

# Complement-mediated Activation of Calcium-independent Phospholipase A<sub>2</sub>γ

## ROLE OF PROTEIN KINASES AND PHOSPHORYLATION\*

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**Background:** Calcium-independent phospholipase A<sub>2</sub>γ (iPLA<sub>2</sub>γ) is a mediator of complement-induced glomerular injury.

**Results:** Complement stimulated iPLA<sub>2</sub>γ through activation of mitogen-activated protein kinases.

**Conclusion:** Phosphorylation of iPLA<sub>2</sub>γ plays a role in activation and signaling.

**Significance:** Understanding the regulation of iPLA<sub>2</sub>γ activity is essential for developing novel therapeutic approaches to glomerular injury and proteinuria.

In experimental membranous nephropathy, complement C5b-9 induces glomerular epithelial cell (GEC) injury and proteinuria. The effects of C5b-9 are mediated via signaling pathways, including calcium-independent phospholipase A<sub>2</sub>γ (iPLA<sub>2</sub>γ), and mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38. The iPLA<sub>2</sub>γ pathway is cytoprotective. This study addresses the mechanisms of iPLA<sub>2</sub>γ activation. iPLA<sub>2</sub>γ activity was monitored by quantifying prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production. In GECs, iPLA<sub>2</sub>γ localized at the endoplasmic reticulum and mitochondria. Complement-mediated production of PGE<sub>2</sub> was amplified in GECs that overexpress iPLA<sub>2</sub>γ, compared with control cells, and was blocked by the iPLA<sub>2</sub>γ inhibitor bromoenol lactone in both iPLA<sub>2</sub>γ-overexpressing and control GECs. In GECs that overexpress iPLA<sub>2</sub>γ, complement-mediated PGE<sub>2</sub> production was reduced by inhibitors of MAP/ERK kinase 1 (MEK1) and p38 but not JNK. In COS-1 cells that overexpress iPLA<sub>2</sub>γ and cyclooxygenase-1, PGE<sub>2</sub> production was induced by co-expression of constitutively active MEK1 or MAPK-interacting kinase 1 (MNK1) as well as by stimulation with epidermal growth factor (EGF) + ionomycin. Complement- and EGF + ionomycin-stimulated iPLA<sub>2</sub>γ activity was attenuated by the S511A/S515A double mutation. Moreover, complement and EGF + ionomycin enhanced phosphorylation of Ser-511. Thus, complement-mediated activation of iPLA<sub>2</sub>γ is mediated via ERK and p38 pathways, and phosphorylation of Ser-511 and/or Ser-515 plays a key role in the catalytic activity and signaling of iPLA<sub>2</sub>γ. Defining the mechanisms by which complement activates iPLA<sub>2</sub>γ provides opportunities for development of novel therapeutic approaches to GEC injury and proteinuria.

Phospholipases A<sub>2</sub> (PLA<sub>2</sub>s)<sup>3</sup> comprise a family of enzymes that hydrolyze the acyl bond at the *sn*-2 position of phospholipids to yield free fatty acids such as arachidonic acid (AA), and lysophospholipids (1, 2). Both products represent precursors for signaling molecules that can exert multiple biological functions. AA can be converted into bioactive eicosanoids by the effect of cyclooxygenases (COX), lipoxygenases, and cytochrome P450 (3). Schaloske and Dennis (4) classified PLA<sub>2</sub> enzymes into 15 groups based on their sequence homologies, and from a functional point of view these groups can be recombined into five principal types of PLA<sub>2</sub>s, secreted PLA<sub>2</sub>, cytosolic PLA<sub>2</sub> (cPLA<sub>2</sub>), calcium-independent PLA<sub>2</sub> (iPLA<sub>2</sub>), platelet-activating factor acetylhydrolases, and lysosomal PLA<sub>2</sub>. iPLA<sub>2</sub>s are members of group VI family of PLA<sub>2</sub> enzymes (4). The first and most extensively studied enzyme in this group is VIA, which has two isoforms, iPLA<sub>2</sub>-VIA-1 and -2 (iPLA<sub>2</sub>β short and iPLA<sub>2</sub>β long). The second iPLA<sub>2</sub> isoform, Group VIB (iPLA<sub>2</sub>γ), is homologous to iPLA<sub>2</sub>β in the C-terminal catalytic domain but shows no similarities in N-terminal region; accordingly, iPLA<sub>2</sub>γ may have distinct regulatory properties from those of iPLA<sub>2</sub>β (5, 6).

Various PLA<sub>2</sub> enzymes have been shown to mediate pathways of cell injury in experimental disease models (7–11). For example, cPLA<sub>2</sub>α and its products are important mediators of complement-induced glomerular epithelial cell (GEC; podocyte) injury in the passive Heymann nephritis model of membranous nephropathy (12, 13). In passive Heymann nephritis, GECs (an important component of the glomerular permselectivity barrier) (14, 15) are targeted by the complement C5b-9 membrane attack complex, which leads to noncytolytic GEC injury (12, 13). Injury is associated with activation of diverse signaling pathways, which include phospholipases as well as protein kinases, proteinases, COX2, endoplasmic reticulum (ER) stress, reactive oxygen species, and others. These pathways

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<sup>3</sup> The abbreviations used are: PLA<sub>2</sub>, phospholipase A<sub>2</sub>; cPLA<sub>2</sub>, cytosolic PLA<sub>2</sub>; iPLA<sub>2</sub>, calcium-independent PLA<sub>2</sub>; AA, arachidonic acid; BEL, bromoenol lactone; COX, cyclooxygenase; ER, endoplasmic reticulum; GEC, glomerular epithelial cell; HIS, heat-inactivated human serum; MEK1, MAP/ERK kinase 1; MEK1, MAP/ERK kinase 1; MK2, MAPK-activated protein kinase-2; MKK3, MAPK kinase 3; MNK1, MAPK-interacting kinase 1; NS, normal human serum; PG, prostaglandin.

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contribute to changes in GEC lipid structure and function, actin cytoskeleton reorganization, and displacement of filtration slit diaphragm proteins, ultimately resulting in proteinuria (12, 13, 16). Recently, we demonstrated that C5b-9 stimulated a Ca<sup>2+</sup>-independent PLA<sub>2</sub> activity in GECs, and complement-induced release of [<sup>3</sup>H]AA and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) was amplified in GECs that overexpress iPLA<sub>2</sub>γ (13, 16). Furthermore, overexpression of iPLA<sub>2</sub>γ attenuated complement-induced GEC injury, and this effect was reversed by the iPLA<sub>2</sub>-directed inhibitor, bromoenol lactone (BEL) as well as indomethacin, suggesting that the cytoprotective effect of iPLA<sub>2</sub>γ was at least in part mediated by generation of prostanoids (16).

Although we and others have shown functional coupling of iPLA<sub>2</sub>γ with COX1, leading to prostanoid production (17), the functions of iPLA<sub>2</sub>γ have not been fully delineated (18). In resting cells, iPLA<sub>2</sub>s, including iPLA<sub>2</sub>β, are involved in the maintenance of membrane phospholipids by generating lysophospholipid acceptors that are reacylated with fatty acids. Therefore, iPLA<sub>2</sub> plays a housekeeping role by facilitating phospholipid remodeling (19–21). Inhibition of iPLA<sub>2</sub> in HEK293 and INS-1 cells altered the amounts of several phospholipids and resulted in decreased cell growth and p53 activation (22–24). During oxidative stress, an ER-associated iPLA<sub>2</sub> in renal proximal tubular cells (iPLA<sub>2</sub>γ) recognizes, cleaves, and removes oxidized phospholipids from the ER membrane (25); thus, iPLA<sub>2</sub>γ may act to repair or prevent lipid peroxidation during oxidative stress (25). Another study demonstrated that expression of shRNA against iPLA<sub>2</sub>γ increased lipid peroxidation and induced apoptosis in renal cells (26). iPLA<sub>2</sub> is involved in signal transduction pathways that include mitogen-activated protein kinase (MAPK) p38, epidermal growth factor (EGF) receptor, the tumor suppressor gene, p53, and cell cycle-regulator, p21 (27). A diverse array of cellular processes has been proposed to be regulated by iPLA<sub>2</sub>γ, including cellular proliferation (28), assembly of very low density lipoprotein (29), apoptosis (30), endothelial cell platelet activating factor synthesis (31), tumorigenesis, cell injury, and chemotaxis (7).

iPLA<sub>2</sub>γ is a membrane-bound enzyme that is reported to localize at the ER, peroxisomes, and mitochondria (25). These distinct sites of localization may be a result of specific domains in the structure of the enzyme (32). iPLA<sub>2</sub>γ gene transcription and translation appear complex, as distinct translation initiation sites, resulting in the production of 88-, 77-, 74-, and 63-kDa forms of the enzyme were reported (33). iPLA<sub>2</sub>γ contains a consensus site for nucleotide binding and a lipase consensus motif in its C-terminal half as well as potential cAMP-dependent protein kinase, protein kinase C, and extracellular signal-regulated kinase (ERK) phosphorylation sites (32). The lipase consensus motif GVSTG (amino acids 481–485 in the C-terminal region) is essential for Ca<sup>2+</sup>-independent PLA<sub>2</sub> catalytic activity, and substitution of Ala for Ser-483 or Asp-627 results in loss of PLA<sub>2</sub> activity (34). To date, it is not known if/how phosphorylation would affect iPLA<sub>2</sub>γ activity.

The goal of the present study was to further characterize the activation of iPLA<sub>2</sub>γ in complement-induced GEC injury. Specifically, we addressed the role of various kinases known to be activated by complement. In GECs, we demonstrate the subcellular localization of iPLA<sub>2</sub>γ at the ER and mitochondria, which

was dependent on the N-terminal region of iPLA<sub>2</sub>γ. Complement-induced activation of iPLA<sub>2</sub>γ was mediated via ERK and p38 pathways. Stimulation of iPLA<sub>2</sub>γ was dependent on phosphorylation of Ser-511 and/or Ser-515 via MAPK-interacting kinase 1 (MNK1).

### EXPERIMENTAL PROCEDURES

**Materials**—Tissue culture media, G418 (Geneticin), plasmid pRc/RSV, and Lipofectamine 2000 were from Invitrogen. Electrophoresis reagents were from Bio-Rad. Mouse monoclonal anti-green fluorescent protein (GFP), sheep anti-COX1, rabbit anti-MEK1 (C-18), and rabbit anti-MAP/ERK kinase kinase 1 (MEKK1) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-calnexin was from Assay Designs and Stressgen (Ann Arbor, MI). Mouse monoclonal anti-α-tubulin was from Sigma. Rabbit phospho-p44/42 MAPK (Thr-202/Tyr-204), rabbit anti-phospho-p38 (Thr-180/Tyr-182), rabbit anti-phospho-JNK (Thr-183/Tyr-185), and rabbit anti-phospho-Ser/Thr Akt substrate antibody (which recognizes the sequence (R/K)XX(pS/T)) were from Cell Signaling Technology (Danvers, MA). MitoTracker Red CMXRos was from Molecular Probes (Eugene, OR). Enhanced chemiluminescence (ECL) reagents were from GE Healthcare. BEL, CGP57380, PLA<sub>2</sub> assay kits and PGE<sub>2</sub> enzyme immunoassay kits were from Cayman Chemical (Ann Arbor, MI). PD98059 was from Calbiochem. Human iPLA<sub>2</sub>γ wild type (WT) in pcDNA 1.1 was kindly provided by Drs. Richard Gross and David Mancuso (Washington University School of Medicine, St. Louis, MO) (16). R4F-MEK cDNA was provided by Dr. Natalie Ahn (University of Colorado, Boulder, CO) (35). pFC-MEKK, the constitutively active form of MEKK1, and pFC-MKK3, the constitutively active form of MKK3, were from Stratagene (La Jolla, CA). C8 and C8-deficient serum were from Complement Technology (Tyler, TX). pcDNA3-myc-MAPK-activated protein kinase-2 (MK2) WT and constitutively active mutant cDNA3-myc-MK2-EE were kindly provided by Professor Matthias Gaestel (Institute of Biochemistry, Medical School, Hannover, Germany) (36). Constructs encoding glutathione S-transferase (GST)-tagged MNK1 (pEBG-MNK1), a constitutively active form of MNK1 (pEBG-T332D), dominant-interfering MNK1 mutant (pEBG-T2A2), and pEBG empty vector were kindly provided by Dr. Jonathan Cooper (Fred Hutchinson Cancer Research Center, Seattle, WA) (37). Other reagents were from Sigma.

