry.

Phosphorylation Studies

At 20 h postinfection, Sf9 cells plated at a density of 2.5×10^6 cells/25 cm^2 flask were labeled for 48 h with [^{32}P]orthophosphoric acid (0.5 mCi/ml) in 2.5 ml of Grace's supplemented, phosphate-free medium containing 10% FBS, 100 μg/ml streptomycin sulfate, and 100 units/ml penicillin G. The labeling medium was removed, and Grace's supplemented phosphate-free medium containing 0.1% HSA was added. For stimulation, okadaic acid (1 μM) or A23187 (0.5 μg/ml) dissolved in Me_2SO was added, and the cells were incubated for 2 h at 27 °C. Controls were incubated with Me_2SO alone. After stimulation, the medium was removed from the cells and replaced with lysis buffer (50 mM Hepes, pH 7.4, 10% glycerol, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 100 mM sodium fluoride, 3 μM *p*-nitrophenyl phosphate, 200 μM sodium orthovanadate, and 10 mM tetrasodium pyrophosphate). Lysates were centrifuged at $16,000 \times g$, and the supernatant was precleared with preimmune serum-coated protein A-Sepharose beads for 1 h at 4 °C. The beads were removed by centrifugation, and ^{32}P-labeled $cPLA_2$ was immunoprecipitated from the Sf9 cell lysates, as described below.

For ^{32}P labeling the mononuclear cells were plated at 70×10^6 cells/100-mm dish, as above, allowed to adhere for 4 h, and then the monolayer was washed three times with phosphate-free RPMI 1640. The cells were labeled overnight with [^{32}P]orthophosphoric acid (1 mCi/dish) in 5 ml of phosphate-free RPMI 1640 containing 10% dialyzed FBS, 100 μg/ml streptomycin sulfate, 100 units/ml penicillin G, and 0.29 mg/ml glutamine. Stimulants were added to the labeling medium for 2 h. The cells were rinsed in Hanks' balanced salt solution and scraped into lysis buffer. The lysates were centrifuged, and the ^{32}P-labeled $cPLA_2$ was immunoprecipitated from the monocyte cell lysates, as described below.

Phosphorylation of $cPLA_2$ in Vitro with MAP Kinase

To 30 μg of $cPLA_2$ in 50 mM Tris, 100 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM $MgCl_2$, 140 μM ATP, 10 μCi [γ-^{32}P]ATP (3,000 Ci/mmol, DuPont NEN), pH 8.0, was added 60 ng of recombinant MAP kinase (obtained as a generous gift from Prof. E. Krebs, University of Washington), and the mixture was incubated for 2.5 h at room temperature. Radiolabeled $cPLA_2$ was submitted to electrophoresis on a Laemmli gel, and the protein was eluted from the gel and digested with trypsin as described below. The digest was submitted to HPLC (218TP52 column) as described below, and fractions containing cpm in the peak 4 region (see Fig. 4) were pooled. Most of the solvent was removed in a Speed-Vac concentrator (Savant Instruments), and half of the sample was submitted to two-dimensional peptide mapping. The remaining half was concentrated to dryness in a Speed-Vac, and the residue was oxidized with 30 μl of performic acid reagent, diluted with 50 μl of water, and lyophilized as described (29). The oxidized peptide was dissolved in 5 μl of glacial acetic acid, and the sample was submitted to two-dimensional peptide mapping.

Immunoprecipitation of ^{32}P-Labeled cPLA$_2$

Sf9 and monocyte cell lysates were incubated with polyclonal antiserum (11683) generated as described previously against recombinant cPLA$_2$ at either a 1:7.5 or a 1:500 dilution, respectively, for 16 h at 4 °C (24). Protein A-Sepharose beads were added and the mixture tumbled for 2 h at 4 °C. The cPLA$_2$ was recovered by centrifugation at 4 °C. The beads were washed five times in lysis buffer and then boiled in 2 × Laemmli buffer containing 40 mM dithiothreitol (30). The eluted cPLA$_2$ was separated on a 7.5% SDS-polyacrylamide gel, the gel was dried, and the cPLA$_2$ band was visualized by autoradiography.

Preparation of Tryptic Peptides for HPLC and Two-dimensional Peptide Maps

^{32}P-Labeled cPLA$_2$ was excised from dehydrated SDS-polyacrylamide gels, and two to three gel pieces were homogenized in 1.2 ml of 50 mM ammonium bicarbonate containing 0.08% SDS and 1% 2-mercaptoethanol (elution buffer). The samples were boiled for 5 min and then tumbled for 16 h at room temperature. Gel pieces were separated from eluant by centrifugation, another 300 μl of elution buffer was added to the gel pellet, and the sample was rocked 2 h at room temperature. The second eluant was collected by centrifugation, combined with the first, and the volume per tube was reduced in a Speed-Vac to 200 μl. The amount of cPLA$_2$ recovered from Sf9 cell lysates was estimated by comparison with known amounts of purified recombinant cPLA$_2$ (determined by measuring the absorbance at 280 nm) on Coomassie-stained, SDS-polyacrylamide gels (24). Unlabeled recombinant cPLA$_2$ (5 μg) was added to monocyte samples as carrier protein. The cPLA$_2$ was precipitated from elution buffer by the addition of 30 μl of 100% trichloroacetic acid. The tubes were incubated on ice for 2 h, the trichloroacetic acid precipitate was collected by centrifugation in a microcentrifuge at full speed for 10 min at 4 °C, washed two times with ice-cold acetone by centrifugation as above, and the residual acetone was removed with a stream of N_2. Based on Cerenkov counting, the yield of precipitated cPLA$_2$ was greater than 90%. The precipitate was resuspended in 200 μl of freshly made 50 mM ammonium bicarbonate, pH 7.8–8.2, and trypsin was added at a trypsin:cPLA$_2$ ratio of 1:10 by weight. The samples were incubated for 3 h at 37 °C and then stored at −20 °C. The amount of trypsin in the stock solution was assayed each time it was used. A tube containing 20 μg of lyophilized trypsin, as supplied by Promega, was hydrated with 100 μl of Promega trypsin dilution buffer. One ml of 50 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.6, was placed in a cuvette, and 4.8 μl of benzoyl-arginine ethyl ester solution (100 mM) was added. The cuvette was placed in the thermostatted holder of the spectrophotometer, and the absorbance at 253 nm was recorded at 29 °C until the base line was flat. Three μl of trypsin solution was added to the assay, and the absorbance at 253 nm was monitored for several minutes. The typical response from this 0.6-μg portion of trypsin was 0.036 absorbance/min, and this specific activity was used to determine the amount of trypsin in other stock solutions. Trypsin stock solutions were stored at −20 °C, and working solutions were kept at 4 °C for no more than a few hours. Cerenkov counting revealed that greater than 90% of the cpm became solubilized following proteolysis.

Two-dimensional Phosphopeptide Mapping

Ammonium bicarbonate buffer was removed from the tryptic digests by the addition of 500 μl of water, and then the sample was dried in a Speed-Vac. Water was added again, and the process was repeated two more times. The sample was then resuspended in glacial acetic acid or buffer 1, pH 1.9 (acetic acid, 88% formic acid, H_2O) (78:25:897, v/v/v). The sample was subjected to thin layer electrophoresis on 20 × 20-cm Kodak cellulose thin layer chromatography plates. Electrophoresis in the first dimension was performed at 1,400 V for 20 min in ammonium carbonate buffer, pH 8.9 (10 g of ammonium carbonate/liter), using a Hunter Thin Layer Peptide Mapping System (CBS Scientific, Del Mar, CA). The plate was air dried at room temperature overnight and then subjected to ascending chromatography in isobutyric acid:pyridine:acetic acid:butanol:H_2O (65:5:3:2:29, v/v/v/v). The plate was then air dried and subjected to autoradiography.

