The Role of cAMP-dependent Signaling in Receptor-recognized Forms of α2-Macroglobulin-induced Cellular Proliferation*

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Ligation of α2-macroglobulin receptors by receptor-recognized forms of α2-macroglobulin (α2M*) activates various signaling cascades and promotes cell proliferation. It also elevates cAMP in murine peritoneal macrophages. We now report that a significant elevation of cAMP-response element-binding protein (CREB) occurs in α2M*-stimulated cells, and this effect is potentiated by isobutylmethylxanthine, dibutyryl-cAMP, or forskolin. An α2M* concentration-dependent rapid increase in phosphorylated CREB at Ser133 also occurred, a necessary event in its activation. Inhibition of Ca2+/calmodulin kinase, protein kinases A and C, tyrosine kinases, ribosomal S6 kinase, farnesyl transferase, extracellular signal-regulated kinases 1/2, phosphatidylinositol 3-kinase, or p38 mitogen-activated protein kinase markedly reduce α2M*-induced phosphorylation of CREB, indicating a role for the p21ras-dependent and phosphatidylinositol 3-kinase signaling pathways in regulating CREB activation by α2M*. Finally, silencing the CREB gene by transfecting cells with a homologous gene sequence double-stranded RNA drastically reduced the expression of CREB and blocked the ability of α2M* to promote macrophage cell division. We conclude that cAMP-dependent signal transduction as well as other signaling cascades are essential for α2M*-induced cell proliferation.

α2-Macroglobulin (α2M) is part of a large superfamily that includes proteinase inhibitors and complement components (1). α2M is a homotramer, and, like C3 and C4, each subunit contains a β-cysteinyl-γ-glutamyl thiolester (2, 3). Upon reaction of α2M with proteinases, the thiolesters rupture, and the molecule undergoes a large conformational change (2, 3). This exposes a cryptic determinant located in the carboxy-terminal domain of each subunit, which constitutes the receptor recognition site (2, 3). Direct reaction of the thiolesters with small nucleophiles, such as NH3 or CH3NH2 also triggers exposure of the receptor recognition sites (2, 3). α2M binds to the low density lipoprotein receptor-related protein and to the α2M signaling receptor (α2MSR), which appears to consist of a coreceptor complexed to lipoprotein receptor-related protein (4–11). Binding of α2M* to α2MSR activates a pertussis toxin-insensitive phospholipase C, which hydrolyzes membrane phosphoinositides, generating two second messengers, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 raises cytosolic free Ca2+, [Ca2+]i, by releasing Ca2+ sequestered in the endoplasmic reticulum, thus triggering the onset of several Ca2+-dependent signaling cascades (4–13). DAG, on the other hand, activates protein kinase C (PKC), thus triggering the activation of phosphorylation-dependent signaling components. Ligation of α2MSR induces DNA and protein synthesis, which is Ca2+-dependent and requires participation of activated tyrosine kinases, p21ras-dependent MAPK, and PI 3-kinase signaling cascades (4–18, 12–17). Treatment of macrophages with α2M* also causes a 2–2.5-fold increase in cell number (10).

cAMP-response element-binding protein (CREB) is a nuclear transcription factor which is a downstream target of cAMP signaling (18, 19). Protein kinase A (PKA) phosphorylates CREB at Ser133 within the kinase-inducible domain (18, 19). This increases its transcriptional activity by promoting its association with CREB-binding protein, leading to activation of the transcriptional machinery. CREB also can be phosphorylated at Ser133 by multiple signaling mechanisms including ERK 1/2, PKC, Ca2+/calmodulin-dependent protein kinases, p38 MAPK, and ribosomal S6 kinase (p70s6k) (18–27).

MAPKs activate CREB kinase (p90s6k), which in turn phosphorylates and activates CREB. To elucidate the role of cAMP signaling in cellular physiology and homeostasis, several studies have used genetic manipulations in intact animals and cell systems. These include gene knockout and gene overexpression. In the last several years, the use of posttranscriptional gene silencing and RNA interference techniques have been employed to block protein expression in a variety of in vitro systems (28–36). The techniques of RNA interference employ sequence-specific posttranslational gene silencing in animals and plants initiated by double-stranded RNA that is homologous in sequence to the silenced gene (28–36). The mediators of sequence-specific messenger RNA degradation are 21–23-nt small interfering RNA fragments generated by ribonuclease III cleavage from longer double-stranded RNAs (dsRNAs) (31, 32). To date, these techniques have not been employed with primary macrophages.

α2M* binding to macrophages significantly raises cAMP levels (12); therefore, we examined the role of CREB in α2M*-induced macrophage proliferation. We studied phosphorylation of CREB and the protein kinases involved in its phosphoryla-

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§ The abbreviations used are α2M, α2-macroglobulin; α2M*, receptor-recognized forms of α2M; α2MSR, the α2M* signaling receptor; IP3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; PI, phosphatidylinositol; HHBSS, Hanks’ balanced salt solution containing 10 mM HEPES, pH 7.4, and 3.5 mM NaHCO3; BSA, bovine serum albumin; CREB, cAMP response element binding protein; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal protein; PKA, protein kinase A; p70s6k, ribosomal S6 kinase; dsRNA, double-stranded RNA; IBMX, isobutylmethylxanthine; SH2 and -3, Src homology 2 and 3, respectively; BAPTA/AM, 1,2-bis-(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid acetoxymethyl ester; DEME, Dulbecco’s modified Eagle’s medium; ECF, enhanced chemiluminescence.
tion, and analyzed Ras family members in murine macrophages treated with either αM or cAMP-elevating agents. We report in the current study that treatment of macrophages with αM elevated the levels of CREB as well as phosphorylated CREB and caused a 1.5–2-fold increase in macrophage cell number at 24 h of incubation. These effects were potentiated by dibutyryl-cAMP, IBMX, or forskolin. The maximal phosphorylation of CREB in αM-treated cells occurred at ~10–20 min of incubation. αM elevated phosphorylation of ERK 1/2 and other MAPKs as well as Rap-1, Raf-1, Raf-B, and p70S6k protein levels. These effects were potentiated by dibutyryl-cAMP or forskolin treatment of cells. Pharmacological intervention with agents that affect various protein kinases affected phosphorylation of CREB, [3H]thymidine incorporation, and cellular growth. To elucidate further the role of CREB, the target of cAMP signaling, in the proliferation of αM-stimulated peritoneal macrophages, we have transfection macrophages with dsRNA homologous in sequence to the CREB gene and have measured various parameters of cell macrophage proliferation.

