Regulation of Cytosolic Phospholipase A2 Activity in Macrophages Stimulated with Receptor-recognized Forms of α2-Macroglobulin

ROLE IN MITOGENESIS AND CELL PROLIFERATION

Macrophages exposed to receptor-recognized forms of α2-macroglobulin (α2M) demonstrate increased DNA synthesis and cell division. In the current study, we have probed the role of cytosolic phospholipase A2 (cPLA2) activity in the cellular response to α2M. Ligand of the α2M signaling receptor by α2M, or its receptor binding fragment, increased cPLA2 activity 2–3-fold in a concentration and time-dependent manner. This activation required a pertussis toxin-sensitive G protein. Cellular binding of α2M also induced transient translocation of cPLA2 activity to nuclei and membrane fractions. Inhibition of protein kinase C activity or chelation of Ca2+ inhibited α2M-induced increased cPLA2 activity. Binding of α2M to macrophages, moreover, increased phosphorylation of MEK 1/2, ERK 1/2, p38 MAPK, and JNK. Incubation of macrophages with inhibitors of MEK 1/2 or p38 MAPK before stimulation with α2M profoundly decreased phosphorylation of MAPKs, blocked cPLA2 activation. α2M-induced increase in [3H]thymidine uptake and cell proliferation was completely abolished if activation of cPLA2 was prevented. The response of macrophages to α2M requires transcription factors nuclear factor κB, and cAMP-responsive element-binding protein as well as expression of the proto-oncogenes c-fos and c-myc. These studies indicate that the activation of cPLA2 plays a crucial role in α2M-induced mitogenesis and cell proliferation.

The plasma proteinase inhibitor α2-macroglobulin (α2M) undergoes a major conformational change when it binds proteinases (1, 2). Each α2M subunit also contains an internal thiol ester, which can be directly attacked by small nucleophiles resulting in a similar conformational change (1). In either event, receptor recognition sites are exposed in each of the subunits (1). These receptor-recognized forms of α2M, termed α2M*, bind to the low density lipoprotein receptor-related protein (LRP) present on a variety of cells including macrophages (1–3). In 1993, we demonstrated that the binding of α2M* to macrophages activated signaling cascades characterized by an inositol 1,4,5-trisphosphate (IP3)-dependent increase in [Ca2+]i (4). Subsequent studies demonstrated that the binding to macrophages activates a pertussis toxin-sensitive phospholipase C, which hydrolyzes membrane phosphoinositides generating both IP3 and diacylglycerol (5, 6). These studies also demonstrated that α2M*-mediated signal transduction was not blocked by addition of receptor-associated protein (RAP). Because RAP blocks the binding of all known ligands to LRP, this observation suggested the presence of a second α2M* receptor on macrophages, which was termed the α2MSR signaling receptor (α2MSR) (1, 5, 6). In support of the identification of a distinct α2M* receptor were several other observations. Two classes of binding sites were identified on macrophages, one of high affinity and low capacity (Kd ~ 50 pm and ~1600 sites/cell) and the other LRP, which demonstrated lower affinity and higher binding capacity (Kd ~ 2–5 ns and ~70,000 sites/cell) (7–10). Binding of other ligands to LRP, moreover, initiated signaling cascades that activated a pertussis toxin-sensitive G protein and were blocked by addition of RAP (4–12). More recent observations, however, suggest that α2MSR-mediated signal transduction requires the presence of LRP on cells (13). Thus, Backsai et al. (13) have shown that α2M* binding to LRP on neuronal cells mediates signaling via N-methyl-d-aspartate receptors. These authors suggest that an adapter protein causes LRP to associate with this receptor, allowing α2M* to activate signal transduction. Furthermore, Herz and colleagues (14, 15) have identified a large number of adapter proteins that can associate with LRP, and Barnes et al. (16) have demonstrated that Tyr-phosphorylated LRP associates with the adapter protein SHC in SRC-transformed cells.

Based on our observations with respect to activation of the p21ras-dependent MAPK and PI 3-kinase signaling cascades and subsequent cell proliferation, we have proposed that α2M* functions like a growth factor (9, 17–20). Known growth factors activate cytosolic phospholipase A2 (cPLA2) and the products of cPLA2 hydrolysis are involved in the growth-promoting effects of these factors (21–26). We, therefore, have studied the effect on cPLA2 activation of ligating α2M* receptors on peritoneal macrophages. Specifically, we studied activation of PKC, MEK 1/2, ERK 1/2, p38 MAPK, JNK, and cPLA2; translocation to nuclei and membranes; modulation of cell division by inhibitors.
of MAPks and cPLA2: activation of transcription factors NF-kB and CREB; and expression of c-fos and c-myc proto-oncogenes.

