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Serine 727 Phosphorylation and Activation of Cytosolic Phospholipase A_2 by MNK1-related Protein Kinases*

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We have previously reported that in thrombin-stimulated human platelets, cytosolic phospholipase A_2 (cPLA2) is phosphorylated on Ser-505 by p38 protein kinase and on Ser-727 by an unknown kinase. Pharmacological inhibition of p38 leads to inhibition of cPLA2 phosphorylation at both Ser-505 and Ser-727 suggesting that the kinase responsible for phosphorylation on Ser-727 is activated in a p38-dependent pathway. By using Chinese hamster ovary, HeLa, and HEK293 cells stably transfected with wild type and phosphorylation site mutant forms of cPLA2, we show that phosphorylation of cPLA2 at both Ser-505 and Ser-727 and elevation of Ca^{2+} leads to its activation in agonist-stimulated cells. The p38-activated protein kinases MNK1, MSK1, and PRAK1 phosphorylate cPLA2 *in vitro* uniquely on Ser-727 as shown by mass spectrometry. Furthermore, MNK1 and PRAK1, but not MSK1, is present in platelets and undergo modest activation in response to thrombin. Expression of a dominant negative form of MNK1 in HEK293 cells leads to significant inhibition of cPLA2-mediated arachidonate release. The results suggest that MNK1 or a closely related kinase is responsible for *in vivo* phosphorylation of cPLA2 on Ser-727.

Cytosolic phospholipase A_2 (cPLA2)[1] specifically releases arachidonic acid from the *sn*-2 position of glycerophospholipids to provide the precursor of the eicosanoids. cPLA2 is ubiquitously distributed in human tissues, which seems to reflect its central role in receptor-regulated release of arachidonic acid (for reviews see Refs. 1–4). It is generally accepted that cPLA2 is activated by a rise in cytosolic calcium, although calcium-independent cPLA2 activation has been observed with the protein phosphatase inhibitor okadaic acid (2). Calcium binds to a membrane binding domain of cPLA2 known as the C2 domain (5–9) and causes the enzyme in the aqueous phase to transfer to the perinuclear region of the cell (10, 11).

A second method for cPLA2 activation is phosphorylation. With the availability of the complete sequence of cPLA2 (12, 13), it became apparent that this enzyme has a consensus phosphorylation motif (containing Ser-505) for members of the mitogen-activated protein kinase (MAPK) family. The 42-kDa MAPK (ERK2) is able to phosphorylate cPLA2 *in vitro* on Ser-505 (14–16), and expression of the Ser-505 to Ala mutant in Chinese hamster ovary (CHO) cells gives substantially lower arachidonate release than does expression of the wild type enzyme in response to Ca^{2+} ionophore plus phorbol 12-myristate 13-acetate (PMA) (14). cPLA2 phosphorylation also produces a characteristic shift in migration of this protein when analyzed by SDS-PAGE (14), and this technique has been used by many investigators to track cPLA2 phosphorylation in a variety of agonist-stimulated cells (2). In thrombin- and collagen-activated human platelets, Ser-505 of cPLA2 is phosphorylated by p38 protein kinase (mainly the 2a isoform, also known as stress-activated protein kinase, SAPK-2a) as well as other p38 isoforms that belong to the MAPK family (16–19).

Rigorous protein chemical analysis of phosphorylation of cPLA2 expressed in the baculovirus/insect cell system established that this enzyme is phosphorylated on 4 serines (437, 454, 505, and 727) (20). Subsequent studies with human platelets and HeLa cells showed that cPLA2 is exclusively phosphorylated on a subset of these serines (Ser-505 and Ser-727) in response to agonists, and no evidence for tyrosine phosphoryl-

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[1] The abbreviations used are: cPLA2, 87-kDa cytosolic (group IV) phospholipase A_2; cPLA2-WT, wild type cPLA2; MAPK, mitogen-activated protein kinase; mbHPLC, microbore high pressure liquid chromatography; MBP, myelin basic protein; PMA, phorbol 12-myristate 13-acetate; pS727-P cPLA2-derived tryptic peptide containing phospho-Ser-727 (likewise for pS437-P, pS454-P, and pS505-P); pS727-Pox, pS727-P with its cysteine oxidized to cysteic acid; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; GST, glutathione *S*-transferase; IL-1, interleukin-1; WT, wild type; MS, mass spectrometry.

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ation could be found (16). These are the only studies to date in which the sites of cPLA2 phosphorylation have been fully mapped, and they underscore the need for detailed protein chemical analysis rather than the SDS-PAGE gel shift to characterize the phosphorylation of this enzyme in cells.

Treatment of platelets with the p38-2a/2b inhibitors SB 203580 and SB 201950 not only decreases phosphorylation of cPLA2 on Ser-505 but also on Ser-727 (16). The degree of inhibition is agonist-dependent, and this appears to be due to the fact that multiple p38s are activated in agonist-specific ways and that only some of these isoforms are sensitive to SB 203580 (19). The inhibition data make it likely that Ser-727 is phosphorylated by a kinase that is downstream of p38s in the activation cascade. The present study was therefore designed to test whether MAPK-regulated kinases phosphorylate cPLA2 *in vitro*, whether they are present in platelets, and to explore the functional significance of Ser-505 and Ser-727 phosphorylation by analysis of three mammalian cell lines stably transfected with phosphorylation site mutants of cPLA2.

MATERIALS AND METHODS

Reagents—Dephosphorylated wild type cPLA2 (cPLA2-WT) (treated with phosphatase) and cPLA2 mutants were prepared as described previously (21, 22). Kinase substrate peptide KKLNRTLSVA was synthesized by Alta Bioscience Ltd. (Birmingham, UK). cPLA2 tryptic phosphopeptides containing phosphoserine at positions 437 (pS437-P), 454 (pS454-P), 505 (pS505-P), and 727 (pS727-P) were obtained as described (16), and the non-phosphorylated Ser-727 tryptic peptide, pS727-P, was prepared by SynPep (Dublin, CA). All synthetic peptides were purified by reverse phase HPLC, and their structures and purities were confirmed by electrospray mass spectrometry. 4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl) imidazole (SB 202190) was purchased from Alexis Corp. (Nottingham, UK), and 2-(2-amino-3-methoxyphenyl)-oxanaphthalen-4-one (PD 98059) was kindly provided by D. T. Dudley (23). Myelin basic protein (MBP) and bovine thrombin were purchased from Sigma, and collagen was from Nycomed Arzneimittel (Munich, Germany).

Polyclonal anti-MNK1 antiserum was obtained as described (24) or from Santa Cruz Biotechnology; polyclonal anti-ERK2 antibody was from Santa Cruz Biotechnology, and polyclonal anti-cPLA2 (R11683) was obtained from C. Leslie (National Jewish Medical and Research Center, Denver, CO). Recombinant PRAK1 (see below) expressed in *Escherichia coli* and the peptide FKRNAAVIDPLQFHMGVER corresponding to residues 384–402 of human MSK1 and conjugated to bovine serum albumin and keyhole limpet hemocyanin were injected into sheep at Diagnostic Scotland (Carluke, Ayrshire, UK). The antibodies against PRAK1 and MSK1 were affinity-purified by chromatography on PRAK1-CH-Sepharose or peptide-CH-Sepharose, respectively.

Activated Protein Kinases—Recombinant glutathione *S*-transferase-tagged human MAPK-interacting kinase 1 (MNK1) was obtained by infecting insect cells with MNK1 baculovirus as described (24). MNK1 was purified and activated, fresh before use, with activated ERK1 (Calbiochem) as described (24). MAPK-activated protein kinase 2 (MAPKAP-K2) purified from rabbit skeletal muscle and activated by p38-2a was purchased from Upstate Biotechnology Inc. (TCS Biologicals Ltd., Bucks, UK) and stored at −70 °C.