To our knowledge, this is the first use of RNA interference in a primary cell line; we find that silencing of the CREB gene in these αM-stimulated cells drastically reduces cell proliferation. We thus show here that the mitogenic and cell proliferative responses of murine peritoneal macrophages treated with αM are primarily mediated by cAMP and cAMP-dependent signaling cascades.

**EXPERIMENTAL PROCEDURES**

**Materials**—Culture media were from Invitrogen. Dibutyryl-cAMP, fatty acid-free bovine serum albumin (BSA), and actinomycin D were from Sigma. Forskolin, IBMX, PD98059, SB203580, wortmannin, LY294002, chelerythrin, genistein, rapamycin, manumycin A, H-89, KN-62, and cycloheximide were procured from Biomol (Plymouth Meeting, PA). [3H]Thymidine (specific activity, 71.5 Ci/mmol) was from American Radiochemicals, Inc. (St. Louis, MO). Antibodies against CREB, Rap-1, Raf-1, Raf-B, p70S6k, Grb2, Sos 1/2, and She were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against CREB phosphorylated at Ser-133 and phosphorylated ERK 1/2, p38 MAPK, and JNK were procured from Cell Signaling Technology, Inc. (Beverly, MA). Antibodies against tyrosine and threonine phosphorylated CREB were procured from Zymed Laboratories, Inc. (South San Francisco, CA). Lipo-ACtIVINE was procured from Invitrogen. αM was prepared as described previously (4–7). Other reagents of the highest available grade were procured locally.

**Determination of CREB by Western Blotting in Macrophages Treated with cAMP-elevating Agents**—This protocol has been described in detail elsewhere (4, 5, 12). Briefly, macrophages (2 × 10⁶ cells/well) were incubated overnight in RPMI 1640 medium containing 0.2% fatty acid-free BSA. The cells were washed twice with HHBSS, and a volume of medium was added, followed by the additions of αM (100 μM), dibutyryl-cAMP (1 μM), forskolin (20 μM), and IBMX (100 μM), either alone or in combination with αM in separate experiments. The cells were incubated for 20 min at 37°C in a humidified CO₂ (5%) incubator. The reaction was terminated by aspirating the medium. The monolayers were washed twice with HHBSS, and a volume of RPMI medium was added, followed by the addition of [3H]thymidine. To the respective wells, αM (100 μM) or forskolin (20 μM) either alone or together were added. In experiments where the effect of KN-62 (1 μM/15 min), rapamycin (50 μM/15 min), PD98059 (50 μM/90 min), SB203580 (15 μM/30 min), manumycin A (10 μM/60 min), genistein (20 μM/16 h), wortmannin (30 μM/30 min), LY294002 (25 μM/15 min), chelerythrin (200 μM/15 min), actinomycin D (5 μg/ml/10 min), cycloheximide (10 μg/ml/10 min), or BAPTA/AM (10 μM/30 min) were studied, these were added to their respective wells, and cells were incubated for the specified time before adding αM or forskolin. The cells were incubated overnight in a humidified CO₂ (5%) incubator. The incubations were terminated by aspirating the medium and washing macrophages twice with HHBSS, followed by tritiated thymidine acid (15 min/3°C) and then incubated with [3H]thymidine for 16 h in the presence of HHBSS. The monolayers were lysed with 1 N NaOH, and an aliquot was used for liquid scintillation counting and protein estimation (8, 10, 17).

**Measurement of [3H]Thymidine Uptake by Macrophages Exposed to Forskolin**—Murine peritoneal macrophages (4 × 10⁶ cells/well) in 48-well plates, 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 incubations were terminated by aspirating the medium and washing macrophages twice with HHBSS, followed by [3H]thymidine acid (15 min/3°C) and then incubated with [3H]thymidine for 16 h in the presence of HHBSS. The monolayers were lysed with 1 N NaOH, and an aliquot was used for liquid scintillation counting and protein estimation (8, 10, 17).
EXPERIMENTAL PROCEDURES

Peritoneal macrophages were harvested and allowed to adhere in four-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 a volume of RPMI 1640 medium containing 0.2% fatty acid-free BSA, and 1-ml aliquots of 1 × 10^6 cells were pipetted into 15-ml siliconized polypropylene tubes. To the respective tubes, αM* (100 µm), forskolin (20 µm), or αM+ (100 µm) with forskolin (20 µm) was added, and the contents were gently mixed and incubated for 24 and 48 h as above. After the specified period of incubation, an aliquot was removed from each tube, trypsin blue was added, and the contents were gently shaken during incubation for 2 min. A 10-µl aliquot was then employed for counting the number of cells in a hemocytometer. The cell numbers were corrected for dead cells. Changes in the morphology and macrophage number before and after treatment with αM*, forskolin, or αM+ with forskolin at 24 and 48 h were determined by phase-contrast microscopy. For these studies, an equal number of macrophages adhered for 2 h were pipetted into six-well plates and incubated as above. After the specified periods of incubation with the agents, the cells were examined under a phase-contrast microscope (20×/10, 47).