Phosphoamino Acid Analysis

A tryptic digest of immunoprecipitated cPLA$_2$ was digested in 100 μl of 6N HCl for 1 h at 110 °C. The HCl was removed in a Speed-Vac, and the pellet was redissolved in buffer 1, pH 1.9. A mixture of phosphoserine, phosphothreonine, and phosphotyrosine (5 μg of each) was added. The samples were analyzed by ascending chromatography on Merck 20 × 20-cm cellulose thin layer chromatography plates with butanol:acetic acid:ethanol:H_2O (1:1:1:1, v/v/v/v) (18). Phosphoamino acids were visualized by spraying the plates with a solution of 0.2% ninhydrin in acetone (w/v), and ^{32}P-labeled phosphoamino acids were identified by autoradiography.

Purification of Tryptic Phosphopeptides for Analysis by Micro-LC/MS, MALDI-TOF, and Edman Sequencing

Trypsin-digested, ^{32}P-labeled cPLA$_2$ was injected onto an HPLC column (Vydac 218TP52 or 218HS52, 0.21 × 25 cm), and the column was developed at a flow rate of 0.15 ml/min with 100% solvent A (0.06% CF_3COOH in water (Milli-Q, Millipore)) for 15 min, to 30% solvent B (0.06% CF_3COOH in CH_3CN (Baker HPLC grade)) over 60 min, to 60% solvent B over 30 min, to 100% solvent B over 10 min, and finally holding at 100% solvent B. Peptides were detected by monitoring the absorbance at 210 nm. Fractions were collected into silanized polypropylene microcentrifuge tubes (VWR 20172-935) and were stored at −20 °C prior to further analysis. These tubes were also used for all manipulations of the HPLC fractions, and Cerenkov counting was used at all stages of the analyses to gauge the solubility of the tryptic phosphopeptides.

HPLC Peak 1—Trypsinized, radiolabeled cPLA$_2$ from unstimulated Sf9 cells (30 μg, 142,000 cpm) was injected onto the HPLC column (218TP52), and fractions containing radioactivity in the peak 1 region (see Fig. 4) were pooled. The solution was concentrated to 30 μl in a Speed-Vac concentrator, 400 μl of water was added, and this process was repeated twice; the final volume was adjusted to 100 μl. Three μl of the sample was injected onto the micro-LC/MS. Some of this sample (10 μl) was esterified with ethanol as follows. The sample was concentrated to dryness in a Speed-Vac, and 20 μl of HCl/ethanol was added. After 2 h at room temperature, 10 μl of 0.5% acetic acid was added, and 5 μl of sample was injected onto the LC/MS. The esterification reagent was prepared by adding 160 μl of acetyl chloride dropwise to 1 ml of ethanol with stirring, and after 5 min at room temperature the reagent was used. Purified water (Milli-Q) was used in all subsequent manipulations of the HPLC fractions.

HPLC Peak 3—Trypsinized, radiolabeled cPLA$_2$ from okadaic acid-treated Sf9 cells (690 μg, 116,000 cpm) was injected in two portions onto the HPLC column (218TP52), and fractions containing radioactivity in the peak 3 region (see Fig. 4) from both runs were pooled. Solvent was removed in a Speed-Vac. To the residue was added 60 μl of glacial acetic acid, the tube was gently shaken, and 0.5 ml of water was added. The mixture was immediately injected onto the 218HS52 column. Virtually all of the cpm eluted in a single peak at 80.5 min. The cpm-containing fractions were pooled, and solvent was removed in the Speed-Vac. To the residue was added 2 μl of glacial acetic acid followed by 18 μl of water, and 2 μl was injected onto the micro-LC/MS. A portion (1–2 μl) was also analyzed by positive ion MALDI-TOF using the instrument described previously (31). The sample was deposited on the sample slide and mixed with an equal volume of α-cyano-4-hydroxycinnamic acid reagent (5 mg/ml in acetonitrile, 0.1% aqueous trifluoroacetic acid (1:2)).

HPLC Peak 2—Trypsinized, radiolabeled cPLA$_2$ from okadaic acid-treated Sf9 cells (900 μg, 240,000 cpm) was injected in three portions onto the HPLC column (218HS52), and fractions containing radioactivity in the peak 2 region (see Fig. 4) from two of the runs were pooled. Peak 2 fractions from the third HPLC run were stored at −20 °C and were not used further. Solvent was removed from the two tubes of pooled fractions in a Speed-Vac, and to each tube was added 20 μl of glacial acetic acid. After gentle mixing, each sample was spotted onto a separate cellulose plate. To ensure complete transfer onto the plate, a second portion of acetic acid (10 μl) was added to each tube, and this material was transferred to the plates. The plates were submitted to electrophoresis and ascending chromatography as described above. Prior to autoradiography (3-h exposure), the plates were air dried overnight at room temperature. The region of each plate containing the radioactive material was scraped with a razor blade, and the cellulose powder was transferred to a microcentrifuge tube. To the cellulose powder from each of the two samples was added 0.5 ml of buffer 1, pH 1.9, the tubes were mixed by vortexing for 2 min and microcentrifuged for 15 min. The supernatants were transferred to new tubes, and this extraction procedure was repeated. After combining the supernatants, the solvent was reduced to 40–50 μl in each sample in a Speed-Vac, and the samples were stored at −20 °C. Each tube contained about 1,200 cpm. Micro-LC/MS was carried out by mixing a 3–4-μl aliquot of two-dimensional map-purified peptide with 5 μl of 0.5% acetic acid and injecting 6 μl of the mixture onto the instrument. Purified material was also analyzed by MALDI-TOF.

A portion of this two-dimensional peptide map-purified phosphopep-

tide (400 cpm) was submitted to Edman sequencing. The peptide was coupled to a Sequelon-AA membrane (MilliGen) according to the procedure provided by the manufacturer. The membrane disc was washed with two 1-ml portions of methanol, and 200 cpm of peptide remained on the disc, which was stored at −20 °C for a few days. The disc was placed in the reaction vessel of a 477A Sequencer (Applied Biosystems). Sequencing reaction cycles were carried out with standard reagents and conditions except that the S3 wash, which is usually *n*-butyl chloride, consisted of 2 mM NaH_2PO_4 in methanol:water (9:1), pH 7 (not adjusted). The entire S3 wash from each cycle was diverted into glass vials.

Micro-LC

The HPLC grade solvents methanol, acetonitrile, and acetic acid were purchased from Fisher Scientific. Microcolumns were made by employing 98-μm inner diameter fused silica capillary tubing obtained from Polymicro Technologies (Tucson, AZ) (32). The columns were packed with PerSeptive Biosystems (Boston) POROS 10 R2, a 10-μm reverse-phase packing material, to a length of 15–20 cm. Samples were injected onto the column using a high pressure packing device, as described previously (32). Micro-LC was performed using Applied Biosystems 140B dual syringe pumps. The flow rate from the pumps was 100 μl/min. The solvent stream was split, ~50:1, precolumn, to produce a final flow rate of 1–2 μl/min. After loading the sample the column was washed with 100% solvent A (0.5% acetic acid) for 5 min. The peptides were eluted with a linear gradient of 0–80% B (80% acetonitrile, 20% water in 0.5% acetic acid) in 15, 30, or 60 min. The outlet of the column was inserted directly into the electrospray needle.

Electrospray Ionization Mass Spectrometry

Mass spectra were recorded on a Finnigan MAT (San Jose, CA) TSQ700 equipped with an electrospray ionization source as described previously (33). Electrospray ionization was performed using the following conditions. The needle voltage was set at 4.6 kV. The sheath and auxiliary gases consisted of nitrogen gas (99.999%) and were set at 20–25 p.s.i. and 5–10 units, respectively. The heated capillary temperature was set at 150 °C. A sheath liquid flowed around the end of the column at a flow rate of 1.5 μl/min and was a methanol:water (70:30) mixture containing 0.1% acetic acid.

