Elevation of intracellular cAMP by forskolin, 8-bromo-adenosine 3',5'-cyclic monophosphate, and prostaglandin E1, in synergy with insulin, stimulated DNA synthesis in quiescent Swiss 3T3 cells to the same level achieved by platelet-derived growth factor (PDGF) or bombesin. Both forskolin and 8-bromo-adenosine 3',5'-cyclic monophosphate stimulated a significant increase in cell number which, in the presence of insulin, reached the same levels achieved with PDGF. Treatment with either PDGF or bombesin caused a marked and persistent stimulation of p42MAPK and p44MAPK. In striking contrast, no activation was seen with mitogenic combinations of cAMP as shown by three different assays. Swiss 3T3 cells stably transfected with a constitutively activated Gs \( \alpha \) subunit were 100-fold more sensitive to the mitogenic effects of forskolin but in this distinct cellular model forskolin did not activate p42MAPK. Swiss 3T3 cells stably transfected with interfering mutants of MEK-1 showed a 60% decrease in PDGF-stimulated p42MAPK activation, but there was no inhibition of the mitogenic effect of forskolin in these cells. Furthermore, the upstream kinases MEK-1/MEK-2 and p74raf-1 were not activated by mitogenic combinations of cAMP while PDGF caused marked stimulation of their activity. Treatment of 3T3 cells with forskolin attenuated PDGF-stimulated p74raf-1 and p42MAPK activation but enhanced the mitogenic effects of this agent. Mitogenic combinations of cAMP strongly stimulated the phosphorylation and activation of p70\( \text{S6K} \) an effect that was inhibited by rapamycin. This agent markedly inhibited cAMP-stimulated DNA synthesis suggesting a critical role for p70\( \text{S6K} \) in cAMP mitogenic signaling. These results demonstrate that cAMP-induced mitogenesis can be dissociated from activation of the mitogen-activated protein kinase cascade and that this is not an obligatory point of convergence in mitogenic signaling in Swiss 3T3 cells.

The mitogen-activated protein (MAP) \(^1\) kinases (ERKs) are a family of highly conserved serine/threonine kinases that are activated in response to a wide range of extracellular signals including growth factors, hormones, and neuropeptides (1–3). The two best characterized isoforms p42\( \text{MAPK} \) (ERK-2) and p44\( \text{MAPK} \) (ERK-1) (4) can be activated through both tyrosine kinase receptors or G-protein-linked receptors (1–3). Once activated, p42\( \text{MAPK} \) and p44\( \text{MAPK} \) phosphorylate an array of cellular proteins including protein kinases such as p90\( \text{rsk} \) (5), transcription factors (6–8), and proteins involved in the regulation of cell growth (9). MAP kinases are themselves activated by phosphorylation on specific threonine and tyrosine residues by the dual-specificity MAP kinase kinase (or MEK) of which at least two isoforms have been identified in mammalian cells (10–12). This kinase is itself regulated by upstream kinases including the Raf family (13) and MEK kinase (14). Studies with dominant-negative and activating mutants have provided evidence that this pathway can lead to the stimulation of DNA synthesis (15, 16). However, it is unclear whether the activation of p42\( \text{MAPK} \) and p44\( \text{MAPK} \) is a point of convergence in the action of all signals that promote DNA synthesis (17–19).

The cAMP-protein kinase pathway links a number of extracellular signals to a range of cell functions including cell proliferation (20). Considerable evidence indicates that an increase in intracellular cAMP can act as a mitogenic signal for Swiss 3T3 cells (21). Agents that promote cAMP production and accumulation, such as forskolin and PGE\(_1\), as well as permeable cAMP analogues, stimulate DNA synthesis in 3T3 cells acting synergistically with insulin and other factors (21–24). Cells expressing a mutated cAMP-protein kinase regulatory subunit show markedly reduced cAMP-protein kinase activation and mitogenesis in response to agents that elevate intracellular cAMP (25). Conversely, Swiss 3T3 cells expressing a constitutively active Gs \( \alpha \) subunit are highly sensitive to the mitogenic effects of cAMP elevating agents (26). Increases in cAMP also lead to early intracellular events associated with cell proliferation including an increase in the expression of the proto-oncogene c-myc (27). However, the exact relationship between the mitogenic effect of cAMP and activation of the MAP kinase cascade is as yet not defined.

