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# Activation of Akt/PDK Signaling in Macrophages Upon Binding of Receptor-Recognized Forms of $\alpha_2$-Macroglobulin to its Cellular Receptor: Effect of Silencing the *CREB* Gene

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**Abstract** Macrophage binding of receptor-recognized forms of $\alpha_2$-macrogobulin ($\alpha_2$M*) significantly increases cAMP, CREB, and activated CREB. We have now examined the participation of the PI 3-kinase/PDK/Akt/p70s6k signaling cascade in $\alpha_2$M*-induced cellular proliferation and also studied the role of CREB in these events. Exposure of cells to $\alpha_2$M* caused an ~2-fold increase in CREB and its phosphorylation at $Ser^{133}$, phosphorylation of the regulatory subunit of PI 3-kinase, Akt phosphorylation at $Ser^{473}$ or $Thr^{308}$, and phosphorylated 70s6k. Silencing of the *CREB* gene with dsRNA homologous in sequence to the target gene, markedly reduced the levels of CREB mRNA activation of CREB, PI 3-kinase, Akt, and p70s6k in $\alpha_2$M*-stimulated macrophages. We conclude that in murine peritoneal macrophages, $\alpha_2$M*-induced increase of cAMP is involved in cellular proliferation and this process is mediated by the PI 3-kinase signaling cascade. J. Cell. Biochem. 93: 1020–1032, 2004. © 2004 Wiley-Liss, Inc.

**Key words:** $\alpha_2$-macroglobulin signaling receptor; PI 3-kinase signaling pathway; regulation of PDK; regulation of Akt; phosphorylated Akt; phosphorylated p70s6k; phosphorylated PI 3-kinase regulatory subunit

Receptor-recognized forms of $\alpha_2$-macroglobulin ($\alpha_2$M*) bind to the low density lipoprotein receptor-related protein (LRP) and to the $\alpha_2$M* signaling receptor ($\alpha_2$MSR) which has been identified as GRP78 [Misra et al., 1994a,b, 1999, 2002; Howard et al., 1996; Backsai et al., 2000; Misra and Pizzo, 2002]. Binding of $\alpha_2$M* to GRP78 ($k_d \sim 50$ pM, 1600 binding sites) activates phospholipase Cβ and γ resulting in $IP_3$ synthesis and mobilization of calcium [Misra et al., 1993, 1994a,b, 1999a,b, 2002; Howard et al., 1996; Misra and Pizzo, 2002]. These second messengers initiate the onset of calcium and phosphorylation-dependent downstream events culminating ultimately in the synthesis of DNA and proteins, as well as cell proliferation [Misra et al., 1993, 1994a,b, 2002; Howard et al., 1996; Misra and Pizzo, 1998, 2002]. A number of intracellular signaling pathways play a role in the regulation of cell growth and proliferation in response to stimulation by growth factors and G protein-coupled receptors. Both the classical Ras-Raf-MEK-ERK and PI 3-kinase/Akt/PDK/p70s6k pathways are involved in growth factor-mediated biological responses [Kolch, 2000; Vanhasebroeck and Alessi, 2000; Blume-Jensen

Abbreviations used: $\alpha_2$M*, receptor-recognized forms of $\alpha_2$M; $\alpha_2$M, $\alpha_2$-macroglobulin; $\alpha_2$MSR, the $\alpha_2$M* signaling receptor; LRP, lipoprotein receptor-related protein; PDK1, phosphoinositide-dependent kinase 1; MAPK, mitogen activate protein kinase; PI 3-kinase, phosphoatidylinositol 3-kinase; HHBSS, Hanks' balanced salt solution containing 10 mM HEPES, pH 7.4 and 3.5 mM $NaHCO_3$; BSA, bovine serum albumin; CREB, cAMP response element binding protein; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal regulated kinase; p70s6k, ribosomal s6 kinase; cAMP, cyclic adenosine monophosphate.

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and Hunter, 2001]. $\alpha_2$M* receptor binding triggers activation of a number of signaling cascades in a manner analogous to growth factors [Misra et al., 2002].

PI 3-kinases are heterodimeric lipid kinases that are composed of an 85 kDa regulatory/adaptor subunit, and a 110 kDa catalytic subunit. PI 3-kinase catalytic activity is tightly regulated by either phosphorylation of the 85 kDa regulatory subunit or through Ras-dependent activation of the catalytic subunit. Activation of several signaling cascades by phosphoinositides are initiated upon their binding to proteins containing plekstrin homology (PH) domains which are found in numerous proteins including phosphoinositide-dependent kinase (PDK) and Akt. Interaction of these lipids with the PH domains of Akt induces a conformational change which exposes two primary phosphorylation sites, $Thr^{308}$ in the activation loop and $Ser^{473}$ in the hydrophobic motif of the enzyme. PDK1 phosphorylates Akt at $Thr^{308}$ whereas PDK2 phosphorylates Akt at $Ser^{473}$ [Alessi et al., 1996; Proud and Denton, 1997; Vanhasebroeck and Alessi, 2000; Blume-Jensen and Hunter, 2001]. Phosphorylation at both of these sites is necessary for maximal activation of Akt. Akt phosphorylates several substrates which are either regulators of cell growth or of apoptosis.

cAMP response element binding protein (CREB) is a nuclear transcription factor which is a downstream target of cAMP signaling [Montminy, 1997; Greenberg and Shaywitz, 1999]. cAMP-induced cell proliferative effects vary with cell type. Increased levels of cAMP may stimulate [Cass et al., 1999; Gonzalez-Robayna et al., 2000; Misra et al., 2002], while in other situations inhibit, cell proliferation [Dumont et al., 1989]. We have reported that, like other cAMP elevating agents, stimulation of murine macrophages with $\alpha_2$M* causes activation of Ras and PI 3-kinase-dependent signaling pathways culminating in enhanced DNA synthesis, cell growth, and cellular proliferation [Misra and Pizzo, 1998, 2002; Misra et al., 1999a, 2002a,b]. PI 3-kinase-dependent mitogenic signaling involves p70s6k which is downstream to PI 3-kinase [Chou and Blenis, 1995; Klippel et al., 1998; Grew et al., 1999]. It phosphorylates the ribosomal s6 protein which promotes increased translation of mRNAs having the polypyrimidine tract motif and is important in growth factor-dependent cellular proliferation [Chou and Blenis, 1995; Klippel et al., 1998; Grew et al., 1999; Kolch, 2000; Vanhasebroeck and Alessi, 2000; Blume-Jensen and Hunter, 2001].

To understand the role of CREB in $\alpha_2$M*-induced activation of the PI 3-kinase/Akt/PDK/p70s6k signaling pathway, we have used post-transcriptional gene silencing by RNA interference [Fire et al., 1999; Elbashir et al., 2001; Sharp, 2001] to silence *CREB* gene expression. Activation of receptors with $\alpha_2$M* causes an approximate 2-fold increase in protein levels and/or phosphorylation of the components of this pathway. Silencing of *CREB* gene expression drastically reduced $\alpha_2$M*-dependent increased levels and/or phosphorylation of these components demonstrating that CREB activation is important in the transcriptional regulation of this pathway.