**EXPERIMENTAL PROCEDURES**

**Materials**—The sources of thioglycollate, cell culture materials, [3H]thymidine, BAPTA/AM, genistein, staurosporine, chelerythrine, manumycin A, SB 203580, PD98059, U0126, ACOCCF6, Wortmannin, and EGF have previously been described (6, 10). 1-6-(C17-3-Methoxyestradi-1,5(10)-tien-17-y1) aminoxyethyl-1H-pyrrrole-2,5-dione (U73122) was purchased from Biomol (Plymouth Meeting, PA). Bromoelonactone (BEL) and pertussis toxin were procured from Sigma. Endotoxin-free αM*, binding site mutants of αM*, and RAP were prepared as described previously (9, 10). Antibodies against phosphorylated MEK 1/2, ERK 1/2, p38 MAPK, and JNK were purchased from Cell Signaling Technology (Massachusetts, MA). Antibodies against c-Fos, c-Myc, CREB, and NFkB were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). [3H]Methylcholine with specific activity of 60–90 Ci/mol was purchased from ARC (St. Louis, MO). Silica gel G plates were from Analytical Technology (Newark, DE). Lipid standards were purchased from Avanti Polar Lipids (Alabaster, AL). Xestospongin C was purchased from Calbiochem (San Diego, CA). GDPβS and GDPγS were obtained from Roche Molecular Biochemicals and AIФ was from Sigma. Culture media were from Invitrogen. All other chemicals and solvents used were of the highest available grade.

**Cell Culture**—Thioglycollate-elicited peritoneal macrophages were obtained from pathogen-free 6-week-old C57BL/6 mice (Charles River Laboratories, Raleigh, NC) in Hank’s balanced salt solution containing 10 mM HEPES, pH 7.4, and 3.5 mM NaHCO3 (HHSBS). The cells were washed with HHSBS and suspended in RPMI 1640 medium containing 2 mM glutamine, 12.5 units/ml penicillin, 6.25 g/ml streptomycin, and 10% fetal bovine serum and plated at a cell density of 3.5 × 10^6 cells/ml. The cells were incubated overnight at 37 °C in RPMI 1640 medium containing 5% fetal bovine serum and kept in a humidified CO2 (5%) incubator. The monolayers were washed with HHSBS three times to remove nonadherent cells, and monolayers were incubated overnight at 37 °C in RPMI 1640 medium containing the additions listed above except that 0.2% fatty acid-free BSA replaced the serum.

**Measurement of cPLA2 Activity**—Macrophage monolayers adhered for 2 h in RPMI 1640 medium were radiolabeled with [3H]methylcholine (2 μCi/ml) for 16–18 h at 37 °C in a humidified CO2 (5%) incubator. The monolayers were washed three times with cold HHSBS, a volume of the buffer (200 μl) added, and monolayers (3 × 10^6 cells/well) incubated for 5 min at 37 °C prior to stimulation with 100 μM/15 min. The 17-kDa receptor-binding fragment of αM* (RBF), or its mutant K1370A for various periods of time. Studies were also performed with EGF (10 ng/ml) as a control. The reaction was stopped by aspirating the medium and adding a volume of chilled methanol. The cells were scraped into screw cap glass tubes and lipid extracts according to Bligh and Dyer (27). The extract was evaporated to dryness under nitrogen, dissolved in a volume of CHCl3/CH2OH/acetate acid:H2O (65:43:1.3, v/v/v) (28). Each sample was co-chromatographed with 10 μg of authentic lyssoPC. The reactions were dried, exposed to I2 vapor, and gel areas corresponding to the standard lyssoPC scraped into scintillation vials and their radioactivity counted. [3H]lysophosphatidylcholine-derived fragments from cPLA2-catalyzed cleavage of [3H]methylcholine were also chromatographed on plates for quantification of radioactivity in [3H]lysophosphatidylcholine fraction were described as above. We also examined the role of IP3 generation after hydrolysis of phosphatidylinositol 4,5-biphosphate by G protein-coupled PI-PLC activity on cPLA2 activation in αM*-stimulated macrophages, we employed U73122, which is a relative inhibitor of G protein-mediated PI-PLC activation and PI-PLC-linked events (33). To [3H]methylcholine-labeled and washed macrophages in RPMI 1640 medium, U73122 (2 μM) was added, cells incubated for 10 min as above and then stimulated with αM* (100 μM/20 min). The reaction was terminated by aspirating the medium and adding a volume of methanol. Other details of lipid extraction, thin layer chromatography fractionation, and determination of radioactivity in the [3H]lysophosphatidylcholine fraction were described as above.