Western Blotting of Phosphorylated ERK 1/2, p38 MAPK, and JNK in Macrophages Stimulated with αM* and Forskolin—Freshly harvested peritoneal macrophages in RPMI 1640 medium containing glutamine, penicillin, streptomycin, and 5% fetal bovine serum were allowed to adhere to the wells of a six-well plate (3 × 10^5 cells/well) for 2 h as above. The monolayers were washed twice with HHBSS; a volume of RPMI 1640 medium containing 0.2% fatty acid-free BSA was added; and the cells were treated with αM* (100 µm/20 min), forskolin (20 µm/20 min), or αM+ with forskolin. The incubations were terminated by aspirating the medium. The lysis of cells, their electrophoresis, and Western blotting with respective antibodies against phosphorylated ERK 1/2, p38 MAPK, or JNK were performed according to the manufacturer’s instructions. In each case, an equal amount of protein was used for electrophoresis. The detection of phosphorylated proteins by ECF and quantification of their distribution were performed as above (10, 47).

Western Blotting of Grb2, Sos, and She Proteins in Macrophages Exposed to αM* and Forskolin—These studies were performed as described above. The detection of Grb2, Sos 1/2, and She by ECF and quantification of their distribution were performed by PhosphorImager.

Western Blotting of p70S6K, Rap-1, Raf-1, and Raf-B Proteins in Macrophages Exposed to αM* and Forskolin—These studies were performed as described above. The detection of p70S6K, Raf-1, Rap-1, and Raf-B by ECF and quantification of their distribution were performed by PhosphorImager.

Western Blotting of c-Fos Protein in Macrophages Treated with αM* and Forskolin—These studies were performed as described above. The detection of a c-Fos protein by ECF and quantification of their distribution were performed by PhosphorImager.

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 nucleotides 324–344 (5'-AGAGACAACAGAGAAUGA(U)-3'; sequence ID 173; Austin, TX). For making dsRNA, the sense (5'-AGAGACAACAGAGAAATGATA-3'; SWISS-PROT, entry name ATFB 344 (5'-AGAGACAACAGAGAAUGA(U)-3'; sequence ID 173; Austin, TX). For making dsRNA, the sense (5'-AGAGACAACAGAGAAATGATA-3'; SWISS-PROT, entry name ATFB) and antisense (5'-AUUA(U)AGUGUCUGCT-3') oligonucleotides were annealed according to the manufacturer’s instructions. Throughout the entire period of experiment, 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 was stored at −20 °C before use.

Transfection of Murine Peritoneal Macrophages, Stimulation with αM*, and Western Blotting of CREB and Thymidylate Synthase Proteins—TG-elicted murine peritoneal (1 × 10^6 cells/well) were plated as above and allowed to adhere for 2 h in RPMI 1640 medium containing 10% fetal bovine serum, penicillin (12.5 units/ml), streptomycin (6.5 µg/ml), and 2 mM glutamine at 37 °C in a 5% humidified incubator at 37 °C. The nonadherent cells were aspirated, monolayers were washed twice with HHBSS, 2 ml of DMEM containing 10% fetal bovine serum and the above antibiotics was added, and the cells were incubated at 37 °C for 48 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, layered in each well with 1 ml of LipofectAMINE-DMEM (10 µl/ml) or lipid-dsRNA.
mixtures, containing different amounts of dsRNA, gently mixed, and incubated for 5 h at 37 °C in a humidified CO₂ incubator. At the end of the incubation, 1 ml of antibiotic-free DMEM containing 10% fetal bovine serum was added to each well, and cells were incubated for 16 h as above. Microscopic observation of the monolayers did not show evidence of toxicity. The medium was replaced with DMEM containing antibiotics and 10% fetal bovine serum 24 h after the start of transfection. The cells were detached with trypsin-EDTA (0.5%) and centrifuged for changes in cell morphology. The cell numbers were corrected for dead cells (10).

**RESULTS**

α₂M* and cAMP-elevating Agents Increase CREB in Macrophages—CREB is a member of a family of factors that regulate transcription by binding to sequences in gene promoters (18, 19). CREB is a downstream target once cAMP is elevated, and it becomes functionally activated upon phosphorylation at Ser-133 (18, 19). CREB is a member of a family of factors that regulate phages—

**Fig. 3. Levels of phosphorylated CREB in macrophages stimulated with different cAMP-elevating agents.** The levels of phosphorylated CREB were determined by Western blotting using antibodies against Ser-133-phosphorylated CREB and quantified by a PhosphorImager. Bar 1, buffer; bar 2, α₂M* (100 μM); bar 3, dibutyryl-cAMP (1 mM); bar 4, dibutyryl-cAMP and then α₂M*; bar 5, IBMX (100 μM); bar 6, IBMX and then α₂M*; bar 7, forskolin (20 μM); bar 8, forskolin, and then α₂M*. A representative Western blot is shown at the bottom of the corresponding bar graph. Values are mean ± S.E. from 3–4 independent experiments performed in triplicate and are expressed as arbitrary units.