An expressed sequence tag clone encoding residues 17–471 of human PRAK1 was kindly provided by the Image consortium, St. Louis (565786-AA137161). The full-length PRAK1 sequence (residues 1–471) was generated by a polymerase chain reaction-mediated repair strategy using the EST clone as a template. The resulting polymerase chain reaction product was subcloned into the pCR/TOPO cloning vector (Invitrogen) and the sequence verified by DNA sequencing. The vector was then digested with *Nde*I/*Xho*I and the cDNA fragment ligated into the same sites of a modified pFastBAC1 vector encoding a hexahistidine tag 5′ to the *Nde*I site. The resulting pFastBAC-PRAK construct was then used to generate recombinant baculovirus using the Bac-to-Bac methodology (Life Technologies, Inc.). The cDNA for FLAG-MSK1 (25) was subcloned as a *Bam*HI-*Kpn*I fragment into pFastBACHTb and recombinant baculovirus generated as described for pFastBAC-PRAK1. The resulting viruses, encoding amino-terminally hexahistidine-tagged PRAK1 and MSK1, were used to infect Sf21 cells (1.5×10^6/ml) at a multiplicity of infection of 5. The infected cells were harvested 72 h post-infection and the His-tagged PRAK1 and MSK1 proteins purified by Ni^{2+}-nitrilotriacetic acid-agarose chromatography as described previously for protein kinase B-β (26). The purified proteins were dialyzed against 50 mM Tris/HCl, pH 7.5, 50% glycerol, 150 mM NaCl, 0.1 mM EGTA, 0.1% β-mercaptoethanol, 0.03% Brij-35, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride and stored at −20 °C. PRAK1 and MSK1 were recovered with a yield of 8 and 20 mg/liter of infected Sf21 cells, respectively, and were >90% pure as judged by SDS-polyacrylamide gel electrophoresis followed by staining with Coomassie Blue.

PRAK1 was activated at a concentration of 2 μM with 2 units/ml active GST-p42MAPK (1 milliunit incorporates 1 nmol of phosphate per min into MBP) in 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 0.1% β-mercaptoethanol, 10 mM magnesium acetate, 0.1 mM ATP for 45 min at 30 °C. MSK1 was activated at 300 μg/ml with 10 units/ml GST-p42 MAPK in 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 0.1% β-mercaptoethanol, 10 mM magnesium acetate, 0.1 mM ATP for 30 min at 30 °C. The GST-p42 MAPK was removed after activation by glutathione-Sepharose chromatography. Activated MSK1 and PRAK1 were stored at −20 °C.

The activity of kinases were assayed in 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 10 mM β-mercaptoethanol, 10 mM magnesium acetate (MSK1 and PRAK1), 20 mM HEPES, pH 7.4, 1 mM dithiothreitol, 10 mM magnesium chloride (MNK1) (24), and 25 mM HEPES, pH 7.4, 1 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM EGTA, 100 μM Na_3VO_4, 50 mM β-glycerophosphate (MAPKAP-K2) (27) all containing 100 μM [γ-^{32}P]ATP (250 Ci/mol) and substrate peptide KKLNRTLSVA ((30 μM for MSK1, PRAK1, and MAPKAP-K2 (25, 27), and 1.5 mM for MNK1 (24)). Reaction mixtures (20 μl) were incubated for 30 min at 37 °C, and phosphopeptide product was quantified as described (16). One microunit of kinase is defined as the amount of enzyme that phosphorylates 1 pmol of substrate peptide per min. With these conditions, maximal velocities at saturating peptide concentration were measured for all kinases (25, 28).

Expression Vector for Mouse cPLA2 and Mutagenesis—The pCDNA3.1/Seo(+) mammalian expression vector for mouse cPLA2 has been described (29). Vectors expressing the cPLA2 mutants (S505A, S727A) were prepared using the QuickChange kit (Stratagene) and the primers CACATCATATCCACTGGCTCCCCTGAGAGACTTCAGC (+ complement) and GAACCCATCTCGTTGCGCTGTTTCCCTCAG (+ complement), respectively. The double mutant S505A/S727A was prepared sequentially. The entire coding regions of all mutant cPLA2 vectors were confirmed by DNA sequencing.

Establishment of Stable Transfected Mammalian Cells, RNA Blotting, and Immunoblotting—CHO-K1 cells and HeLa S3 cells (from ATCC) were transfected using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer's instructions. Stable transfectant clones were isolated by limiting dilution in the presence of 250 and 100 μg/ml zeocin for CHO and HeLa cells, respectively. cPLA2 immunoblots were carried out with 4 μg of cell protein per gel lane (10% SDS-PAGE) using a 1:1000 dilution of the R11683 antiserum and ECL detection (Amersham Pharmacia Biotech). Establishment of HEK293 cell stable transfectants and analysis of cPLA2 expression by RNA blotting and immunoblotting were carried out as described (29, 30).

In order to establish HEK293 transfectants stably co-expressing $cPLA_2$ and a dominant negative form of MNK-1, cells expressing cPLA2-WT were subjected to a second transfection as described above with mouse dominant negative MNK-1 cDNA, which had been subcloned into pcDNA3.1/Zeo(+) (Invitrogen) at the *Bam*HI site. The cells were cloned by limiting dilution in 96-well plates in culture medium supplemented with 50 μg/ml zeocin (Invitrogen). After culture for 1–2 months, wells containing a single colony were chosen, and the expression of each protein was assessed by immunoblotting using anti-cPLA2 and anti-MNK-1 antibodies (Santa Cruz Biotechnology). The established clones were expanded and used for subsequent experiments.

Expression of cPLA2-WT and MNK-1 proteins in HEK293 transfectants was assessed by immunoblot analysis using lysates from 10^5 cells applied to a SDS-PAGE using a 7.5% gel under reducing conditions. The separated proteins were electroblotted onto nitrocellulose membranes (Schleicher & Schuell) using a semi-dry blotter (MilliBlot-SDE system; Millipore). The membranes were probed with the respective antibodies (1:5000 dilution for cPLA2-WT and 1:2000 dilutions for MNK-1) for 2 h, followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1:5,000 dilution) (Amersham Pharmacia Biotech) for 2 h, and visualized using the ECL Western blot system (PerkinElmer Life Sciences).

Arachidonate Release Studies—CHO were grown to 80% confluence in Ham's/F-12 medium containing 10% fetal bovine serum and 2 mM glutamine in a 24-well plate. Medium was removed and replaced with fresh medium containing 0.1 μCi/ml [^{3}H]arachidonic acid (200 Ci/mmol,

American Radiochemicals). After 20 h at 37 °C, medium was removed, and cells were washed three times with complete medium. Cells were stimulated in 1 ml of complete medium with 1 μM A23187 for 10 min at 37 °C. In some experiments, cells were pretreated for 5 min with 1 μM PMA prior to stimulation with ionophore or treated with PMA alone for 15 min. Culture medium was collected, briefly microcentrifuged, and 0.5 ml was submitted to scintillation counting. Cells were dissolved in 1 ml of $CHCl_3$:CH_3OH (2:1); solvent was removed, and the residue was submitted to scintillation counting to obtain total cell associated counts/min. Arachidonate release is expressed as the percentage of total counts/min released into the medium. HeLa cells were grown to 70% confluence in high glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 2 mM glutamine. Cells were radiolabeled as for CHO cells. For stimulation, HeLa cells were treated with 1 μM A23187 for 10 min at 37 °C. In some experiments, cells were treated for 20 min with 1000 units/ml interferon-α (Accurate Chemicals) at 37 °C. Release of counts/min into the medium was quantified as described for CHO cells after the cell suspension was submitted to low speed centrifugation. Arachidonic acid release from A23187-stimulated HEK293 cells was carried out as described (29) except that 48-well plates were used (125 μl/well).

Intracellular Calcium Measurements—CHO and HeLa cells were prepared for fluorimetric monitoring of intracellular calcium as described previously (10) except that the fluorophore Fluo-3/AM (Molecular Probes) was used, and cells were loaded by incubation with 4 μM fluorophore for 1 h at 37 °C. Cells were stimulated with A23187 (1 or 10 μM) while monitoring fluo-3 fluorescence with stirring at 37 °C.

In Vitro Phosphorylation of cPLA2 and cPLA2-derived Peptides—pS727 or pS727-P (100 μM) or MBP (100 μM) in the presence of MNK1 kinase buffer (see above) containing 100 μM [γ-^{32}P]ATP (250 Ci/mol) in a total volume of 50 μl for 10 min at 30 °C and radiophosphorylated peptide were quantified as described (16). For cPLA2 phosphorylation, aliquots of activated kinase (3 microunits or as noted in the figure legends) and 2 μg of purified dephosphorylated wild type cPLA2 or cPLA2 mutant were incubated with kinase buffer, 100 μM [γ-^{32}P]ATP (250 Ci/mol) in a total volume of 20 μl. After incubation for 30 min at 37 °C (or as noted in the figure legends), the reaction was terminated by the addition of 5 μl of 5× Laemmli sample buffer. Samples were boiled for 5 min, and proteins were separated by 8 or 10% SDS-PAGE. Autoradiographs were taken from dried gels. Radioactivity was quantified by Cerenkov counting of gel slices or by densitometry analysis of exposed x-ray films. To generate radiophosphorylated cPLA2 for peptide mapping, reactions were carried out as above except higher specific activity [γ-^{32}P]ATP was used (1250 Ci/mol) along with 4 μg of cPLA2.