**Modulation of Phosphatidylinositol-dependent Phospholipase C (PI-PLC) Activity and cPLA2 Activation**—To understand the role of G protein-coupled PI-PLC activity on cPLA2 activation in αM*-stimulated macrophages, we employed U73122, which is a relative specific inhibitor of G protein-mediated PI-PLC activation and PI-PLC-linked events (33). To [3H]methylcholine-labeled macrophages in RPMI 1640 medium was added xestospongin C (5 μM) and the cells were incubated for 10 min as above prior to addition of αM* (100 μM/20 min). The reaction was terminated by aspirating the medium and adding a volume of methanol. Other details of determining radioactivity in [3H]lysophosphatidylcholine fraction were described as above.

**Measurement of [3H]Thymidine Uptake by Macrophages**—Murine peritoneal macrophages harvested as above were allowed to adhere for 2 h in RPMI 1640 medium containing 0.2% fatty acid-free BSA, penicillin, streptomycin, and glutamine at 37 °C in a humidified CO2 (5%) incubator. The monolayers were washed twice with HHSBS and a volume of above RPMI medium added, followed by the incubation of [3H]thymidine (2 μCi/ml) (9, 17). To the respective wells αM* (100 μM) or PDGF (10 ng/ml) were added. In experiments where the effect of ACOCCF6, (20 μM/15 min), PD98059 (50 μM/10 min), or SB 203580 (15 μM/15 min) were studied, these were added to their respective wells and cells incubated for the specified time before adding αM* or PDGF. The cells were incubated overnight in a humidified CO2 (5%) incubator. The incubations were terminated by aspirating the medium and washing macrophages twice first with 5% trichloroacetic acid (15 min/4 °C) and then three times with HHSBS. The monolayers were lysed with 1 N NaOH and an aliquot used for liquid scintillation counting and protein estimation (35).

**Determination of Macrophage Cell Number**—Because increased DNA synthesis is generally associated with an increase in total cellularity, the number of macrophages were counted before and after overnight exposure to varying concentrations of M2 agonist. Macrophages were harvested and allowed to adhere in six-well plates in RPMI 1640 medium containing 5% fetal bovine serum for 2 h as described above. The adhered cells were carefully scraped, centrifuged at 1200 rpm for 5 min, and suspended in 15 ml of RPMI 1640 medium containing 0.2% fatty acid-free BSA, and 0.5 ml aliquots (2 × 10^6 cells) were pipetted into 15 ml siliconized polypropylene tubes. To the respective
FIG. 1. α2M*-induced increase in cPLA2 activity as measured by quantification of [3H]lysoPC in macrophages. Panel A, effect of time of incubation on cPLA2 activity of α2M* (100 pM) (●), native α2M (100 pM) (○), or buffer (□). Panel B, column 1, buffer; column 2, α2M* (100 pM/20 min); column 3, RBF (100 pM/20 min); column 4, K1370A (100 pM/20 min); column 5, EGF (10 ng/ml/20 min). Panel C, effect of concentration of α2M* on cellular cPLA2 activity. Details are described under “Experimental Procedures.” Values in each panel are mean ± S.E. from three to five independent experiments and are expressed as percentage of change over basal value, which is taken as 100%.

tubes, a specified concentration of α2M* was added, the contents mixed gently, and the tubes incubated overnight as above. After overnight incubation, 10 μl of trypan blue solution was added to each tube, the tubes gently shaken during incubation for 2 min, and a 10-μl aliquot employed for counting the number of cells in a hemocytometer. In experiments where the modulation in cell numbers of α2M*-exposed macrophages (2 × 10⁶ cells/tube) was studied, SR203580, a specific inhibitor of p38 MAPK (15 μM/30 min) (36); PD98059, a specific inhibitor of MEK 1/2 (50 μM/30 min) (37); U0126, a specific inhibitor of MEK 1/2 (1 μM/10 min) (38); AAOCCF, (20 μM/15 min) (39); and wortmannin, a specific inhibitor of PI 3-kinase (30 μM/30 min) (40) were added to the respective tubes, and tubes incubated for the specified time before adding α2M* (100 pM). The tubes were incubated and cell numbers counted as described above.