**Cloning and Construction of iPLA<sub>2</sub>γ Mutants**—WT, full-length (782 amino acid; M1) human iPLA<sub>2</sub>γ in pcDNA 1.1 was digested with SalI and SacII and was subcloned into pEGFP-C1 vector (Clontech) at SalI and SacII restriction sites to produce M1 GFP-iPLA<sub>2</sub>γ WT (M1 denotes that the iPLA<sub>2</sub>γ cDNA sequence begins at the codon for the first methionine). Mutations in potential ERK phosphorylation sites (Ser-271 and Ser-168 to Ala as well as the double mutation) were constructed by PCR-based mutagenesis (primers are presented in Table 1). A double mutation in two other potential phosphorylation sites (Ser-511 and Ser-515 to Ala) was constructed using an analogous approach (Table 1). For construction of N-terminally truncated (M4) GFP-iPLA<sub>2</sub>γ (*i.e.* iPLA<sub>2</sub>γ cDNA sequence beginning at the codon for the 4th methionine, amino acid 221)

TABLE 1

PCR primers employed to construct iPLA<sub>2</sub>γ mutants

Bold letters denote base substitutions encoding for mutated amino acids.

GFP-iPLA <sub>2</sub> γ	Primer	Primer sequence (5' to 3')
GFP-iPLA <sub>2</sub> γ S271A	F1	CCGGAGCTCCTGCAGTCGACATGTCTATTAATCT
	R2	CGCAGAAAGGAGCTGTAGGCTTG
	F2	CAAGCTACAGCTCCTTCTGCG
	R1	CCTTGTTCTCCACCATCAA
GFP-iPLA <sub>2</sub> γ S168A	F1	CCGGAGCTCCTGCAGTCGACATGTCTATTAATCT
	R3	GGAAAAGGAGCCTTTTCTGCTGAT
	F3	GCAGAAAAGGCTCCTTTCCAGAA
GFP-iPLA <sub>2</sub> γ S511A/S515A	R1	CCTTGTTCTCCACCATCAA
	F1	GCGAGAAAAGATTATCGCAAG
	R4	TTGTGCAAATACATCTGCTCCTAATTTTC
	F4	AATTAGGAGCAGATGTATTTCACAAAATGT
	R1	GGGGTTTCTTGCTGTTTCAA
M4 GFP-iPLA <sub>2</sub> γ	M4-F1	CCGGAGCTCAAATGAAATGTCTCAACAAAAGGAAAATG
	M4-R1	CCTTGTTCTCCACCATCAA

PCR reactions were performed with primers M4-F1 in combination with R1 (Table 1). All GFP-iPLA<sub>2</sub>γ mutant cDNAs were verified by DNA sequencing.

**Cell Culture and Transfection**—Rat GEC culture and characterization have been described previously (38). GECs were maintained in K1 medium on plastic substratum. Cells were stably transfected with M1 or M4 GFP-iPLA<sub>2</sub>γ WT plasmids using Lipofectamine 2000 reagent according to the manufacturer's instructions. After selection with G418 and expansion, cells were sorted by flow cytometry to obtain cells with the highest expression of GFP-iPLA<sub>2</sub>γ WT. Fluorescence microscopy and immunoblotting were used to confirm GFP-iPLA<sub>2</sub>γ overexpression. A clone of GECs containing the neomycin-resistance gene was used as a control (GEC-Neo). COS-1 cells were cultured in DMEM, 10% fetal bovine serum and were transfected transiently with GFP-iPLA<sub>2</sub>γ and/or COX1 cDNAs using Lipofectamine 2000.

**Incubation of GECs with Complement**—GECs in monolayer culture were washed twice and incubated with rabbit anti-GEC antiserum (5% v/v) in modified Krebs-Henseleit buffer containing 145 mM NaCl, 5 mM KCl, 0.5 mM MgSO<sub>4</sub>, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.5 mM CaCl<sub>2</sub>, 5 mM glucose, and 20 mM Hepes, pH 7.4, for 30 min at 22 °C. The cells were then incubated for 40 min at 37 °C with normal human serum (NS, 2% v/v; with full complement activity) or heat-inactivated (decomplemented) human serum (HIS, 2% v/v; incubated at 56 °C for 60 min) in controls (39, 40).

**PGE<sub>2</sub> Assay**—Stimulated iPLA<sub>2</sub> enzymatic activity was monitored by measuring PGE<sub>2</sub> production. After incubation, supernatants were collected to quantify PGE<sub>2</sub>. The amount of PGE<sub>2</sub> released into supernatants was equivalent to that from cells plus supernatants, indicating that most PGE<sub>2</sub> was released from cells into supernatants. PGE<sub>2</sub> was quantified using an enzyme immunoassay kit according to the manufacturer's instructions. The range of the standard curve in the assay was 4–1000 pg of PGE<sub>2</sub>/100 μl of sample (41). PGE<sub>2</sub> concentration was calculated according to standard formulas.

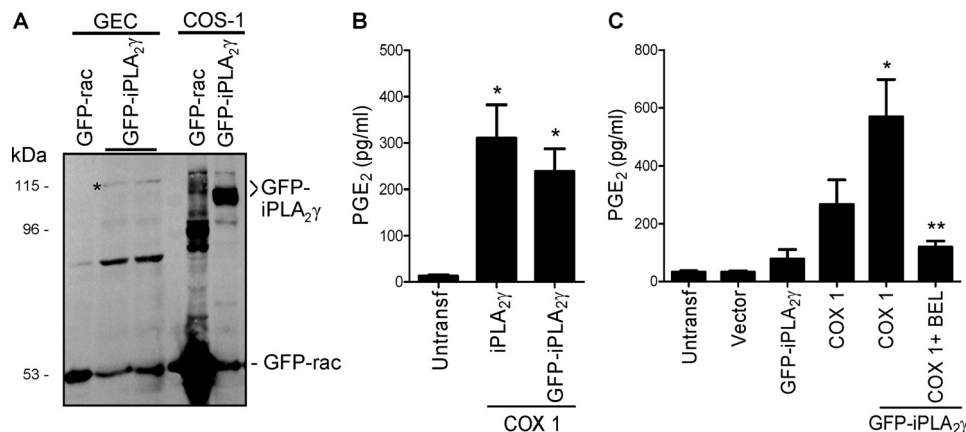
**PLA<sub>2</sub> Assay**—PLA<sub>2</sub> activity was measured in COS-1 cell extracts using a PLA<sub>2</sub> activity assay kit according to the manufacturer's instructions and as described previously (42). In this assay, hydrolysis of arachidonoyl thiophosphatidylcholine at the sn-2 position by PLA<sub>2</sub> releases a free thiol that is detected by 5,5'-dithio-bis-2-nitrobenzoic acid. Briefly, cells were homogenized in 50 mM Hepes, pH 7.4, containing 1 mM EDTA. Cell

homogenates were cleared by centrifugation at 8500 × g for 10 min at 4 °C. The reaction was initiated by the addition of 2-arachidonoyl thiophosphatidylcholine to cell extracts in buffer containing 80 mM Hepes, pH 7.4, 150 mM NaCl, 4 mM Triton X-100, 30% glycerol, and 1 mg/ml BSA. Duplicate samples were incubated with and without 10 μM BEL. After 60 min at 22 °C, the reaction was terminated by the addition of 1 mM 5,5'-dithio-bis-2-nitrobenzoic acid, and the absorbance was measured at 450 nm. To determine iPLA<sub>2</sub> activity, the optical density obtained in the presence of BEL was subtracted from the total optical density (42) (in control cells, ~20% of PLA<sub>2</sub> activity was inhibited by BEL). The value of the group with maximum iPLA<sub>2</sub> activity was set to 1.0, and the iPLA<sub>2</sub> activities of the other groups were calculated as percent of maximum.

**Immunoblotting**—Cells were lysed in ice-cold buffer containing 1% Triton X-100, 125 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EGTA, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM sodium pyrophosphate, 25 mM NaF, and protease inhibitor mixture (Roche Diagnostics). Equal amounts of lysate proteins were dissolved in Laemmli buffer and subjected to SDS-PAGE under reducing conditions. Proteins were then electrophoretically transferred onto a nitrocellulose membrane and blocked at room temperature for 60 min with 5% dry milk in buffer containing 10 mM Tris, pH 7.5, 50 mM NaCl, 2.5 mM EDTA, and 0.05% Tween 20. The membrane was then incubated with primary and secondary antibodies and developed with ECL.

**Immunofluorescence Microscopy**—GECs expressing GFP-iPLA<sub>2</sub>γ WT (M1 or M4) and GEC-Neo (control) were cultured on glass coverslips for 24 h. All reactions were carried out at 22 °C. To examine the localization of GFP-iPLA<sub>2</sub>γ WT at the ER, cells were fixed with 3% paraformaldehyde in PBS for 30 min and permeabilized with 0.1% Triton-X 100 in PBS for 30 min. After washing with PBS, GECs were incubated with rabbit anti-calnexin antiserum or normal rabbit serum (negative control) diluted in 3% BSA for 30 min. Cells were washed and incubated with rhodamine-conjugated goat anti-rabbit IgG in 3% BSA for 30 min. Nuclei were counter-stained with 4',6-diamidino 2-phenylindole (DAPI, 30 nM) in PBS for 4–5 min just before mounting the coverslips onto glass slides. Staining was visualized with a Zeiss AxioObserver fluorescence microscope with visual output connected to an AxioCam digital camera. To visualize mitochondria, GECs expressing GFP-iPLA<sub>2</sub>γ WT (M1 or M4) and GEC-Neo (control), on coverslips were incu-

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**FIGURE 1. Expression and activity of M1 GFP-iPLA<sub>2</sub>γ WT.** A, GECs were stably transfected, and COS-1 cells were transiently transfected with M1 GFP-iPLA<sub>2</sub>γ WT or a GFP-rac fusion protein for comparison. Lysates were immunoblotted with antibody to GFP. \* denotes M1 GFP-iPLA<sub>2</sub>γ in GECs, which migrated slower than M1 GFP-iPLA<sub>2</sub>γ in COS-1 cells, possibly because of differential post-translational modifications in the two cell lines. B, COS-1 cells were transiently transfected with untagged full-length iPLA<sub>2</sub>γ, M1 GFP-iPLA<sub>2</sub>γ WT, and COX1. PGE<sub>2</sub> production was measured in cell supernatants 24 h after transfection. Both GFP-tagged and untagged enzymes increased PGE<sub>2</sub> release. \*,  $p < 0.001$  iPLA<sub>2</sub>γ + COX1 and GFP-iPLA<sub>2</sub>γ + COX1 versus untransfected (Untransf) cells, three experiments. C, COS-1 cells were untransfected or were transiently transfected with vector, M1 GFP-iPLA<sub>2</sub>γ WT, COX1, or M1 GFP-iPLA<sub>2</sub>γ WT + COX1. Expression of COX1 alone increased PGE<sub>2</sub> release compared with control, whereas GFP-iPLA<sub>2</sub>γ + COX1 substantially amplified the increase in PGE<sub>2</sub> production. This increase was inhibited by coinubation with 30 μM BEL (6 h). \*,  $p < 0.001$  GFP-iPLA<sub>2</sub>γ + COX1 versus untransfected or vector and  $p < 0.001$  GFP-iPLA<sub>2</sub>γ + COX1 versus GFP-iPLA<sub>2</sub>γ,  $p < 0.05$  GFP-iPLA<sub>2</sub>γ + COX1 versus COX1, \*\*,  $p < 0.001$  GFP-iPLA<sub>2</sub>γ + COX1 + BEL versus GFP-iPLA<sub>2</sub>γ + COX1, 7 experiments.

bated for 15 min at 37 °C with MitoTracker Red CMXRos (25 nM). Cells were then fixed with 3% (w/v) paraformaldehyde in PBS for 30 min. After washing, coverslips were mounted onto glass slides and visualized with a fluorescence microscope.

**Statistics**—Data are presented as the mean ± S.E. One-way analysis of variance was used to determine significant differences among groups. Where significant differences were found, individual comparisons were made between groups using the t-statistic and by adjusting the critical value according to Tukey's or Bonferroni's method. Statistical significance was considered to be  $p < 0.05$ .