**Fig. 4. Effect of various protein kinase inhibitors on α₂M*-induced levels of Ser-133-phosphorylated CREB in macrophages.** The levels of phosphorylated CREB were determined by Western blotting using antibodies against Ser-133-phosphorylated CREB and quantified by a PhosphorImager. Representative corresponding Western blots are shown below their respective bar graphs. A, bar 1, buffer; bar 2, α₂M* (100 μM/20 min); bar 3, chelerythrin (200 nM/15 min) and then α₂M*; bar 4, H-89 (10 μM/90 min) and then α₂M*; bar 5, KN-62 (1 μM/1 h) and then α₂M*; bar 6, BAPTA/AM (10 μM/30 min) and then α₂M*; bar 7, okadaic acid (50 nM/15 min) and then α₂M*; bar 8, B, 1, buffer; bar 2, α₂M* (100 μM); bar 3, PD98059 (50 μM/90 min) and then α₂M*; bar 4, SB 203580 (25 μM/30 min) and then α₂M*; bar 5, wortmannin (30 μM/30 min) and then α₂M*; bar 6, LY294002 (15 μM/15 min) and then α₂M*; bar 7, genistein (20 μM/16 h) and then α₂M*; bar 8, manumycin A (15 μM/60 min) then α₂M*. Values are means ± S.E. from three independent experiments performed in triplicate expressed as arbitrary units.

with α₂M* promoted phosphorylation of Ser-133 in CREB. The effect of stimulating macrophages with increasing concentrations of α₂M* (0–20 nM) on the levels of phosphorylated CREB is shown in Fig. 2B. The maximal phosphorylation of CREB occurred at α₂M* concentrations of 50–100 pM (Fig. 2B). Stimulation of macrophages with α₂M* after pretreatment with dibutyryl-cAMP, IBMX, or forskolin elevated the levels of CREB phosphorylated at Ser-133 significantly when compared with buffer-treated cells (Fig. 3).

**Modulation of CREB Phosphorylation at Ser-133**—The maximal increase in cAMP levels in α₂M*-treated cells was observed between 15 and 20 min after α₂M* treatment (12); however, maximal CREB phosphorylation also occurred between 10 and 15 min (Fig. 2A). These data suggest that in addition to PKA, other protein kinases activated via IP₃-dependent signaling cascades functioning before the levels of cAMP are elevated are involved in the phosphorylation of CREB (18–27). We therefore next studied the modulation of CREB phosphorylation. Chelation of intracellular Ca²⁺ with BAPTA/AM prevented agonist-induced CREB phosphorylation (Fig. 4A). Treatment of macrophages with chelerythrin, a specific inhibitor of PKC, with H-89, a specific inhibitor of PKA, or
with KN-62, a specific inhibitor of Ca\textsuperscript{2+}/calmodulin kinase, before stimulation with \(\alpha\text{M}^*\) nearly abolished \(\alpha\text{M}^*\)-induced CREB phosphorylation at Ser-133 (Fig. 4A). Likewise, treatment of macrophages with PD98059, a specific inhibitor of ERK 1/2, with SB203580, a specific inhibitor of p38 MAPK, and manumycin A, an inhibitor of farnesyl transferase, before stimulation with \(\alpha\text{M}^*\) significantly inhibited CREB phosphorylation compared with buffer-stimulated cells (Fig. 4B).

\(\alpha\text{M}^*\) and Forskolin Treatment of Cells Elevates the Levels of Phosphorylated ERK 1/2, p38 MAPK, JNK, and p70s6k Protein—Both \(\alpha\text{M}^*\) and forskolin raised levels of phosphorylated ERK 1/2 (Fig. 5A), phosphorylated p38 MAPK (Fig. 5B), and phosphorylated JNK (Fig. 5C) by about 1.5–2-fold. Cell stimulation with \(\alpha\text{M}^*\) or forskolin also raised the levels of ribosomal kinase p70s6k (Fig. 5D).

\(\alpha\text{M}^*\) and Forskolin Elevate \(^{3}\text{H}\)Thymidine Uptake into DNA—We studied the contribution of cAMP signaling to \(\alpha\text{M}^*\)-induced cell proliferation and DNA synthesis by quantifying the incorporation of \(^{3}\text{H}\)thymidine into DNA. We also determined macrophage cell number and studied the morphology of cells treated as above and incubated for 24 and 48 h under identical conditions. We compared these effects with those induced by forskolin, an established cAMP-elevating agent (Figs. 6–8). \(\alpha\text{M}^*\)-induced macrophages, like forskolin, increased \(^{3}\text{H}\)thymidine uptake by about 2-fold as compared with buffer-treated cells (Fig. 6A). When macrophages were stimulated with both \(\alpha\text{M}^*\) and forskolin, the \(^{3}\text{H}\)thymidine uptake was nearly additive (Fig. 6A). The \(^{3}\text{H}\)thymidine incorporation into DNA of macrophages stimulated with \(\alpha\text{M}^*\) and forskolin was significantly reduced by pretreating the cells with KN-62, an inhibitor of Ca\textsuperscript{2+}/calmodulin kinase (40); rapamycin, an inhibitor of p70s6k (48); PD98059, an inhibitor of ERK 1/2; SB 203580, an inhibitor of p38 MAPK; manumycin A, an inhibitor of farnesyl transferase required for membrane attachment of Ras; genistein, an inhibitor of tyrosine kinases, chelerythrin, an inhibitor of PKC; BAFA/AM, a chelator of intracellular Ca\textsuperscript{2+}; actinomycin D; and cycloheximide (Fig. 6B).

These results suggest that cAMP-dependent signaling as well as the p21\textsuperscript{rca1} and PI 3-kinase-dependent pathways (9, 10, 12) are involved in \(\alpha\text{M}^*\)-induced macrophage proliferation.