Western Blotting of Phosphorylated MEK 1/2, ERK 1/2, p38 MAPK, and JNK in Macrophages Stimulated with α2M*—Freshly harvested peritoneal macrophages in RPMI 1640 medium containing penicillin, streptomycin, and 0.2% fatty acid-free BSA were allowed to adhere in six-well plates (3 × 10⁶ cells/well) for 2 h as above. The monolayers were washed twice with HBBSS, a volume of above RPMI 1640 medium added, and plates incubated overnight as above. The monolayers were washed twice, a volume of RPMI medium containing 0.2% fatty acid-free BSA added, and the cells pretreated with specific inhibitors/modulators of MAPKs for the specified time period before exposing to α2M* (100 pM/20 min) or buffer. The incubations were terminated by aspirating the medium. The lysis of cells, their electrophoresis, and Western immunoblotting were performed according to the manufacturer’s instruction. In each case, an equal amount of protein was employed for electrophoresis. The detection of phosphorylated MAPKs by enhanced chemifluorescence and quantification of their distribution was performed by phosphorimaging (Storm®).

Western Blotting of c-Fos, c-Myc, CREB, and NFκB Proteins in Macrophages Exposed to α2M*—Freshly harvested peritoneal macrophages in RPMI 1640 medium containing penicillin, streptomycin, glutamine, and 0.2% BSA were allowed to adhere in six-well plates (3 × 10⁶ cells/well) for 2 h as above. The monolayers were washed twice with HBBSS, a volume of above RPMI 1640 medium added, and plates incubated overnight as above. The monolayers were washed, a volume of above RPMI medium added, and the cells incubated with specific inhibitors/modulators of MAPKs or a Ca²⁺ chelator, for the specified time period before exposing to α2M* (100 pM/20 min) or buffer. The incubations were terminated by aspirating the medium. The lysis of cells, their electrophoresis, and Western immunoblotting were performed according to the manufacturer’s instruction. In each case, an equal amount of protein was employed for electrophoresis. The detection of immunoblots was performed by enhanced chemifluorescence, and quantitation of their distribution was done by phosphorimaging (Storm®).
Translocation of α2M*-induced cPLA2 to Membrane and Nuclear Fractions of Macrophage—Two-h adhered cells (3 × 10^6 cells/well) in above RPMI 1640 medium were radiolabeled with [3H]methylocholine (2 μCi/ml) for 16–18 h at 37 °C in a humidified CO2 (5%) incubator. The radiolabeled monolayers were washed three times with cold HBSS, a volume of the buffer added, and monolayers incubated for 5 min at 37 °C prior to stimulation with α2M* (100 μg) for various periods of time. The reaction was stopped by aspirating the medium, and a volume of “buffer A” containing 20 mM Tris-HCl, pH 7.4, 10 mM KCl, 2 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 0.3 mM CaCl2, 1 mM NaF, and 1 mM sodium orthovanadate was added. Cells were allowed to swell for 10 min on ice, followed by the addition of three volumes of “buffer B” containing 50 mM Tris-HCl, pH 7.4, 25 mM KCl, 5 mM MgCl2, 0.25 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 0.2 mM EGTA, 0.2 mM CaCl2, 1 mM NaF, 1 mM sodium orthovanadate, and 100 mM benzamidine. The cells were scraped into glass tubes containing sodium orthovanadate and 100 mM benzamidine and homogenized in a Potter-Elvehjem homogenizer by 15 up-and-down strokes at 4 °C. The homogenates were centrifuged for 10 min at 800 × g, 4 °C. Supernatant was carefully removed, nuclear pellets washed...
FIG. 5. Modulation of MEK 1/2 and ERK 1/2 phosphorylation in macrophages stimulated with αM*. See “Experimental Procedures” for details. Panel A, MEK 1/2 phosphorylation. Column/lane 1, buffer; column/lane 2, αM* (100 pM); column/lane 3, BAPTA/AM (10 μM/30 min) then αM*; column/lane 4, chelerythrine (200 nM/15 min) then αM*. The results shown are representative of at least four individual experiments. Quantification of immunoblots was performed by PhosphorImager (Storm®), and the results are expressed as changes in phosphorylated MEK 1/2 levels in arbitrary units.