Phosphopeptide Analysis—Microbore high performance liquid chromatography (mbHPLC) and two-dimensional thin layer chromatography were performed as described previously (16). Briefly, cPLA2 was eluted from gel slices using 50 mM NH_4HCO_3, 0.1% SDS, 1% β-mercaptoethanol, and 20 μg of carrier cPLA2 protein. Protein was precipitated with trichloroacetic acid and digested with trypsin (20). Samples were analyzed on mbHPLC, and synthetic phosphopeptide standards were co-injected with each run. Radioactivity was monitored by Cerenkov counting. For two-dimensional phosphopeptide maps, tryptic digests were separated by electrophoresis followed by ascending thin layer chromatography. In some experiments, peptides were oxidized with performic acid (16).

Mass Spectrometry—Five samples each containing 1 μg of cPLA2 were incubated with 70 microunits of activated MNK1 (+MNK1 sample) or with buffer (−MNK1 sample) containing [γ-^{32}P]ATP for 90 min at 30 °C as described above. Samples were pooled in a wide well of a preparative gel, and proteins were separated by SDS-PAGE. The phosphorylation reaction gave 5600 cpm (+MNK1) and 1800 cpm (−MNK1) (Cerenkov counting) which corresponds to a substrate phosphorylation of 4.6 and 1.5%, respectively. cPLA2 was eluted from gel slices in the absence of carrier protein as described above. Yield of cPLA2 after purification was 4.23 μg (+MNK1 sample) and 3.18 μg (−MNK1 sample) based on Cerenkov counting.

The analysis of the synthetic peptide standard and the native protein tryptic digest was performed by micro-column HPLC/tandem mass spectrometry. The micro-column consisted of a 100 × 365 μm fused silica capillary (J & W Scientific, Folsom, CA) in which one end was pulled to a 2-μm tip using a laser puller (Sutter Instruments Co., Novato, CA) (31). Poros R2 reverse phase material (10 μm, Perspective Biosystems, Framinghan, MA) was packed against the tip to a length of approximately 14 cm. The peptide samples were loaded onto the column according to the method described by Yates *et al.* (32). An HP 1100 binary HPLC pump (Hewlett-Packard Co., Wilmington, DE) was used to deliver the gradient to the column. Buffer A consisted of 0.5% acetic acid, and buffer B consisted of 80:20 acetonitrile:water with 0.5% acetic acid. A flow rate of 150 nl/min through the column was obtained by using a pre-column split. The gradient was ramped from 2% buffer B to 60% buffer B in 30 min. Throughout the gradient, the mass spectrometer (Finnigan-MAT, San Jose, CA) was programmed for selected ion monitoring of m/z 938.3 followed by MS/MS of m/z 938.3 and selected ion monitoring of m/z 914.4 followed by MS/MS of m/z 914.4. Authentic pS727-P and cPLA2 tryptic digest were analyzed on different HPLC columns to avoid carry-over contamination.

Immunoprecipitation of MNK1 and PRAK1 from Human Platelets—Blood was drawn from healthy volunteers into acidic citrate dextrose as anticoagulant, and washed platelets were prepared as described previously (33). Samples (4–5 × 10^8 platelets/ml) were stimulated with thrombin (1 unit/ml) or collagen (100 μg/ml) as described (33) and were lysed in ice-cold lysis buffer (20 mM Tris, pH 8, 1% Triton X-100, 40 mM $Na_4P_2O_7$, 50 mM NaF, 5 mM $MgCl_2$, 100 mM Na_3VO_4, 10 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, 100 nM okadaic acid, 20 μg/ml leupeptin, and 20 μg/ml aprotinin). After pre-clearing lysates with protein A-Sepharose CL-4B for 30 min at 4 °C, samples were incubated with polyclonal anti-MNK1 antiserum (24) and fresh protein A-Sepharose CL-4B overnight. Immunoprecipitates were washed twice in TBS-T (20 mM Tris, 137 mM NaCl, 0.1% Tween 20, pH 7.6) containing 1 mM EGTA and an additional 500 mM NaCl and were washed twice more in TBS-T/EGTA. A final wash was carried out in 50 mM Tris, pH 7, containing 100 μM EGTA. All supernatants were taken off, and pellets were incubated with 50 μl of MNK1 kinase buffer (see above) containing 100 μM MBP and 100 μM [γ-^{32}P]ATP (500 Ci/mol) as substrates. Samples were incubated for 10 min at 30 °C on a shaking platform. The reaction was terminated by spotting 40-μl aliquots onto P-81 phosphocellulose filter. Filter papers were washed and submitted to scintillation counting as described (16). Alternatively, immunoprecipitates were applied onto 10% SDS-polyacrylamide gels containing 0.5 mg/ml MBP. The in-gel renaturation kinase assay was performed as described previously (33).

For PRAK1 immunoprecipitation, platelets were prepared and activated with thrombin (1 unit/ml) as described for MNK1. The *in vitro* kinase assay was identical to that used for MNK1 except that affinity purified anti-PRAK1 antibody and protein G-Sepharose were used to immunoprecipitate PRAK1. The PRAK1 substrate peptide KKLRRTLSVA (Upstate Biotechnology, Inc.) was used in place of MBP to assay PRAK1 activity.

Immunoprecipitation of cPLA2 from [^{32}P]Phosphate-labeled Platelets—Platelets were labeled with 25 mCi of $^{32}P_i$ as described previously (16) and were resuspended at 1 × 10^9 platelets/ml. After stimulation of 500-μl aliquots, cPLA2 was immunoprecipitated (34) and purified on 10% SDS-PAGE and, after elution, by trichloroacetic acid precipitation (see above). Tryptic digests were analyzed on mbHPLC, and radioactivity of eluting fractions was monitored by Cerenkov counting.

Analysis of cPLA2 Phosphorylation with [^{32}P]Phosphate-labeled CHO Cells—CHO cells stably transfected with mouse cPLA2 (one T25 flask at 80% confluence) were labeled with 1 mCi of $^{32}P_i$ (~9000 Ci/mmol) per flask in phosphate-free Dulbecco's modified Eagle's medium (Life Technologies, Inc., catalog number 11971-025) containing 0.5% bovine serum albumin for 4 h. Cells were activated for 10 min with 1 μM A23187 and then washed 2 times with ice-cold culture medium. Cells were lysed, and cPLA2 was immunoprecipitated exactly as described for HeLa cell cPLA2 (16). Gel-purified cPLA2 was processed for trypsin digestion, and radiolabeled tryptic peptides were analyzed by mbHPLC as described above.

RESULTS

Phosphorylation of cPLA2 in CHO-K1 Cells—Previous studies have shown that overexpression of cPLA2 in CHO cells leads to enhanced arachidonate release in response to calcium ionophore. Furthermore, phosphorylation of cPLA2 in response to PMA in the absence of a rise in intracellular calcium is insufficient to cause arachidonate release but augments fatty acid release induced by calcium ionophore (14). This effect requires MAPK phosphorylation of cPLA2 on Ser-505 since expression of cPLA2-S505A in CHO cells leads to a loss of the increase in arachidonate release that is observed following expression of cPLA2-WT in cells and treatment with A23187 alone or in combination with PMA (14).