## RESULTS

**M1 GFP-iPLA<sub>2</sub>γ WT Is Enzymatically Active**—To study the effect of complement on iPLA<sub>2</sub>γ activation, we first established a subclone of GECs that stably overexpresses M1 GFP-iPLA<sub>2</sub>γ WT (M1 GEC-iPLA<sub>2</sub>γ). By immunoblotting, M1 GEC-iPLA<sub>2</sub>γ was expressed as a 115-kDa protein, consistent with GFP (27 kDa) fused with the 88-kDa isoform of iPLA<sub>2</sub>γ (Fig. 1A). When M1 GFP-iPLA<sub>2</sub>γ WT or untagged iPLA<sub>2</sub>γ was transfected in COS-1 cells together with COX1, PGE<sub>2</sub> production was markedly increased compared with untransfected control (Fig. 1B) or cells transfected with COX1 alone (Fig. 1C). This increase was attenuated by the addition of BEL (Fig. 1C). Together the results indicate that M1 GFP-iPLA<sub>2</sub>γ WT is enzymatically active.

**iPLA<sub>2</sub>γ Localizes at the ER and Mitochondria**—The expression and localization of iPLA<sub>2</sub> isoforms varies considerably among different cell types, and the role of iPLA<sub>2</sub> may be determined by the isoform present in a specific cell or organelle (43). We examined the localization of M1 GFP-iPLA<sub>2</sub>γ WT in stably transfected GECs. By fluorescence microscopy, confluent monolayers of resting GECs demonstrated green fluorescent staining mainly in the perinuclear region (Fig. 2, A and C). A significant portion of the M1 GFP-iPLA<sub>2</sub>γ WT co-localized with calnexin (Fig. 2, B and D), indicating localization at the ER.

In resting cells not expressing M1 GFP-iPLA<sub>2</sub>γ WT, calnexin staining showed a similar distribution in the perinuclear region, indicating that expression of M1 GFP-iPLA<sub>2</sub>γ WT did not affect the structure of the ER (data not shown).

A portion of the M1 GFP-iPLA<sub>2</sub>γ WT did not appear to co-localize with calnexin but localized at the mitochondria (Fig. 2, E–G). The appearance of the mitochondria was unaffected by the expression of M1 GFP-iPLA<sub>2</sub>γ WT (data not shown). Finally, we treated the GECs expressing M1 GFP-iPLA<sub>2</sub>γ WT with digitonin to permeabilize the plasma membranes and release cytosolic proteins into cell supernatants. Distribution of the GFP fluorescence in the digitonin-treated cells was similar to that seen in untreated (intact) cells (Fig. 2H), further supporting the association of iPLA<sub>2</sub>γ with intracellular organelles. Together the results shown in Fig. 2 indicate that in GECs M1 GFP-iPLA<sub>2</sub>γ WT is associated with at least two organelles, the ER and mitochondria.

**N-terminally Truncated iPLA<sub>2</sub>γ (M4 GFP-iPLA<sub>2</sub>γ) Is Mislocalized**—The catalytic domain of iPLA<sub>2</sub>γ is located in the C-terminal region of the protein, whereas the role of the N-terminal region is poorly defined. Indeed, a previous study showed that iPLA<sub>2</sub>γ contains four N-terminal methionine residues that may act as translation initiation sites, resulting in 88-, 77-, 74-, and 63-kDa forms of iPLA<sub>2</sub>γ in SF9 insect cells (33). To determine if the N-terminal region may be involved in localization and/or regulation of iPLA<sub>2</sub>γ catalytic activity, we deleted the 220 N-terminal amino acids (spanning between the 1st and 4th methionine) to generate a short form of iPLA<sub>2</sub>γ in which the GFP-iPLA<sub>2</sub>γ fusion would be at the 4th methionine (M4 GFP-iPLA<sub>2</sub>γ). Expression of M4 GFP-iPLA<sub>2</sub>γ in COS-1 cells showed a prominent band at ~92 kDa (Fig. 3A) that represents GFP (27 kDa) fused with the 63-kDa form of iPLA<sub>2</sub>γ. When both M1 and M4 isoforms were expressed at serially increasing concentrations in COS-1 cells (together with COX1), PGE<sub>2</sub> production by M1 GFP-iPLA<sub>2</sub>γ WT was markedly greater compared with

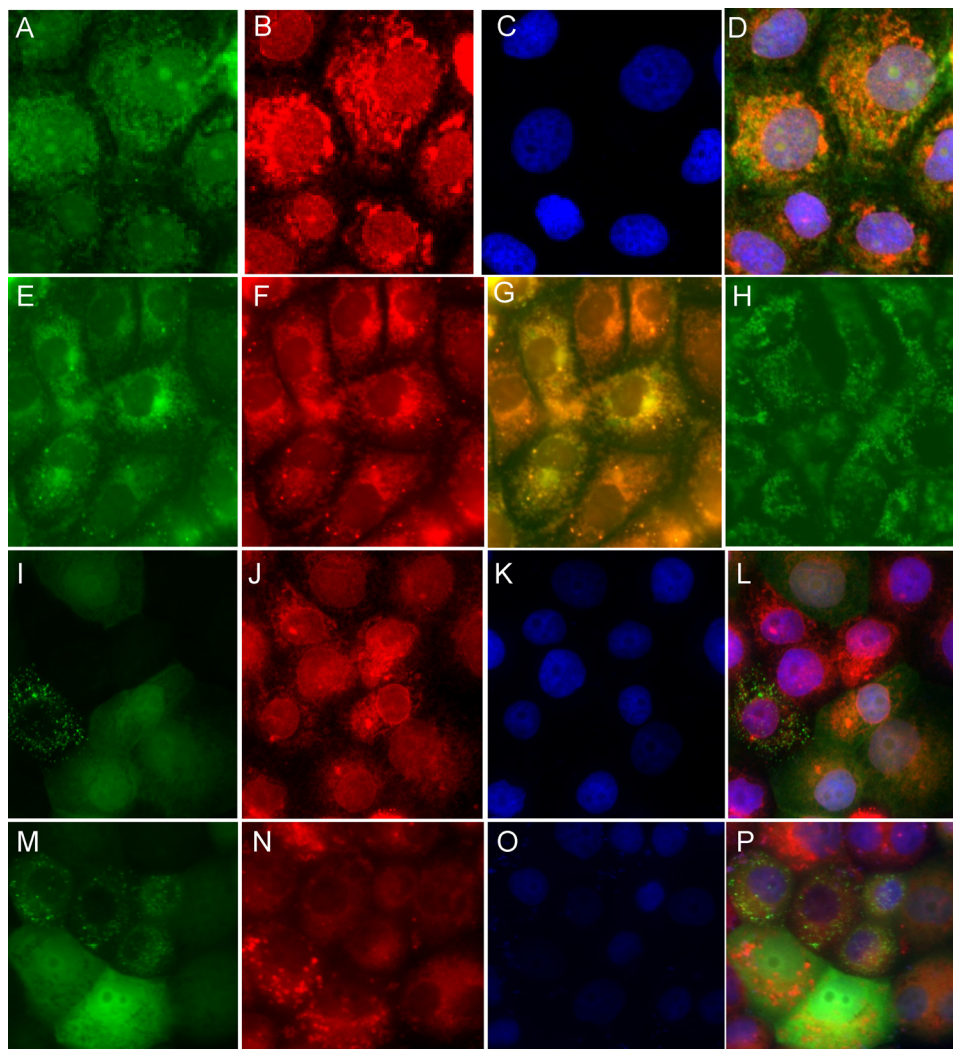


FIGURE 2. **Subcellular localization of M1 GFP-iPLA<sub>2</sub>γ WT and N-terminally truncated GFP-iPLA<sub>2</sub>γ (M4).** GECs stably transfected with M1 GFP-iPLA<sub>2</sub>γ WT show predominantly perinuclear green fluorescent staining (A). Cells were labeled with antibody to calnexin (red staining; B) to localize the ER, whereas nuclei were counterstained with DAPI (blue fluorescence; C). Calnexin staining was perinuclear (B). Panel D shows co-localization of M1 GFP-iPLA<sub>2</sub>γ WT and calnexin (yellow-orange staining). GECs that express M1 GFP-iPLA<sub>2</sub>γ WT (E) were stained with Mitotracker red, a marker of the mitochondria (F). Panel G shows co-localization of GFP-iPLA<sub>2</sub>γ with Mitotracker red. GECs expressing M1 GFP-iPLA<sub>2</sub>γ WT were treated with digitonin to permeabilize the plasma membranes (H). Digitonin treatment did not affect the localization of M1 GFP-iPLA<sub>2</sub>γ WT. GECs stably transfected with M4 GFP-iPLA<sub>2</sub>γ mainly show cytoplasmic green fluorescent staining (I and M). There is some minor perinuclear accentuation, and there appear to be aggregates in occasional cells. GECs were stained with anti-calnexin antibody (red staining; J), Mitotracker red (N), and DAPI (blue fluorescence; K and O). Panel L (merge of I–K) shows only minor co-localization of M4 GFP-iPLA<sub>2</sub>γ with calnexin. Panel P (merge of M–O) shows an absence of co-localization of M4 GFP-iPLA<sub>2</sub>γ with Mitotracker red.

M4 GFP-iPLA<sub>2</sub>γ despite weaker expression (Fig. 3, A–C). PGE<sub>2</sub> production by M4 GFP-iPLA<sub>2</sub>γ was trivial, as it was not significantly greater compared with control cells.

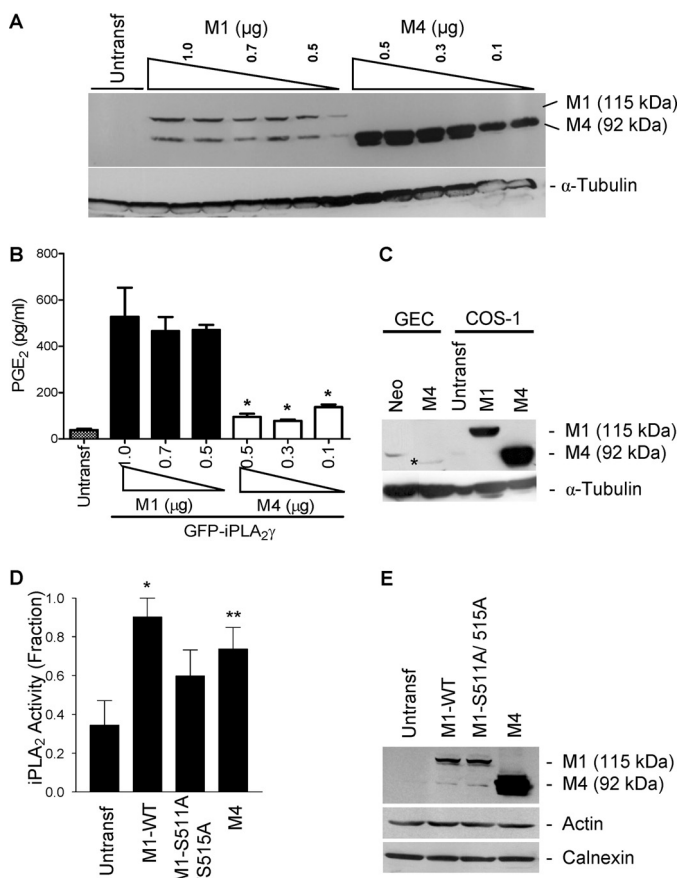
We also employed an *in vitro* assay to measure iPLA<sub>2</sub> enzymatic activity in extracts of control COS-1 cells and COS-1 cells expressing M1 GFP-iPLA<sub>2</sub>γ WT or M4 GFP-iPLA<sub>2</sub>γ. Using 2-arachidonoyl phosphatidylcholine as substrate, M1 GFP-iPLA<sub>2</sub>γ WT activity was significantly greater than control. M4 GFP-iPLA<sub>2</sub>γ also demonstrated significant activity (Fig. 3D). Thus, M4 GFP-iPLA<sub>2</sub>γ is active *in vitro* but not in intact cells.