Here we report that the mitogenic effects of cAMP are not associated with detectable activation of p42\( \text{MAPK} \) and p44\( \text{MAPK} \), MEK-1/2, or of p74raf-1. Interfering mutants of MEK-1 stably transfected into Swiss 3T3 cells significantly inhibited PDGF-stimulated p42\( \text{MAPK} \) activation but did not inhibit cAMP-induced mitogenesis. Further dissociation of mitogenesis from the MAPK cascade is demonstrated by the finding that elevating intracellular cAMP inhibits PDGF-stimulated p74raf-1 and p42\( \text{MAPK} \) activation but enhances PDGF-stimulated DNA synthesis. Mitogenic combinations of cAMP strongly stimulated the phosphorylation and activation of p70\( \text{S6K} \), an effect that was

PGE\(_1\), prostaglandin E1; cpm, counts/minute; WT, wild type.
inhibited by rapamycin. This agent markedly inhibited cAMP-stimulated DNA synthesis identifying this as a distinct pathway in cAMP mitogenic signaling.

**EXPERIMENTAL PROCEDURES**

Cell Culture—Stock cultures of Swiss 3T3 fibroblasts were maintained in DMEM supplemented with 10% FBS in a humidified atmosphere containing 10% CO₂ and 90% air at 37°C. For experimental purposes, cells were plated out either in 33-mm Nunc Petri dishes at 10⁵ cells/dish or in 90-mm dishes at 6 x 10⁵ cells/dish in DMEM containing 10% FBS and used after 6–8 days when the cells were confluent and quiescent.

DNA Synthesis Measurements—Quiescent cultures of cells were washed twice with DMEM and incubated with DMEM/Waymouth’s medium (1:1, v/v) containing [³²P] thymidine (1 μCi/ml) and various factors as indicated. After 40 h, the cultures were washed twice with ice-cold phosphate-buffered saline and incubated in 5% trichloroacetic acid for 30 min at 4°C. Trichloroacetic acid was then removed and the cultures washed twice with ethanol and extracted in 1 ml of 2% Na₂CO₃, 0.1 M NaOH, 1% SDS. Incorporation of [³²P] thymidine was determined by scintillation counting in 6 ml of scintillation fluid.