In stimulated platelets and HeLa cells, we have shown that cPLA2 is phosphorylated on Ser-505 and additionally on Ser-727 (16). In order to analyze completely the sites of cPLA2

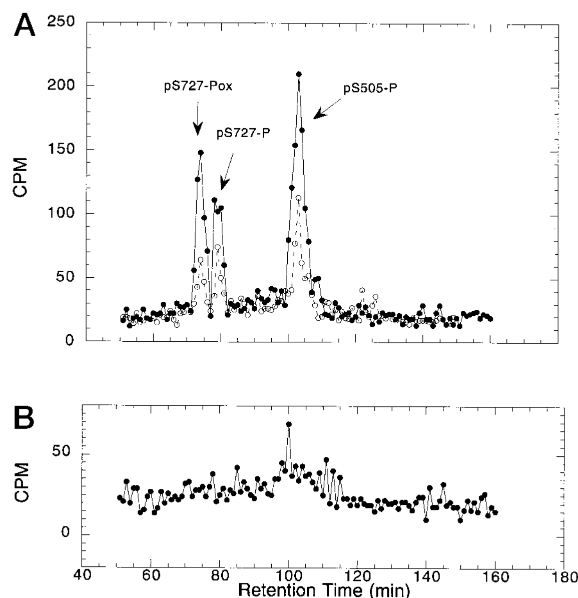

FIG. 1. **Phosphorylation of cPLA2 in CHO cells.** CHO cell clones expressing cPLA2-WT (*A*) or cPLA2-S505A/S727A (*B*) were labeled with $^{32}P_i$ and then either not stimulated (○) or stimulated (●) with 1 μM A23187 for 10 min. Cells were lysed, and cPLA2 was immunoprecipitated and further purified by SDS-PAGE. cPLA2 was eluted from the gel and trypsinized. The peptides were submitted to mbHPLC analysis, and fractions were submitted to Cerenkov counting. Retention times in minutes (cpm/peptide standard): oxidized pS727-P (pS727-Pox), 73.2/73.8; pS727-P, 79.2/79.1; pS505-P, 102.5/102.8.

phosphorylation in CHO cells, cells stably overexpressing mouse cPLA2 were labeled with [^{32}P]orthophosphate and stimulated. cPLA2 was immunoprecipitated from cell lysates and further purified by SDS-PAGE. Gel-eluted cPLA2 was digested with trypsin, and radiophosphorylated peptides were analyzed by mbHPLC. Results in Fig. 1*A* show that wild type cPLA2 is phosphorylated solely on Ser-505 and Ser-727. As reported previously (16), the Ser-727 phosphopeptide (pS727-P) becomes partially oxidized (cysteine to cysteic acid, pS727-Pox) and runs as a pair of peptides that co-elute with authentic phosphopeptide standards (Fig. 1*A*). pS505-P is identified based on co-elution of the radiolabeled phosphopeptide derived from CHO cell cPLA2 with authentic pS505-P generated by trypsin digestion of cPLA2 treated with MAPK *in vitro*. As shown in Fig. 1*A*, A23187 treatment causes a 2.5-fold increase in the counts/min incorporated at Ser-505 and at Ser-727. As also shown, the double mutant cPLA2-S505A/S727A is essentially not phosphorylated (Fig. 1*B*). This latter result not only confirms that cPLA2 is phosphorylated on Ser-505 and Ser-727 in these cells, but it shows that mouse cPLA2 is significantly overexpressed in these cells such that phosphorylation of endogenous CHO cell cPLA2 contributes very little to phosphopeptide counts/min. Based on immunoblotting data (not shown), it is estimated that the cPLA2 levels in the transfected clones are 5–10-fold higher than in non-transfected cells.

Functional Significance of Ser-727 cPLA2 Phosphorylation—In order to investigate the functional significance of cPLA2 phosphorylation at Ser-727 and Ser-505 for arachidonate release, CHO, HeLa, and HEK293 cell clones were established that stably express cPLA2-WT and the following phosphorylation site mutants: cPLA2-S505A, cPLA2-S727A, and cPLA2-S505A/S727A, and arachidonate release was studied after agonist stimulation.

For all three cell lines, multiple clones were isolated and screened for cPLA2 expression by immunoblotting so that clones with comparable levels of cPLA2 could be selected for arachidonate release studies. Shown in Fig. 2 are results for single clones expressing the different cPLA2s; similar results were found among a group of 3–4 independent clones that expressed comparable levels of the different cPLA2s. Overexpression of cPLA2-WT in CHO cells leads to enhanced arachidonate release compared with non-transfected cells (Fig. 2*A*). A23187 stimulation leads to more arachidonate release than does stimulation with PMA, and stimulation with both agonists leads to a synergistic effect (Fig. 2*A*). This behavior is virtually identical to that reported previously for CHO cells expressing human cPLA2 (14). Mutation of either Ser-505 or Ser-727 to alanine leads to diminished arachidonate release induced by all three stimulation conditions. Cells expressing cPLA2-S505A/S727A release amounts of arachidonate comparable to that produced in non-transfected cells. When CHO cells are stimulated with a higher concentration of A23187 (10 μM), the effect of the cPLA2 double mutation is rescued (Fig. 2*A, right panel*) showing that the mutant is fully functional in these cells and that higher intracellular calcium allows non-phosphorylated cPLA2 to function efficiently. Studies with fluo-3-loaded CHO cells revealed that the increase in fluorescence is 8–11-fold higher in cells stimulated with 10 μM A23187 *versus* 1 μM A23187 (not shown), thus confirming that intracellular calcium rises to a higher level in the presence of increased ionophore concentration.

Previous studies have shown that cPLA2 becomes phosphorylated in interferon-α-simulated HeLa cells (35). In these cells, cPLA2 is phosphorylated exclusively on Ser-505 and Ser-727 in response to interferon-α or upon addition of sodium arsenite as a stimulator of p38s (16). Several HeLa cell clones stably expressing cPLA2 and its phosphorylation site mutants were established and submitted to arachidonate release analysis in the absence and presence of A23187 or interferon-α. Results in Fig. 2*B* (*left panel*) show that 1 μM A23187 or 1000 units/ml interferon-α cause significant and comparable amounts of arachidonate release. Each of the single site cPLA2 mutants is much less efficient in producing arachidonate, and cells expressing the double mutant fail to produce arachidonate in excess of that produced in the non-transfected cells. As with CHO cells, stimulation with 10 μM A23187 can rescue the effect of the double phosphorylation site mutation (Fig. 2*B, right panel*), and studies with fluo-3-loaded HeLa cells confirm a higher intracellular calcium concentration obtained with 10 *versus* 1 μM A23187 (not shown).

We have previously shown that overexpression of cPLA2 in HEK293 leads to enhanced arachidonate release induced by A23187 over 30 min (immediate release) or by IL-1 in combination with serum over 4 h (delayed release) (29, 30). HEK293 clones expressing cPLA2-WT and its phosphorylation site mutants were established to study the role of cPLA2 phosphorylation in modulating arachidonate release in this cell model. Results in Fig. 2*C* (*left* and *middle panels*) show that mutation of either Ser-505 or Ser-727 or both leads to a marked reduction of arachidonate release to the level seen with non-transfected cells. Note that about 5-fold more arachidonate is produced during the delayed response over 4 h than during the immediate response during 30 min, and cPLA2 phosphorylation is important for both release phases. Again, a high concentration of A23187 can rescue the response with the cPLA2 double-site mutant (Fig. 2*C, right panel*).

MNK1 and Related Kinases Phosphorylate cPLA2 on Ser-727 in Vitro—Inhibition of cPLA2 phosphorylation on both Ser-505 and Ser-727 by p38 inhibitors suggests that the Ser-727 kinase may be one of the growing family of protein kinases that are activated by MAPK family members (16, 19). cPLA2 in thrombin- and collagen-stimulated platelets is phosphorylated on Ser-505 by one or more p38 isoforms and not by ERK1 or ERK2