In the next set of experiments, we employed GECs stably transfected with M4 GFP-iPLA<sub>2</sub>γ (Fig. 3C) to examine the localization. M4 GFP-iPLA<sub>2</sub>γ was found mainly in the cytosol, and only a minor portion was co-localized with calnexin (Fig. 2, I–L). Some cells contained what appeared to be aggregates of M4 GFP-iPLA<sub>2</sub>γ (Fig. 2J). M4 GFP-iPLA<sub>2</sub>γ did not co-localize

with Mitotracker red (Fig. 2, M–P). Thus, deletion of the N-terminal region of iPLA<sub>2</sub>γ leads to mislocalization of the enzyme away from the ER and mitochondria. Taken together, M4 GFP-iPLA<sub>2</sub>γ is enzymatically active but may not be functional in releasing AA and PGE<sub>2</sub> in intact cells due to the mislocalization of the enzyme from the membrane containing the substrate.

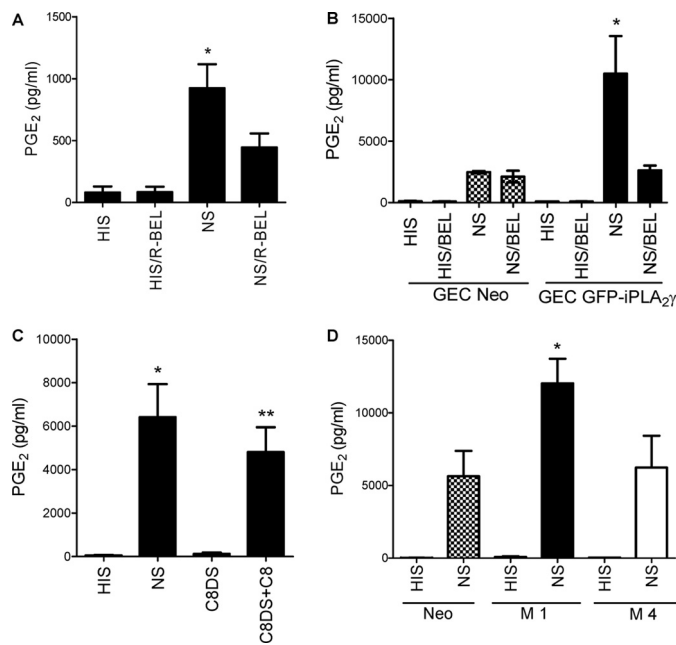
*Complement Induces Release of PGE<sub>2</sub> in GECs That Overexpress iPLA<sub>2</sub>γ*—A previous study demonstrated that in GECs, complement can induce release of [<sup>3</sup>H]AA via activation of iPLA<sub>2</sub>γ (16). Consistent with the previous results, when GEC-Neo cells were incubated with antibody and sublytic NS (to form C5b-9) or HIS in controls, PGE<sub>2</sub> production increased significantly, which was inhibited by R-BEL, a specific inhibitor of iPLA<sub>2</sub>γ (Fig. 4A). Next, we compared PGE<sub>2</sub> release in M1 GEC-iPLA<sub>2</sub>γ and GEC-Neo. Overexpression of M1 GFP-iPLA<sub>2</sub>γ WT did not affect the basal PGE<sub>2</sub> production (during

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**FIGURE 3. Expression and activity of GFP-iPLA<sub>2</sub>γ WT and mutants.** COS-1 cells were co-transfected with N-terminally truncated (M4) GFP-iPLA<sub>2</sub>γ (0.1–0.5 μg of plasmid DNA) and for comparison with M1 GFP-iPLA<sub>2</sub>γ WT (0.5–1.0 μg of plasmid DNA), both with COX1. *A*, anti-GFP antibody immunoblot shows greater expression of M4 GFP-iPLA<sub>2</sub>γ compared with M1 GFP-iPLA<sub>2</sub>γ WT. Expression tended to increase with increasing doses of plasmid DNA. The lower band in the M1 lanes is nonspecific. *B*, PGE<sub>2</sub> release in COS-1 cells expressing M4 GFP-iPLA<sub>2</sub>γ or M1 GFP-iPLA<sub>2</sub>γ WT (both with COX1) was normalized for corresponding protein expression. In these experiments basal PGE<sub>2</sub> release (untransfected cells) was 38.2 pg/ml. PGE<sub>2</sub> production by M1 GFP-iPLA<sub>2</sub>γ WT was markedly greater compared with M4 GFP-iPLA<sub>2</sub>γ. \*, *p* < 0.0001 M4 + COX1 (0.5, 0.3, and 0.1 μg) versus M1 + COX1 (1, 0.7, and 0.5 μg), three experiments. *C*, GECs were stably transfected, and COS-1 cells were transiently transfected with M4 GFP-iPLA<sub>2</sub>γ. GEC-Neo and COS-1 cells transiently transfected with M1 GFP-iPLA<sub>2</sub>γ WT are presented for comparison. Lysates were immunoblotted with antibody to GFP. \* denotes M4 GFP-iPLA<sub>2</sub>γ in GECs. The bands in GEC-Neo and untransfected COS-1 cells are nonspecific. *D*, PLA<sub>2</sub> activity in untransfected (control) COS-1 cells and COS-1 cells expressing M1 GFP-iPLA<sub>2</sub>γ WT, M1 GFP-iPLA<sub>2</sub>γ S511A/S515A double mutant, and M4 GFP-iPLA<sub>2</sub>γ is shown. Cell extracts were prepared 24 h after transfection, and iPLA<sub>2</sub> activity was monitored by release of AA from 2-arachidonoyl-phosphatidylcholine ("Experimental Procedures"). iPLA<sub>2</sub> activities of M1 GFP-iPLA<sub>2</sub>γ WT and M4 GFP-iPLA<sub>2</sub>γ were significantly greater compared with control. iPLA<sub>2</sub> activity of M1 GFP-iPLA<sub>2</sub>γ S511A/S515A tended to be greater than control. \*, *p* < 0.01 M1 GFP-iPLA<sub>2</sub>γ WT versus control, \*\*, *p* < 0.05 M4 GFP-iPLA<sub>2</sub>γ WT versus control, four experiments. In these experiments basal iPLA<sub>2</sub> activity (control cells) was 0.69 nmol/min/ml. *E*, cell lysates were immunoblotted with antibodies to GFP (iPLA<sub>2</sub>γ), actin, or calnexin (marker of ER).

incubation with HIS). However, after incubation with complement, PGE<sub>2</sub> release was significantly amplified in M1 GEC-iPLA<sub>2</sub>γ compared with GEC-Neo (Fig. 4*B*), and the complement-mediated PGE<sub>2</sub> release in M1 GEC-iPLA<sub>2</sub>γ was almost completely inhibited by BEL (racemic mixture, non-selective inhibitor of iPLA<sub>2</sub>β and -γ) (Fig. 4*B*), in keeping with earlier results (16). Therefore, activation of complement is coupled with stimulation of iPLA<sub>2</sub>γ activity. To verify that the PGE<sub>2</sub>

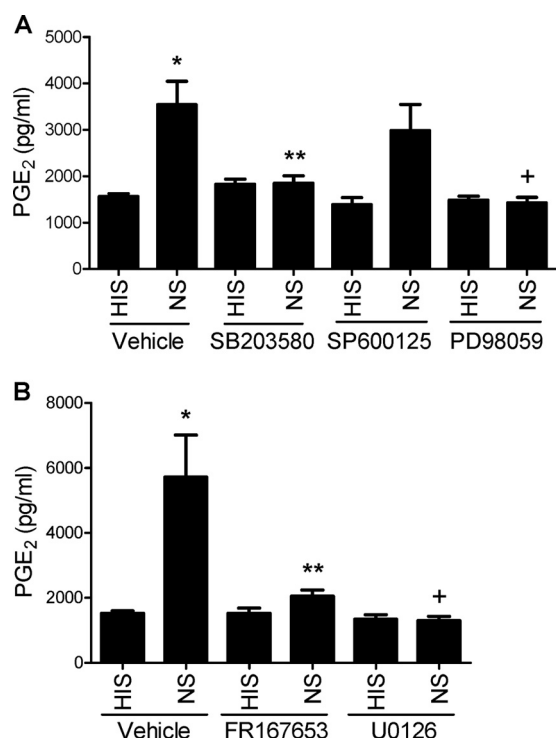


**FIGURE 4. Complement induces production of PGE<sub>2</sub> via iPLA<sub>2</sub>γ.** *A*, shown is the role of endogenous iPLA<sub>2</sub>γ. Neo GECs were incubated with anti-GEC antiserum for 30 min at 22 °C in the presence or absence of the iPLA<sub>2</sub>γ-directed inhibitor R-BEL (10 μM). Cells were then incubated at 37 °C with 2% NS (to form C5b-9) or HIS in controls with or without R-BEL for 40 min. Then PGE<sub>2</sub> production was measured in cell supernatants. Complement stimulated PGE<sub>2</sub> production, and the increase was significantly attenuated by R-BEL. \*, *p* < 0.0001 NS versus HIS and *p* < 0.001 NS versus NS/R-BEL, three experiments. *B*, complement-induced production of PGE<sub>2</sub> is amplified in GECs that overexpress M1 GFP-iPLA<sub>2</sub>γ WT (GEC GFP-iPLA<sub>2</sub>γ). GECs that express M1 GFP-iPLA<sub>2</sub>γ WT were incubated with antibody and complement with or without BEL as above. M1 GFP-iPLA<sub>2</sub>γ WT markedly amplified complement-induced PGE<sub>2</sub> production, and the increase was attenuated by BEL (30 μM). \*, *p* < 0.001 GEC-GFP-iPLA<sub>2</sub>γ (NS) versus GEC-Neo (NS), *p* < 0.001 GEC-GFP-iPLA<sub>2</sub>γ (NS/BEL) versus GEC-GFP-iPLA<sub>2</sub>γ (NS), three experiments. *C*, PGE<sub>2</sub> production is dependent on C5b-9 assembly. GECs that express M1 GFP-iPLA<sub>2</sub>γ WT were incubated with antibody and C8-deficient serum (C8DS) with or without purified C8. When C8DS was reconstituted with C8, PGE<sub>2</sub> production amplified significantly. \*, *p* < 0.0001 NS versus HIS; \*\*, *p* < 0.001 C8DS+C8 versus C8DS, 3 experiments. *D*, M4 GFP-iPLA<sub>2</sub>γ is inactive in intact GECs. Neo GECs or GECs that stably express M1 GFP-iPLA<sub>2</sub>γ WT or M4 GFP-iPLA<sub>2</sub>γ were incubated with antibody and complement as above. \*, *p* < 0.001 GEC-M1 GFP-iPLA<sub>2</sub>γ WT (NS) versus GEC-Neo (NS) and *p* < 0.001 GEC-M1 GFP-iPLA<sub>2</sub>γ WT (NS) versus GEC-M4 GFP-iPLA<sub>2</sub>γ (NS), three experiments.

release produced by antibody and NS was actually due to formation of C5b-9, antibody-sensitized M1 GEC-iPLA<sub>2</sub>γ were exposed to C8-deficient serum or C8-deficient serum reconstituted with C8 (40). C8-deficient serum alone had no significant effect on PGE<sub>2</sub> production, whereas C8-deficient serum reconstituted with C8 increased PGE<sub>2</sub> release significantly (Fig. 4*C*).

We also tested the effect of complement on PGE<sub>2</sub> release in the GECs stably expressing M4 GFP-iPLA<sub>2</sub>γ. In contrast to M1 GEC-iPLA<sub>2</sub>γ, the effect of complement on PGE<sub>2</sub> production in the M4-expressing cells was not significantly different from GEC-Neo (Fig. 4*D*). The result indicates that M4 GFP-iPLA<sub>2</sub>γ is less active in the cell and is in keeping with the experiments involving transient transfection of the M1 and M4 forms of iPLA<sub>2</sub>γ in COS-1 cells (Fig. 3*B*).