Mass spectra were acquired as peptides eluted from the LC by scanning Q3 at a rate of 500 u/s over the range 400-1,500 *m/z*. Peak widths ranged from 1.5 to 2.0 u. Signal was detected with a conversion dynode/electron multiplier. Sequence analysis of peptides was performed during a second HPLC analysis by selecting the precursor ion with a 2–3-u (full width at half height) wide window in the first mass analyzer and passing the ions into a collision cell filled with argon to a pressure of 3–4 millitorr. Collision energies were on the order of 10–50 eV (E_{lab}). The fragment ions produced in Q2 were transmitted to Q3, which was scanned at 500 u/s over a mass range of 50 u to the molecular weight of the precursor ion to record the fragment ions. Peak widths in the second mass analyzer ranged from 1.5 to 2.0 u. The electron multiplier setting was 200–400 V higher than that used to record the molecular weight.

Data Base Searching

Amino acid sequence data bases were searched directly with tandem mass spectra by a computer algorithm previously described (34–36). All computer algorithms were written on a DEC station 5000/200 computer by using the C programming language under the Ultrix operating system. The OWL data base, version 24.0, was obtained as ASCII text files in the FASTA format from the National Center for Biotechnology Information (NCBI) by anonymous file transfer protocol. A species-specific data base was created by removing the protein sequences derived from *Homo sapiens* to create subsets of the OWL.

RESULTS

Stimulated Arachidonic Acid Release by Sf9 Cells Expressing $cPLA_2$—Previous studies have demonstrated that $cPLA_2$ is expressed to 3–5% of the cellular protein in Sf9 insect cells using the baculovirus expression system, and the enzyme is phosphorylated on Ser-505, the MAP kinase consensus site (24, 27, 37). Experiments were carried out to determine if this expression system could be used to study the regulation of $cPLA_2$ activity. Sf9 cells were found to readily incorporate radiolabeled arachidonic acid into membrane phospholipid. When Sf9 cells were infected with recombinant baculovirus containing $cPLA_2$ and

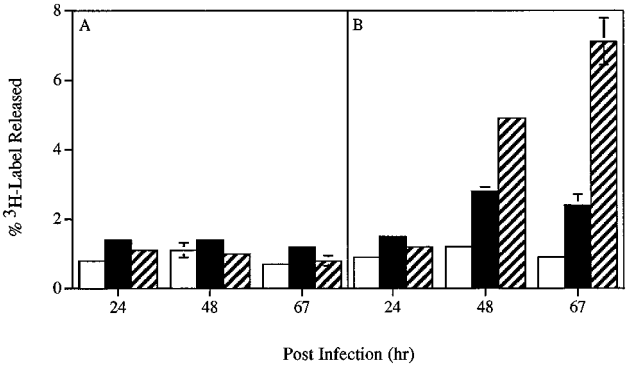

FIG. 1. **[^{3}H]Arachidonic acid release from Sf9 cells.** Sf9 cells were infected with either wild-type virus (*panel A*) or recombinant virus containing the gene for $cPLA_2$ (*panel B*). Cells were labeled with [^{3}H]arachidonic acid 18 h prior to the addition of stimuli. At the indicated times postinfection [^{3}H]arachidonic acid-labeled Sf9 cells were treated with A23187 (0.5 μg/ml, *solid bars*), okadaic acid (1 μM, *striped bars*), or vehicle (0.1% Me_2SO, *open bars*) for 2 h. ^{3}H label released into the medium is expressed as a percent of the total radioactivity (cell associated plus media). The results are expressed as mean ± S.D. of triplicate values from a representative experiment.

then labeled with [^{3}H]arachidonic acid, the cells could be stimulated to release arachidonic acid with either A23187 or okadaic acid (Fig. 1). The degree of stimulated arachidonic acid release correlated with the level of $cPLA_2$ expression which we have previously demonstrated is maximal between 48 and 67 h postinfection (24). In contrast, when Sf9 cells were infected with wild-type baculovirus there was only a small increase in arachidonic acid release in response to A23187 treatment, but the degree of release did not change at different times after infection. Consequently, the stimulated release of arachidonic acid in Sf9 cells infected with recombinant virus could be attributed to the specific activation of $cPLA_2$ by either A23187 or okadaic acid. The time course of [^{3}H]arachidonic acid release in response to A23187 and okadaic acid from Sf9 cells expressing $cPLA_2$ is shown in Fig. 2. An increase in arachidonic acid release above control levels could be detected 15 min after treatment with either A23187 or okadaic acid. The label continued to accumulate gradually in the medium up to 2 h after A23187 treatment reaching 3-fold the control levels. A greater level of free arachidonic acid, 7–8 times the level in unstimulated cells, was released in the medium up to 2–4 h after okadaic acid treatment.

Phosphorylation of $cPLA_2$ in Sf9 Cells—It has been demonstrated that site-specific phosphorylation of $cPLA_2$ by MAP kinase reduces its electrophoretic mobility on SDS gels (21). $cPLA_2$ expressed in unstimulated Sf9 cells 67 h after viral infection runs as a doublet on a SDS-polyacrylamide gel, with approximately equal amounts in both forms (Fig. 3). Treatment with A23187 did not affect this pattern, whereas okadaic acid induced a complete gel shift. The ability of okadaic acid to induce a complete gel shift suggests a correlation of $cPLA_2$ phosphorylation with activation of $cPLA_2$ leading to arachidonic acid release. To explore further the role that phosphorylation plays in $cPLA_2$ activation in this model system, the phosphorylation sites in control and okadaic acid-treated Sf9 cells were identified.

HPLC Analysis of Tryptic Phosphopeptides from $cPLA_2$ Expressed in Sf9 Cells—A typical HPLC profile of tryptic peptides (absorbance at 210 nm) and phosphopeptides (cpm) of ^{32}P-labeled $cPLA_2$ from okadaic acid-stimulated Sf9 cells is shown in Fig. 4. Four major peaks of radioactivity were seen. The distribution of counts in the four peaks obtained from $cPLA_2$ immunoprecipitated from unstimulated Sf9 cells and the change in labeling of the peaks in response to agonist treat-

ment are shown in Table I. Peaks 1 and 4 were the major ^{32}P-labeled phosphopeptides of cPLA$_2$ from unstimulated cells. Treatment of Sf9 cells with A23187 induced only a small increase in labeling of the peptides. The most dramatic change was observed after stimulation with okadaic acid, which resulted in a 4.5-fold increase in ^{32}P labeling of peak 2. Only small increases in labeling of peaks 1, 3, and 4 occurred in response to okadaic acid. In a few HPLC runs, a small peak of radioactivity preceded peak 4 by 6 min (not shown). As described below, this peak is an oxidized derivative of peak 4. Importantly, the total cpm eluted from the HPLC column was typically 70–80% of that applied, and <0.5% of the cpm eluted in the void volume. In addition, greater than 90% of the cPLA$_2$-associated cpm was solubilized following trypsinization. The fact that yields of cpm are high in all steps rules out the possibility that a major phosphopeptide was lost during sample manipulations.

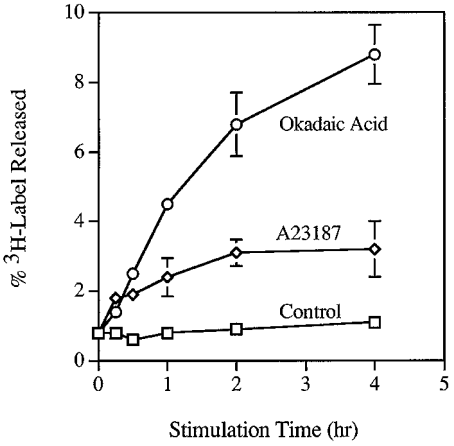

FIG. 2. **Time course of [^{3}H]arachidonic acid release from Sf9 cells.** [^{3}H]Arachidonic acid-labeled Sf9 cells infected with recombinant virus were stimulated at 67 h postinfection for the times indicated with A23187 (0.5 μg/ml, ◇), okadaic acid (1 μM, ○), or vehicle (0.1% Me_2SO, □). ^{3}H label released into the media is expressed as a percent of the total radioactivity (cell associated plus media). The results are expressed as the mean ± S.D. of triplicate values from a representative experiment.