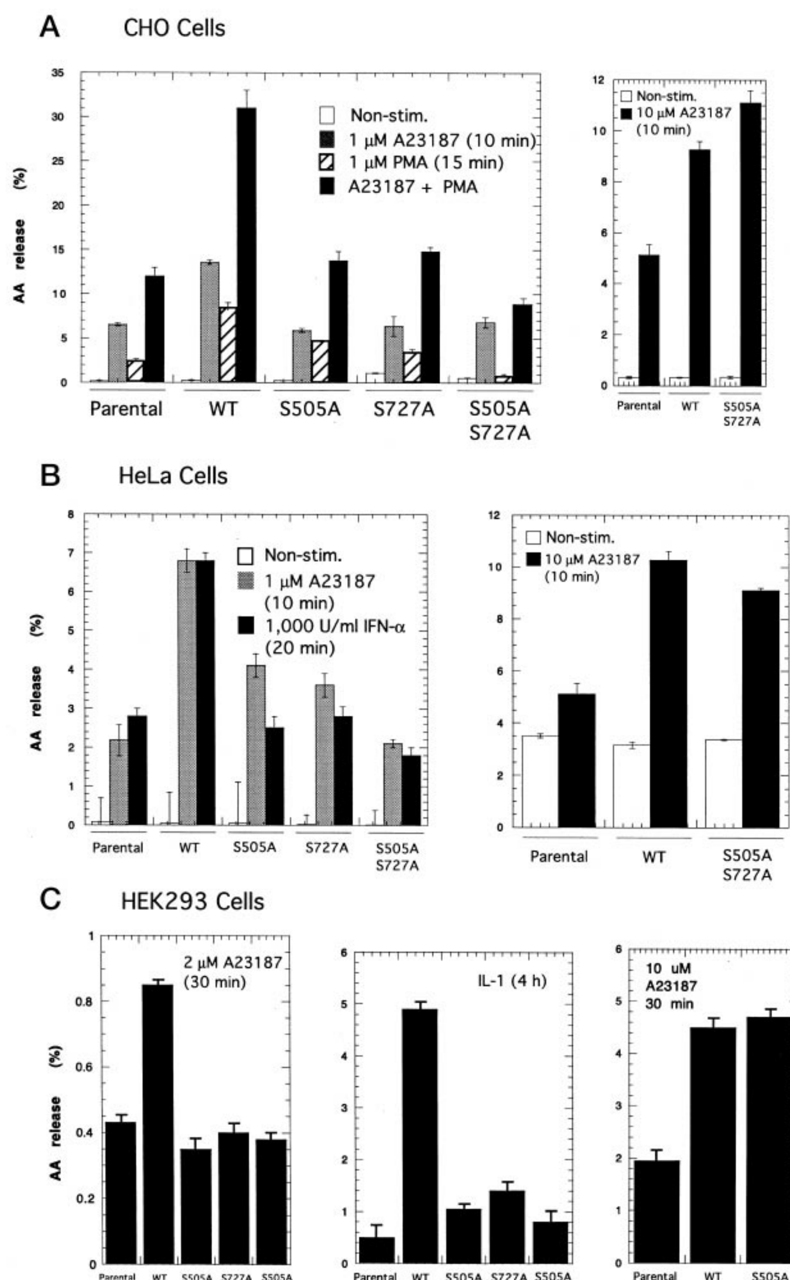

FIG. 2. **Functional significance of cPLA2 phosphorylation in mammalian cells.** *A,* CHO cells were either unstimulated or stimulated with 1 μM A23187 for 10 min or pretreated for 5 min with 1 μM PMA prior to ionophore stimulation or treated with PMA alone for 15 min (*left panel*) or treated with 10 μM A23187 for 10 min (*right panel*). Arachidonate release is reported as the counts/min of released tritium into the culture medium as a percentage of total cell-associated counts/min. The average and S.E. from three independent experiments for each clone are shown. Results are shown for non-transfected cells (*parental*) or cells transfected with cPLA2-WT (*WT*), cPLA2-S505A (*S505A*), cPLA2-S727A (*S727A*), or the double mutant (*S505A/S727A*). *B,* HeLa cells were either unstimulated or treated with 1 μM A23187 for 10 min or with 1,000 units/ml interferon-α for 20 min (*left panel*) or with 10 μM A23187 for 10 min (*right panel*). The average and S.E. from three independent experiments for each clone are shown. *C,* HEK293 cells were treated with 2 μM A23187 for 30 min (*left panel*) or with interleukin-1 (*IL-1*) plus serum for 4 h (29) (*middle panel*) or with 10 μM A23187 for 30 min (*right panel*). Note the difference in the *y* axis scales.

(17, 19). We therefore examined the ability of MNK1, MSK1, PRAK1, and MAPKAP-K2 to phosphorylate cPLA2 *in vitro* since these kinases are reported to be activated by p38s (24, 25, 36–38). Furthermore, the sequence surrounding Ser-727 of cPLA2, . . . Arg-Cys-Ser^{727}-Val-Ser . . . , is conserved in all known cPLA2 sequences (human, rat, mouse, horse, chick, and zebrafish) and bears some similarities with the substrate peptide sequence, . . . RTLSVA. . . . , for this group of kinases. As shown in Fig. 3, MNK1, MSK1, and PRAK1, but not MAPKAP-K2, are able to phosphorylate cPLA2-WT *in vitro*. In all cases, the mutant of cPLA2 in which all four sites of Sf9 cell phosphorylation (cPLA2 mutant S437A/S454A/S505A/S727A) is phosphorylated much less than is cPLA2-WT, suggesting that most of the phosphorylation is occurring on one or more serines from the group Ser-437, -454, -505, or -727.

To map the phosphorylation sites, cPLA2-S505A (available in larger quantities than cPLAZ-WT) was treated with MNK1 *in vitro*, isolated by SDS-PAGE, digested with trypsin, and submitted to mbHPLC together with synthetic tryptic phosphopeptide standards. As shown in Fig. 4*A*, a single major radiolabeled phosphopeptide elutes from the column at the same position as the synthetic standard pS727-P (92 min). pS437-P and pS454-P elute earlier from the mbHPLC column. Total counts/min eluting from the column was 85% of that applied indicating that the 92-min peak accounts for the major phosphorylation product. When the mbHPLC-purified phosphopeptide derived from cPLA2 was oxidized with performic acid and reinjected onto the column, elution occurred ~3 min earlier, at the same position of the synthetic standard pS727-Pox (pS727-P with its single cysteine oxidized to cysteic acid) (Fig. 4*A*). pS454-P lacks cysteine, and its retention time is not altered by performic acid treatment (16).

Tryptic peptides pS437-P, pS454-P, pS505-P, and pS727-P can also be resolved by two-dimensional chromatography on thin layer plates (16, 20), and we analyzed the radioactive fraction eluting from the mbHPLC column in this way. The non-oxidized and oxidized peptides derived from cPLA2 that were radiophosphorylated by MNK1 *in vitro* co-migrate with

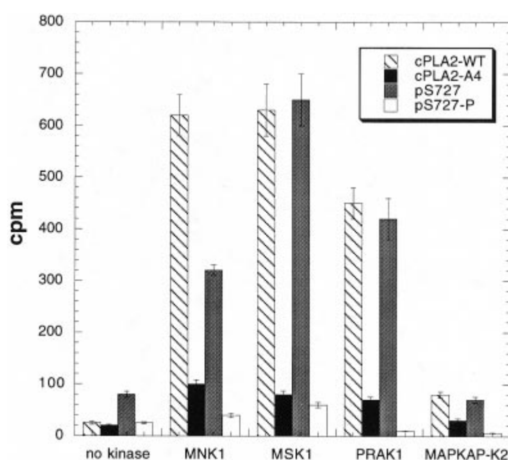

FIG. 3. ***In vitro* phosphorylation of cPLA2 and cPLA2-derived peptides.** Kinases were present at 10 milliunits (see "Materials and Methods" for definition) in all cases. Other conditions are given under "Materials and Methods." The average and S.D. from three independent experiments are shown. cPLAZ-A4 designates the S437A/S454A/S505A/S727A mutant.