\(^{3}\text{H}\)Thymidine uptake may indicate enhanced DNA synthesis, but there are potential mechanisms of enhanced uptake independent of new synthesis of nucleic acids. We therefore also studied the effect of \(\alpha\text{M}^*\), dibutyryl-cAMP, and forskolin on cell morphology (Fig. 7) and macrophage cell number (Fig. 8 and Table I) at 24 and 48 h of incubation. Like \(^{3}\text{H}\)thymidine uptake, macrophages treated with \(\alpha\text{M}^*\) or forskolin showed a 1.5–2-fold increase in cell numbers compared with buffer-stimulated cells at 24 h (Fig. 8 and Table I). The decrease in cell numbers observed at 48 h of incubation was largely due to cell death as evident by increased trypan blue uptake (Fig. 7 and Table I). Pretreatment of cells treated with H-89 or KN-62 inhibited \(\alpha\text{M}^*\)- or forskolin-induced increase in cell number (Table I). The \(\alpha\text{M}^*\)- or forskolin-treated macrophages showed increased numbers, were enlarged, and exhibited a stellate morphology at 24 h compared with buffer-treated macrophages, but by 48 h the cell number decreased drastically, and morphology also changed (Figs. 7 and 8 and Table I).

\(\alpha\text{M}^*\) and Forskolin Induce Expression of the c-fos Gene—Expression of c-fos is part of a mitogenic response that is required for cell proliferation. Transcription of the c-fos gene is regulated in part by CREB (18, 19). Increased [Ca\textsuperscript{2+}], can activate c-fos transcription through CREB phosphorylation. To understand the role of cAMP signaling in early response gene expression, we tested the expression of the c-Fos protein by Western blotting in macrophages stimulated with \(\alpha\text{M}^*\) or forskolin (Fig. 9A). Both \(\alpha\text{M}^*\) and forskolin, which increased levels of phosphorylated CREB (Fig. 3), also increased the levels of c-Fos protein by about 2-fold compared with buffer-treated cells (Fig. 9A).

\(\alpha\text{M}^*\) and Forskolin Elevate the Levels of Grb2, Sos, Shc, and the Small G Protein Rap-1 in Macrophages—Receptor tyrosine kinases propagate intracellular signals by coupling to multiple signal transduction pathways. Many of these pathways are mediated by interactions with SH2 and SH3 domain-contain-
buffer; bar 2

pressed as fmol of [3H]thymidine uptake/mg of protein. [3H]Thymidine incorporation in macrophages treated with various inhibitors only was either equal to the basal uptake or slightly lower.

incorporation into DNA and its modulation by inhibitors of protein kinases. Experimental details are described under “Experimental Procedures.” A, bar 1, buffer; bar 2, αM* (100 pM); bar 3, dibutyryl-cAMP (1 mM); bar 4, dibutyryl-cAMP and then αM*; bar 5, forskolin (20 μM); bar 6, forskolin and then αM*; bar 7, H-89 (10 μM/2 h) and then forskolin; bar 8, KN-62 (1 μM/1 h) and then forskolin. B, bar 1, buffer; bar 2, forskolin (20 μM/25 min); bar 3, rapamycin (100 nM/20 min) and then forskolin; bar 4, wortmannin (30 nM/30 min) and then forskolin; bar 5, PD98059 (50 μM/90 min) and then forskolin; bar 6, BAPTA/AM (10 μM/30 min) and then forskolin; bar 7, chelerythrin (200 nM/20 min) and then forskolin; bar 8, genistein (20 μM/16 h) and then forskolin; bar 9, actinomycin D (5 μg/ml/20 min) and then forskolin; bar 10, cycloheximide (10 μg/ml/20 min) and then forskolin; bar 11, SB203580 (20 μM/30 min) and then forskolin; bar 12, manumycin A (20 μM/60 min) and then forskolin. The values are means ± S.E. from two independent experiments performed in quadruplicate and are expressed as fmol of [3H]thymidine uptake/mg of protein. [3H]Thymidine incorporation in macrophages treated with various inhibitors only was either equal to the basal uptake or slightly lower.

ing proteins (49–51). Molecules implicated in signal transduction pathways containing the SH2 domain include phospholipase Cγ, PT 3-kinase, and GTPase-activating proteins of Ras (51–53). Ras plays a central role in signaling a variety of cellular responses including cell proliferation and differentiation (51, 53–56). Ras is connected to receptor tyrosine kinase through adaptor protein Grb2, containing two SH3 domains and one SH2 domain, and Sos, a guanine nucleotide exchange factor (51, 53–56). The SH2 domain of Grb2 provides a site for interaction with tyrosine-phosphorylated proteins, and Sos functions as an activator of Ras (53–57). Another SH2 domain-containing docking protein, Shc, primarily a cytosolic protein that becomes tyrosine-phosphorylated and translocates to membranes in response to growth factors, associates with Grb2/Sos (58–61). Shc, therefore, could provide an alternative mechanism of coupling to Ras and may amplify or modulate the signaling input from receptor tyrosine kinases to Ras. To understand the mechanism of cAMP-induced proliferation of macrophages, under our experimental conditions, we assayed the levels of Grb2, Sos, Shc, Raf-1, Rap-1, and Raf-B by Western blotting (Figs. 9 and 10). αM* and forskolin either alone or in combination increased the levels of Grb2, Sos, and Shc (Fig. 9, B–D). We have shown earlier that exposure of peritoneal macrophages to αM* elevated the levels of RAS-GTP by about 2–2.5-fold (16). In the next series of experiments, we quantified the levels of signaling components downstream to Ras, namely Raf-1, Rap-1, and Raf-B (Fig. 10, A–C). Treatment of macrophages with αM* raised the levels of Raf-1, whereas forskolin treatment either alone or with αM* decreased Raf-1 expression (Fig. 10, A and B). Since Ras and Raf-1 are physically associated, one possible explanation for the decreased levels of Raf-1 in the forskolin group may lie in the decreased stability of Raf-1 due its uncoupling from Ras as a result of PKA phosphorylation of Raf-1 (55, 62–67). Treatment of macrophages with αM* raised the levels of Rap-1 and Raf-B by about 1.5–2-fold, but forskolin either alone or with αM* raised the levels of Rap-1 by about 4–5.5-fold (Fig. 10, B and C). These results show that αM*-induced macrophage proliferation uti-
lizes predominantly MAPK activation through Ras/Raf-1 signaling, whereas forskolin-induced cell proliferation utilizes largely Rap-1/Raf-B signaling for MAPK activation. Thus, H9251 2M* utilizes both the IP3/Ca2+ dependent signaling (early phase) as well as cAMP-dependent signaling (late phase) to achieve mitogenesis and cell proliferation.