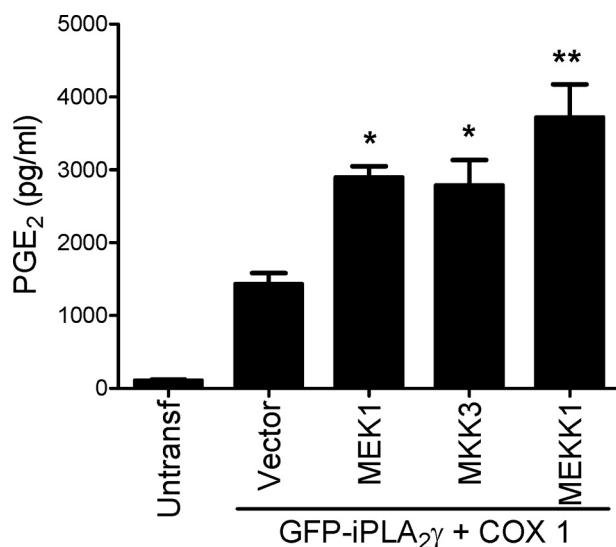
**Role of MAPKs in iPLA<sub>2</sub>γ Activation**—The C5b-9 complex is assembled in the plasma membranes of cells, whereas iPLA<sub>2</sub>γ is localized at the ER and mitochondria, *i.e.* at organelles generally separated from the plasma membrane. It is, therefore, unlikely that C5b-9 interacts with iPLA<sub>2</sub>γ directly. Instead, it is reason-



**FIGURE 5. Complement-induced PGE<sub>2</sub> production in GECs that stably express M1 GFP-iPLA<sub>2</sub>γ WT is mediated by ERK and p38.** A, GECs were incubated with antibody and complement (as in Fig. 4) in the presence of inhibitors of p38 (SB203580; 10 μM), MEK1 (PD98059; 50 μM), or JNK (SP600125; 10 μM). Complement-induced PGE<sub>2</sub> production was reduced significantly by the p38 and MEK1 inhibitors. \*,  $p < 0.0001$  NS versus HIS (vehicle); \*\*,  $p < 0.01$  SB203580 versus vehicle (NS); +,  $p < 0.001$  PD98059 versus vehicle (NS), six experiments. B, GECs were incubated with antibody and complement in the presence of inhibitors of p38 (FR167653; 10 μM) and MEK1 (U0126; 50 μM). Complement-induced PGE<sub>2</sub> production was reduced significantly by both drugs. \*,  $p < 0.0001$  NS versus HIS (vehicle); \*\*,  $p < 0.001$  FR167653 versus vehicle (NS); +,  $p < 0.0001$  U0126 versus vehicle (NS), four experiments.

able to propose that C5b-9 may activate iPLA<sub>2</sub>γ via intermediary signals. C5b-9 has been shown to increase the cytosolic Ca<sup>2+</sup> concentration and activate MAPKs, including ERK, JNK, and p38 (39). In the next series of experiments we examined if complement-induced activation of iPLA<sub>2</sub>γ was mediated through these MAPK pathways. We employed several MAPK pathway inhibitors including the MEK1 inhibitor, PD98059, the p38 inhibitor SB203580, and the JNK inhibitor SP600125 (44). M1 GEC-iPLA<sub>2</sub>γ were preincubated with each inhibitor and were then incubated with antibody and complement (Fig. 5A). The complement-induced release of PGE<sub>2</sub> was inhibited significantly by SB203580 and PD98059. SP600125 tended to decrease the complement-mediated production of PGE<sub>2</sub>, but the effect was not significant. PD98059 and SB203580 were reported to cross-react and inhibit COX1 and COX2 activities (45). We, therefore, tested FR167653 and U0126, inhibitors of p38 and MEK1, respectively, which do not inhibit COX1 and COX2 (46) and are structurally distinct from SB203580 and PD98059. Both FR167653 and U0126 inhibited the complement-mediated production of PGE<sub>2</sub> (Fig. 5B), confirming a role for p38 and ERK pathways in iPLA<sub>2</sub>γ activation by complement.

**MAPKs Enhance iPLA<sub>2</sub>γ Activity in COS-1 Cells**—To confirm the role of MAPK pathways in the regulation of iPLA<sub>2</sub>γ

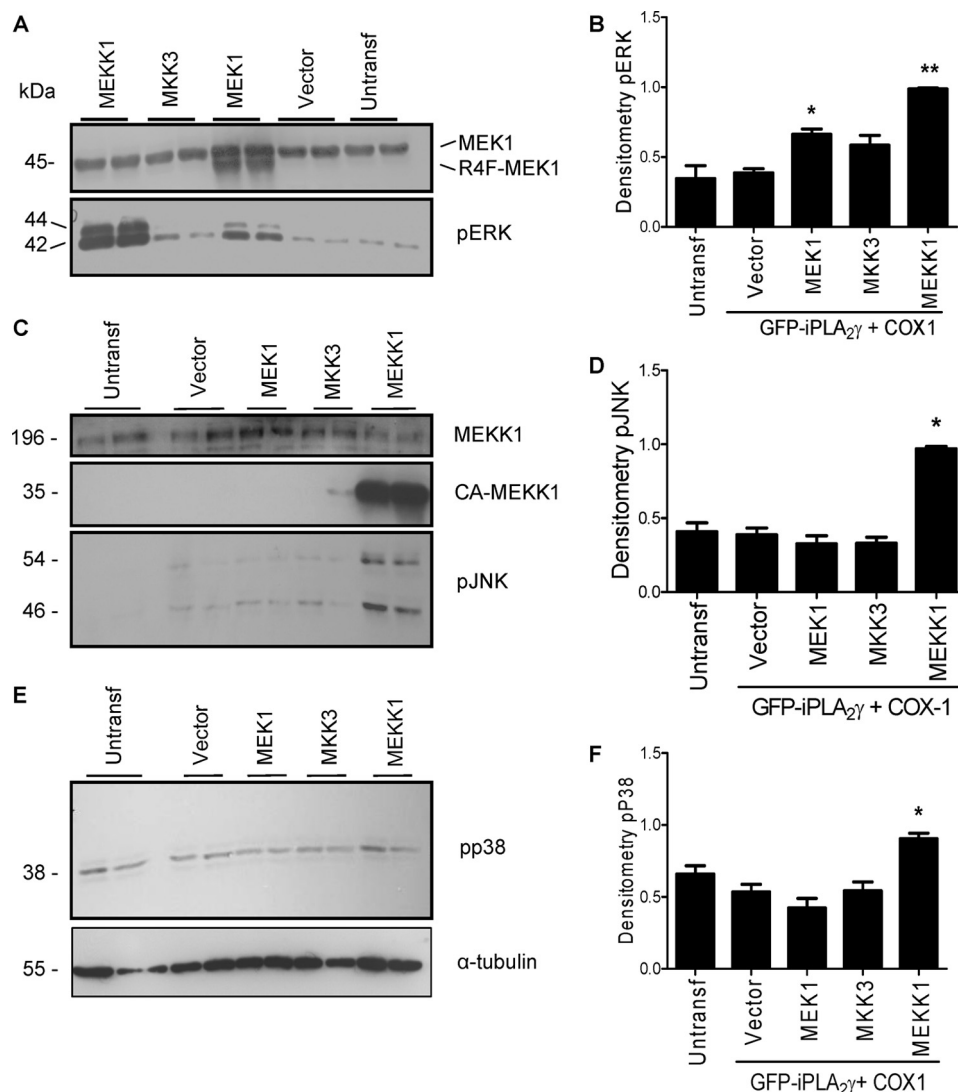


**FIGURE 6. Activation of MAPK pathways stimulates iPLA<sub>2</sub>γ-mediated PGE<sub>2</sub> production.** COS-1 cells were co-transfected with M1 GFP-iPLA<sub>2</sub>γ WT, COX1, and constitutively active mutants of MEK1, MKK3, or MEKK1 or with empty vector. Untransfected cells are additional controls. PGE<sub>2</sub> release was measured 48 h after transfection. \*,  $p < 0.01$ ; \*\*,  $p < 0.0001$  versus vector, three experiments.

activity, we transiently co-transfected COS-1 cells with M1 GFP-iPLA<sub>2</sub>γ WT, COX1, and constitutively active mutants of MEK1 (kinase upstream of ERK), MEKK1 (kinase upstream of JNK and possibly p38), and MKK3 (kinase upstream of p38). All three constitutively active mutants enhanced PGE<sub>2</sub> production compared with control (vector) (Fig. 6).

The expression and function of the constitutively active mutants were evaluated in the same sets of experiments. Constitutively active MEK1 migrated slightly faster than the endogenous MEK1 (45 kDa) (Fig. 7A). ERK phosphorylation (which reflects ERK activation) was increased 1.7-fold by the constitutively active MEK1 (Fig. 7, A and B), confirming functional activity. Constitutively active MEKK1 was expressed as a protein of ~35 kDa (Fig. 7C), and it stimulated phosphorylation of JNK as expected by ~3-fold (Fig. 7, C and D). In addition, the constitutively active MEKK1 activated the ERK pathway by 2.5-fold (Fig. 7B) and showed modest but significant activation of the p38 pathway (Fig. 7, E and F), consistent with earlier studies showing that when overexpressed, the MEKK1 mutant can activate ERK and p38 (47). Unexpectedly, we could not detect phosphorylation of p38 after transfection of the constitutively active mutant of MKK3 (Fig. 7, E and F) even though MKK3 increased PGE<sub>2</sub> release (Fig. 6). Possibly, phosphorylation of p38 was very transient, limiting its detectability. In summary, the results support a role for ERK in the activation of iPLA<sub>2</sub>γ, as constitutively active protein kinases that stimulated ERK phosphorylation also stimulated iPLA<sub>2</sub>γ activity, and complement-stimulated iPLA<sub>2</sub>γ activity was blocked by ERK pathway-directed inhibitors. By analogy, p38 may also stimulate iPLA<sub>2</sub>γ, as constitutively active MEKK1 stimulated iPLA<sub>2</sub>γ and p38 phosphorylation, and complement-stimulated iPLA<sub>2</sub>γ activity was blocked by p38 inhibitors. However, the role of MKK3 could not be established definitively and will require additional study. Finally, the results do not support a role for JNK in the activation of iPLA<sub>2</sub>γ.

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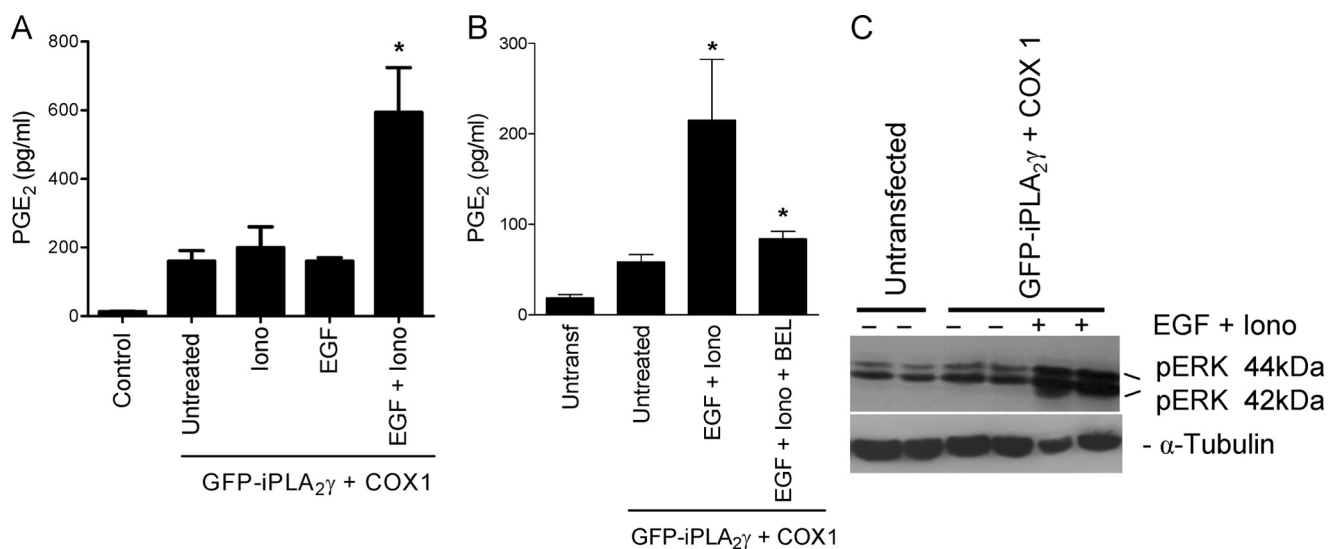
**FIGURE 7. Effects of constitutively active kinases on ERK, JNK, and p38 phosphorylation.** COS-1 cells were co-transfected with M1 GFP-iPLA<sub>2</sub>γ WT, COX1, and constitutively active mutants of MEK1, MKK3, and MEKK1 or with empty vector. *A*, lysates were immunoblotted with antibodies to MEK1 (upper panel) or phospho-ERK (pERK; lower panel). Endogenous MEK1 is present in all samples; constitutively active MEK1 (R4F-MEK1) migrates ahead of endogenous MEK1 (MEK1; 45 kDa). Both constitutively active MEK1 and MEKK1 stimulated ERK phosphorylation. *B*, densitometric quantification of pERK is shown. \*,  $p < 0.01$  MEK1 versus vector; \*\*,  $p < 0.0001$  MEKK1 versus vector, three experiments. *C*, lysates were immunoblotted with antibodies to MEKK1 (upper and middle panels) or phospho-JNK (pJNK; lower panel). Endogenous MEKK1 is present in all samples; constitutively active MEKK1 migrates at 35 kDa. Constitutively active MEKK1 stimulated JNK phosphorylation. *D*, densitometric quantification of pJNK is shown. \*,  $p < 0.0001$  MEKK1 versus vector, three experiments. *E*, lysates were immunoblotted with antibodies to phospho-p38 (pp38; upper panel) or tubulin (lower panel). Constitutively active MEKK1 stimulated p38 phosphorylation. *F*, densitometric quantification of pp38. \*,  $p < 0.001$  MEKK1 versus vector, three experiments.