## MATERIALS AND METHODS

### Materials

Culture media were from Life Technologies, Inc. (Grand Island, NY) Antibodies against the phosphorylated and unphosphorylated 85 kDa subunit of PI 3-kinase and HSP70 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against unphosphorylated Akt and Akt phosphorylated at $Ser^{473}$ or $Thr^{308}$, PDK, phosphorylated and unphosphorylated p70s6k, phosphorylated and unphosphorylated CREB, $ATF_2$, c-Jun were purchased from Cell Signaling Technology, Inc. (Beverley, MA). Antibodies against actin were purchased from Sigma Chemicals (St. Louis, MO). $\alpha_2$M* was prepared as described previously [Misra et al., 1994a,b, 1999]. The PI 3-kinase inhibitors LY294002 and Wortmannin, and the farnesyl transferase inhibitor manumycin A were procured from Biomol (Plymouth Meeting, PA). LY303511 was from Sigma Chemicals. Other reagents of the highest available grade were procured locally.

### Determination of PDK1, Akt Phosphorylated at $Ser^{473}$ or $Thr^{308}$, the Phosphorylated 85 kDa Regulatory Subunit of PI 3-Kinase, and Phosphorylated p70s6k

This protocol has been described in detail elsewhere [Misra et al., 2002a,b]. In brief, macrophages ($2 \times 10^6$ cells/well) were incubated overnight in RPMI 1640 medium containing 0.2% fatty acid free BSA. The cells were washed

twice with Hanks' balanced salt solution containing HEPES and $NaHCO_3$ (HHBSS) and a volume of medium added, followed by the additions of $\alpha_2$M* (100 pM). The cells were incubated for 20 min at 37°C in a humidified $CO_2$ (5%) incubator. The reaction was terminated by aspirating the medium. The monolayers were washed once with cold HHBSS and the cells lysed in lysis buffer containing 20 mM Tris-HCl (pH 8.6), 0.1 M NaCl, 1 mM EDTA, 50 mM NaF, 30 mM Na pyrophosphate, 1 mM Na orthovanadate, 1 mM PMSF, 20 μg/ml leupeptin, and 0.5% Nonidet® P40 for 10 min on ice. The DNA strands were broken by passing the lysates through a 27 gauge needle and syringe several times. The lysates were centrifuged at $800 \times g$ for 5 min at 4°C to remove cell debris. The supernatants were transferred to clean tubes and their protein contents determined. Equal amounts of lysate proteins were electrophoresed according to Lemmlie [Misra et al., 2002]. Proteins from gels (10%) were transferred to Hybond P® membranes (Amersham Biosciences, Pisctaway, NJ) and immunoblotted with antibody against the phosphorylated and unphosphorylated 85 kDa regulatory subunit of PI 3-kinase, PDK1, Akt and Akt phosphorylated at $Ser^{473}$ or $Thr^{308}$, and phosphorylated and unphosphorylated p70s6k according to the manufacturer's instructions. Protein bands on the membrane were visualized by ECF (Amersham Biosciences) and quantified using a Storm® 860 Phosphorimager (Molecular Dynamics, Sunnyvale, CA). The effect of $\alpha_2$M* on unphosphorylated signaling proteins, namely the 85 kDa regulatory subunit of PI 3-kinase, PDK, and p70s6k was evaluated by either reprobing the respective membranes according to the manufacturer's instruction or by probing a new gel. To evaluate protein loading on gels the respective immunoblots were also reprobed for actin and the proteins visualized by ECF.

### Inhibition of Phosphorylation of the Regulatory Subunit of PI 3-Kinase and Akt Phosphorylated at $Thr^{308}$ or $Ser^{473}$ by Wortmannin and LY294002 in $\alpha_2$M*-Stimulated Cells

In experiments where the effects of PI 3-kinase inhibition by Wortmannin and LY294002 were evaluated, these specific inhibitors were added at varying concentrations to macrophages adhered for 2 h in RPMI 1640 medium containing antibiotics and 0.2% fatty acid free BSA in ($2 \times 10^6$ cells/well, 6-well plates) in separate experiments. The plates were incubated for the specified time period at 37°C before adding $\alpha_2$M* (100 pM). The cells were incubated for 20 min longer as above. The reaction was stopped by aspirating the medium and cells lysed in the lysis buffer as above. The lysates were electrophoresed and transferred to Hybond P® membranes and immunoblotted with antibodies (diluted 1:2000) against the phosphorylated 85 kDa subunit of PI 3-kinase, and Akt phosphorylated at $Ser^{483}$ or $Thr^{308}$. Protein bands on the membrane were visualized by ECF and quantified as described above. To monitor the protein loading on the gels, the immunoblots were reprobed as above for actin. Protein bands on the membranes were visualized as above.

### Manumycin A Treatment and Levels of PDK, Phosphorylated PI 3-Kinase, Phosphorylated Akt at $Ser^{473}$ or $Thr^{308}$, and Phosphorylated 70s6k in $\alpha_2$M*-Stimulated Cells

Macrophages adhered for 2 h in RPMI 1640 medium containing 0.2% fatty acid free BSA and antibiotics ($2 \times 10^6$ cells/well, 6-well plates) were exposed to the farnesyl transferase inhibitor manumycin A (20 μM/16 h) which blocks membrane attachment of Ras and thus its activation before adding $\alpha_2$M* (100 pM). The cells were incubated for 20 min longer as above. The reaction was stopped by aspirating the medium, cells lysed in the lysis buffer as above, and their protein contents determined. Equal amounts of lysate protein were then electrophoresed. The protein bands on the gel were transferred to Hybond P® membrane and immunoblotted with antibodies against the phosphorylated 85 kDa subunit of PI 3-kinase, Akt phosphorylated at $Ser^{473}$ or $Thr^{308}$, PDK1, and phosphorylated p70s6k, respectively. Protein bands on the membrane were visualized by ECF and quantified as described above. The respective immunoblots were reprobed as above for actin.

In experiments where the effects of manumycin A (20 μg/16 h), Wortmannin (30 nM/30 min), LY294002 (20 μM/15 min), and rapamycin (100 nM/15 min), were examined, these inhibitors were added to $\alpha_2$M* unstimulated cells and cells incubated as described for $\alpha_2$M*-stimulated cells. The cell lysates were processed as above for quantitation of 85 regulatory subunits of PI 3-kinase, PDK, Akt, and p70s6k.

### Chemical Synthesis of dsRNA Homologous in Sequence to the Target *CREB* Gene Sequence

The chemical synthesis of dsRNA homologous to the target mouse *CREB* gene sequence nb 324–344 (5′-AAGAGACAACAGAGAATGATA-3′; SWISS-PROT, Entry name–ATFB MOUSE. Primary accession number (35,451) was performed by Ambion (sequence ID 173; Austin, TX) [Misra et al., 2002]. For making dsRNA, the sense (5′-GAGACAACAGAGAAUGAUtt-3′) and antisense-(5′-UAUCAUUCUGUUGUCUCtt-3′) oligonucleotides were annealed according to the manufacturers instructions. Throughout the entire period of experimentation, handling of reagents was performed in an RNAse-free environment. Briefly, equal amounts of sense and antisense oligonucleotides were mixed and heated at 90°C for 1 min and then for 1 h at 37°C in an incubator. The dsRNA preparation were stored at −20°C before use [Misra et al., 2002].