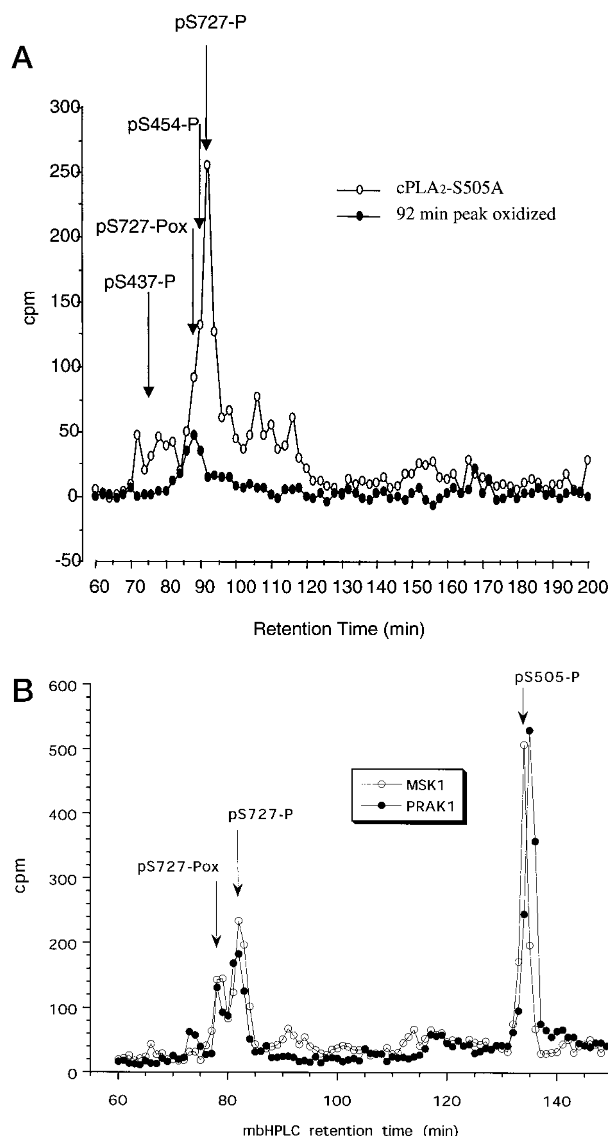

FIG. 4. **mbHPLC analysis of *in vitro* phosphorylated cPLA2.** *A,* cPLA2-S505A was phosphorylated with MNK1, and the trypsin digest was analyzed by mbHPLC. The phosphopeptide eluting at 92 min was oxidized with performic acid and re-analyzed by mbHPLC. The positions of synthetic phosphopeptide standards are shown by *arrows. B,* cPLA2-WT was phosphorylated with MSK1 and PRAK1, and the trypsin digest was analyzed by mbHPLC. Radioactivity eluting in the first 60 min amount to less than 5% of the applied counts/min. Retention time in minutes (cpm MSK1 reaction/cpm PRAK1 reaction/peptide standard): pS727-Pox, 79.0:78.7:78.9; pS727-P, 83.2:83.2:83.0; pS505A-P, 133.5:134.8:133.4.

the synthetic standards pS727-P and pS727-Pox (not shown).

To demonstrate rigorously that MNK1 phosphorylates Ser-727 within the tryptic peptide residues 721–736 and not any of the other serines present in this peptide or elsewhere in the protein, we analyzed the cPLA2-MNK1 reaction mixture by micro-column HPLC/tandem mass spectrometry. After *in vitro* phosphorylation of wild type cPLA2 with activated MNK1, the enzyme was freed of radiolabeled ATP by SDS-PAGE, and protein was prepared for trypsin digestion as described for mbHPLC and two-dimensional chromatography. The analysis of the authentic synthetic standard pS727-Pox showed that elution of the oxidized peptide, m/z 938.3, occurred at 11.30 min. In a separate HPLC run, a peptide from the tryptic digest of cPLA2 with m/z 938.3 eluted at 12.06 min. Fig. 5 shows the tandem mass spectrum of this peptide in comparison to pS727-Pox. The fragment ions of the y type, y_3-y_8, y_{10}-y_{12}, and y_{14}, and of the b type, b_5-b_7 and b_9-b_{13}, were identical in the cPLA2-derived peptide and authentic pS727-Pox. This phosphopeptide was not seen in the tryptic digest of a cPLA2 reaction mixture lacking MNK1 (not shown).

MNK1 is also capable of phosphorylating the synthetic peptide pS727, which corresponds to the tryptic cleavage product residues 721–736 of cPLA2 (Fig. 3). The synthetic peptide that had Ser-727 already phosphorylated (pS727-P) was not a substrate for MNK1, and MAPKAP-K2 failed to phosphorylate pS727 (Fig. 3).

As shown in Fig. 4*B*, treatment of cPLA2-WT *in vitro* with MSK1 and PRAK1 followed by trypsin digestion and mbHPLC resulted in major radioactive peaks eluting at the positions of pS727-P and pS727-Pox. In these experiments, pS727-P underwent partial spontaneous oxidation in the absence of performic acid treatment; this was observed previously with platelet-derived cPLA2 (16). A late eluting phosphopeptide (~135 min) co-elutes with authentic pS505-P. This is the result of contaminating p38 and ERK used to activate MSK1 and PRAK1, respectively, since treatment of cPLA2-WT with p38-2a and ERK1 alone resulted only in Ser-505 phosphorylation (16). In addition, MSK1 and PRAK1 phosphorylate pS727 but not pS727-P (Fig. 3). All together, these results provide strong evidence that MSK1 and PRAK1, like MNK1, phosphorylate cPLA2 on Ser-727.

MNK1 and PRAK1 Activity in Human Platelets—To find out whether a MNK1-related kinase could be the kinase phosphorylating Ser-727 of cPLA2 in an intact cell, we tested their presence in human platelets and activity before and after stimulation with thrombin. As shown in Fig. 6, MNK1 was detected in non-stimulated platelets by immunoblot analysis. To explore further the presence and activation of MNK1 in platelets, this kinase was immunoprecipitated with a specific antiserum (24), and *in vitro* kinase assays using MBP as substrate were performed (24, 37). MNK1 was active under basal conditions and underwent a further 2.5-fold increase after stimulation with thrombin (Fig. 7*A*). The increase in activity was transient, returning to the near basal value after 5 min (Fig. 7*A*).

Since MBP is a nonspecific substrate for serine/threonine kinases, it was important to check whether other kinases co-precipitated with MNK1. We therefore performed an in-gel renaturation kinase assay after immunoprecipitation of MNK1. In this assay, we detected strong MBP kinase activity at 50–55 kDa which co-migrated with purified and activated

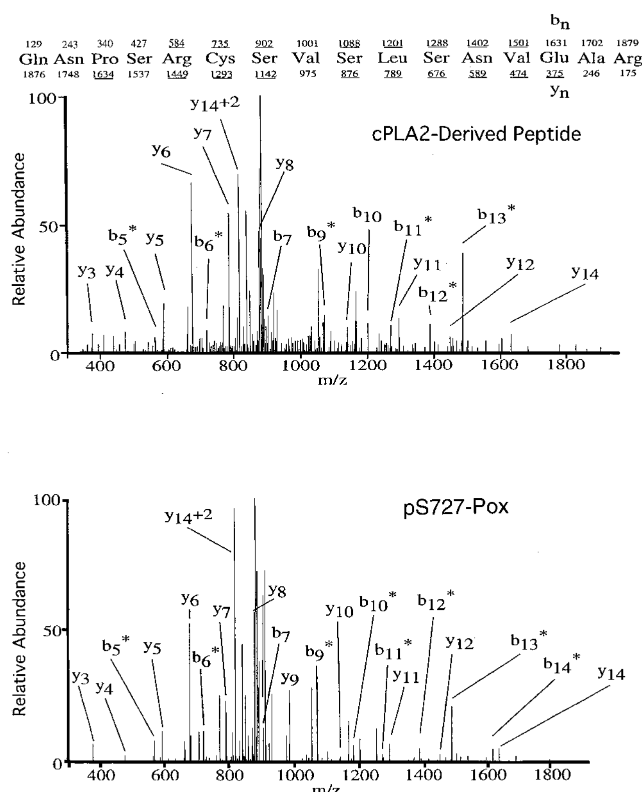

FIG. 5. **Tandem mass spectrum of tryptic phosphopeptide derived from cPLA2-WT treated with MNK1 *in vitro*.** Tandem mass spectrum of the $(M + 2H)^{2+}$ ion at m/z 938.3 of authentic pS727-Pox (*bottom panel*) and of the phosphopeptide derived from MNK1-treated cPLA2-WT (*top panel*) eluting from the HPLC column at the same time as pS727-Pox. This cPLA2-derived phosphopeptide was not seen in the tryptic digest of cPLA2-WT from the minus MNK1 control reaction. The *asterisks* in the spectrum denote the loss of water from an ion.

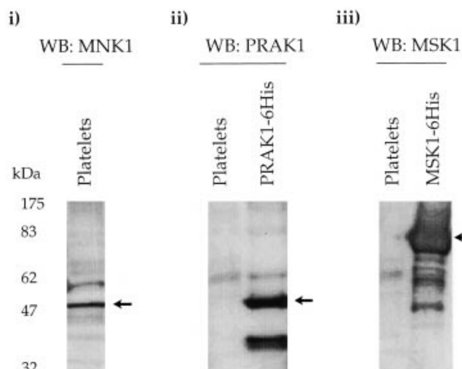

FIG. 6. **Presence of MNK1 in platelets.** Washed platelets (2×10^7) were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane for immunoblotting analysis. PRAK1–6His and MSK1–6His (1 μg each) were included in *gels ii* and *iii*, respectively, as positive controls. Immunoblot (*WB*) analyses were carried out for MNK1 (anti-MNK1, Santa Cruz Biotechnology, 0.75 μg/ml) (*gel i*), PRAK1 (anti-PRAK1, 50 ng/ml) (*gel ii*), and MSK1 (anti-MSK1, 50 ng/ml) (*gel iii*).