FIG. 6. Regulation of phosphorylated p38 MAPK and JNK in macrophages stimulated with αM*. See “Experimental Procedures” for details. Panel A, p38 MAPK phosphorylation. Column/lane 1, buffer; column/lane 2, αM* (100 pM); column/lane 3, BAPTA/AM (10 μM/30 min) then αM*; column/lane 4, chelerythrine (200 nM/15 min) then αM*. Results shown are representative of three to four individual experiments and are expressed as changes in phosphorylated p38 MAPK levels in arbitrary units. Panel B, JNK phosphorylation. Column/lane 1, buffer; column/lane 2, αM* (100 pM); column/lane 3, BAPTA/AM (10 μM/30 min) then αM*. Results shown are representative of three or four individual experiments and are expressed as changes in phosphorylated JNK levels in arbitrary units.

Platelet-activating factor (PAF) receptor stimulation endotoxins do not contribute to agonist-induced stimulation of cPLA2 activity. The results indicate that endotoxin stimulation of cPLA2 activity parallels those observed in αM*-induced increase in [Ca2+]i, IP3 (17), phospholipase D activation, PI 3-kinase activation (10), and p21ras activation reported previously (20).

αM*-induced Activation of cPLA2 Is RAP- and BEL-insensitive—RAP, the 39-kDa receptor-associated protein does not block αM*-induced signal transduction, but does block signaling by all other LRP ligands so far studied (5, 17). The results presented in Fig. 2A show that the ligation of macrophage receptors with either αM* or the RBF binding site mutant K1370A, activates cPLA2 activity in the presence of RAP, as expected from this fact. Chelation of [Ca2+]i, with BAPTA/AM, completely abolished αM*-stimulated cPLA2 activity (Fig. 2B). We further evaluated the Ca2+ sensitivity of αM*-induced increase in cPLA2 activity by using BEL, a very potent inhibitor of Ca2+–dependent cytosolic PLA2 (Fig. 2C). Preincubation of labeled cells with BEL had no effect on cPLA2 activity stimulated by αM* or K1370A (Fig. 2C), which demonstrates that αM* induces the activity of a Ca2+-dependent cytosolic PLA2. To rule out the contribution of endotoxin in αM* or K1370A-induced activation of cPLA2 activity, the labeled cells were treated with boiled αM* and cPLA2 activation measured (Fig. 2C). In addition all preparations were endotoxin-free as determined by assay (data not shown). The results indicate that endotoxins do not contribute to agonist-induced stimulation of cPLA2 activity.

αM*-induced cPLA2 Activity Is Coupled to a Pertussis Toxin-insensitive G Protein—Addition of αM* and GTPγS
(20 μM) to saponin-permeabilized cells caused a nearly 2-fold increase in cPLA₂ activity compared with that observed with α₂M* alone (Fig. 3A). Preincubation of cells with GDPβS prior to addition of GTPγS and α₂M* nearly abolished α₂M*-stimulated cPLA₂ activity (Fig. 3B). Further, treatment of cells with ALFβ, as expected, potentiated the α₂M*-induced increase in cPLA₂ activity (Fig. 3C). These results strongly support the involvement of a heterotrimeric G protein in the activation of cPLA₂ by α₂M*. We propose that cPLA₂ activation in α₂M*-stimulated cells requires a heterotrimeric G protein coupled to PI-PLC. If this hypothesis is correct, inhibition of PI-PLC activation and blocking the binding of second messenger generated consequent to PI-PLC activation to the effector molecules should inhibit agonist-induced cPLA₂ activation. The results shown in Fig. 3C demonstrate that, indeed, either the inhibition of PI-PLC with U73122 or the inhibition of binding of newly generated IP₃ to IP₃R by xestospongin C inhibited c-PLA₂ activation in α₂M*-stimulated cells. We next treated pertussis toxin preincubated cells with α₂M* and quantified the release of [³H]lysoPC (Fig. 3D). The results demonstrate that the G protein involved in α₂M*-mediated stimulation of cPLA₂ activity is pertussis toxin-insensitive. Activation of cPLA₂ by receptor-coupled G proteins may be mediated by direct interaction of these G proteins with cPLA₂ (21-26). In addition, G protein-dependent activation of PLC increases DAG and IP₃, the latter of which raises [Ca²⁺]. Activation of PKC, activation of MAPKs, and subsequent phosphorylation and activation of cPLA₂ then occur (21, 26).