Cell Proliferation—Confluent, quiescent Swiss 3T3 cells were treated with various factors added directly to the culture medium. After 72 h, the cultures were washed twice with 0.02% EDTA in phosphate-buffered saline and then lysed in SDS-PAGE sample buffer. Lysates were then subjected to a kinase assay in SDS-polyacrylamide gels using a modification of the method described by Kameshita and Fujisawa (28). Briefly, samples were subjected to SDS-PAGE in 10% polyacrylamide minigels containing 0.5 mg/ml MBP. After electrophoresis, SDS was removed from the gels by three 20-min washes with 20% (v/v) propan-2-ol in 50 mM Tris-HCl (pH 8.0) followed by three washes with 5 mM Tris-HCl (pH 8.0). Proteins were denatured by two 30-min washes with 6 M guanidine hydrochloride in 50 mM Tris-HCl (pH 8.0) and then reneutralized by incubation at 4°C in 5 changes of 50 mM Tris-HCl containing 0.04% (v/v) Tween-40 and 5 mM b-mercaptoethanol over 12–18 h. After preincubation of the gels at room temperature for 1 h in 40 mM HEPES (pH 8.0), 2 mM dithiothreitol, 10 mM MgCl₂, in gel phosphorylation of MBP was performed in 40 mM HEPES (pH 8.0), 0.5 mM EGTA, 10 mM MgCl₂, 2 μM CAMP-dependent protein kinase inhibitor peptide, 50 μM ATP, 2.5 μCi/ml of [γ-³²P]ATP, for 1 h at room temperature. After extensive washing in 5% trichloroacetic acid (w/v) with 1% (w/v) sodium pyrophosphate, the gels were dried and autoradiographed. The same preparation was used to assess the relative protein levels of MEK and p42MAPK as substrates. Overnight cultures of Escherichia coli strain BL21 DE3 transformed with GST-p42MAPK and GST-MEK expression vectors (pGEX-2T) were diluted 1 in 10 and grown for 1 h. GST-p42MAPK was induced with 1 μM isopropyl-β-D-thiogalactopyranoside for 4 h at 37°C and GST-MEK induced with 30 μM isopropyl-1-thio-β-D-galactopyranoside at 27°C overnight. The cells were then pelleted, frozen-thawed. The kinase reaction was performed by centrifugation, and GST fusion proteins were purified by adding 0.5 ml of glutathione-Sepharose beads and rotating for 30 min at 4°C. The GST-p42MAPK was cleaved from the GST in thrombin buffer while the GST-MEK was eluted from the glutathione-Sepharose beads with 5 mM glutathione in 50 mM Tris (pH 8). Both preparations were then dialyzed against several washes in phosphate-buffered saline, then rotating the proteins to SDS-PAGE and staining the gels with Coomassie Blue. For the GST-p42MAPK typical yields were 10 μg/ml of culture, with a purity of >95%. For the GST-MEK typical yields were 5 μg/ml of culture with a purity of >95%. For the kinase assay quiescent cells were treated as indicated and lysed in lysis buffer as above. p74[ERK1,2] immune precipitation was performed with an affinity purified rabbit polyclonal antibody for 2 h with protein A-agarose added for the second hour. Immune complexes were collected by centrifugation and then washed three times in lysis buffer with no phenylmethylsulfonyl fluoride and once with buffer A (50 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, 0.5 mM Na₂VO₄, and 0.1% β-mercaptoethanol). Pellets were then resuspended in 30 μl of buffer C (6.5 mM b-D-glucose-1-phosphate, 0.03% Brij-35, 10 mM MgCl₂, and 20 mM NaOCl-β-D-glucopyranoside) and incubated at 30°C for 30 min. The reaction was then terminated by diluting the supernatant in 40 μl of buffer A with 1 mg/ml of BSA and after mixing 10 μl of the supernatant to a fresh tube. p42MAPK activation was then measured using the MBP peptide phosphorylation assay essentially as above.

**MEK Assay—Activation of MEK-1 and MEK-2 was assayed using a modification of the above technique. Lysates from treated cells were incubated with a rabbit polyclonal antibody which recognized both MEK-1 and MEK-2. Immune complexes were incubated with GST-p42MAPK as above, and the resultant activation of this enzyme was measured as above.**

p70S6K Immune Complex Kinase Assay—Quiescent cells were treated with various factors as above and lysed at 4°C in 1 ml of a solution containing 10 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM NaVO₃, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM dithiothreitol, and filtered through 0.22-μm filters. The samples were clarified by centrifugation at 15,000 x g for 10 min at 4°C. Immunoprecipitation was performed using the polyclonal anti-p70S6K antibody as above incubating the samples on a rotating wheel for 2 h. Washed protein A-agarose beads (50 μl 1:1 slurry) were added for the second hour. Immune complexes were collected by centrifugation and washed twice in lysis buffer and twice in kinase buffer (15 mM Tris-HCl, 15 mM MgCl₂). The kinase reaction was performed by resuspending the assay mixture containing kinase buffer with 0.5 mM EGTA, 1 mg/ml MBP-peptide (APRTPGGGR), 50 μM ATP, 50 μCi/ml of [γ-³²P]ATP, 2 μM CAMP-dependent protein kinase inhibitor peptide, and 100 nm microcystine LR. Incubations were performed under linear assay conditions at 30°C and, following centrifugation for 10 s, terminated by spotting 25 μl of the supernatant onto Whatman P81 chromatography paper. Filters were washed four times for 5 min in 0.5% orthophosphoric acid, immersed in acetone, and dried before scintillation counting. The average radioactivity of two blank samples containing no immune complex was subtracted from the result of each sample. Results are expressed as cpm/1.5 x 10⁵ cells. The specific activity of [γ-³²P]ATP used was 900-1200 cpm/pmol.