### The Effect of $\alpha_2$M* Stimulation on Murine Peritoneal Macrophages Transfected With dsRNA Homologous in Sequence to CREB

Thioglycolate-elicited murine peritoneal macrophages ($1 \times 10^6$ cells/well in a 6-well plate) were lavaged as above and allowed to adhere for 2 h in RPMI 1640 medium containing 10% FBS, penicillin (12.5 U/ml), streptomycin (6.5 μg/ml), and 2 mM glutamine at 37°C in a $CO_2$ (5%) humidified incubator at 37°C. The non-adherent cells were aspirated, monolayers washed twice with HHBSS, 2 ml of DMEM medium containing 10% FBS and above antibiotics added, and cells incubated as above for 15 h. For each transfection, 2 μg of dsRNA was diluted into 100 μl of serum-free DMEM in a tube. In another tube 10 μl of lipofectamine was diluted into 100 μl of serum-free medium. The two solutions were combined, mixed gently, and incubated for 45 min at room temperature followed by the addition of 800 μl of serum-free and antibiotic-free medium to each tube. The monolayers were washed twice with serum-free DMEM medium, layered in each well with 1 ml of lipofectamine–DMEM (100 μl/ml) or lipid–dsRNA mixtures, containing different amounts of dsRNA, gently mixed, and incubated for 5 h at 37°C in a humidified $CO_2$ incubator. At the end of the incubation, 1 ml antibiotic-free DMEM containing 10% FBS was added to each well and cells incubated for 16 h as above. Microscopic observation of the monolayers did not show evidence of toxicity. The medium was replaced with DMEM medium containing antibiotics and 10% FBS 24 h following the start of transfection [Misra et al., 2002]. The monolayers were washed with the above DMEM medium once, a volume of the same medium added to the dsCREB RNA transfected cells in the respective wells and cells incubated overnight as above. The cells were washed with DMEM medium, and exposed to buffer, or $\alpha_2$M* (100 pM/20 min). The reaction was terminated by aspirating the medium and cells lysed in lysis buffer as above. Equal amounts of lysate protein were electrophoresed (10% gel), protein transferred to Hybond P® membrane. The phosphorylated 85 kDa subunits of PI 3-kinase, PDK1, Akt phosphorylated at $Ser^{473}$ or $Thr^{308}$, and phosphorylated p7056K on the membranes were detected by Western blotting with the respective antibodies in separate experiments and quantified by Storm® Phosphorimager as detailed above. To control for the amount of protein loaded on to the gels, the respective membranes were reprobed for actin as described above. To examine whether silencing of the *CREB* gene with ds CREB RNA affects other genes, gels were electrophoresed, proteins transferred to membranes, and the membranes immunoblotted with antibodies against GRP78, HSP70, and HSP90. Protein bands were visualized as above.

### Transfection of Macrophages With a dsRNA Negative Control

To demonstrate that the transfection of macrophages with dsRNA homologous in sequence to the target *CREB* gene, does not produce non-specific effects on target gene expression, macrophages were transfected with equimolar concentrations of a negative control dsRNA (Silencer™ negative control, catalog number 4610 or 4612, Ambion) and exposed to $\alpha_2$M* (100 pM) under identical conditions as described above for transfection with CREB dsRNA. The monolayers were washed with the above DMEM medium once, a volume of the same medium added to the monolayers followed by the addition of 100 pM of $\alpha_2$M*. The reaction was terminated by aspirating the medium, cells lysed in lysis buffer, and protein contents of lysates determined. Studies were then performed as described above.

### Effect of Silencing *CREB* Gene on mRNA Levels of CREB

Macrophages incubated overnight in RPMI1640 medium containing 10% FBS, penicillin (12.5 U/ml), streptomycin (6.5 μg/ml), and 2 mM glutamine were washed twice with serum and antibiotic-free DMEM medium. The monolayers in the respective wells were layered with 1 ml of either lipofectamine-DMEM, lipofectamine + dsCREB RNA, or lipofectamine + scrambled dsRNA. The contents were gently mixed and monolayers incubated for 5 h at 37°C in a humidified $CO_2$ incubator. At the end of the incubation, 1 ml antibiotic-free medium containing 10% FBS was added to each well and cells incubated for 16 h as above. The medium was replaced with DMEM medium containing antibiotic and cells further incubated overnight. The monolayers were washed with DMEM once, a volume of DMEM added to each well, and the respective wells treated with either medium or $\alpha_2M^*$ (100 pM/20 min) and cells incubated as above. The incubation was terminated by aspirating the medium and adding a volume of DMEM to each well. Total RNA from the respective monolayers was extracted by a single step method using an Rneasy® mini kit (Quigen, Chatsworth, CA) according to manufacturer's instructions. Total RNA was reverse transcribed with 1 μg of RNA in a 20 μl reaction mixture, using Malony murine leukemia virus reverse transcriptase (200 U) and oligo (dt) as the primer for 1 h at 42°C. The resulting of cDNA (5 μl) was used as a template and a 350 bp segment of CREB cDNA was amplified using a 21-mer upstream primer (5′-CAA CAA CTT GGT TGC TGG GCA-3′) identical to positions corresponding to amino acids 840–846 and a 21-mer downstream primer (5′-CAA AAA CGA AGG GAA ATC CTT-3′) complimentary to positions 480–486 amino acids encoded by CREB mRNA. A 302 bp segment of mouse β-actin (constitutive internal control) c-DNA was co-amplified using a set of PCR primers provided in a R&D system kit (Minneapolis, MN). Amplification was carried out in a Techne Thermal Cycler PHC for two cycles, one cycle: 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s. PCR products were analyzed on a 1.2% (w/v) agarose-ethidinium bromide gel. The gels were photographed and the intensity of the individual CREB and β-actin mRNA bands quantified on a Phosphorimager (Molecular Dynamics marketed by Amersham Biosciences).

## RESULTS

### Time-Dependent Changes in the Levels of Activated PI 3-Kinase, PDK1, Akt Phosphorylated at $Ser^{473}$ or $Thr^{308}$, and Phosphorylated p70s6k in Cells Exposed to $\alpha_2M^*$

We have previously reported a 2- to 3-fold increase in intracellular $IP_3$ and free cytosolic calcium levels within 1 min after stimulation of murine peritoneal macrophages with $\alpha_2M^*$ [Howard et al., 1996; Misra et al., 2002] which was followed by a similar increase in the levels of cAMP [Misra et al., 1993, 1994a,b] at about 15–20 min post stimulation. Exposure of cells to $\alpha_2M^*$ (100 pM) for varying periods of time increased the levels of phosphorylated 85 kDa regulatory subunit of PI 3-kinase (ranging from 0.75- to 3-fold) at about 10–20 min of incubation. Thereafter activation declined slowly as compared to buffer controls at zero time of incubation (Fig. 1). $\alpha_2M^*$ did not affect the protein levels of PDK1 at early periods of incubation; however, a slight decline in its levels was observed at longer periods of incubation (Fig. 1). Full activation of Akt, a downstream effector of PI 3-kinase, occurs by its phosphorylation on $Thr^{308}$ and $Ser^{473}$ residues as catalyzed by PDK1 and PDK2, respectively. A differential effect of $\alpha_2M^*$ on phosphorylation of Akt at $Thr^{308}$ and $Ser^{473}$ residues was observed in murine peritoneal macrophages (Fig. 1). $\alpha_2M^*$ increased the phosphorylation of Akt at $Ser^{473}$ by 10–20 min of incubation which varied from 2- to 3-fold. Activation declined rapidly at longer periods of incubation compared to buffer controls at zero time of incubation (Fig. 1). On the other hand, $\alpha_2M^*$-induced enhanced-phosphorylation of Akt at $Thr^{308}$ was observed only at longer periods of incubation compared to buffer controls (Fig. 1). $\alpha_2M^*$-induced enhanced-phosphorylation of p70s6k also was maximal by 10–20 min of incubation (range 1.5- to 2.5-fold), but it declined rapidly at longer periods of incubations compared to buffer controls (Fig. 1). In preliminary experiments the protein levels of components of the PI 3-kinase signaling pathway in buffer controls remained unchanged up to 90 min of incubations under identical experimental conditions. Since $\alpha_2M^*$ only negligibly affected the levels of the unphosphorylated PI 3-kinase regulatory