MNK1 (not shown). This confirms MNK1 as the major kinase underlying phosphorylation of MBP. A second, very weak kinase activity was detected in long exposures. Its activation time course corresponds to the time course of platelet ERK2 activity under similar stimulation conditions (33).

Pretreatment of platelets with the p38-2a inhibitor SB 202190 or the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor PD 98059 resulted in a partial inhibition of thrombin-stimulated MNK1 activity (Fig. 7*B*). It is possible, that both p38-2a and ERK2 regulate thrombin-stimulated MNK1 activity, as both kinases are active under these conditions (17, 33). This corresponds with the original reports describing MNK1 activation downstream of p38-2a and ERK2 (24, 37) and with our finding that the phosphorylation of Ser-727 on cPLA2 is partially inhibited by the p38-2a inhibitor SB 203580 in thrombin-activated platelets (16).

As shown in Fig. 6, PRAK1 could not be detected in platelets by immunoblotting analysis, but PRAK1 activity was detectable after immunoprecipitation from thrombin-stimulated platelets followed by assaying for kinase activity using a peptide substrate (Fig. 7*C*). The rise in PRAK1 kinase activity after thrombin stimulation was more modest than that for MNK1, reaching only about 1.4-fold. Like for MNK1, the increase in PRAK1 activity was transient. We were not able to detect MSK1 in platelets either by immunoblot analysis using a specific antibody (Fig. 6) or by immunoprecipitation followed by assaying for kinase activity (not shown).

Studies with Dominant Negative MNK1—To explore further the involvement of MNK1 in Ser-727 phosphorylation of cPLA2, HEK293 cells expressing both cPLA2-WT and a dominant negative form of MNK1 (39) were prepared and used for arachidonate release studies. The expression levels of cPLA2-WT and dominant negative MNK-1 proteins in the transfectants, as assessed by immunoblotting, are shown in Fig. 8*A*. Expression of endogenous cPLA2 was barely detectable, and endogenous MNK-1 was detected faintly in control 293 cells (MNK1 was readily detected when 10-fold more protein from non-transfected HEK293 cells was analyzed, not shown).

As shown in Fig. 8*B*, when cells were stimulated with 10 μM A23187 for 30 min, arachidonate release by cells expressing cPLA2-WT alone and those co-expressing cPLA2-WT and dominant negative MNK-1 were almost comparable. In marked contrast, when replicate cells were stimulated for 4 h with 1 ng/ml IL-1 in the presence of 10% fetal calf serum, arachidonate release by cPLA2-WT was suppressed by ~70% in cells that also express dominant negative MNK-1 (Fig. 8*C*). These observations are consistent with MNK-1 or a closely related kinase being the cPLA2 Ser-727 kinase *in vivo*. They also confirm observations shown in Fig. 2 that cPLA2 phosphorylation is less important for promoting arachidonate release in the presence of high calcium ionophore.

DISCUSSION

In this study, we report, for the first time, that phosphorylation of cPLA2 on Ser-727 is functionally important for arachidonate release in mammalian cells. Poor arachidonate release in response to A23187 following overexpression of cPLA2-S727A was seen in three mammalian cell models, CHO, HeLa, and HEK293 cells. In these cells, phosphorylation of cPLA2 at Ser-505 is also important, and this is consistent with the previous study with CHO cells (14). Mutation of either serine alone reduces arachidonate release almost as much as mutating both residues, showing that phosphorylation of cPLA2 at either Ser-505 or Ser-727 alone is insufficient to allow for optimal arachidonate release.

These studies contribute in an important way to our understanding of the regulation of cPLA2 function in mammalian cells, and they underscore the need for rigorous protein chemical analysis for determining the full pattern of phosphorylation. Many investigators have reported that cPLA2 undergoes a shift in electrophoretic mobility following agonist stimulation of cells, an indication of cPLA2 phosphorylation, but phosphopeptide analysis of radiophosphorylated cPLA2-derived peptides has only been carried out with insect cells (Sf9) expressing cPLA2 (20), platelets (16, 19), HeLa cells (16), and

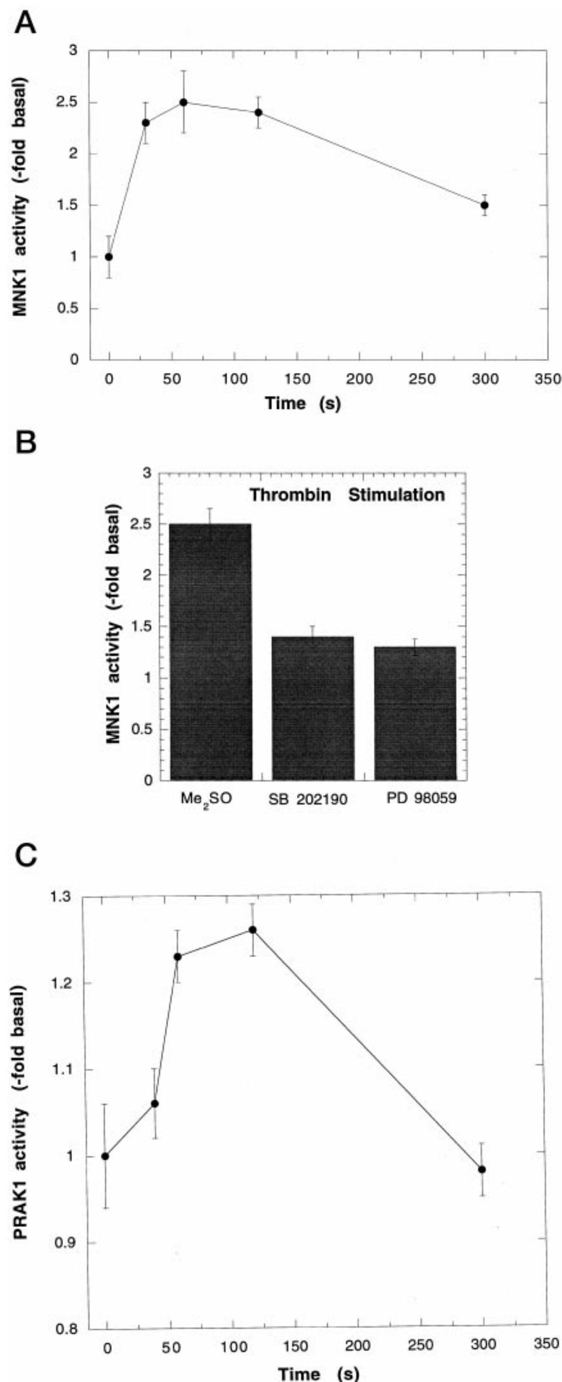

FIG. 7. **MNK1 and PRAK1 activity in human platelets.** Indomethacin-treated platelets (1×10^9/ml) were stimulated with thrombin (1 unit/ml) under stirred conditions at 37 °C, and MNK1 was immunoprecipitated with the previously described antiserum (24) from lysates (stimulation times given in seconds). *A*, immunoprecipitates were incubated with kinase buffer containing 100 μM MBP, 100 μM ATP, and 50 μCi/ml [γ-^{32}P]ATP. After 10 min at 30 °C, an aliquot was spotted onto P-81 phosphocellulose filter papers that were washed and scintillation counted for radioactivity. MNK1 activity is expressed in -fold over basal after subtraction of values from "mock" immunoprecipitates performed without cell lysate. The basal value was 3900 cpm. Data are presented as mean ± range from duplicate determination. Similar data were obtained in two independent experiments with platelets prepared from different blood donors. *B*, platelets were pretreated with Me$_2$SO vehicle (0.5%), SB 292190 (20 μM), or PD 98059 (20 μM) at 37 °C for 20 min and were stimulated with thrombin (1 unit/ml) for 2 min under stirred conditions. MNK1 was immunoprecipitated, and kinase activity was determined as described for *A*. *C*, platelets were prepared and stimulated with thrombin as described for *A*, and PRAK1 was immunoprecipitated from lysates (stimulation times given in seconds). Immunoprecipitates were incubated in kinase buffer containing 30 μM PRAK1 substrate peptide, and the kinase assay was performed as described in *A*. The data are presented as mean ± S.D. from duplicate determination in n = three experiments.