Transfection of Interfering Mutants of MEK-1—MEK-1 mutants with alanine substitutions at serine 217 or serine 221 were used as interfering factors to block MAP kinase activation in vivo (15). Wild type and MEK-1 mutants in the retroviral expression vector pBABEpuro (30) were transfected into Swiss 3T3 cells by co-culturing with the retrovirus producer cell line GP+E pre-treated with mitomycin C to render cells non-viable (30). Resistant clones were selected using puromycin (5 μg/ml). Expression of MEK-1 was determined by Western blotting with a specific anti-MEK-1 monoclonal antibody. All clones selected for subsequent experiments had comparable levels of expression of exogenous MEK-1. Results shown are for one of the wild type (WT), alanine 217 (Ala217) and alanine 221 (Ala221) mutant expressors but are typical of those obtained with two independent clones for each transfectant.
CAMP Stimulates Mitogenesis but Not MAP Kinase Cascade

µCi/ml of [γ-32P]ATP, 2 µM CAMP-dependent protein kinase inhibitor peptide and 100 nm microcystine LR. Incubations were performed under linear assay conditions at 30°C for 20 min and, following centrifugation for 10 s, terminated by spotting 25 µl of the supernatant onto Whatman P81 chromatography paper. Filters were washed four times for 5 min in 0.5% orthophosphoric acid, immersed in acetic acid, and dried before scintillation counting. The average radioactivity of two blank samples containing no immune complex was subtracted from the result of each sample.

p70s6k Mobility Shift Assay—Activation of p70s6k was determined by the appearance of slower migrating forms in SDS-PAGE as a result of phosphorylation on several clustered serine and threonine residues (32). Immunoblot analysis on cell lysates was performed using a rabbit polyclonal antibody which recognized both 70 and 68 kDa isoforms of p70s6k (33).

Materials—Forskolin, 8-BrcA, IBMX, PGE1, bombesin, puromycin, myelin basic protein, and insulin were obtained from Sigma. Protein A-agarose conjugate was obtained from Boehringer Mannheim, Germany. CAMP-dependent protein kinase inhibitor peptide was obtained from Bachem (U.K.) Ltd., Saffron Walden, United Kingdom. Rapamycin was obtained from Calbiochem-Novabiochem (U.K.) Ltd., Nottingham, U.K. The anti-p44MAPK, anti-p70s6k, and anti-p74akt affinity purified rabbit polyclonal antibodies were obtained from Santa Cruz Biotechnology Ltd. Anti-MEK-1 monoclonal antibody and anti-MEK-1/MEK-2 polyclonal antibody was obtained from Affiniti Research Products Ltd, Nottingham, U.K. The N terminally directed anti-p70s6k rabbit polyclonal antibody was obtained from Upstate Biotechnology Inc. The anti-p42MAPK antibody was a kind gift from Dr J. Van Lint, Katholieke Universiteit Leuven, Belgium. GST-MEK and GST-p42MAPK expression vectors and MEK-1 mutants in pBABE puro were kind gifts from Professor C. Marshall, Chester Beattie Laboratory, Institute for Cancer Research, London, U.K. and F. Obermuller and Dr. H. Land, Imperial Cancer Research Fund. The Gαs (Q227L) 3T3 cell line was a kind gift from Dr. I. Zachary, Department of Medicine, King's College School of Medicine and Dentistry, London, U.K. Recombinant PDGF (BB homodimer), 125I-protein A, and [γ-32P] ATP were obtained from Amer sham Corp. U.K. All other reagents used were of the purest grade available.

RESULTS

Agents Which Elevate cAMP Are Mitogenic Signals for Swiss 3T3 Cells but Do Not Activate p42MAPK or p44MAPK—Quiescent cultures of Swiss 3T3 cells can be induced to initiate DNA synthesis through distinct signaling pathways. As shown in Fig. 1A, the addition of PDGF, which acts through a receptor with intrinsic tyrosine kinase activity or bombesin, which acts via a G-protein-linked receptor, induced striking stimulation of [3H] thymidine incorporation into these cells, the effect of bombesin being further enhanced by the presence of insulin. Forskolin, a direct activator of adenyl cyclase that increases intracellular CAMP in Swiss 3T3 cells and 8-BrcA, a cell-permeable CAMP analogue, also induced DNA synthesis in combination with insulin. As shown in Fig. 1A both these agents stimulated DNA synthesis to a level comparable to that achieved with PDGF or bombesin confirming that an increase in cAMP is a potent mitogenic signal for Swiss 3T3 cells.