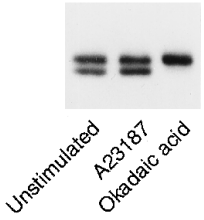

FIG. 3. **Effect of stimulation of Sf9 cells on mobility of recombinant cPLA$_2$ on a SDS-polyacrylamide gel.** Sf9 cells infected with recombinant virus were stimulated at 67 h postinfection for 2 h with Me_2SO (0.1%), A23187 (0.5 μg/ml), or okadaic acid (1 μM). Cell lysates (400 ng/lane) were separated on a 10% SDS-polyacrylamide gel prepared as described previously (24). Immunoblots were incubated with a polyclonal antibody against the recombinant cPLA$_2$, and the Amersham ECL system was used for detection.

The ^{32}P-labeled tryptic phosphopeptides were also analyzed on two-dimensional phosphopeptide maps. A representative phosphopeptide map of ^{32}P-labeled cPLA$_2$ from okadaic acid-treated Sf9 cells is shown in Fig. 5. Five major spots were observed. To determine the correspondence between the HPLC peaks and the spots on the two-dimensional peptide maps, each of the HPLC-purified phosphopeptides derived from cPLA$_2$ expressed in Sf9 cells was individually analyzed by two-dimensional peptide mapping. Since phosphopeptide spots 1, 2, and 3 run close together, pairs of HPLC peaks (1 + 2, 1 + 3, and 2 + 3) were also analyzed by two-dimensional phosphopeptide mapping to make the assignments unequivocal. In this way phosphopeptide spots 1–4 were assigned. There still remained a significant phosphopeptide spot near spot 1 (labeled 5 in Fig. 5) which had no corresponding peak in the HPLC. Since HPLC peaks 1–4 account for virtually all of the eluted cpm, it was suspected that phosphopeptide spot 5 was the result of decomposition of one or more phosphopeptides during analysis. This was indeed the case. The intensity of spot 5 relative to its neighboring spot 1 was highly variable in independent analyses. When spot 5 was eluted from the cellulose plate and then injected onto the HPLC column, all of the cpm eluted in the void volume, and as described above, virtually no cpm was detected in the void volume of trypsin-digested cPLA$_2$ that was injected onto HPLC without prior submission to two-dimensional peptide mapping. Further evidence indicated that phosphopeptide spot 5 comes completely from phosphopeptide 1. When the phosphopeptide spot 1 was eluted from the cellulose plate and reanalyzed by two-dimensional peptide mapping, both spot 1 and 5 were seen. The nature of this decomposition was not investigated further.

Identification of cPLA$_2$ Phosphorylation Sites by Mass Spectrometry—Analysis of HPLC peak 1 material by micro-LC/MS showed several peptides (data not shown). The most prominent ion was accompanied by another ion 33 u smaller, which is indicative of the loss of neutral phosphoric acid from a triple-charged ion. The loss of phosphoric acid is often seen in phosphoserine- and phosphothreonine-containing peptides and is thus used as a marker for phosphopeptides (however, see below). The observed molecular weight of this putative phosphopeptide, 2,079 (not shown), is consistent with the tryptic peptide of amino acid residues 428–445 from cPLA$_2$ containing a single phosphate. To confirm the identity of this peptide and to locate the phosphorylation site, micro-LC/MS/MS analysis was carried out. The resulting product ion spectrum (not shown) was sufficient to confirm the identity of the peptide and to narrow down the site of phosphorylation to either Ser-435 or Ser-437. However, it was not possible to determine confidently which of these two serines was phosphorylated, although the

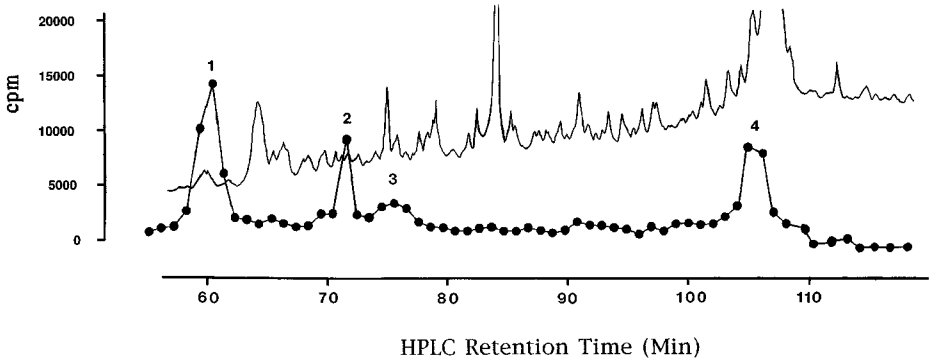

FIG. 4. **HPLC of tryptic peptides derived from cPLA$_2$ from okadaic acid-stimulated Sf9 cells.** After trypsinization of trichloroacetic acid-precipitated cPLA$_2$, the digest was applied to a C_{18} reverse-phase HPLC column (Vydac 218TP52). Other details are given under "Experimental Procedures." Eluted cpm were determined by Cerenkov counting (*dotted line*), and the peptide elution was monitored by absorbance at 210 nm.

TABLE I
^{32}P-Labeling pattern of tryptic phosphopeptides of recombinant cPLA$_2$ from stimulated and unstimulated Sf9 cells

Tryptic digests of ^{32}P-labeled recombinant PLA$_2$ from unstimulated, A23187-stimulated, and okadaic acid-stimulated Sf9 cells were separated by HPLC. Fractions were collected and radioactivity determined by Cerenkov counting. Data represent the mean ± S.E. from three separate experiments.

Distribution of ^{32}P label in phosphopeptides[a]		Increase of ^{32}P label in phosphopeptides[a]	
Peaks	Unstimulated	A23187	Okadaic acid
	%	*-fold*	
1	49 ± 4	1.4 ± 0.2	1.3 ± 0.3
2	7 ± 2	1.5 ± 0.2	4.5 ± 0.8
3	9 ± 2	1.2 ± 0.1	1.6 ± 0.3
4	34 ± 3	1.3 ± 0.1	1.4 ± 0.1

[a] The cpm from the different HPLC runs were normalized to a constant mass of trypsinized cPLA$_2$. The latter was obtained from the peak areas of the 210 nm absorbance trace for each HPLC run.