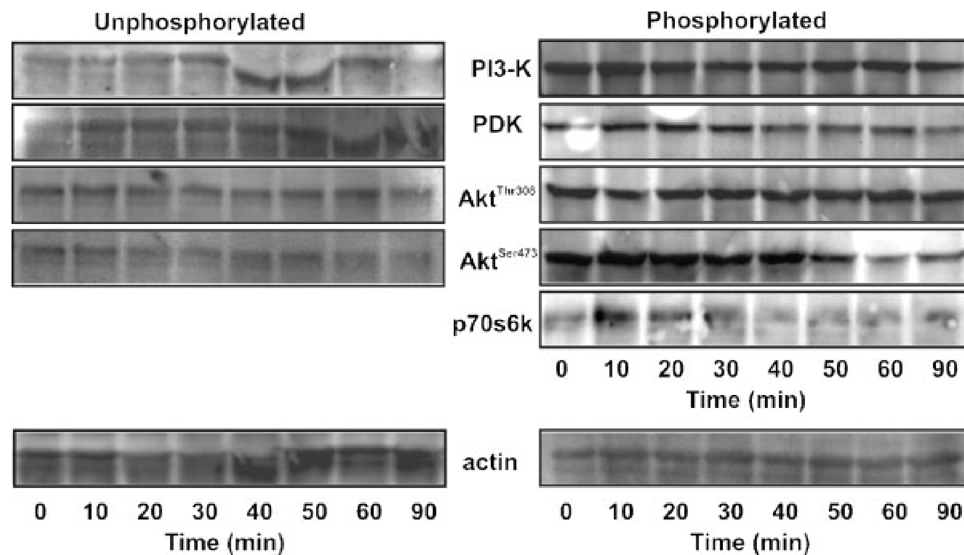


**Fig. 1.** Effect of time of incubation on $\alpha_2$M* (100 pM) on the levels of unphosphorylated and phosphorylated components of the PI 3-kinase/Akt-signaling pathway and actin. Details of quantifying unphosphorylated and phosphorylated components are described under "Materials and Methods." Immunoblots shown are representative of at least 4–5 individual experiments. Representative immunoblots of actin for unphoshporylated and phosphorylated 85 kDa regulatory subunit of PI 3-kinase, PDK, Akt, and p70s6k is shown below its respective panel.

subunit, Akt, and p70s6k (Fig. 1), these studies demonstrate the activation of this limb of intracellular signaling by $\alpha_2$M*. The levels of actin, a protein loading control in each immunoblot in both the groups, were nearly comparable. A representative actin immunoblot in both the groups is shown in Figure 1.

### Modulation of PI 3-Kinase, PDK1, and Akt Activation by $\alpha_2$M*

Recruitment of cytosolic Ras protein to the plasma membrane is a prerequisite for its biological activation and this is achieved by its lipidation. It is not clear whether activated Ras is involved in the recruitment of PI 3-kinase, PDK1, and Akt to membranes, but incubation of cells with manumycin A (20 μM/16 h) before stimulating with $\alpha_2$M* nearly abolished $\alpha_2$M*-induced elevations in the levels of PI 3-kinase, PDK, and Akt, phosphorylated at $Thr^{308}$ or $Ser^{473}$ (Figs. 2 and 3, Table I). These results suggest a possible role of Ras protein in membrane localization and activation of PI 3-kinase signaling pathway components in macrophages. $\alpha_2$M*-induced increases in 85 regulatory subnit of PI 3-kinase, Akt phosphorylated at $Thr^{308}$ or $Ser^{473}$ were drastically reduced by the specific PI 3-kinase inhibitors LY294002 and Wortmannin (Fig. 3, Table I). Consistent with our previous studies, these inhibitors

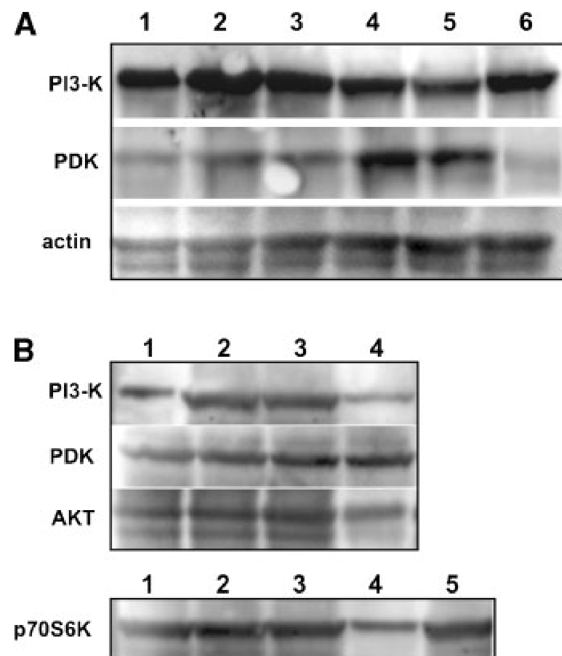


**Fig. 2.** Modulation of $\alpha_2$M*-induced changes in the phosphorylated protein levels of the 85 kDa regulatory subunit of PI 3-kinase, PDK1, and actin. **Panel A**: The lanes are: (1) buffer; (2) $\alpha_2$M* (100 pM/20 min); (3) LY393511 (20 μM/15 min) then $\alpha_2$M*; (4) LY294002 (20 μM/15 min) then $\alpha_2$M *; (5) Wortmannin (30 nM/30 min) then $\alpha_2$M*; and (6) manumycin A (20 μm/16 h) then $\alpha_2$M*. **Panel B**: The results obtained from several such experiments are shown in Table I. Values are expressed in arbitrary units and are the mean + SE from at least 2–4 experiments in each group.