To assess whether treatment of Swiss 3T3 cells with CAMP-elevating agents resulted in cell proliferation, cultures of 3T3 cells in conditioned medium were treated for 72 h with the various factors and cell number determined. As shown in Table I addition of either forskolin or 8-BrcA alone both caused a statistically significant increase in cell number compared to control cultures. In the presence of insulin, the stimulatory effect of forskolin was comparable to that achieved with PDGF.

To examine the effects of these mitogenic factors upon the activity of p42MAPK and p44MAPK, cell lysates from parallel cultures were analyzed by Western blotting using specific polyclonal antibodies to these proteins. Activation was determined by the appearance of slower migrating forms which results from the phosphorylation of specific threonine and tyrosine residues in these kinases (29). Treatment of cells with PDGF or bombesin, either alone or in combination with insulin, stimu-

![Fig. 1. cAMP stimulates DNA synthesis in Swiss 3T3 cell but does not activate p44MAPK and p44MAPK. A, confluent and quiescent cultures of Swiss 3T3 cells were washed and incubated at 37°C in 2 ml of DMEM/Waymouth's medium containing 1 µC/ml of [3H]thymidine and various factors: 10 µM forskolin with 50 µM IBMX (FSK), 2.5 µM 8-BrcA (BrcA), 10 ng/ml bombesin (Bom), 10 ng/ml PDGF, or no addition (--), either in the absence or presence of 1 µg/ml of insulin (Ins). After 40 h, DNA synthesis was assessed by measuring the [3H] thymidine incorporated into acid-precipitable material. Results are expressed as a percentage of the incorporation induced by 10% FBS, and data are shown as mean ± S.E. for four independent experiments each performed in duplicate. The specific activity of [γ-32P] ATP used was 900-1200 cpm/pmol.](image-url)lated p42MAPK as judged by the mobility shift (Fig. 1B, upper panel). In contrast, no slower migrating form was seen with the mitogenic combinations of either forskolin or 8-BrcA with insulin or with insulin alone. Similar findings were obtained with p44MAPK (data not shown).

The activation of p42MAPK and p44MAPK in response to mi-
Table 1

| Addition | -Ins | +Ins |
|----------|------|------|
| FSK      | 3.69 ± 0.07 | 4.01 ± 0.14 |
| BMPcA    | 4.95 ± 0.12* | 9.70 ± 0.14* |
| PDGF     | 4.42 ± 0.06* | 6.59 ± 0.07* |
| Bm      | 6.22 ± 0.06* | 9.47 ± 0.05* |
| 44MAPK   | 4.30 ± 0.04* | 6.01 ± 0.13* |

**cAMP Stimulates Mitogenesis but Not MAP Kinase Cascade**

Effect of various mitogens on the proliferation of Swiss 3T3 cells

Six-day-old cultures of Swiss 3T3 cells in conditioned medium were treated with various factors: 10 μM forskolin with 50 μM IBMX (FSK), 2.5 mM 8-BrcA, 10 nM PDGF, 10 ng/ml bombesin (Bom), or control (−) in the absence or presence of 1 μg/ml of insulin (+Ins). Cell number was then determined after 72 h as described under "Experimental Procedures." Results shown are the means ± S.E. of two experiments performed in quadruplicate (*p < 0.001 in Student’s t test compared with control without insulin for factors in the absence of insulin and with control with insulin for factors in the presence of insulin).

Addition | Cell number (×10³)
---|---
FSK | 3.69 ± 0.07
BMPcA | 4.95 ± 0.12*
PDGF | 6.22 ± 0.06*
Bom | 4.30 ± 0.04*

To further assess the kinetics of p42MAPK activation, immobilized kinase assays were performed over a time course of up to 120 min. As shown in Fig. 2B, bombesin stimulated a persistent activation in p42MAPK activity beyond 120 min while forskolin and insulin did not induce a significant increase in activity over the same time period compared to untreated control cells.