*EGF Together with Ionomycin Enhances iPLA<sub>2</sub>γ Activity in COS-1 Cells*—EGF is a well known activator of the ERK pathway, and COS-1 cells express abundant EGF receptor. To further substantiate the role of the ERK pathway in the activation of iPLA<sub>2</sub>γ, we investigated the effect of EGF on PGE<sub>2</sub> release in COS-1 cells that were transiently transfected with M1 GFP-iPLA<sub>2</sub>γ WT (and COX1). Treatment of COS-1 cells with EGF alone did not affect PGE<sub>2</sub> release (Fig. 8A). Although iPLA<sub>2</sub> is Ca<sup>2+</sup>-independent (does not require Ca<sup>2+</sup> for its catalytic activity), iPLA<sub>2</sub> activity may nonetheless be regulated by Ca<sup>2+</sup> or a Ca<sup>2+</sup>-dependent factor (48). For this reason, we used the Ca<sup>2+</sup> ionophore ionomycin to induce a Ca<sup>2+</sup> influx. Interestingly, stimulation with EGF in the presence of ionomycin increased PGE<sub>2</sub> release by more than 4-fold compared with EGF alone, ionomycin alone, or untreated (Fig. 8A). Further-

more, BEL inhibited iPLA<sub>2</sub>γ activity induced by EGF + ionomycin significantly (Fig. 8B). Finally, we confirmed that incubation of COS-1 cells with EGF + ionomycin induced ERK phosphorylation (Fig. 8C). Thus, the effect of ionomycin + EGF on iPLA<sub>2</sub>γ activation is analogous to the effect of C5b-9, which also induces a Ca<sup>2+</sup> influx and activation of ERK.

*Mutations in Putative ERK Phosphorylation Sites Do Not Affect iPLA<sub>2</sub>γ Activity*—Analysis of the iPLA<sub>2</sub>γ protein sequence by the Scansite program (49) suggested that Ser-168 (EKSP amino acid motif) and Ser-271 (PTSP motif) may be ERK phosphorylation sites. Given the substantial evidence for the activation of iPLA<sub>2</sub>γ via the ERK pathway, in the next series of the studies we examined if iPLA<sub>2</sub>γ may be a direct target of ERK. We constructed three mutant forms of M1 GFP-iPLA<sub>2</sub>γ, including S168A, S271A, and S168A/S271A double mutation,





**FIGURE 8. EGF together with ionomycin enhances iPLA<sub>2</sub>γ activity.** COS-1 cells were co-transfected with M1 GFP-iPLA<sub>2</sub>γ WT and COX1. After 24 h COS-1 cells were incubated with EGF (100 ng/ml) and/or ionomycin (*iono*, 1.0–1.5 μM) for 40 min. **A**, stimulation by EGF and ionomycin significantly increased PGE<sub>2</sub> release compared with unstimulated cells or cells stimulated with each agonist alone. \*,  $p < 0.001$  EGF + ionomycin versus untreated,  $p < 0.05$  EGF + ionomycin versus ionomycin alone,  $p < 0.001$  EGF + ionomycin versus EGF (alone), four experiments. **B**, COS-1 cells were co-transfected with M1 GFP-iPLA<sub>2</sub>γ WT and COX1. After 24 h 1 group of cells was preincubated with BEL (30 μM) for 6.5 h. Then cells were incubated for 30 min with EGF and ionomycin with or without BEL. The increase of PGE<sub>2</sub> release was inhibited almost completely in presence of BEL. \*,  $p < 0.01$  EGF + ionomycin versus untreated. \*,  $p < 0.01$  EGF + ionomycin versus EGF + ionomycin + BEL, 3 experiments. **C**, cell lysates were immunoblotted with antibodies to phospho-ERK (*pERK*) or tubulin. EGF and ionomycin enhanced phosphorylation of ERK.

and tested their activities in COS-1 cells. PGE<sub>2</sub> production stimulated by EGF + ionomycin with the iPLA<sub>2</sub>γ mutants did not differ from the WT (Fig. 9A), whereas the expression levels of all constructs were comparable (Fig. 9B). Thus, single or double mutations of putative ERK phosphorylation sites did not affect iPLA<sub>2</sub>γ activation by EGF + ionomycin, suggesting that the effect of ERK on the stimulation of iPLA<sub>2</sub>γ is indirect.

**Mutations in Ser-511 and Ser-515 Inhibit iPLA<sub>2</sub>γ Activation—**A recent phosphoproteomic analysis of mitochondrial proteins in murine heart revealed two iPLA<sub>2</sub>γ phosphorylation sites, Ser-505 and Thr-509, corresponding to Ser-511 and Ser-515 in human iPLA<sub>2</sub>γ (50). The authors suggested that the two phosphorylation sites (RKLGP<sub>SDV</sub>FpSQNV) may be in the context of MK2 or casein kinase I substrate motifs (50). Based on these results, we constructed a S511A/S515A double mutant form of M1 GFP-iPLA<sub>2</sub>γ and tested its activity in COS-1 cells. The *in vitro* enzymatic activity of M1 GFP-iPLA<sub>2</sub>γ S511A/S515A in COS-1 cells tended to be greater compared with control but was lower compared with M1 GFP-iPLA<sub>2</sub>γ WT (Fig. 3D). In addition, PGE<sub>2</sub> production stimulated by EGF + ionomycin was attenuated significantly in the COS-1 cells expressing the S511A/S515A double mutant compared with WT (Fig. 10A), whereas the expression levels of WT and double mutant were comparable (Fig. 10B). Basal PGE<sub>2</sub> levels in iPLA<sub>2</sub>γ WT and S511A/S515A-expressing cells appeared comparable (Fig. 10A).

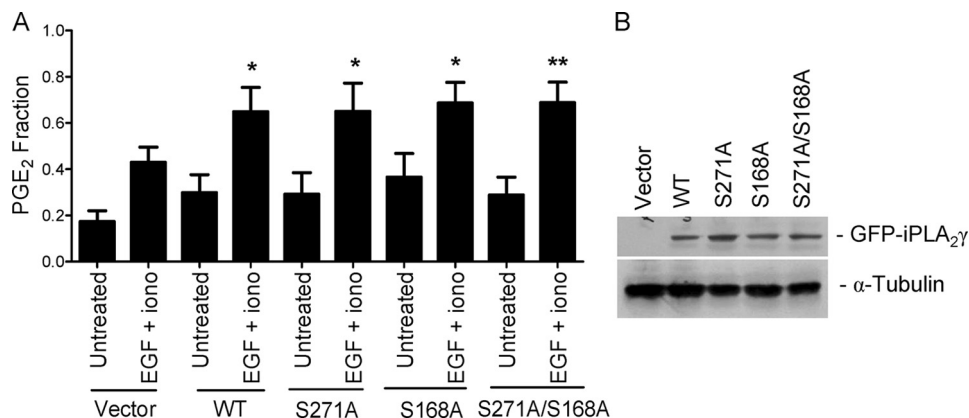
Next, we tested if Ser-511 in iPLA<sub>2</sub>γ WT was phosphorylated by EGF + ionomycin stimulation using an antibody that identifies the (R/K)XX(pS/T) motif, corresponding to KLGpS in iPLA<sub>2</sub>γ. Phosphorylation of Ser-511 was evident in stimulated cells; in some experiments, faint phosphorylation was detected in unstimulated cells (Fig. 10C). In contrast, the S511A/S515A mutant iPLA<sub>2</sub>γ was not phosphorylated by EGF + ionomycin

(Fig. 10C), confirming that in the WT enzyme Ser-511 is the relevant phosphorylation site. These results suggest that the iPLA<sub>2</sub>γ activation by EGF + ionomycin is mediated by the direct phosphorylation of iPLA<sub>2</sub>γ by a kinase, which is downstream of ERK. Phosphorylation of Ser-515 in iPLA<sub>2</sub>γ WT was not monitored due to the unavailability of an antibody that identifies the DVFP<sub>SQ</sub> motif.

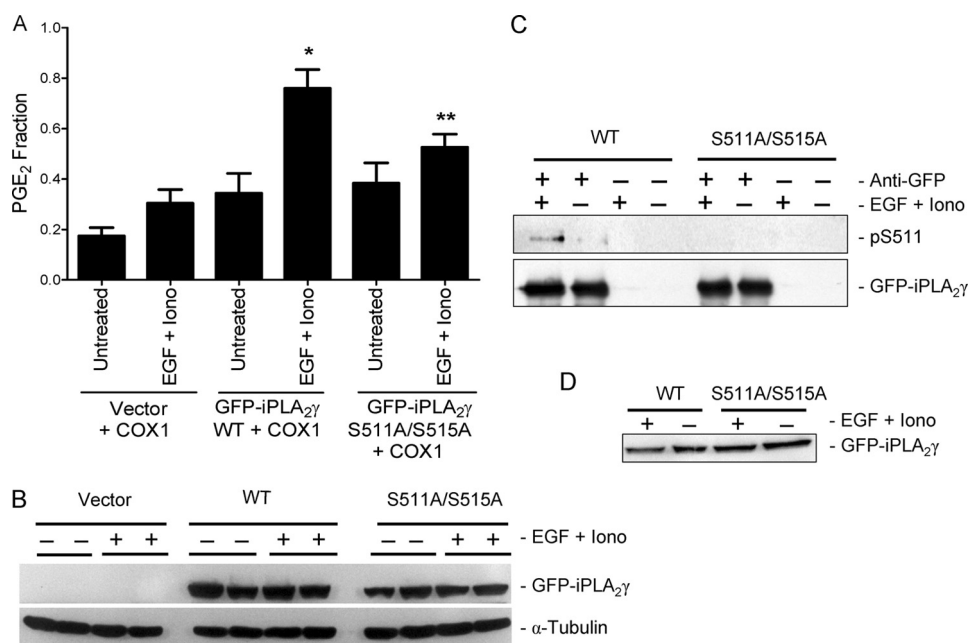
In a previous study we demonstrated that complement induced a robust activation-specific phosphorylation of MK2 (~4-fold above control) and that activation of MK2 was mediated by both ERK and p38 kinase pathways (51). Indeed, MK2 is regarded primarily as a substrate of p38 kinase but is also reported to be a substrate of ERK (44, 51). Given that Ser-511 and Ser-515 may represent a MK2 phosphorylation motif, we investigated if expression of MK2 would stimulate the activity of iPLA<sub>2</sub>γ WT. By analogy to constitutively active MEK1 and MEKK1 (as shown in Fig. 6), WT and constitutively active MK2 were expressed with iPLA<sub>2</sub>γ WT in COS-1 cells. Despite robust expression, neither WT nor constitutively active MK2 stimulated PGE<sub>2</sub> production consistently (data not shown).

Another protein kinase that is activated by ERK and p38 is MNK1 (44). To determine if the effect of the ERK pathway on iPLA<sub>2</sub>γ activation was mediated by MNK1, we expressed GFP-iPLA<sub>2</sub>γ in COS-1 cells and examined PGE<sub>2</sub> production after stimulation with EGF + ionomycin in the presence or absence of the MNK1-directed inhibitor CGP57380 (44). Stimulated PGE<sub>2</sub> production was blocked completely by CGP57380 (Table 2). CGP57380 was reported to have some inhibitory activity against MEK1 (44); however, we verified that EGF-induced phosphorylation of ERK was not reduced in the presence of CGP57380 (result not shown). Therefore, inhibition of the EGF + ionomycin-induced activation of iPLA<sub>2</sub>γ was most likely due to the inhibition of MNK1.