CHO cells (Fig. 1). In all cases, cPLA2 is phosphorylated on Ser-505 and Ser-727 to a similar degree.

In thrombin-stimulated platelets, cPLA2 undergoes a complete electrophoretic mobility shift (34). Furthermore, after thrombin stimulation, virtually all of the cPLA2 elutes later from an ion exchange column compared with cPLA2 from non-stimulated cells (34). All together, the data show that cPLA2 is stoichiometrically phosphorylated on Ser-505 and Ser-727 in thrombin-stimulated platelets. When assayed with phospholipid vesicles *in vitro*, the enzymatic activity of Ser-505/727 phosphorylated cPLA2 from thrombin-stimulated platelets is 3–4-fold higher than that of cPLA2 partially purified from the same number of platelets but in the absence of thrombin (34). This increase in specific activity of the enzyme could explain the dependence of optimal arachidonate release on cPLA2 phosphorylation that we observe (Fig. 2), but other modes of activation of cPLA2 by phosphorylation in cells cannot be ruled out (see below). It is interesting to note that the importance of cPLA2 phosphorylation for maximal arachidonate release is much less when CHO, HeLa, and HEK293 cells are stimulated with sufficient calcium ionophore to provide optimal arachidonate release (Fig. 2). Results in the literature are consistent with the idea that phosphorylation of cPLA2 is less important for activation when intracellular calcium rises to a high and sustained level (2). These results suggest that phosphorylation becomes most important when the rise in intracellular calcium is insufficient to cause all of the cPLA2 to translocate to its membrane target. The fact that cPLA2 phosphorylation does not further increase the amount of arachidonate release at high calcium does not fit well with the model whereby phosphorylation only activates cPLA2 *in vivo* by increasing its catalytic efficiency. Rather the data suggest that cPLA2 phosphorylation or high intracellular calcium can independently allow cPLA2 either to bind to its membrane target or promote release of cPLA2 from a non-membrane target so it can transfer to the membrane for the lipolysis reaction. Further studies are needed to understand the mechanism of cPLA2 activation *in vivo*.

We have recently reported that cPLA2 is phosphorylated to a similar degree on Ser-505 and Ser-727 in human platelets in response to collagen and thrombin and through a pathway that is partially sensitive to inhibitors of p38-2a/2b (16, 19). The inhibition data suggest that the Ser-727 kinase is activated by p38-2a/2b in platelets. We therefore examined whether MNK1, MSK1, MAPKAP-K2, and PRAK1 could be the responsible kinases since these are known to be activated by p38-2a.

MAPKAP-K2 was not able to phosphorylate cPLA2 *in vitro*, even though the kinase was active on MPB and a peptide substrate. A similar result was seen for MAPKAP-K3, which lies downstream of ERK2, p38-2a, and JNK (40), following immunoprecipitation and exposure to cPLA2 (not shown). MAPKAP-K1 (p90^{rsk}) is not activated by p38-2a but lies downstream of ERK2 (38, 40).

MNK1, PRAK1, and MSK1 were able to phosphorylate cPLA2 *in vitro.* We provide unambiguous structural results (cPLA2 mutagenesis, mbHPLC, two-dimensional phosphopeptide mapping, and tandem mass spectrometry) showing that MNK1 phosphorylates cPLA2 uniquely at Ser-727 *in vitro*. mbHPLC results provide strong evidence that other members of the MAPK-activated protein kinase family, MSK1 and PRAK1, are also able to phosphorylate cPLA2 uniquely at Ser-727. Another example of different substrate specificities of

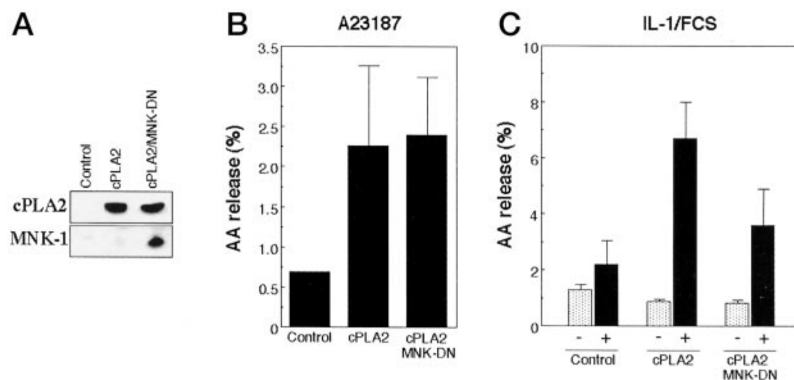

FIG. 8. **Studies with dominant negative MNK1.** *A*, expression of cPLA2-WT (cPLA2), MNK1, and dominant negative MNK1 (MNK-DN) proteins in HEK293 transfectants. Lysates from 10^5 cells were subjected to immunoblot analysis using anti-cPLA2 and anti-MNK1 antisera. *B* and *C*, cells prelabeled with [^{3}H]arachidonate were stimulated for 30 min with 10 μM A23187 (*B*) or for 4 h with 1 ng/ml IL-1 plus 10% fetal calf serum (*C*), and [^{3}H]arachidonate release was assessed as described under "Materials and Methods." Values are mean ± S.D. of three independent experiments.

MNK1 and MAPKAP-K2 *in vitro* is eIF-4E, which is phosphorylated by MNK1 and not by MAPKAP-K2 (37).

In order to investigate whether MNK1, MSK1, and PRAK1 are candidate kinases for mediating Ser-727 phosphorylation in platelets, we looked for their presence and monitored their activity in response to stimulation. Although MNK1 and PRAK1 are present in platelets, we were unable to detect the presence of MSK1 by immunoblotting or by immunoprecipitation using a specific antibody. Furthermore, MSK1 is inhibited by Ro 31-8220 (25), but this compound did not inhibit cPLA2 phosphorylation in platelets (17), thus emphasizing that MSK1 is an unlikely candidate for cPLA2 Ser-727 phosphorylation.

MNK1 and PRAK1 become activated in platelets stimulated with the same agonists (thrombin) that lead to phosphorylation of cPLA2 on Ser-727 (and on Ser-505). The time courses of MNK1 and PRAK1 activation (Fig. 7, *A* and *C*) fit with the temporal increase in cPLA2 phosphorylation (17) and with the time course of thrombin- and collagen-dependent ERK2 and p38-2a activation (17, 18, 33). Basal activities of MNK1 and PRAK1 were high compared with activities of ERK2, p38-2a, and MAPKAP-K2 in unstimulated platelets and underwent a modest increase in kinase activity following stimulation. It is likely that MNK1 and PRAK1 need to translocate to bind to their substrates and that this translocation is regulated by extracellular stimuli in intact cells. In fact, MNK1 is found in a tight-binding complex with ERK2 or p38-2a (37), and this interaction could regulate MNK1-substrate binding.

Stimulation of platelets by the non-physiological agonist phorbol ester leads to phosphorylation and activation of cPLA2, and this is mediated by ERK2, which differs from the p38-2a-regulated cPLA2 response to thrombin or collagen (41). cPLA2 from platelets that were stimulated with phorbol 12,13-dibutyrate was phosphorylated at Ser-505 and at Ser-727 to a similar degree based on mbHPLC analysis (not shown). Pretreatment with the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor PD 98059 substantially decreased Ser-505 and Ser-727 phosphorylation by 80%. In addition, we observed a small increase of MNK1 activity after stimulation with phorbol 12,13-dibutyrate (maximal 1.4-fold over basal, 5 min stimulation). MNK1 activity was reduced toward basal levels in the presence of either PD 98059 or SB 202190, which is consistent with results from transfected HeLa and HEK293 cells (24, 37). Given that MNK1 can be activated *in vivo* by EKR2 and p38-2a, these results provide further evidence that MNK1 or a closely related kinase is responsible for Ser-727 phosphorylation of cPLA2 in platelets.

All together, our results suggest that cPLA2 is phosphorylated at Ser-727 either by MNK1 or a closely related isoform in thrombin- and collagen-stimulated human platelets and that this supports activation by low concentrations of agonists. Studies with a dominant negative MNK1 construct show that MNK1 becomes accessible to cPLA2 *in vivo*. Studies with MNK1-deficient mammalian cells will be required to determine whether it is MNK1 or a closely related isoform that is responsible for cPLA2 phosphorylation.

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