Transfection of Cells with dsRNA Homologous in Sequence to CREB Gene Blocks H9251 2M*-induced Cell Proliferation—At both concentrations of dsRNA (10 and 50 μg/ml) employed, expression of the CREB gene was significantly inhibited (70%) (Fig. 11), as was its phosphorylation (Fig. 12). Macrophages in which CREB gene expression was silenced no longer were responsive to H9251 2M* stimulation with respect either to CREB level or its phosphorylation (Figs. 11 and 12). Similar results were observed when forskolin was employed as a stimulant (data not shown). We next evaluated the role of cAMP-CREB signaling in α2M*-induced cell proliferation in macrophages after silencing the CREB gene with sequence-homologous dsRNA. Cell number was determined 24 h after α2M* treatment of transfected cells (Table II). Transfection of cells with dsRNA (10 or 50 μg/ml) nearly abolished α2M*-induced cell proliferation (Table II). Microscopic examination of the cells as well as trypan blue uptake did not show toxic effects secondary to transfection. Cells transfected with LipofectAMINE alone showed no effects on cell morphology, cell shape, or spreading. In contrast, cells transfected with the LipofectAMINE-dsRNA complex were largely round and showed no spreading (Fig. 13). These changes in cell morphology are similar to those observed in other cell types transfected with dsRNA (29–36). These results conclusively demonstrate that CREB and cAMP signaling are of crucial importance in H9251 2M*-induced proliferation of murine peritoneal macrophages.

Silencing the CREB Gene Blocks Up-regulation of Thymidylate Synthetase Induced by H9251 2M*—To further examine the cell-proliferative role that CREB plays in macrophages stimulated with α2M*, we quantified the levels of thymidylate synthetase, a critical enzyme involved in DNA synthesis under these experimental manipulations (Fig. 14). An appreciable amount of thymidylate synthetase protein is observed in macrophage, and H9251 2M* stimulation nearly doubles the amount

Table I

| Treatment                      | 0-h incubation | 24-h incubation | 48-h incubation |
|-------------------------------|----------------|-----------------|-----------------|
| Buffer alone                  | 325 ± 28       | 254 ± 28        | 301 ± 29        |
| α2M* (100 pM)                 | 605 ± 45       | 240 ± 28        | 220 ± 25        |
| Dibutyryl-cAMP (1 mM)         | 478 ± 35       | 220 ± 35        | 210 ± 25        |
| Dibutyryl-cAMP then α2M*M     | 550 ± 50       | 245 ± 29        | 230 ± 30        |
| Forskolin (20 μM)             | 506 ± 43       | 270 ± 30        | 250 ± 29        |
| Forskolin then α2M*M          | 680 ± 55       | 325 ± 28        | 290 ± 30        |
| H-89 (10 μM)                  | 350 ± 35       | 283 ± 25        | 260 ± 25        |
| H-89 then forskolin           | 362 ± 30       | 243 ± 25        | 230 ± 25        |
| H-89 then α2M*M               | 362 ± 35       | 220 ± 26        | 210 ± 25        |
| KN-62 (100 nM)                | 372 ± 38       | 240 ± 24        | 230 ± 24        |
| KN-62 then forskolin          | 300 ± 31       | 240 ± 20        | 230 ± 20        |
| KN-62 then α2M*M              | 310 ± 35       | 270 ± 25        | 260 ± 25        |

FIG. 9. Effect of α2M* and forskolin on the levels of c-Fos, Grb2, Sos 1/2, and She in macrophages. A, c-Fos; B, Grb2; C, Sos 1/2; D, She proteins in macrophages. The proteins were detected by Western blotting and quantified by a PhosphorImager as described under "Experimental Procedures." All panels, bar 1, buffer; bar 2, α2M* (100 pM); bar 3, forskolin (20 μM); bar 4, forskolin and then α2M*. The corresponding gel blots are shown at the bottom of the respective bar graphs. The values are expressed in arbitrary units and are the means ± S.E. from two or three independent experiments performed in triplicate.
of thymidylate synthase protein. However, thymidylate synthase protein is not up-regulated in \( \alpha_2M^- \)-treated macrophages upon silencing of the CREB gene with sequence-homologous dsRNA. Thymidylate synthase is clearly an important enzyme in regulating the intracellular thymidine pool necessary to provide precursors for DNA synthesis. These results further suggest that CREB signaling is involved in \( \alpha_2M^- \)-induced mitogenesis and cell proliferation.