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**FIGURE 9. Mutations in putative ERK phosphorylation sites do not affect iPLA<sub>2</sub>γ activity.** COS-1 cells were transiently transfected with M1-GFP-iPLA<sub>2</sub>γ WT, the S168A and S271A mutants, or the S168A/S271A double mutant together with COX1. *A*, after 24 h, cells were untreated or were incubated with EGF (100 ng/ml) + ionomycin (*Iono*, 1.5 μM) for 40 min. PGE<sub>2</sub> release was stimulated significantly by EGF + ionomycin in the cells expressing M1 GFP-iPLA<sub>2</sub>γ WT and all mutants. \*, *p* < 0.05; \*\*, *p* < 0.01 versus corresponding untreated groups, seven experiments. In these experiments basal PGE<sub>2</sub> release (vector + COX1-transfected, untreated cells) was 827 pg/ml. *B*, cell lysates were immunoblotted with antibodies to GFP or tubulin. The blot shows comparable levels of expression.



**FIGURE 10. The S511A/S515A double mutation inhibits stimulated iPLA<sub>2</sub>γ activity.** COS-1 cells were transiently transfected with M1-GFP-iPLA<sub>2</sub>γ WT, GFP-iPLA<sub>2</sub>γ S511A/S515A mutant, or empty vector together with COX1. *A*, after 24 h cells were untreated or were incubated with EGF (100 ng/ml) + ionomycin (*Iono*, 1.5 μM) for 40 min. PGE<sub>2</sub> release was stimulated significantly by EGF + ionomycin in the cells expressing M1 GFP-iPLA<sub>2</sub>γ WT. \*, *p* < 0.0001 versus corresponding untreated group. The increase in PGE<sub>2</sub> release was smaller in cells expressing GFP-iPLA<sub>2</sub>γ S511A/S515A mutant. \*\*, *p* < 0.01 versus cells expressing M1 GFP-iPLA<sub>2</sub>γ WT and treated with EGF + ionomycin, four experiments. In these experiments basal PGE<sub>2</sub> release (vector + COX1-transfected, untreated cells) was 191 pg/ml. *B*, cell lysates were immunoblotted with antibodies to GFP or tubulin. The blot shows comparable levels of expression. *C*, cell lysates were immunoprecipitated with anti-GFP antibody (+) and were immunoblotted with anti-(R/K)XX(pS/T) or anti-GFP antibodies. The blot shows enhanced phosphorylation of Ser-511 (pS511) in the M1-GFP-iPLA<sub>2</sub>γ WT in EGF + ionomycin-stimulated cells. Phosphorylation of Ser-511 is faint in unstimulated WT-expressing cells and is absent in the mutant. *D*, shown are total lysates of the above immunoprecipitation experiments blotted with anti-GFP antibody.

To confirm that MNK1 can activate and phosphorylate iPLA<sub>2</sub>γ, COS-1 cells were transfected with WT GFP-iPLA<sub>2</sub>γ and COX1 in the presence of WT, constitutively active (T332D), or dominant interfering (T2A2) forms of MNK1 or with empty vector. Both MNK1 WT and T332D enhanced PGE<sub>2</sub> production compared with control (vector), whereas MNK1 T2A2 did not show a significant effect (Fig. 11, *A* and *B*). We tested Ser-511 phosphorylation by constitutively active MNK1 in iPLA<sub>2</sub>γ WT using the anti-(R/K)XX(pS/T) antibody. Phosphorylation of Ser-511 was enhanced when COS-1 cells

were cotransfected with MNK1 T332D compared with empty vector (Fig. 11, *C* and *D*). Together these results support the view that phosphorylation of iPLA<sub>2</sub>γ on Ser-511 in response to EGF stimulation is mediated by MNK1.

**Complement Induces Phosphorylation of iPLA<sub>2</sub>γ on Ser-511**— In these experiments we assessed if complement-mediated activation of iPLA<sub>2</sub>γ involves phosphorylation. First, we tested if Ser-511 in iPLA<sub>2</sub>γ WT is phosphorylated by complement in GECs using the anti-(R/K)XX(pS/T) antibody. Phosphorylation of Ser-511 was stimulated in complement-treated GECs (NS)

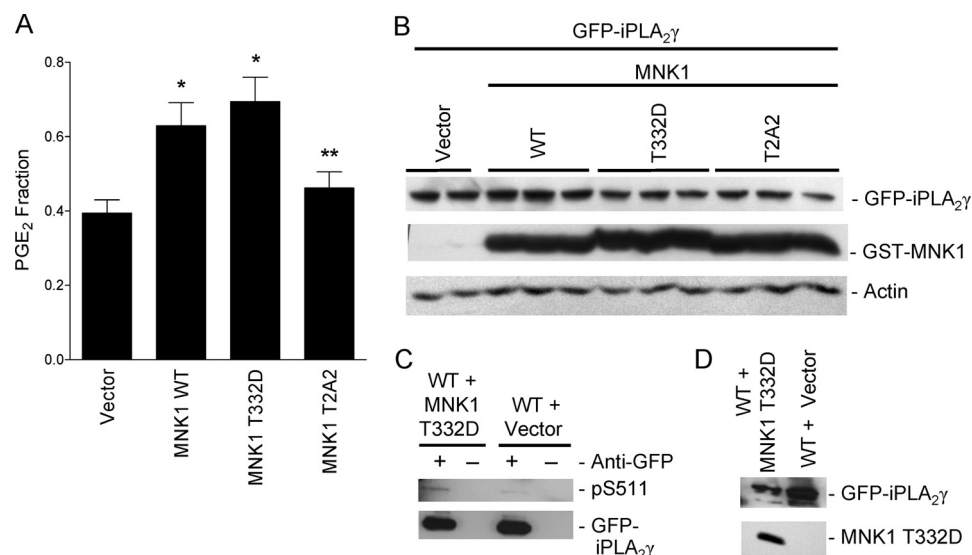
compared with control (HIS; Fig. 12A). In some experiments ionomycin was included together with NS and HIS incubations; however, ionomycin did not modulate Ser-511 phosphorylation either in complement-treated cells or control (Fig. 12A). Because complement increases the cytosolic Ca<sup>2+</sup> concentration (16, 52), ionomycin would not be expected to provide any additional stimulatory effect. Second, we compared complement-induced PGE<sub>2</sub> release in GECs expressing M1 GFP-iPLA<sub>2</sub>γ WT or GFP-iPLA<sub>2</sub>γ S511A/S515A and GEC-Neo. Overexpression of M1 GFP-iPLA<sub>2</sub>γ WT or the S511A/S515A mutant did not affect the basal PGE<sub>2</sub> production (during incubation with HIS). After incubation with complement (NS), PGE<sub>2</sub> release was significantly amplified in cells expressing M1 GFP-iPLA<sub>2</sub>γ compared with GEC-Neo (Fig. 12B), in keeping with previous experiments (Fig. 4, B and D). PGE<sub>2</sub> release in complement-treated cells expressing the S511A/S515A mutant was significantly lower compared with WT (Fig. 12B) despite comparable levels of expression (Fig. 12C). Together, these results indicate that complement at least in part activates iPLA<sub>2</sub>γ via direct phosphorylation on Ser-511, most likely by MNK1.

**TABLE 2**  
Effect of the MNK1 inhibitor, CGP57380, on PGE<sub>2</sub> production

COS-1 cells were co-transfected with M1 GFP-iPLA<sub>2</sub>γ WT and COX1. After 24 h some cells were treated with CGP57380 (20 μM) for 16 h. Then cells were incubated with EGF + ionomycin (see the legend to Fig. 8) for 40 min. Stimulated PGE<sub>2</sub> production was inhibited by CGP57380. Basal PGE<sub>2</sub> release (untransfected, untreated cells) was 51 pg/ml.

Transfection/treatment	PGE <sub>2</sub> fraction
Untransfected, untreated	0.13 ± 0.02
Untransfected + CGP57380	0.17 ± 0.04
GFP-iPLA <sub>2</sub> γ + COX, untreated	0.65 ± 0.04
GFP-iPLA <sub>2</sub> γ + COX, EGF + ionomycin	0.91 ± 0.04
GFP-iPLA <sub>2</sub> γ + COX, EGF + ionomycin + CGP57380	0.56 ± 0.07 <sup>a</sup>

<sup>a</sup> *p* < 0.001 EGF + ionomycin + CGP57380 vs. EGF + ionomycin, three experiments.



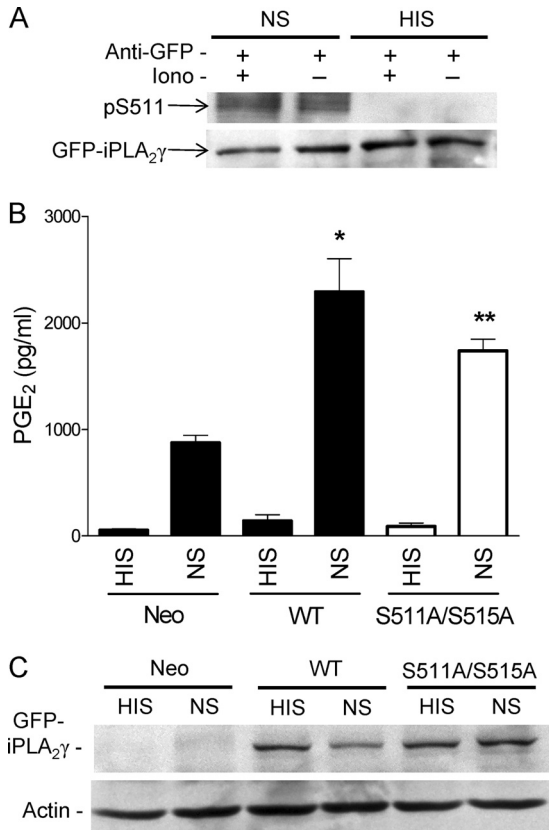
**FIGURE 11. Constitutively active MNK1 activates and phosphorylates iPLA<sub>2</sub>γ.** COS-1 cells were transiently co-transfected with M1 GFP-iPLA<sub>2</sub>γ WT, COX1 and GST-MNK1 WT, GST-MNK1 T332D, and GST-MNK1 T2A2 or with empty vector. **A**, PGE<sub>2</sub> release was measured 48 h after transfection. \*, *p* < 0.05 MNK1 WT versus vector; \*, *p* < 0.01 MNK1 T332D versus vector and \*\*, *p* < 0.05 MNK1 T332D versus MNK1 T2A2, five experiments. In these experiments basal PGE<sub>2</sub> release (vector + M1 GFP-iPLA<sub>2</sub>γ WT + COX1-transfected cells) was 164 pg/ml. **B**, lysates were immunoblotted with antibodies to GFP, GST, or actin. The blot shows comparable levels of expression. **C**, COS-1 cells were transiently co-transfected with M1 GFP-iPLA<sub>2</sub>γ WT and GST-MNK1 T332D or vector. After 48 h cells were treated with ionomycin (10 μM, 40 min) (ionomycin was included in these experiments to enhance the phosphorylation signal, although ionomycin did not independently induce phosphorylation; see Fig. 12). Cell lysates were immunoprecipitated with anti-GFP antibody (+) or nonimmune IgG in controls (-) and were immunoblotted with anti-(R/K)XX(pS/T) or anti-GFP antibodies. The blot shows enhanced phosphorylation of iPLA<sub>2</sub>γ Ser-511 (pS511) in MNK1 T332D transfected cells. **D**, total lysates of the above immunoprecipitation experiments blotted with anti-GFP or anti-GST (MNK1) antibodies are shown.