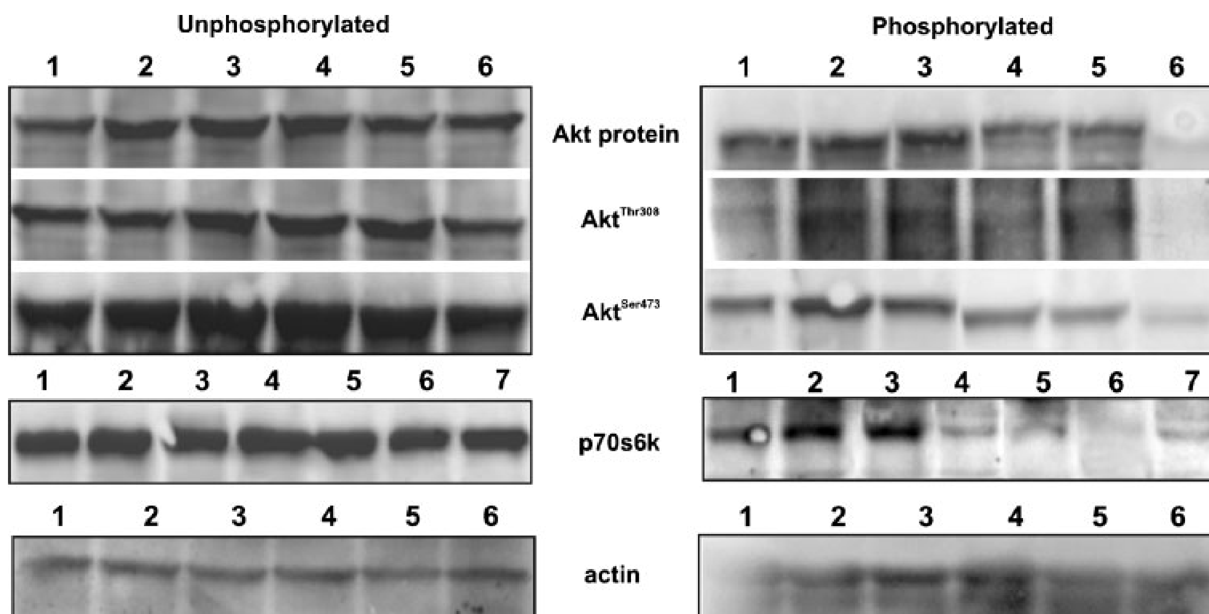


**Fig. 3.** Modulation of $\alpha_2M^*$-induced changes in the levels/activation of signaling components. The lanes are: (1) buffer; (2) $\alpha_2M^*$ (100 pM/20 min); (3) LY39351 (20 μM/15 min) then $\alpha_2M^*$; (4) LY294002 (20 μM/15 min) then $\alpha_2M^*$; (5) Wortmannin (30 nM/30 min) then $\alpha_2M^*$; (6) manumycin A (20 μM/16 h) then $\alpha_2M^*$; and (7) rapamycin (100 nM/15 min) then $\alpha_2M^*$. The results obtained from several such experiments are shown in Table I. Values are expressed as arbitrary units.

affected the protein levels of PI 3-kinase, PDK1, Akt, and p70s6k in unstimulated cells negligibly (Fig. 2, Table I) [Misra et al., 2002] (data not shown). In contrast, LY393511, an inactive analogue of LY294002, did not affect $\alpha_2M^*$-induced levels of these proteins demonstrating both the specificity of inhibition and that Akt is a downstream effector of PI 3-kinase signaling (Fig. 3, Table I).

In Figure 4 are shown the effects of increasing concentrations of Wortmannin and LY294002 on the activation of regulatory subunit of PI 3-

**TABLE I. Modulation of Macrophage Signaling Components and Their Phosphorylation by $\alpha_2M^*$**

| Additions | Phosphorylated PI 3-kinase regulatory subunit | PDK1 | Akt protein | Akt Phosphorylated at Thr$^{308}$ | Akt Phosphorylated at Ser$^{473}$ | Phosphorylated p70s6k |
|---|---|---|---|---|---|---|
| | Arbitrary units × $10^4$ | | | | | |
| None | 715 ± 50 | 87 ± 18 | 473 ± 102 | 151 ± 20 | 381 ± 98 | 111 ± 5 |
| $\alpha_2M^*$ (100 pM/20 min) | 1538 ± 34[a] | 179 ± 13[a] | 1282 ± 71[a] | 410 ± 53[a] | 1428 ± 71[a] | 240 ± 21[a] |
| Manumycin (20 μM/16 h then $\alpha_2M^*$) | 658 ± 107 | 8 ± 2[b] | 65 ± 18[b] | 60 ± 10[b] | 149 ± 33[b] | 10 ± 3[b] |
| LY393511 (20 μM/15 min then $\alpha_2M^*$) | 1059 ± 53[a] | 165 ± 13[a] | 1150 ± 202[a] | 330 ± 10[a] | 1003 ± 59[a] | 304 ± 27[a] |
| LY294002 (20 μM/15 min then $\alpha_2M^*$) | 600 ± 71[b] | 189 ± 7[a] | 375 ± 32[b] | 40 ± 15[b] | 149 ± 33[b] | 28 ± 11[b] |
| Wortmannin (30 nM/30 min then $\alpha_2M^*$) | 422 ± 112[b] | 132 ± 35[a] | 262 ± 12[b] | 22 ± 8[b] | 93 ± 72[b] | 52 ± 9[b] |
| None | 1770 ± 160 | 1245 ± 130 | 1260 ± 118 | n.d. | n.d. | 1770 ± 175 |
| Mannumycin A (20 μM/16 h) | 1751 ± 150 | 1718 ± 160 | 2069 ± 350 | n.d. | n.d. | 1831 ± 175 |
| LY294002 (20 μM/15 min) | 1649 ± 180 | 1495 ± 160 | 2287 ± 210 | n.d. | n.d. | 2368 ± 250 |
| Wortmannin (30 nM/30 min) | 1898 ± 200 | 1775 ± 160 | 1140 ± 160 | n.d. | n.d. | 1708 ± 150 |
| Rapamycin | n.d. | n.d. | n.d. | n.d. | n.d. | 2156 ± 225 |

Values are then mean ± SE from at least 8–10 experiments analyzed by Western blot in triplicate in each case.
n.d., not done.
[a]Significantly different from buffer treated controls at the 5% level.
[b]Significantly different from $\alpha_2M^*$-treated cells at the 5% level.

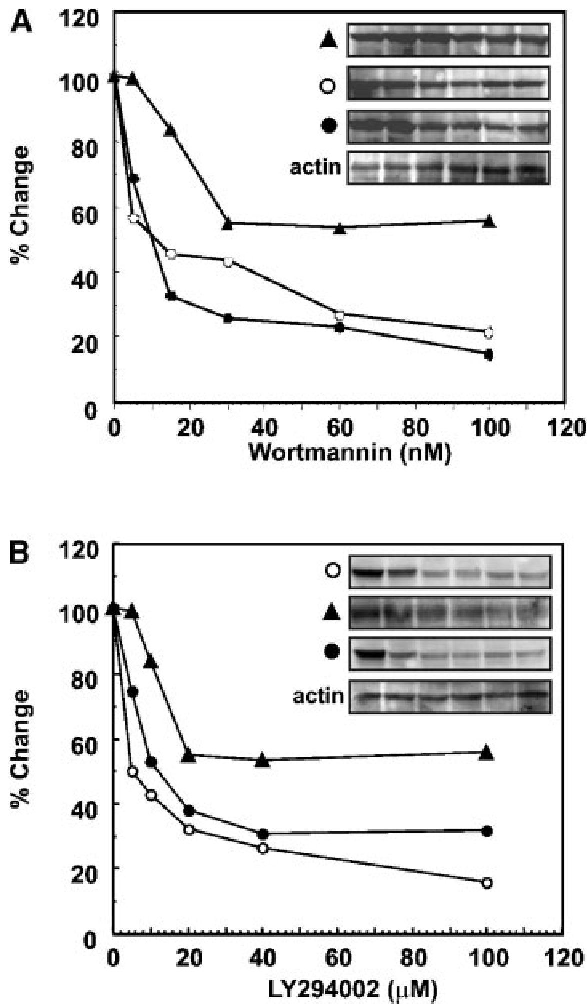


**Fig. 4.** Effect of increasing concentrations of LY294002 and Wortmannin on $\alpha_2$M*-induced changes in the levels of phosphorylated 85 kDa regulatory subunit of PI 3-kinase; and Akt phosphorylated at Thr$^{308}$ or Ser$^{473}$. **Panel A**: Effect of Wortmannin and (**Panel B**) the effect of LY29002 on: PI 3-kinase (–●–); Akt phosphorylated at Thr$^{308}$ (–▲–) or Ser$^{473}$ (–○–). The corresponding gels are shown in the inset. Values are expressed as percent change over $\alpha_2$M*-stimulated but uninhibited cells which has been considered as 100%. The graph is representative of at least 3 independent experiments. $\alpha_2$M*-stimulated PI 3-kinase, p-Akt$^{308}$, and p-Akt$^{473}$ activation in the absence of any inhibitor after 20 min of incubation has been considered as 100%. This activation is usually 2- to 3-fold higher than buffer treated cells (see Figs. 1 and 2 above) and the inhibition by LY294002 and Wortmannin has been calculated on this basis.