**Silencing of the CREB Gene with Sequence-homologous dsRNA Inhibits \( \alpha_2M^- \)-induced c-Fos Expression**—In order to investigate the relationship between CREB and c-Fos expression, we examined the effect of silencing the CREB gene on the expression of c-Fos in \( \alpha_2M^- \) stimulated-macrophages. dsRNA treatment of macrophages profoundly reduced the expression of c-Fos protein in \( \alpha_2M^- \)-stimulated cells (Fig. 15). These results suggest that CREB modulates c-Fos-mediated cellular events.

**DISCUSSION**

We have studied the role of cAMP-dependent signaling pathways in \( \alpha_2M^- \)-induced macrophage proliferation. The binding of \( \alpha_2M^- \) to its receptors causes a significant increase in CREB
Activated α2-Macroglobulin and CREB Regulation

**TABLE II**

Effect of LipofectAMINE/LipofectAMINE-RNA complex on α2M*-stimulated macrophage cell number

Two studies were performed in triplicate.

| Treatments                                      | Cell number (× 10⁶) |
|------------------------------------------------|---------------------|
|                                                | At start | 24 h after the addition of α2M* to transfected cells |
| Buffer                                         | 5.45 ± 0.65 | 4.45 ± 0.39 |
| Buffer + LipofectAMINE (10 μl/ml)              |          |             |
| Buffer + α2M* (100 pm) + LipofectAMINE (10 μl/ml) |          |             |
| Buffer + 10 μg/ml dsRNA + α2M* (100 pm)        |          |             |
| Buffer + 50 μg/ml dsRNA + α2M* (100 pm)        |          |             |

**Fig. 13.** Morphological changes in macrophages before and 24 h after transfection with dsRNA. A, macrophages before transfection; B, macrophages transfected with 50 μg of dsRNA for 24 h and then stimulated with α2M* (100 pm for 24 h). The images shown are representative of two independent experiments.

expression and phosphorylation of CREB at Ser-133. α2M*-induced phosphorylation of CREB was reduced by inhibitors of PKA, PKC, Ca²⁺/calmodulin kinase, ERK 1/2, p38 MAPK, tyrosine kinases, PI 3-kinase, and p70s6k as well as by BAPTA/AM, actinomycin D, and cycloheximide. Binding of α2M* to macrophages elevated the levels of phosphorylated ERK 1/2, p38 MAPK, JNK, and p70s6k, comparable with levels induced by forskolin. α2M* and forskolin both increased the uptake of [³H]thymidine by macrophages as well as cell number. Like CREB phosphorylation, [³H]thymidine uptake was reduced by inhibitors of PKA, PKC, Ca²⁺/calmodulin kinase, ERK 1/2, p38 MAPK, tyrosine kinases, and PI 3-kinase, p70s6k, or BAPTA/AM, actinomycin D, and cycloheximide. Both α2M* and forskolin elevated the levels of the docking proteins Grb2 and Shc and the guanine nucleotide exchange factor Sos. Both α2M* and forskolin significantly raised the levels of Rap-1 and Raf-B either alone or in combination, whereas only α2M* elevated the levels of Raf-1. These results demonstrate that α2M* triggers both IP3- and cAMP-dependent pathways, culminating in enhanced mitogenesis and increased cell proliferation. By contrast, forskolin is known to act only through elevating cAMP (59–64). These observations are schematically depicted in Fig. 16.

Beginning, in 1993, we reported that binding of α2M* to cells including macrophages activated signaling cascades (4, 5, 8, 10, 12, 14). These signaling events are mediated by α2M* binding to α2MSR, which consists of lipoprotein receptor-related protein.
protein in complex with a coreceptor (11); moreover, this signaling pathway requires a number of adapter proteins (68, 69). Based on these and other observations, we hypothesized that α2MSR functions like a growth factor receptor and that α2M* functions as a growth factor (10). Binding of α2M* to α2MSR induces tyrosine phosphorylation of phospholipase C, which is induced by the tyrosine phosphorylation of α2MSR (70, 71). Tyrosine-phosphorylated receptor recruits docking protein Grb2 and Shc and guanine nucleotide exchange factor Sos (49–51). The Grb2-Sos or Grb2-Sos-Shc complex activates membrane binding and formation of Ras-GTP (51, 53–56), activation of Raf-1 by PKC (55), and phosphorylation of downstream MEK and MAPKs (55). The activated MAPKs translocate to nuclei and phosphorylate several genes involved in mitogenesis and cell proliferation. In addition, α2M* binding to α2MSR activates membrane phosphatidylinositol 4,5-bisphosphate hydrolysis by phosphatidylinositol-dependent phospholipase C, which raises [Ca2+]i, and DAG membrane PKC as well as several other Ca2+-dependent protein kinases are activated, culminating ultimately in the onset of several intracellular signaling cascades and cellular responses. We show here further that ligation of α2MSR activates cAMP-dependent signaling, which contributes to α2M*-induced mitogenesis and cell proliferation (72). cAMP stimulates the proliferation of many cell types, but in some cases cAMP may actually inhibit cellular proliferation (65, 66, 72–75). The ability of growth factors to stimulate cell proliferation requires the small G protein Ras and MAPKs. Ras activates the serine/threonine kinases Raf-1 and Raf-B, which in turn activate MEK 1/2, and ERK 1/2. The activities of Raf kinases are regulated by the Ras family of small GTP-binding proteins including Ras and Rap-1. Growth-inhibitory effects of cAMP are proposed to be mediated in part through cAMP-dependent inactivation of MAPKs (65, 66, 72–75). cAMP inhibits the binding of Raf-1 to Ras, thus blocking the activation of MAPK pathways (65, 66, 72–75). The growth-stimulatory effects of cAMP are mediated by the activation of Rap-1, which is a selective activator of Raf-B and an inhibitor of Raf-1 (65–67, 72–77). Rap-1 is a small, Ras-like GTPase that is activated by several extracellular stimuli and has been shown to be involved in cellular processes such as cell proliferation and differentiation. Rap-1 is activated by DAG, Ca2+, and cAMP, which promote the release of the GDP and Rap-1 binding to GTP (78). We show here that stimulation of macrophages with α2M* raises cAMP levels, promotes mitogenesis, and raises cell numbers. Thus, in murine peritoneal macrophages, cAMP promotes cell growth by activating MEK 1/2 phosphorylation and activation of downstream MAPKs through activation of a Rap-1/Raf-B pathway.