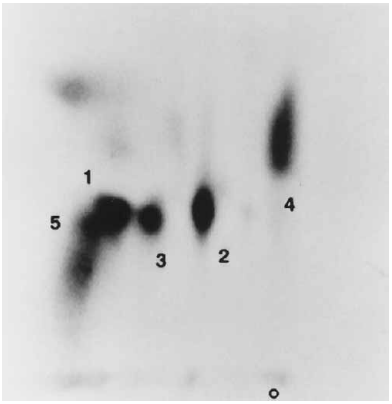

FIG. 5. **Two-dimensional tryptic phosphopeptide maps of ^{32}P-labeled recombinant cPLA$_2$ from Sf9 cells.** A tryptic digest of immunoprecipitated, gel-purified recombinant cPLA$_2$ from okadaic acid-stimulated Sf9 cells was separated by two-dimensional phosphopeptide mapping as described under "Experimental Procedures." Phosphopeptides were detected by autoradiography. The sample was spotted at the position marked by ○. Electrophoresis was run in the horizontal dimension, with the anode on the left, and chromatography was run in the vertical dimension. Phosphopeptides are labeled 1, HPLC-purified tryptic peptide 1; 2, peptide 2; 3, peptide 3; 4, peptide 4 and were identified by comigration with HPLC-purified tryptic peptides (see "Results" for a description of spot 5).

results were more consistent with the phosphate being located on the Ser-437. This is because the predicted b-type and y-type product ions that are different between the the Ser-435 *versus* the Ser-437 were not observed, and such a negative result cannot be used as definitive proof of structure. Thus, the HPLC peak 1 phosphopeptide was treated with ethanol/HCl to convert all of its carboxyl groups to ethyl esters. The micro-LC/MS/MS analysis is shown in Fig. 6*A*, and the site of phosphorylation was identified as Ser-437. The y ions, y_1–y_9, are observed, and at Ser-437 the mass of the y series increases by 80 u, consistent with a phosphate on Ser-437. The y ions y_9–y_{12} and the double-charged y ions, y_{15}–y_{17}, show the characteristic loss of neutral phosphoric acid. The b ions, b_5–b_9, are also observed, as well as a series of b ions resulting from the loss of phosphoric acid (b_{10}–b_{12}).

Analysis of HPLC peak 3 by MALDI-TOF gave two major peaks of molecular weights 2,483 and 2,562 (not shown). These differ in mass by 79, suggesting that the lighter peptide is the nonphosphorylated version of the heavier peptide. The mass corresponds to the tryptic peptide 446–467. Analysis of this material by micro-LC/MS also showed the presence of these two peptides. Only a very weak neutral loss ion was seen for the phosphopeptide. The micro-LC/MS/MS spectrum of the putative phosphopeptide is shown in Fig. 6*B*. A series of y ions, y_1–y_{11}, as well as a series of double-charged y ions, y_5–y_{21}, was seen, indicating that Ser-454 is phosphorylated. Loss of phosphoric acid from double-charged y ions, y_{15}–y_{21}, was observed.

Analysis of HPLC peak 2 by micro-LC/MS was problematic in that this HPLC fraction contained multiple peptides, and no major peak was accompanied by a fragment resulting from the neutral loss of phosphoric acid. Furthermore, no pair of peaks that differed by the mass of the phosphate group was found. However, phosphoamino acid analysis of ^{32}P-labeled peak 2 material confirmed that phosphoserine was present (Fig. 7, *lane 2*). Consequently, HPLC peak 2 material was purified further by HPLC and two-dimensional peptide mapping (see "Experimental Procedures"). Even after further purification, a candidate phosphopeptide could not be identified. As a result, the peptide was submitted to automated Edman sequencing. This analysis revealed a major component that was identified as the tryptic peptide starting at residue 446, which suggests that it is the tryptic peptide 446–467. Analysis of the original micro-LC/MS data set revealed the presence of this peptide in its nonphosphorylated form. Thus, remarkably, HPLC peak 2 material is contaminated with HPLC peak 3 material even after extensive purification. The sequencing also revealed a minor component with the sequence QNPS*XXX*VSLSNVEA*X*, where *X* designates amino acids that could not be identified in the sequencing cycle. The sequencing data suggested that this peptide (721–736) is the result of incomplete trypsin digestion. The original micro-LC/MS data set was analyzed for the presence of this peptide and its phosphorylated derivative. Although ions corresponding to this peptide were not observed, a major peptide in the mixture had a mass corresponding to the 721–736 peptide with one phosphate and three additional oxygens (calculated 1,876.0 from the observed *m/z* values of the +2 and +3 ions of 626 and 939, respectively). It should be stressed that no loss of phosphoric acid was seen from this peptide. The mass data suggested that the cysteine residue of the 721–736 peptide had become oxidized during isolation. A major peak of mass 1,876.2 was also seen in the MALDI-TOF analysis of this phosphopeptide (not shown). Fig. 6*C* shows the micro-LC/MS/MS spectrum of the +3 ion. The y ions y_1–y_{14} were observed, and the series indicates that Ser-727 is phosphorylated. An incomplete series of b ions was observed, as well as fragments that are the result of multiple losses of water and ammonia. This b ion series is also consistent with Ser-727 as the phosphorylation site since the high negative charge near the internal trypsin cleavage site is probably the reason that trypsin did not cleave at this site. Finally, an authentic sample of the Ser-727 phosphopeptide was prepared by oxidation (29) of a machine-synthesized phosphopeptide and was found to have the same HPLC retention time as cPLA$_2$-derived peak 2.

Data Base Searching—The MS/MS spectra in Fig. 6, *A-C*, were used to search the human protein sequence data base of 17,903 entries (see "Experimental Procedures") using the algorithm SEQUEST. This procedure resulted in the correct identification of the three cPLA$_2$ phosphopeptides described above as well as their correct phosphorylation sites.

MAP Kinase Phosphorylation Site—Previous studies have shown that cPLA$_2$ expressed in Sf9 cells is phosphorylated at its MAP kinase site (Ser-505) (37). To determine which, if any, of the HPLC peaks 1–4 is the MAP kinase-phosphorylated tryptic peptide, purified cPLA$_2$ was phosphorylated *in vitro* with recombinant MAP kinase (see "Experimental Procedures"). HPLC analysis of the trypsin digest revealed a major peak of radioactivity which comigrated exactly with peak 4 material from the cPLA$_2$ phosphorylated in Sf9 cells (not