**Phosphorylation of MEK 1/2, ERK 1/2, PKC, p38 MAPK, and JNK in α₂M* Induces Activation of cPLA₂**—We next examined the involvement of MEK 1/2, ERK 1/2, p38 MAPK, and JNK in cPLA₂ activation in two ways, namely by quantifying the levels of phosphorylated (activated) forms of these MAPKs by Western blotting and by inhibiting MEK 1/2 or p38 MAPK with their specific inhibitors before stimulating macrophages with α₂M* and quantifying [³H]lysoPC. Inhibition of p38 MAPK with SB203580, and of MEK 1/2 with PD98059 or U0126, nearly abolished α₂M*-induced increase in cPLA₂ activity and release of [³H]lysoPC (Fig. 4A). Treatment of labeled macrophages with genistein, a tyrosine kinase inhibitor, had no effect on α₂M*-induced increase in cPLA₂ activity (Fig. 4B). The binding of α₂M* to α₂MSR activates the hydrolysis of membrane phosphatidylinositol bisphosphate via both PLCβ and PLCγ (4, 12). We have shown...
that the ligation of α2MSR with α2M* stimulates PLCγ activity, which is sensitive to genistein (12). Because genistein treatment did not affect α2M*-induced cPLA2 activity, the studies suggest that PLCβ is involved in the activation of cPLA2. Inhibition of PI 3-kinase with wortmannin, however, did not affect cPLA2 activation. These results are consistent with published studies demonstrating that both ERK 1/2 and p38 MAPK are involved in the phosphorylation of cPLA2 at Ser-505, which is essential for its activation (21–26).

Stimulation of murine peritoneal macrophages with α2M* caused −2-fold increase in the expression of phosphorylated MEK 1/2 protein (Fig. 5A), phosphorylated ERK 1/2 protein (Fig. 5B), phosphorylated p38 MAPK protein (Fig. 6A), and phosphorylated JNK protein (Fig. 6B). α2M*-induced increase phosphorylation of MEK 1/2 was inhibited by chelation of Ca2+ with BAPTA/AM or inhibition of PKC with chelerythrine (Fig. 5A). α2M*-induced increase in ERK 1/2 phosphorylation was drastically inhibited by PD98059, U0126, chelation of [Ca2+]i with BAPTA/AM, or inhibition of PKC with chelerythrine (Fig. 5B). Inhibition of PKC as well as chelation of [Ca2+]i, abrogated α2M*-induced increase in phosphorylated p38 MAPK protein (Fig. 6A). The increase in JNK protein phosphorylation in α2M*-stimulated macrophages was reduced by BAPTA/AM or chelerythrine (Fig. 6B).

In many cells, PKC regulates MAPK pathways either alone or in combination with other mechanisms (21–26, 45–47). To further examine the involvement of PKC, we quantified the activity of cPLA2 in macrophages in which PKC activity was down-regulated with PMA, or inhibited by chelerythrine or staurosporine, respectively (Fig. 4B). Inhibition of PKC with its inhibitors or its down-regulation nearly abolished α2M*-induced increase in cPLA2 activity, as determined by quantifying of [3H]lysoPC (Fig. 4B). In contrast, up-regulation of PKC significantly increased cPLA2 activity in α2M*-treated cells (Fig. 4B).

α2M*-induced Translocation of cPLA2 to Nuclei and Membranes—cPLA2 activity as determined was present both in nuclei and the membrane fractions (Fig. 7). Immediately after α2M* stimulation, cPLA2 activity increased in the nuclear fraction reaching a maximal level at 15 min and plateauing at longer periods of incubation (Fig. 7A). In contrast, the peak cPLA2 activity in the membrane fraction occurred between 2 and 5 min after α2M* stimulation and then declining to a steady state level. Very little cPLA2 activity was present in the cytosol after α2M* stimulation. The results demonstrate that, in α2M*-stimulated macrophages, the nuclear membranes are the main target for cPLA2 translocation, although some activity is associated with other membranes. Treatment of macrophages with PD98059 or SB203580 before stimulating them with α2M* inhibited cPLA2 activity in nuclei (Fig. 7B). No inhibition in α2M*-induced increase in [Ca2+]i, was observed in these studies; hence, the reduced nuclear cPLA2 activity most likely reflects phosphorylation by MAPKs.