Ras supports mitogenic signaling and cell survival through the sequential activation of PI 3-kinase and protein kinase B (79, 80). PI 3-kinase-dependent mitogenic signaling involves p70s6k, which is downstream to PI 3-kinase and phosphorylates ribosomal S6 protein and increases translation of mRNAs having the polypyrimidine tract motif in a growth factor-sensitive manner (81–87). Activation of p70s6k is involved in the regulation of protein and DNA synthesis (87, 88). Injection of antibodies against the enzyme prevents the progression of cells from G1 into S phase (89). Trophic hormones, which elevate cAMP, activate PI 3-kinase, Akt, and/or p70s6k in thyroid cells and ovarian cells, thus mediating cAMP-induced growth of these cells (90, 91). However, in some cells, such as Swiss 3T3 and COS, cAMP inhibits the PI 3-kinase/Akt/p70s6k pathway by an unknown mechanism (92). Activation of Rap-1 has been suggested to mediate cAMP-induced activation (65–67, 75–78). The direct targets of ERK 1/2 include serine/threonine kinases of the families of mitogen/stress kinases and ribosomal S6 kinase (90, 91). Ribosomal S6 kinases phosphorylate CREB at Ser-133 and are involved in cell survival and proliferation (24, 93). Inhibition of PI 3-kinase with wortmannin or p70s6k with rapamycin resulted in significant reductions in cAMP-induced [3H]thymidine uptake. We conclude, therefore, that cAMP activates the PI 3-kinase/p70s6k pathway for enhanced mitogenesis and cell proliferation in peritoneal macrophages. cAMP-elevating agents increased the levels of Rap-1 and Raf-B and decreased that of Raf-1, which demonstrates that cAMP acti-

\[\text{FIG. 16. A schematic representation of the involvement of signaling cascades and CREB in } \alpha_2\text{M*-dependent macrophage regulation.}\]
vates MAPKs and cell proliferation by activating a Rap-1/Raf-B cascade.

Growth-inhibitory effects of cAMP are believed to be mediated partly by PKA, whereas PI 3-kinase activity may be required for cAMP-stimulated cell proliferation in certain cells (90). The mitogenic effects of cAMP correlate well with the PI 3-kinase/protein kinase B pathway, which is also cell-specific (90). The mitogenic effects of cAMP, which are PKA-independent, are mediated by the phosphorylation of protein kinase B and membrane ruffling (90). Recent findings indicate that multiple cAMP-mediated pathways exist and that only some are PKA-dependent. A AMP receptor, Epac (exchange protein directly by cAMP) or cAMP-regulated guanine nucleotide exchange factor, is involved in cAMP-mediated signaling (78, 94). Epac contains a cAMP binding domain that is homologous to the R subunit of PKA and a guanine nucleotide exchange factor domain. Epac binds to cAMP with high affinity and activates the downstream target Rap-1, which is antagonistic to the cell-transforming function of Ras (24). PKA can phosphorylate Rap-1, but mutations in this domain do not impair its biological functions, which suggests that cAMP-mediated activation of Rap-1 may be PKA-independent (95).

dsRNA-mediated interference of gene expression is currently being widely used to disrupt the expression of specific genes to understand their underlying cellular functions (see Refs. 28–36). Available data show that dsRNA serves as the initial trigger of RNA silencing and, after recognition, is processed by the RNAs into short fragments 21–23 nucleotides in length. These short interfering RNAs are then incorporated into a dsRNA-induced silencing complex to guide cycles of specific RNA degradation. Using this technique, we have shown that the silencing of CREB expression with dsRNA homologous to the target gene drastically reduces cell proliferation and mitogenesis, which is paralleled by drastic reductions in the expression of CREB protein as well as its phosphorylation in murine peritoneal macrophages stimulated with αMβ2. Thymidylate synthase is an enzyme involved in DNA synthesis and is responsible for maintaining thymidine pools, required for DNA synthesis in cells. Since the CREB gene transactivates many genes, one would expect that silencing of CREB expression would also affect the expression of CREB-transactivated genes as well as downstream signaling events. Silencing of the CREB gene profoundly reduced the expression of thymidylate synthase, causing drastic reductions in DNA synthesis. The reduced thymidylate synthase expression in macrophages where the CREB gene is silenced supports other studies in this report that define the role of CREB in αMβ2-stimulated macrophages. Finally, silencing of the CREB gene also profoundly reduced the expression of c-Fos in αMβ2-treated cells. These results show that CREB modulates the expression of c-Fos and cellular proliferation as suggested by other studies in this report.

In summary, we show here that αMβ2 in murine peritoneal macrophages, like other growth factors, utilizes more than one intracellular signaling module to transmit its message to the nucleus. Subsequent events may include various cellular responses, mitogenesis, cell growth, and differentiation. The cross-talk between various intracellular signaling pathways appears to be of crucial importance for achieving the maximal expression and translation of the message contained in αMβ2.

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The Role of cAMP-dependent Signaling in Receptor-recognized Forms of α₂-Macroglobulin-induced Cellular Proliferation
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