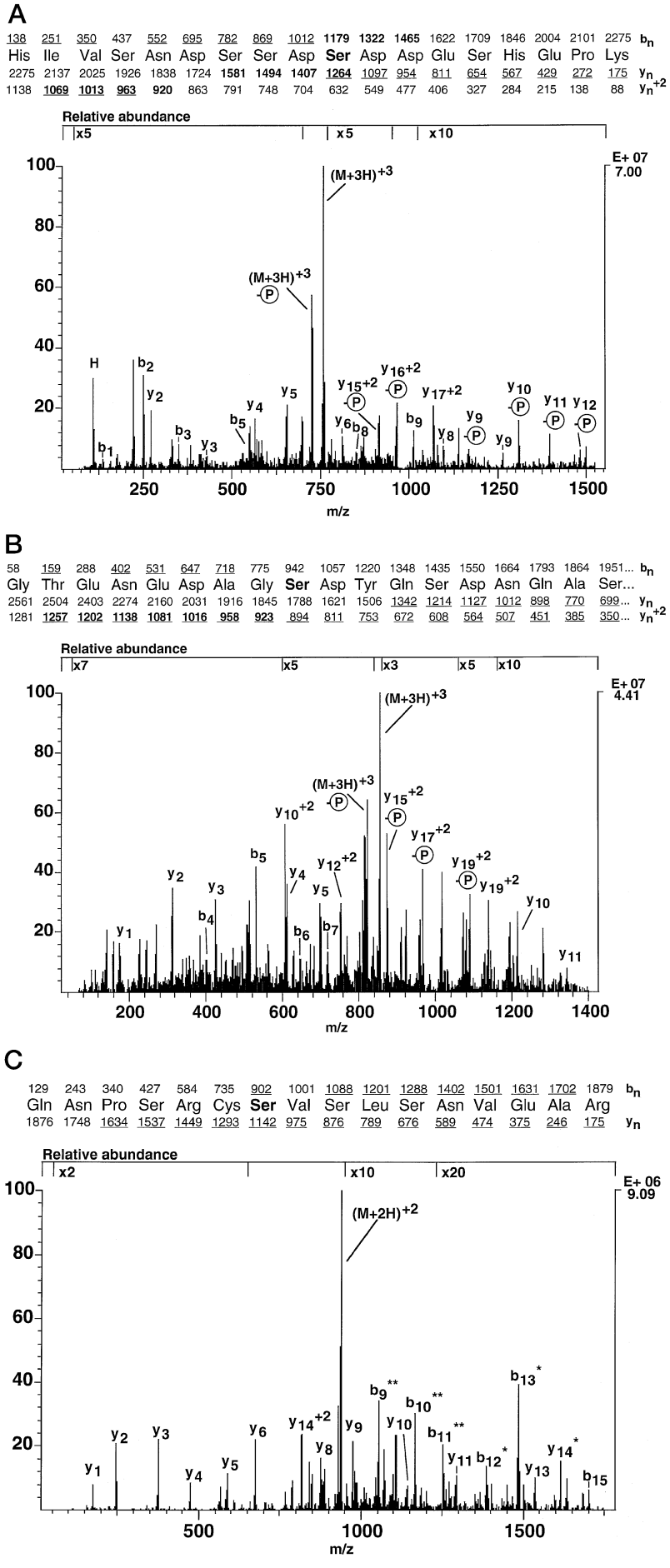

FIG. 6. **Collision-induced dissociation mass spectra recorded on ions generated from cPLA$_2$ tryptic peptides.** Fragments of types b and y having the general formulas $H(NHCHRCO)_n^+$ and $H_2(NHCHRCO)_nOH^+$, respectively, are shown above and below the amino acid sequence at the *top* of the figure. In addition, double-charged y ions are also shown. All ions correspond to average masses. Ions observed in the mass spectrum are *underlined*, and ions resulting from neutral loss of phosphoric acid are shown in *boldface* type. The serine in *boldface* type was determined to be phosphorylated. Leu and Ile were assigned by correspondence to the known sequence. In the spectrum, ions resulting from neutral loss of phosphoric acid are denoted by $-P$. *Panel A*, collision-induced dissociation mass spectrum recorded on the $(M+3H)^{+3}$ ions at *m/z* 759 of a tryptic fragment, residues 428–445, in the ethyl-ester form. *Panel B*, collision-induced dissociation mass spectrum recorded on the $(M+3H)^{+3}$ ions at *m/z* 854 of a tryptic fragment, residues 446–467. Only residues 446–463 are shown at the *top* of the figure. *Panel C*, collision-induced dissociation mass spectrum recorded on the $(M+2H)^{+2}$ ions at *m/z* 939 of a tryptic fragment, residues 721–736. In the spectrum, *asterisks* are used to denote single or multiple neutral losses of water (or ammonia) from an ion.

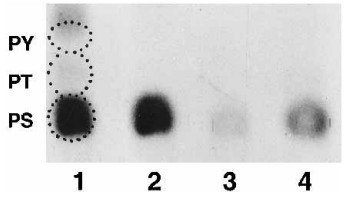

FIG. 7. **Phosphoamino acid analysis.** HPLC-purified ^{32}P-labeled tryptic peptides derived from cPLA$_2$ from okadaic acid-stimulated Sf9 cells (*lane 1*, peak 4; *lane 2*, peak 2) or ^{32}P-labeled tryptic digests derived from cPLA$_2$ from human monocytes (*lane 3*, unstimulated monocytes; *lane 4*, okadaic acid-stimulated monocytes) were hydrolyzed with HCl and separated along with phosphoserine (*PS*), phosphothreonine (*PT*), and phosphotyrosine (*PY*) standards on cellulose thin layer plates as described under "Experimental Procedures." Phosphoamino acid standards were visualized with ninhydrin, and the ^{32}P-labeled phosphoamino acids were detected by autoradiography.

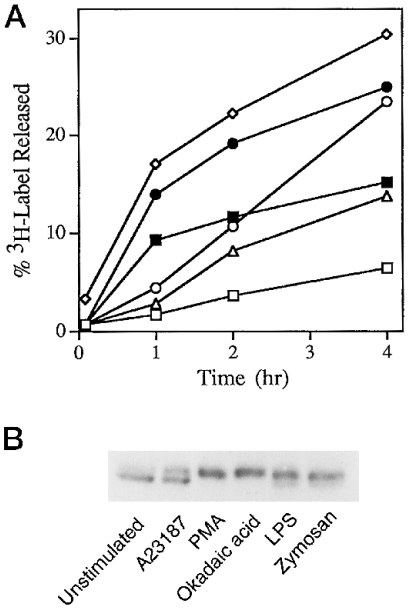

FIG. 8. **Effect of stimulation of human monocytes on [^{3}H]arachidonic acid release and on the mobility of cPLA$_2$ on a SDS-polyacrylamide gel.** *Panel A*, [^{3}H]arachidonic acid-labeled monocytes were incubated with Me_2SO (0.1%, □), okadaic acid (1 μM, △), LPS (1 μg/ml, ■), PMA (500 nM, ○), opsonized zymosan (60 particles/cell, ●), or A23187 (0.5 μg/ml, ◇) for the indicated times. ^{3}H label released into the media is expressed as a percent of the total radioactivity (cell associated plus media). The results are the average of duplicate values from a representative experiment. *Panel B*, monocytes were stimulated for 2 h with Me_2SO (0.1%), okadaic acid (1 μM), LPS (1 μg/ml), PMA (500 nM), opsonized zymosan (60 particles/cell), or A23187 (0.5 μg/ml). Cell lysates (20 μg/lane) were separated on a 7.5% SDS-polyacrylamide gel. Immunoblots were incubated with a polyclonal antibody against the recombinant cPLA$_2$, and the Amersham ECL system was used for detection.

shown). The MAP kinase peptide also comigrated with HPLC peak 4 material when analyzed on two-dimensional peptide maps. Phosphoamino acid analysis showed that peak 4 material contained phosphoserine (Fig. 7, *lane 1*). Repeated attempts to confirm the structure of this *in vitro* phosphorylated MAP kinase peptide by micro-LC/MS were unsuccessful. However, MALDI-TOF analysis of this material revealed a major signal at mass 5,107, which agrees well with the calculated mass of 5,114 for this tryptic phosphopeptide. It is clear from this analysis that HPLC peak 4 is the MAP kinase-phosphorylated tryptic peptide (residues 489–533).

Phosphorylation of cPLA$_2$ in Human Monocytes—cPLA$_2$ plays a key role in releasing arachidonic acid for prostaglandin production in human monocytes, and previous studies revealed that monocyte cPLA$_2$ is regulated by phosphorylation (2, 38). Therefore, monocytes were used to determine if similar sites were phosphorylated on cPLA$_2$ in a mammalian system, as identified on the cPLA$_2$ expressed in Sf9 cells. Experiments were initially carried out to compare the effect of a variety of agonists on inducing arachidonic acid release (Fig. 8*A*). In response to A23187, LPS, and opsonized zymosan, arachidonic acid accumulated in the medium at a linear rate up to 1 h after stimulation and then started to plateau. Release of arachidonic acid with okadaic acid and PMA showed an initial lag compared with the other stimuli. The ability of each stimulus to induce a shift in electrophoretic mobility of the cPLA$_2$ on immunoblots was also investigated (Fig. 8*B*). Most of the cPLA$_2$ from unstimulated monocytes migrated on the gel as the faster migrating form, although a small amount of the phosphorylated form was present, and this varied depending on the donor. After stimulation with PMA, okadaic acid, LPS, or zymosan, the majority of the monocyte cPLA$_2$ shifted to the slower migrating phosphorylated form, whereas A23187 caused a smaller portion of the cPLA$_2$ to gel shift.