α2M*-induced Increase in [3H]Thymidine Uptake and Peritoneal Macrophage Proliferation Are Attenuated by Inhibitors of cPLA2, MEK 1/2, and p38 MAPK—In view of the role of cPLA2 in cell proliferation (21–26), we have examined the involvement of cPLA2 in α2M*-induced [3H]thymidine uptake and cell proliferation (Fig. 8). Like PDGF, α2M* and K1370A increased [3H]thymidine uptake by macrophages by ∼2-fold (Fig. 8A). The agonist-induced increase in [3H]thymidine uptake was abolished by AACOCF3, a specific inhibitor of cPLA2, by PD98059, or by SB 203580 (Fig. 8B). These results demonstrate that cPLA2 activity and MAPK activation is intimately involved in α2M*-induced increases in [3H]thymidine uptake. [3H]Thymidine uptake may indicate enhanced DNA synthesis, but there are other potential mechanisms of enhanced uptake.
independent of new nucleic acid synthesis. Because new DNA synthesis is normally associated with an increase in total cellularity, we determined the macrophage cell number before and after overnight incubation with varying concentrations of α2M* (Fig. 8B). The maximal increase in macrophage numbers occurred at 50–100 pM α2M*, and the cell numbers plateaued at higher concentrations of α2M* (Fig. 8B). The kinetics of α2M*-induced increase in cell number is similar to that observed for α2M*-induced increase in protein and DNA synthesis (17). Inhibition of cPLA2 by AACOCF3 or inhibition of MAPK-dependent phosphorylation of cPLA2 abolished α2M*-induced increase in macrophage cell number (Fig. 8C).

α2M* Exposure Elevates the Levels of NFκB and CREB Transcription Factors in Peritoneal Macrophages—Stimulation of macrophages with α2M* (100 pM/20 min) caused a 2-fold increase in the expression of NFκB (Fig. 9A). Ligation of α2MSR on macrophages elevates cyclic AMP (cAMP) levels by ~50% (4). cAMP, an important intracellular second messenger, mediates the transcriptional induction of many genes through PKA-dependent phosphorylation of transcription factor CREB (48–50). This reaction modulates its nuclear transport, and DNA binding affinity, thus enhancing its transactivation potential (49, 50). Stimulation of macrophages with α2M* (100 pM/20 min) increased the expression of CREB by ~2-fold (Fig. 9B), suggesting thereby the involvement of CREB in α2M*-induced induced responses in macrophages. Inhibition of MEK 1/2 or p38 MAPK in α2M*-stimulated macrophages reduced α2M*-induced increased expression of NFκB protein by ~25–30% (Fig. 9A) and profoundly reduced α2M*-induced increase in CREB protein expression (Fig. 9B). These results suggest that both translational and transcriptional regulation of α2M*-induced cellular responses are mediated by cPLA2 activation.

c-Fos and c-Myc Protein Levels in Macrophages—To understand the involvement of two early genes, namely c-fos and c-myc, in α2M*-induced mitogenesis and cell proliferation, we next quantified the c-Fos and c-Myc proteins in α2M*-stimulated macrophages by Western blotting and phosphorimaging (Fig. 10, A and B). Incubation of macrophages with inhibitors of MEK 1/2 or p38 MAPK, or chelation of [Ca2+]i, before stimulating with α2M* caused inhibition in the expression of c-Fos and c-Myc proteins. The results presented show that the early genes c-fos and c-myc participate in α2M*-induced new protein and DNA synthesis and MAPKs regulate these activities both posttranslationally and transcriptionally.