kinase and Akt phosphorylated at Ser$^{473}$ or Thr$^{308}$ in $\alpha_2$M*-stimulated macrophages. Increased phosphorylation of the 85 kDa regulatory subunit of PI 3-kinase was significantly inhibited by Wortmannin ($IC_{50} \sim 10$ nM) and LY294002 ($IC_{50} \sim 12$ μM) in a dose-dependent manner. These observations in $\alpha_2$M*-stimulated cells are similar to those reported in other cell systems. Increasing concentrations of Wortmannin and LY294002 also inhibited $\alpha_2$M*-induced elevations in the levels of Akt phosphorylated at Ser$^{473}$ or Thr$^{308}$ in a dose-dependent manner with nearly comparable $IC_{50}$ of ~10 nM and ~15 μM for Wortmannin and LY294002, respectively. These results are similar to those recently reported in L6 myocytes where insulin-induced elevation of Akt phosphorylation at both Ser$^{473}$ or Thr$^{308}$ exhibited a similar sensitivity to Wortmannin and LY294002 [Somwar et al., 2001]. Pretreatment of cells with Wortmannin (30 nM/30 min) or LY294002 (20 μM/15 min) did not inhibit $\alpha_2$M*-induced increase in the levels of PDK1 (Fig. 2, Table I) and these results are similar to those reported by others [Greenberg and Shaywitz, 1999; Vanhasebroeck and Alessi, 2000]. LY393511 (20 μM/15 min), did not affect $\alpha_2$M*-induced increase of the phosphorylated 85 kDa regulatory subunit of PI 3-kinase or Akt phosphorylated at Ser$^{473}$ or Thr$^{308}$ demonstrating the specificity of the PI 3-kinase inhibitors.

### Binding of $\alpha_2$M* to its Receptors Elevates the Levels of Phosphophorylated p70s6k

Since p70s6k is a critical component of the PI 3-kinase-dependent mitogenic signaling pathway, we next examined the levels of phosphorylated p70s6k in $\alpha_2$M*-stimulated cells (Fig. 3). $\alpha_2$M* increased the phosphorylation of p70s6k by 2- to 3-fold and this increase was significantly reduced by pretreatment of cells with manumycin A, LY294002, Wortmannin, or rapamycin (Fig. 3, Table I). These results demonstrate that activation of p70s6k by $\alpha_2$M* in macrophages is dependent upon PI 3-kinase activation.

### Involvement of CREB in $\alpha_2$M-Induced PI 3-Kinase/Akt/PDK/p70s6k Activation

We next probed the role of PI 3-kinase/Akt signaling in cAMP/CREB-dependent mitogenesis and cell proliferation observed in $\alpha_2$M*-stimulated macrophages by using RNA interference. This involved silencing the *CREB* gene with dsRNA homologous in sequence to the target *CREB* gene. The degree of transfection was evaluated by quantifying the levels of CREB mRNA and CREB phosphorylation at Ser$^{133}$ (Fig. 5, Table II). To demonstrate that transfection does not produce any non-specific effects on target gene expression, we have used transfection of cells with negative control

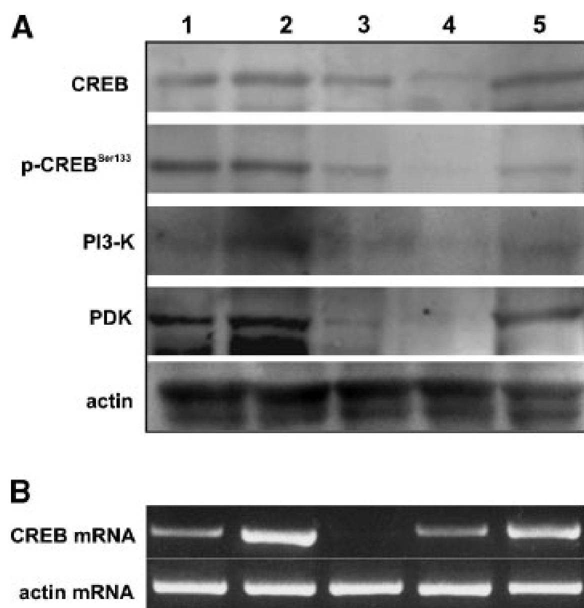


**Fig. 5.** Effect of silencing *CREB* gene expression with dsRNA homologous in sequence to the target gene on the levels/activation of signaling components. **Panel A**: The lanes are: (1) lipofectamine + buffer; (2) lipofectamine + $\alpha_2$M* (100 pM/20 min); (3) lipofectamine + CREB dsRNA (40 µg/ml); (4) lipofectamine + CREB dsRNA + $\alpha_2$M*; and (5) lipofectamine scramble RNA (40 µg) + $\alpha_2$M*. **Panel B**: CREB and mRNA levels in cells. The lanes are: (1) lipofectamine + buffer; (2) lipofectamine + $\alpha_2$M* (100 pM/20 min); (3) CREB dsRNA (40 µg/ml); (4) lipofectamine + CREB dsRNA + $\alpha_2$M*; and (5) lipofectamine + scrambled RNA (40 µg/ml) + $\alpha_2$M*. The results obtained from several such experiments are shown in Table II. Values are expressed as arbitrary units.

siRNA under identical experimental conditions (Fig. 5, Table II). Silencing of the *CREB* gene caused at least a 2- to 3-fold reduction of the levels and/or activation of PI 3-kinase, PDK1, Akt, and p70s6k (Figs. 5 and 6, Table II). These results suggest that CREB is regulating the activation of PI 3-kinase signaling pathway at transcriptional levels and/or there is also a cross-talk between CREB-induced mitogenic signaling and other signaling cascades. We also examined if silencing of *CREB* gene with RNA interference also affects the expression of other genes by probing the lysates from lipofectamine and lipofectamine + dsCREB RNA transfected cells for CREB, $ATF_2$, c-Jun, and heat shock protein HSP70 (Fig. 7). As expected silencing of *CREB* gene also suppressed expression of the related *$ATF_2$* gene, but had negligible effect on the expression of *c-Jun* and *HSP70* genes (Fig. 7). As judged by the levels of CREB mRNA, CREB and phosphorylated CREB protein levels