Monocytes were ^{32}P labeled and stimulated with okadaic acid and opsonized zymosan to determine if agonist treatment induced an increase in phosphorylation of the cPLA$_2$. Incubation with okadaic acid for 2 h induced a 2.5 ± 0.97-fold (mean ± S.E., three experiments) increase in phosphorylation of the cPLA$_2$, whereas in one experiment opsonized zymosan increased phosphorylation by 2.7-fold. Phosphoamino acid analysis showed that phosphorylation occurred exclusively on serine residues in unstimulated and okadaic acid-stimulated monocytes (Fig. 7, *lanes 3* and *4*). Initial attempts to obtain enough ^{32}P-labeled monocyte cPLA$_2$ to prepare tryptic digests for HPLC to compare with cPLA$_2$ phosphorylated in Sf9 cells were unsuccessful because of inefficient labeling of the monocyte cPLA$_2$. Consequently, tryptic phosphopeptides of the ^{32}P-labeled cPLA$_2$ from monocytes and Sf9 cells were compared by two-dimensional phosphopeptide mapping. The migration pattern of the phosphopeptides derived from monocyte cPLA$_2$ was similar to that of phosphopeptides derived from recombinant cPLA$_2$ except for novel phosphopeptides that appeared in monocyte samples in the spot 7 region. Two principal phosphopeptides, 4 and 7, were observed on peptide maps of tryptic digests of cPLA$_2$ derived from unstimulated monocytes (Figs. 9*A* and 10*A*). After stimulation with okadaic acid phosphopeptide 2 became very prominent, and new phosphopeptides, 3 and 6, appeared (Fig. 9*B*). After zymosan stimulation phosphopeptides 4 and 7 were most prominent, and also evident were phosphopeptides 2 and 6 (Fig. 10*B*). The yields of phosphopeptides transferred from the tubes to the cellulose plates as gauged by Cerenkov counting were variable, and thus the increases in the phosphorylation of the phosphopeptides on the maps could only be considered in a qualitative manner. The yields were nearly quantitative and consistent in the HPLC separations, and only these data were used for obtaining quantitative data after stimulation. To confirm the identity of the monocyte phosphopeptides, samples from stimulated monocytes were cospotted with samples from okadaic acid-stimulated Sf9 cells (Figs. 9*D* and 10*D*). Comparison of the maps with the cospotted samples with the maps of the samples run alone showed that the phosphopeptides 2, 3, and 4 derived from monocyte cPLA$_2$ comigrated with those derived from cPLA$_2$ expressed in Sf9 cells. In addition, weak spots in the 1 and 5 positions were seen in the sample from zymosan-stimulated monocytes. Spot 7 is probably an oxidized derivative of the MAP kinase peptide (spot 4). This spot was seen only on some of the phosphopeptide maps. In addition, oxidation of pure phosphopeptide 4, which was eluted from the cellulose plate, with performic acid gave rise to material that migrated in the

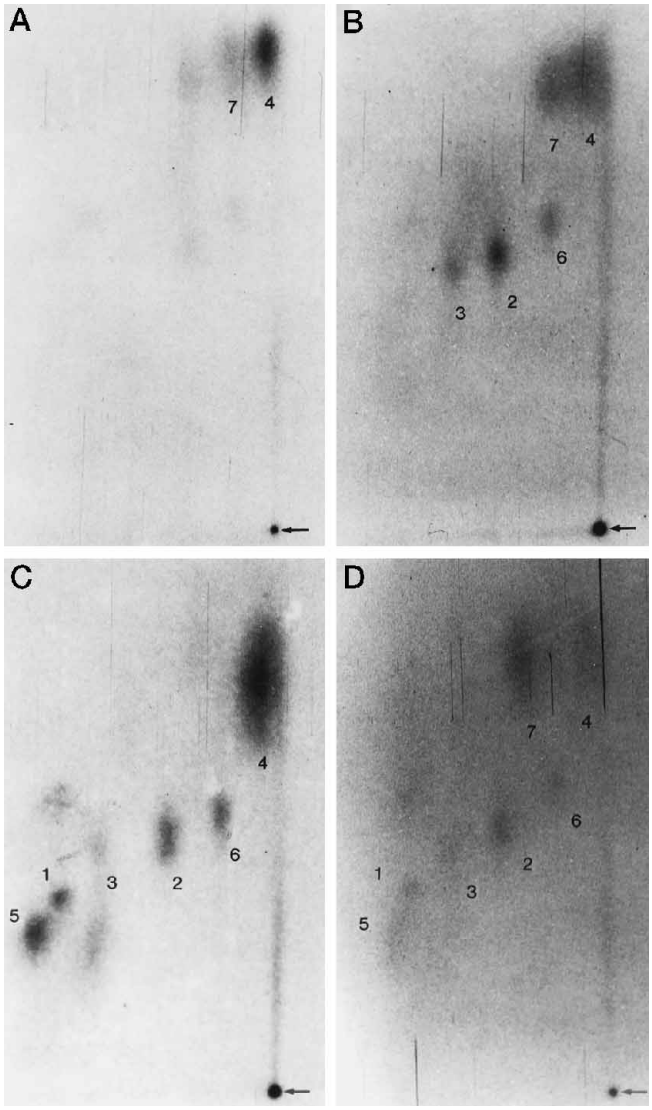

FIG. 9. **Two-dimensional tryptic phosphopeptide maps of ^{32}P-labeled cPLA$_2$ from human monocytes.** Tryptic digests of immunoprecipitated, gel-purified cPLA$_2$ from unstimulated monocytes (*panel A*), from okadaic acid-stimulated monocytes (*panel B*), from okadaic-stimulated Sf9 cells (*panel C*), or from okadaic acid-stimulated monocytes cospotted with okadaic acid-stimulated Sf9 cells (*panel D*) were separated by two-dimensional phosphopeptide mapping as described under "Experimental Procedures." Phosphopeptides were detected by autoradiography and are labeled as described under "Results." The samples were applied at the spots marked by *arrows*. Electrophoresis was run in the horizontal dimension, with the anode on the left, and chromatography was run in the vertical dimension.

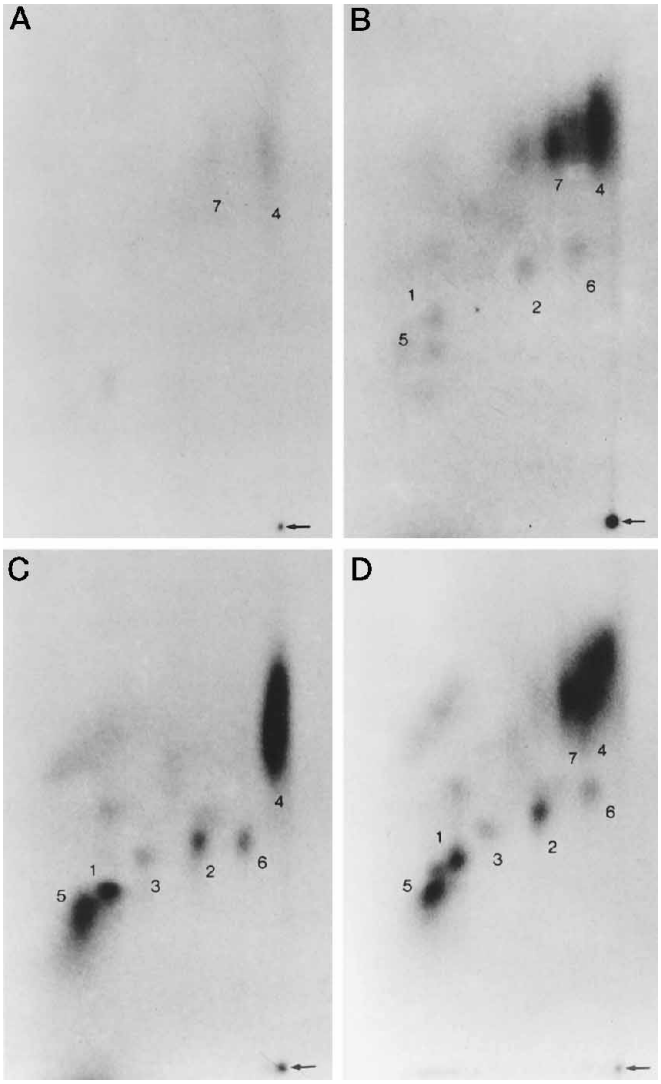

FIG. 10. **Two-dimensional tryptic phosphopeptide maps of ^{32}P-labeled cPLA$_2$ from human monocytes.** Tryptic digests of immunoprecipitated, gel-purified cPLA$_2$ from unstimulated (*panel A*), from zymosan-stimulated monocytes (*panel B*), from okadaic-stimulated Sf9 cells (*panel C*), or from okadaic-stimulated Sf9 cells cospotted with zymosan-stimulated monocytes (*panel D*) were separated by two-dimensional phosphopeptide mapping as described under "Experimental Procedures." Phosphopeptides were detected by autoradiography and are labeled as described under "Results." The samples were applied at the spots marked by *arrows*. Electrophoresis was run in the horizontal dimension, with the anode on the left, and chromatography was run in the vertical dimension.