**DISCUSSION**

In this study we show that ligation of macrophage receptors with α2M*, RBF, or its mutant K1370A up-regulates the Ca2+-dependent 85-kDa cPLA2 activity in a concentration- and time-dependent manner. cPLA2 activity is RAP- and BEl-insensitive and Ca2+-dependent. A pertussis toxin-insensitive G protein is involved in α2M*-induced activation of cPLA2 activity. Binding of α2M* to its receptors on peritoneal macrophages activates MEK 1/2, ERK 1/2, p38 MAPK, and JNK by inducing their phosphorylation. Incubation of macrophages with inhibitors of MEK 1/2 or p38 MAPK before stimulation with α2M* profoundly decreased the expression of the respective phosphorylated MAPKs and nearly abolished α2M*-induced increase in cPLA2 activity. Similar effects were observed by inhibition of PKC or chelation of [Ca2+]i. Binding of α2M* to macrophages induced the transient translocation of cPLA2 activity primarily to nuclei and also to other membrane fractions. Concomitant with these results, inhibition of cPLA2, PKC, or the MAPKs blocked α2M*-induced increase in [3H]thymidine uptake and cell proliferation. Finally, ligation of α2M* receptors increased the levels of transcription factors NFκB and CREB, as well as c-Fos and c-Myc proteins. α2M* induced increased expression of transcription factors, and these proto-oncogene proteins were also affected to varying degrees by inhibitors of MAPKs and chelation of [Ca2+]i.

Multifactorial regulation of cPLA2 activity has been reported, which is cell- and agonist-specific. These factors include transcriptional regulation involving heterotrimetric G proteins, increase in [Ca2+]i, activation of PKC, cPLA2 translocation and membrane localization, and phosphorylation by MAPKs (21–26). Involvement of G proteins in cPLA2 activation may occur directly through interaction of heterotrimetric G proteins with the enzymes or indirectly by activation of signaling cascades subsequent to receptor ligation (21–26). Phosphorylation at Ser-505 of cPLA2 is essential for agonist-induced arachidonic acid release in many, but not all, types of cells (21–26). MEK 1/2, ERK 1/2, p38 MAPK, and JNK are involved in this phosphorylation reaction (45–47). Certain agonists such A23187 or okadaic acid can stimulate cPLA2 activity in a Ca2+- and PKC-
considerably clarified by recent observations. Although initial PLC activity, it appears that PLC activity is involved in the activation of cPLA2 in macrophages stimulated with αM2. PLCβ-catalyzed hydrolysis of phosphatidylcholine bisphosphate generates IP3 and DAG (5, 6, 12). The former elevates [Ca2+]i, and the latter activates PKC. Soluble cPLA2 binds Ca2+ at CaLB domains, and translocates to nuclei and membrane fractions (21–26). Phosphorylation-dependent activation of αM2-induced cPLA2 activity involves the participation of PKC because the inhibition of PKC severely reduced the release of lysoPC as well as activation of MEK 1/2, ERK 1/2, p38 MAPK, and JNK.

Agonists may regulate cPLA2 activity by two possible mechanisms, namely agonist-induced de novo protein synthesis of the enzyme or activation of the enzyme. The data collected in this study suggest that triggering of signaling cascades by αM2 promotes activation of cPLA2. Various agonists, however, also increase steady state cPLA2 mRNA levels and promote this mRNA (57–59). In some types of cells, these agents may also promote translocation of cPLA2 to nuclei (56).

The nature of αM2-mediated signal transduction has been considerably clarified by recent observations. Although initial studies suggested that a distinct receptor accounted for αM2-dependent signaling, more recent studies suggest that LRP in association with one or more adapter proteins constitutes the αM-MSR (13–16). There are two good models, which could account for αM2-dependent signal transduction. The Wingless/ Fzrissed (Wnt/Frz) signaling system was first identified in Drosophila, but is now known to exist in mammals (63–65). The Wnt ligand binds to Frz and a variant of the LRP-5 recruits a group of adapter and signaling components, which promote transcriptional activation (66). The glucocorticoid receptor offers a second potential model for αM2-dependent signal transduction. Although a glucocorticoid receptor protein exists that can bind its ligand, the glucocorticoid receptor must be bound to Hsp 90 to acquire a high affinity steroid binding conformation involved in activating signal transduction (67, 68). Studies are currently under way to determine whether these systems are models for the αM-MSR.
Regulation of Cytosolic Phospholipase A_2 Activity in Macrophages Stimulated with Receptor-recognized Forms of α_2-Macroglobulin: ROLE IN MITOGENESIS AND CELL PROLIFERATION

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