**TABLE II. Silencing of CREB and its Effects in $\alpha_2$M*-Treated Macrophages**

| Treatment | CREB protein | CREB phosphorylated at $Ser^{133}$ | Phosphorylated PI 3-kinase regulatory subunit | PDK1 | Akt protein | Akt Phosphorylated at $Thr^{308}$ | Akt Phosphorylated at $Ser^{473}$ | Phosphorylated p70s6k | CREB mRNA |
|---|---|---|---|---|---|---|---|---|---|
| | Arbitrary units at $\times 10^3$ | | | | | | | | Arbitrary units at $\times 10^4$ |
| Lipofectamine + buffer | $205 \pm 27$ | $383 \pm 12$ | $177 \pm 34$ | $161.0 \pm 16$ | $290 \pm 42$ | $867 \pm 59$ | $39 \pm 27$ | $772 \pm 37$ | 3236 |
| Lipofectamine + $\alpha_2$M* (100 pM) | $1469 \pm 66^a$ | $698 \pm 103^a$ | $1003 \pm 53^a$ | $396 \pm 35^a$ | $703 \pm 63^a$ | $1921 \pm 161^a$ | $252 \pm 158^a$ | $2873 \pm 60^a$ | 7784 |
| dsCREB RNA | $115 \pm 10^a$ | $188 \pm 10^b$ | $107 \pm 12^b$ | $84 \pm 9^b$ | $110 \pm 11^b$ | $77 \pm 18^b$ | $50 \pm 10^b$ | $806 \pm 100$ | 1091 |
| dsCREB RNA + $\alpha_2$M | $428 \pm 11^b$ | $122 \pm 37^b$ | $177 \pm 23^b$ | $74 \pm 9^b$ | $63 \pm 18^b$ | $498 \pm 154^b$ | $37 \pm 85^b$ | $938 \pm 151^b$ | 3709 |
| Scrambled RNA + $\alpha_2$M | $1071 \pm 204^a$ | $487 \pm 37$ | $1003 \pm 138^a$ | $331 \pm 90^a$ | $588 \pm 27^a$ | $1300 \pm 16^a$ | $227 \pm 151^a$ | $3031 \pm 305^a$ | 7219 |

Values are mean ± SE from 4 independent experiments analyzed by Western blot in triplicate in each case.
[a]Significantly different from buffer treated controls at the 5% level.
[b]Significantly different from $\alpha_2$M*-stimulated cells at the 5% level.

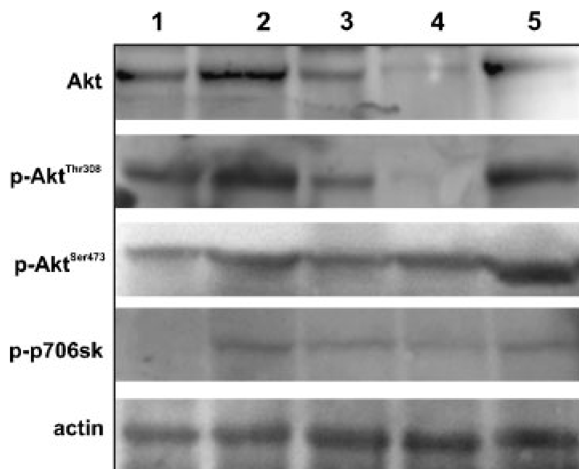


**Fig. 6.** Effect of silencing the expression of *CREB* gene with dsRNA homologous in sequence to the target gene on protein levels/activation of signaling components. The lanes are: (1) lipofectamine + buffer; (2) lipofectamine + $\alpha_2$M* (100 pM/20 min); (3) lipofectamine + CREB dsRNA (40 μg); (4) lipofectamine + CREB dsRNA (40 μg) + $\alpha_2$M*; and (5) lipofectamine + scrambled dsRNA (40 μg) + $\alpha_2$M*. The results obtained from several such experiments are shown in Table II. Values are expressed as arbitrary units.

in transfected cells (Fig. 5, Table II), the transfection of cells with dsRNA is ~60% and we conclude that the effects observed on signaling components were specifically due to the silencing of CREB.

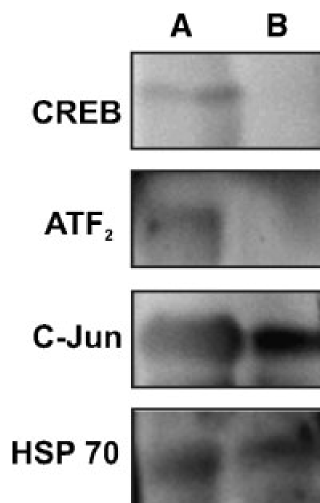


**Fig. 7.** Effect of silencing *CREB* gene on the expression of $ATF_2$, c-Jun, and HSP70. Overnight adhered macrophages were transfected with either lipofectamine (10 μl/ml) (**A**) or lipofectamine + dsCREB RNA (40 μg/ml) (**B**). The transfected cells were lysed and equal amounts of protein were used for PAGE. The gels were transferred onto membranes and the respective membranes were immunobloted with antibodies against CREB, c-Jun, and $ATF_2$ and heat shock protein HSP70 and protein bands visualized as described in the "Materials and Methods."

## DISCUSSION

In this study, we have employed RNA interference to suppress expression of the *CREB* gene as a means to examine the role of cAMP/CREB in PI 3-kinase/PDK/Akt/p70s6k signaling and its participation in $\alpha_2$M*-induced mitogenesis and cell proliferation. The major findings of this study are: (1) binding of $\alpha_2$M* to its receptors caused a 2- to 3-fold increase in the level of CREB mRNA, CREB, CREB phosphorylated at $Ser^{133}$, phosphorylation of the 85 kDa regulatory subunit of PI 3-kinase, Akt phosphorylated at $Ser^{473}$ or $Thr^{308}$, and phosphorylated p70s6k; (2) these effects were maximal between 10 and 20 min of incubation but declined at longer periods of incubation; (3) these effects were Wortmannin- and LY294002-sensitive, but not sensitive to LY303511, an inactive analogue of LY294004; (4) PDK1 levels were Wortmannin- and LY294002-insensitive but greatly reduced by manumycin A; (5) manumycin A nearly abolished $\alpha_2$M*-induced increases/activation of PI 3-kinase, Akt, and p70s6k; (6) pretreatment of cells with rapamycin greatly inhibited $\alpha_2$M*-dependent phosphorylation of p70s6k; and (7) silencing of the *CREB* gene with dsRNA homologous in sequence to the target *CREB* gene significantly reduced CREB mRNA and protein levels and suppressed $\alpha_2$M*-induced increases/activation of CREB, PI 3-kinase, and the remaining components of this pathway. The results unequivocally show that cAMP exerts its mitogenic and cell proliferative activity in $\alpha_2$M*-stimulated macrophages in a PI 3-kinase-dependent manner and these effects require the nuclear factor CREB. However, the interplay of a "cross-talk" between this pathway and other signaling pathways in the elicitation of $\alpha_2$M*-induced changes is not ruled out.

In 1993, we demonstrated that ligation of receptors on the surface of macrophages by $\alpha_2$M*-induced various signaling events [Misra et al., 1993]. We recently demonstrated that GRP78 in complex constitutes the $\alpha_2$MSR, however [Misra et al., 2002]. Activation of $\alpha_2$MSR by its ligation activates both Ras and PI 3-kinase-dependent signal transduction [Misra et al., 1994a,b, 1999a,b, 2002a,b; Howard et al., 1996; Misra and Pizzo, 1998a,b, 2002].

On the basis of the results presented in this communication, we hypothesize that binding of $\alpha_2$M* to GRP78 induces translocation of inac-

tive, cytoplasmic PI 3-kinase to the plasma membrane [Montminy, 1997; Downward, 1998; Du and Montminy, 1998; Chan et al., 1999; Kolch, 2000; Vanhasebroeck and Alessi, 2000; Blume-Jensen and Hunter, 2001]. Membrane localization is followed by PI 3-kinase activation and the synthesis of phosphatidyl inositol (3,4,5-) trisphosphate and phosphatidylinositol (3,4-) bisphosphate. These phosphoinositides then bind to PDK1 and Akt which induces their translocation and co-localization at the plasma membrane as well as a conformational change in Akt exposing the phosphorylation sites $Thr^{308}$ and $Ser^{473}$. For maximal $\alpha_2M^*$-induced activation of Akt both these sites are phosphorylated by PDK1 and PDK2, respectively. PDK1 phosphorylates PKC as well as several other $Ca^{2+}$-dependent protein kinases culminating ultimately in the onset of several intracellular signaling cascade and cellular responses [Misra et al., 1994a,b, 1999a,b, 2002a,b; Howard et al., 1996; Misra and Pizzo, 1998, 2002].