## DISCUSSION

The present study demonstrates that complement C5b-9 activates endogenous and ectopic iPLA<sub>2</sub>γ (Fig. 4). Activation of iPLA<sub>2</sub>γ occurs via ERK and p38 pathways (Fig. 5–9) and is dependent on an increase in cytosolic Ca<sup>2+</sup> concentration and phosphorylation of the enzyme on Ser-511 and/or Ser-515 most likely via MNK1 (Fig. 11, Table 2). To our knowledge this is the first demonstration of phosphorylation-dependent activation of iPLA<sub>2</sub>γ. Stimulation of iPLA<sub>2</sub>γ by complement was coupled with production of PGE<sub>2</sub>. Similar to this result in the present study, iPLA<sub>2</sub>γ was shown to augment AA release and PGE<sub>2</sub> production, which was associated with increased cell growth in a human colorectal adenocarcinoma cell line (HAC-7) (17). Coupling of iPLA<sub>2</sub>γ with COX1 was also shown in HEK293 cells (17).

In the present study we examined the cellular localization of iPLA<sub>2</sub>γ to better understand the mechanisms by which iPLA<sub>2</sub>γ hydrolyzes membrane phospholipids. M1 GFP-iPLA<sub>2</sub>γ WT was found mainly in the perinuclear region and co-localized with markers of the ER and mitochondria (Fig. 2). Deletion of the 220-amino acid N-terminal region (M4 GFP-iPLA<sub>2</sub>γ) altered the localization of the enzyme such that a significant portion of M4 GFP-iPLA<sub>2</sub>γ shifted to the cytosol (Fig. 2). Our result is in keeping with the study of Tanaka *et al.* (34), which showed that deletion of a 362-amino acid N-terminal region of iPLA<sub>2</sub>γ caused a portion of the enzyme to shift from the membrane to the cytosol in a cell fractionation study. Moreover, the N-terminal fragment localized solely in the membrane but not in the cytosolic fraction (34). Deletion of the N-terminal region of iPLA<sub>2</sub>γ did not abolish iPLA<sub>2</sub>γ enzymatic activity *in vitro* (Fig. 3D), consistent with the study by Tanaka *et al.* (34). However, the N-terminal truncated form of iPLA<sub>2</sub>γ was unable to

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**FIGURE 12. Complement induces phosphorylation of iPLA<sub>2</sub>γ on Ser-511.** GECs were transiently transfected with M1 GFP-iPLA<sub>2</sub>γ WT. After 24 h cells were incubated with ionomycin (*Iono*, 5 μM) for 30 min (+) and then incubated with antibody and NS (3%, 40 min) or HIS in controls. *A*, cell lysates were immunoprecipitated with anti-GFP antibody (+) and were immunoblotted with anti-(R/K)XX(pS/T) or anti-GFP antibodies. The blot shows phosphorylation of Ser-511 (*pS511*) in NS-stimulated cells (with or without ionomycin). Phosphorylation is absent in the HIS-stimulated cells. *B*, GEC neo and GECs that express M1 GFP-iPLA<sub>2</sub>γ WT or GFP-iPLA<sub>2</sub>γ S511A/S515A (transient transfection) were incubated with antibody and complement as above. PGE<sub>2</sub> production was amplified in NS-stimulated M1 GFP-iPLA<sub>2</sub>γ WT-expressing cells, whereas the amplification was smaller in GECs expressing the double mutant. \*, *p* < 0.001 M1 GFP-iPLA<sub>2</sub>γ WT (NS) versus GEC-Neo (NS) and \*\*, *p* < 0.05 M1 GFP-iPLA<sub>2</sub>γ WT (NS) versus GFP-iPLA<sub>2</sub>γ S511A/S515A (NS), three experiments. *C*, cell lysates were immunoblotted with antibodies to GFP or actin. The blot shows comparable levels of GFP-iPLA<sub>2</sub>γ expression.

induce significant PGE<sub>2</sub> production when expressed in cells (Fig. 3). Taken together, these results indicate that the N-terminal region is involved in the membrane association of iPLA<sub>2</sub>γ, thereby allowing the enzyme access to phospholipid substrate intracellularly. In addition, the N-terminal region may have positive regulatory elements that could enhance iPLA<sub>2</sub>γ activity. Mislocalization of M4 GFP-iPLA<sub>2</sub>γ from the membrane may be expected to reduce PGE<sub>2</sub> production, as COX1 is localized in the ER membrane and would couple with iPLA<sub>2</sub>γ-mediated AA release (17).

In previous studies it was demonstrated that C5b-9 can activate cPLA<sub>2</sub>α to augment production of [<sup>3</sup>H]AA and prostanoids (12, 13). Complement did not stimulate group IIA-secreted PLA<sub>2</sub>, and in contrast to iPLA<sub>2</sub>γ, overexpression of iPLA<sub>2</sub>β in GECs did not amplify complement-dependent release of [<sup>3</sup>H]AA (16, 52). Thus, both cPLA<sub>2</sub>α and iPLA<sub>2</sub>γ can contribute to complement-dependent release of AA. Previous studies in GECs demonstrated that complement induced an

increase in cPLA<sub>2</sub>α catalytic activity, in association with Ser-505 phosphorylation, although this phosphorylation was not essential for cPLA<sub>2</sub>α activation (12, 13). In addition, glomerular cPLA<sub>2</sub>α was phosphorylated *in vivo* in passive Heymann nephritis (51).

By analogy to cPLA<sub>2</sub>α, complement activated iPLA<sub>2</sub>γ through intermediate signals, including protein kinases. C5b-9 can activate MAPK pathways in GECs (12, 13). In GECs overexpressing GFP-iPLA<sub>2</sub>γ WT, the complement-induced release of PGE<sub>2</sub> was blocked by two distinct chemical inhibitors of both the ERK and p38 pathways but not JNK (Fig. 5). Conversely, constitutively active MAPK pathway mutants (in particular MEK and MEKK1) increased iPLA<sub>2</sub>γ-dependent PGE<sub>2</sub> production (Fig. 6). Both MEK and MEKK1 induced activation-specific phosphorylation of ERK (Fig. 7). Taken together, the ERK and p38 pathways can mediate the activation of iPLA<sub>2</sub>γ by complement. A role for MAPKs in the activation of iPLA<sub>2</sub> has been reported previously. Thrombin stimulated both ERK and p38 and iPLA<sub>2</sub> activity in vascular smooth muscle cells and ventricular myocytes (53, 54). In mouse neural cells, p38 facilitated iPLA<sub>2</sub> activity during hypoxia (55). These studies did not, however, differentiate between iPLA<sub>2</sub>β and iPLA<sub>2</sub>γ. To further substantiate the role of the ERK pathway in the activation of iPLA<sub>2</sub>γ, we showed that EGF + ionomycin stimulated PGE<sub>2</sub> release in COS-1 cells expressing GFP-iPLA<sub>2</sub>γ WT in association with ERK activation (Figs. 8 and 9). Interestingly, unlike the constitutively active mutants of MEK1, MKK3, or MEKK1, the stimulatory effect of EGF in cells required the addition of ionomycin (to increase the cytosolic Ca<sup>2+</sup> concentration) despite the Ca<sup>2+</sup>-independent catalytic properties of iPLA<sub>2</sub>γ *in vitro*. In keeping with previous reports, this finding suggests that activation of iPLA<sub>2</sub>γ in agonist-stimulated cells may involve a Ca<sup>2+</sup>-regulated process (5, 6), possibly activation of Ca<sup>2+</sup>-dependent protein kinases, such as calmodulin (56). Alternatively, Ca<sup>2+</sup> may enhance activation of iPLA<sub>2</sub>γ directly. A recent study showed that divalent cations (Ca<sup>2+</sup> or Mg<sup>2+</sup>) can activate iPLA<sub>2</sub>γ in heart mitochondria, leading to release of eicosanoids and lysolipids, possibly by facilitating mitochondrial phospholipid hydrolysis by iPLA<sub>2</sub>γ (57).

Tanaka *et al.* (6) suggested that iPLA<sub>2</sub>γ may have multiple potential phosphorylation sites. We carried out a mutagenesis analysis of iPLA<sub>2</sub>γ to determine the regulation of iPLA<sub>2</sub>γ activity by the ERK pathway. Mutation of two putative ERK phosphorylation sites, *i.e.* S168A and S271A, and S168A/S271A double mutation did not abrogate the stimulated activity of iPLA<sub>2</sub>γ WT (Fig. 9), implying that iPLA<sub>2</sub>γ was not a direct target of ERK. Another report suggested that the MAPK pathway might be involved in iPLA<sub>2</sub>γ activation indirectly, but no supporting data were presented (17). Based on a phosphoproteomic analysis of murine cardiac mitochondrial proteins (50), we then mutated Ser-511 and Ser-515 to Ala and showed that this double mutation significantly attenuated EGF + ionomycin- as well as complement-stimulated iPLA<sub>2</sub>γ-dependent PGE<sub>2</sub> production (Figs. 10 and 12). Moreover, phosphorylation of Ser-511 was induced by EGF + ionomycin and by complement (Figs. 10 and 12). Phosphorylation of Ser-511 and/or Ser-515 could induce a conformational change in the enzyme, lead-

ing to an increase in catalytic activity. Further studies will be required to define the mechanism more precisely.

The Ser-511 and Ser-515 phosphorylation sites were proposed to be a phosphorylation motif for MK2 (50), and given that complement was shown to activate MK2 via the ERK or p38 pathway (51), it was reasonable to examine if MK2 may be the kinase downstream of ERK that phosphorylates iPLA<sub>2</sub>γ. However, we were not able to show stimulation of iPLA<sub>2</sub>γ after MK2 overexpression. The protein kinase MNK1 was also activated by ERK and p38, and the amino acid sequence preceding Ser-511 in iPLA<sub>2</sub>γ (KLSG) resembles the MNK1 phosphorylation motif in eukaryotic translation initiation factor 4E (KSGS) (44). In the present study the EGF + ionomycin-stimulated activity of iPLA<sub>2</sub>γ was blocked by a MNK1-directed inhibitor (Table 2). Expression of WT and constitutively active MNK1 stimulated PGE<sub>2</sub> release via iPLA<sub>2</sub>γ, and constitutively active MNK1 enhanced Ser-511 phosphorylation (Fig. 11). Thus, activation of iPLA<sub>2</sub>γ by complement most likely involves an ERK-MNK1 pathway, although an additional role of another kinase downstream of ERK remains a possibility. Interestingly, a previous study has shown that phosphorylation and activation of cPLA<sub>2</sub>α can occur via p38 and MNK1 (58).

Various PLA<sub>2</sub> enzymes have been shown to regulate pathways leading to cell injury in experimental disease models. These effects may be associated with generation of prostanoids (59), p38 activation (60), and induction of ER stress (40). There are both cytoprotective and injurious consequences related to the complement-mediated activation of PLA<sub>2</sub> enzymes and production of prostanoids (13, 16). Overexpression of iPLA<sub>2</sub>γ attenuated complement-mediated injury in cultured GECs, and the cytoprotective effect was in part mediated through prostaglandin production (16). Further studies will be required to determine if activation of iPLA<sub>2</sub>γ is cytoprotective in C5b-9-mediated GECs *in vivo*, *i.e.* in attenuating development of proteinuria in experimental membranous nephropathy. Another potential mechanism of iPLA<sub>2</sub>γ cytoprotection may be related to the localization and action of iPLA<sub>2</sub>γ at the ER (Fig. 2). Such actions could include changes in ER membrane lipid composition, alterations in ER Ca<sup>2+</sup> transporters, or modification of ER Ca<sup>2+</sup> stores. Moreover, iPLA<sub>2</sub>γ could perturb the ER membrane to initiate an adaptive ER stress response as a feedback mechanism to limit complement-induced cell injury.

In GECs, a portion of iPLA<sub>2</sub>γ was localized at the mitochondria (Fig. 2). iPLA<sub>2</sub>γ may protect renal cortical mitochondria from oxidant-induced lipid peroxidation and dysfunction (43). Thus, in the presence of oxidized phospholipid acyl chains, iPLA<sub>2</sub>γ may hydrolyze damaged acyl chains and allow for reesterification with normal fatty acids, thereby repairing mitochondrial membrane phospholipids. So far, we have not conclusively defined the site of phospholipid hydrolysis by complement-stimulated iPLA<sub>2</sub>γ. Both ER and mitochondrial lipids in GECs contain AA (61); however, because COX isoenzymes are localized at the ER and the nuclear membranes but not at the mitochondria, the production of PGE<sub>2</sub> suggests a coupling of AA release with COX at the ER. Definition of the subcellular sites of phospholipid hydrolysis and the functional role, including the cytoprotective mechanisms of iPLA<sub>2</sub>γ, will require further investigation.

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