spot 7 region (not shown). In the analysis of some samples of trypsin-digested cPLA$_2$ from Sf9 cells by HPLC, a small peak of cpm eluted just prior to peak 4. Two-dimensional phosphopeptide mapping analysis of this pre-peak 4 revealed a spot in the 7 region, showing that it is the oxidized phosphopeptide 4 (not shown). Spot 6, seen in Figs. 9 and 10, requires additional comment. As seen in these figures, spot 6 is seen with cPLA$_2$ derived from monocytes and Sf9 cells. However, as shown in Fig. 5, in this map spot 6 (not labeled, between 2 and 4) represents a small percentage of the total phosphopeptides from cPLA$_2$ expressed in Sf9 cells. Spot 6 intensity was highly variable in comparison with the other phosphopeptides and was seen in only 5 out of 25 independent tryptic digests of cPLA$_2$ from Sf9 cells. This suggests that spot 6 is either a partial tryptic fragment or a derivative of one of the other phosphopeptides. Altogether, these results suggest that the major phosphorylation sites on cPLA$_2$ from Sf9 cells and monocytes are the same.

DISCUSSION

The Sf9 baculovirus expression system has been used to study phosphorylation of mammalian proteins using numerous approaches. Coexpression of protein along with its upstream activating kinase has resulted in the expression of activated phosphoprotein (39). Insect cell kinases activated during viral infection also phosphorylate expressed proteins (26). In addition, expressed proteins can undergo stimulus-dependent phosphorylation in insect cells (40). In this study expression of cPLA$_2$ in insect cells enabled these cells to release arachidonic acid, but only after stimulation with either A23187 or okadaic acid, demonstrating that in this model pathways for the activation of cPLA$_2$ appear to exist which are similar to those in mammalian cells. Previously, it was found that cPLA$_2$ is phos-

phorylated on Ser-505 during expression in Sf9 cells (27, 37). In this study we identified three additional serines that are phosphorylated on $cPLA_2$ during expression in insect cells. Stimulation of insect cells with okadaic acid resulted in an increase in phosphorylation of Ser-727 on $cPLA_2$. Phosphorylation of this site also appeared to be increased in monocytes after stimulation with okadaic acid and opsonized zymosan, suggesting that this site may have a regulatory role in activation of $cPLA_2$ resulting in arachidonic acid release in mammalian cells.

Although previous studies have suggested that the $cPLA_2$ is phosphorylated on multiple sites after cell activation, only one site, Ser-505, which is phosphorylated by MAP kinase, has been identified (13, 21). Phosphorylation of $cPLA_2$ on Ser-505 also occurs during expression in Sf9 cells possibly by an insect MAP kinase homolog resulting in activated enzyme and retardation in electrophoretic mobility on SDS-polyacrylamide gels (24, 27). The recombinant $cPLA_2$ mutant, which has an alanine replacing Ser-505, does not exhibit this gel shift when expressed in Sf9 cells (27). However, although $cPLA_2$ is partially phosphorylated on Ser-505 in unstimulated Sf9 cells, we found that this is not sufficient to cause these cells to release arachidonic acid since stimulation with A23187 or okadaic acid was required to induce arachidonic acid release. Since neither of these agonists induced a large increase in phosphorylation at Ser-505, this suggests that other mechanisms are required for full activation of $cPLA_2$ leading to arachidonic acid release. It has now been established that in some systems in addition to phosphorylation an increase in calcium is required for full activation (21). For example, stimulation of macrophages with colony-stimulating factor-1, which does not increase calcium levels in these cells, can induce phosphorylation of $cPLA_2$ and activation of MAP kinase without inducing arachidonic acid release[2] (41). However, agonists that induce an increase in calcium act synergistically to affect arachidonic acid release when added with agonists that fully induce a gel shift, such as colony-stimulating factor-1. These results have led to the hypothesis that an increase in intracellular calcium, to promote association of the $cPLA_2$ with the membrane, as well as phosphorylation of $cPLA_2$ is required for full activation (21, 41). However, an increase in intracellular calcium does not appear to be required in some cells as some agonists that can stimulate arachidonic acid release do not raise intracellular calcium levels. For example, okadaic acid induces arachidonic acid release in mouse peritoneal macrophages and activates MAP kinase but does not raise intracellular calcium levels[2] (23). Similarly, okadaic acid did not raise intracellular calcium levels in Sf9 cells, unlike the calcium ionophore bromo-A23187 (data not shown). Consequently, the results suggest that phosphorylation of Ser-727 on $cPLA_2$ in okadaic acid-stimulated Sf9 cells allows association of the $cPLA_2$ with the membrane in the absence of an increase in intracellular calcium levels. Additional experiments are required to explore this possibility fully.

Many investigators use the retardation of $cPLA_2$ on SDS-polyacrylamide gels as an indicator of phosphorylation of $cPLA_2$ on Ser-505. Recently, MAP kinase (ERK1/ERK2)-independent pathways have been implicated in phosphorylation of the $cPLA_2$ leading to a gel shift (22). The okadaic acid-induced gel shift (Fig. 3) was independent of the phosphorylation of the MAP kinase peptide. Okadaic acid slightly increased the level of phosphorylation of the MAP kinase peptide to levels similar to those of A23187, but only okadaic acid induced a gel shift, suggesting that the okadaic acid-induced gel shift was caused by the phosphorylation of Ser-727.

[2] Z.-H. Qiu and C. L. Leslie, unpublished results.

The kinase that phosphorylates the $cPLA_2$ in insect cells on Ser-727 has not been identified, but this serine lies within a consensus sequence for a basotrophic kinase (R*X*S), such as protein kinase C or protein kinase A (42, 43). These kinases can phosphorylate $cPLA_2$, *in vitro*, but phosphorylation has not resulted in a consistent increase in enzyme activity (20, 21). Protein kinase C-dependent pathways have been implicated in the activation of the $cPLA_2$. PMA, an activator of protein kinase C, can stimulate phosphorylation of $cPLA_2$ in mammalian cells (13, 19). PMA can also stimulate phosphorylation of the $cPLA_2$ in insect cells (27). However, since PMA can also activate MAP kinase in mammalian cells further studies are needed to determine whether protein kinase C can directly phosphorylate $cPLA_2$ or whether this kinase produces an indirect effect (23, 44). The results of this study suggest that Ser-727 is also phosphorylated in okadaic acid- and opsonized zymosan-stimulated human monocytes, indicating that this site may be regulated in monocytes during cell activation. The conservation of Ser-727 (as is seen with Ser-505), even in an evolutionary distant species such as zebrafish, suggests that this serine may have an important regulatory role. Ser-454, on the other hand, which is phosphorylated only to a small extent in Sf9 cells and monocytes, is not conserved even in the mouse, whereas Ser-437 is conserved in mouse and chicken (6). In conclusion, the Sf9 baculovirus expression system is a useful system for studying $cPLA_2$ phosphorylation and activation and has led to the identification of multiple novel phosphorylation sites on $cPLA_2$. Further studies will determine whether these sites play a functional role in the activation of $cPLA_2$.

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