The present studies demonstrate a complex interplay between CREB and the PI 3-kinase signaling pathway with respect to the role of $\alpha_2M^*$ in promoting cellular proliferation. CRE-regulated gene transcription is a complex process involving CREB binding to CRE, CREB phosphorylation, and interaction with the coactivator protein CBP/p300 [Montminy, 1997; Greenberg and Shaywitz, 1999; Goodman and Smolik, 2000]. Upon phosphorylation at $Ser^{133}$, CREB can facilitate transcriptional activation of genes containing CRE motifs. Additional cell-specific or signal-specific regulation exists at the level of CRE-binding protein competition for the occupancy of CRE sites [Kelmm et al., 2001]. Furthermore, cAMP-induced cellular responses are affected by cross-coupling of the cAMP signaling pathway with other pathways. Several examples of such cross-talk between signaling pathways have been described [Montminy, 1997; Bornfeldt and Krebs, 1999; Greenberg and Shaywitz, 1999]. The current studies demonstrate the necessity of CREB expression and activation in transcriptional regulation of genes involved in the PI 3-kinase/PDK/Akt/p70s6k signaling pathway.

A number of growth factors and hormones stimulate the expressoin of cellular genes by inducing the phosphorylation of CREB at $Ser^{133}$ by PKA as well as other protein kinases [see Misra et al., 2002]. Genetic studies using transgenic and knock out mice demonstrate that CREB is important for cell survival [Dumont et al., 1989]. Effects of elevated intracellular cAMP levels are cell specific and are PKA-dependent or PKA-independent [Bornfeldt and Krebs, 1999]. The transcription factor CREB, is the target of PKA phosphorylation on $Ser^{133}$, and phosphorylated CREB binds to CBP, a general transcriptional coactivator for various signaling pathway which stimulates the transcription of cAMP responsive genes [Greenberg and Shaywitz, 1999]. cAMP exerts its mitogenic and cell proliferative effects via PI 3-kinase-dependent signaling [Cass et al., 1999; Greenberg and Shaywitz, 1999]. Interference with PKA activity or p70s6k activation impairs cAMP-stimulated DNA synthesis. Microinjection of PKA catalytic subunit fails to stimulate proliferation suggesting that additional PKA-independent signals contribute to cAMP-stimulated cell cycle progression [Cass et al., 1999]. cAMP elevating agents stimulate Akt activity through a PI 3-kinase-dependent pathway distinct from PKA-dependent pathway [Cass et al., 1999]. Treatment with PKA inhibitors does not impair cAMP-dependent Akt activation though these inhibitors abolish p70s6k phosphorylation. Wortmannin, a PI 3-kinase inhibitor, abolishes cAMP effects on Akt activation, but has no effect on p70s6k phosphorylation [Bolster et al., 2002]. The other cAMP-dependent but PKA-independent pathway involved in the activation of PI 3-kinase and its downstream effects is Epac (exchange proteins activated by cAMP) [Grewal et al., 2000; Richard, 2001]. We have examined the involvement of PI 3-kinase-dependent signaling in cAMP-induced mitogenic signaling in macrophages by silencing the expression of *CREB* gene using RNA interference. The reductions in the levels of components of PI 3-kinase pathway do show the transcriptional upregulation of this pathway by CREB. However, the role of cross-talk between cAMP signaling and various other signaling pathways in the regulation of $\alpha_2M^*$-induced mitogenic and cell proliferative signaling is not ruled out.

In summary, employing RNA interference, we have established that $\alpha_2M^*$-induced regulation of CREB is coupled to the activation of the PI 3-kinase/Akt/PDK/p70s6k signaling cascade in macrophages and is an essential mechanism amplifying our understanding of the recently established relationship between $\alpha_2M^*$ induction of macrophage proliferation and CREB

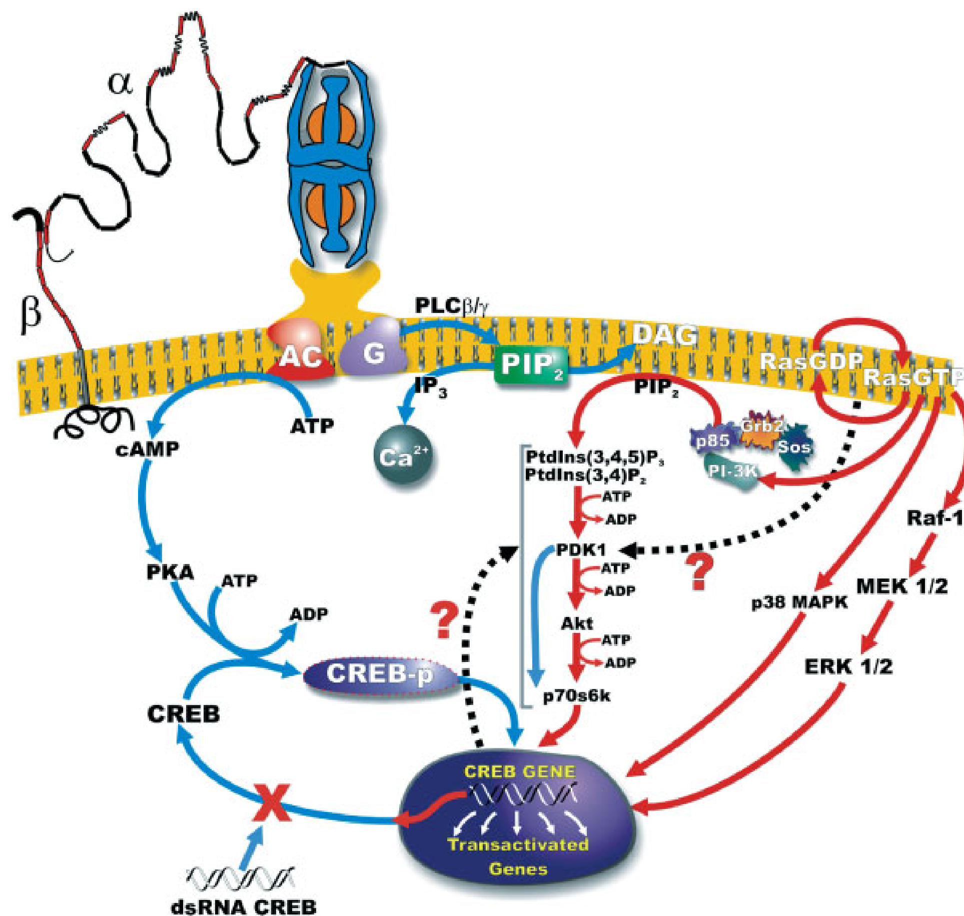


**Fig. 8.** A schematic representation of the relationship between CREB and the PI 3-kinase PDK/Akt/p70s6k signaling in $\alpha_2$M* stimulated cells. The role of the MAPK pathway has been established previously in this system [Misra et al., 2002].

[Misra et al., 2002]. Specifically, we propose that CREB activation regulates one or more components of this pathway. Figure 8 is a schematic representation of these results.

## ACKNOWLEDGMENTS

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