**Fig. 2. cAMP does not activate p42MAPK and p44MAPK over short and long time courses.** A, confluent and quiescent cultures of Swiss 3T3 cells were washed and treated with either 10 ng/ml bombesin (Bom) or 10 μM forskolin and 50 μM IBMX with 1 μg/ml of insulin (FSK) for the times indicated, lysed in sample buffer, and analyzed by Western blotting with anti-p42MAPK and anti-p44MAPK antibodies. The positions of non-phosphorylated (p42MAPK) and the slower migrating phosphorylated form (p44MAPK) are indicated. B, confluent and quiescent cultures of Swiss 3T3 cells were washed and treated with either 10 ng/ml bombesin (●), 10 μM forskolin and 50 μM IBMX with 1 μg/ml of insulin (●), or control medium (serum-free DMEM) (□) for the times indicated, lysed in lysis buffer, immunoprecipitated with anti-p42MAPK antibody, and the immune complexes analyzed in an immune complex kinase assay using MBP peptide as a substrate (see "Experimental Procedures"). Results are the means of duplicates and are expressed as percent of bombesin-stimulated activation (1400–1600 cpm/1.5 × 10⁶ cells at 5 min) and are representative of three independent experiments. The specific activity of [γ-32P]ATP used was 900–1200 cpm/pmol.

PGE₁ Is a Potent Mitogen but Does Not Activate p42MAPK

To investigate the possibility that a receptor-mediated increase in intracellular cAMP, as distinct from that triggered by forskolin or 8-BrcA, might activate p42MAPK. Swiss 3T3 cells were treated with PGE₁ at a dose of 50 ng/ml together with IBMX and insulin. This combination stimulates intracellu-
expressing the interfering mutants. These results provide con-
striking finding shown in Fig. 5

activation by 50 and 60%, respectively (Fig. 5

p42MAPK activation in cells expressing interfering MEK-1 mutants with alanine substitu-
tions at serine217 or serine221 have been shown to block MAP

expression of interfering MEK-1 mutants with alanine substitu-
tions at serine217 or serine221 have been shown to block MAP

kinase activation

interfering mutants of MEK-1 should not prevent [3H]thymidine

overexpressing wild-type MEK-1 was similar to that achieved

with PDGF and bombesin led us to explore the effect of cAMP upon up-
stream components of the MAP kinase cascade. This was

achieved using highly sensitive assays in which the activation of

immunoprecipitated kinases from stimulated cells was

determined using the [3H]thymidine incorporated into acid-pre-
ceptible material. Results are expressed as a percentage of the incor-

p42MAPK activity. Results are expressed as cpm/1.5 × 10^6 cells, and the
da data are shown as the mean ± S.E. for three independent experiments each performed in
duplicate. The specific activity of γ-32P]ATP used was 900-1200
cpm/pmol.

0.1 μM of forskolin. The addition 0.1 μM forskolin to the trans-
ferred cells failed to activate p42MAPK as determined using the

mobility shift (Fig. 4B) and immune complex kinase assays

(Fig. 4C). In contrast bombesin-stimulated p42MAPK activation

could be detected using either assay. Thus, the dissociation of
mitogenesis from MAP kinase activation could also be docu-
mented in Swiss 3T3 cells expressing Q227L MAPK α which are 100-fold more sensitive to forskolin than wild type cells.

Interfering Mutants of MEK-1 Stably Transfected into Swiss

3T3 Cells Significantly Attenuate PDGF-stimulated p42MAPK

Activation but Do Not Inhibit cAMP-induced Mitogenesis—
Expression of interfering MEK-1 mutants with alanine substitu-
tions at serine217 or serine221 have been shown to block MAP
kinase activation in vivo (15). If, as indicated by the preceding
results, cAMP induces DNA synthesis through a MAP kinase-

independent pathway in Swiss 3T3 cells, expression of interfering
mutants of MEK-1 should not prevent [3H]thymidine

incorporation into acid-precipitable material. Results are expressed as a percentage of the incorporation induced by 10% FBS, and data are shown as mean ± S.E. (n = 6). B, confluent and quiescent cultures of Swiss 3T3 cells were washed and treated with PGE2 with 25 μM IBMX and 1 μg/ml of insulin (PGE2) for the times indicated at 37 °C, lysed in sample buffer, and analyzed by Western blotting with anti-p42MAPK polyclonal antibody. Cells stimu-
lated with 10 ng/ml bombesin for 5 min (Bom) were used as a positive control. The positions of non-phosphorylated p42MAPK and the slower migrating phosphorylated form pp42MAPK are indicated. C, confluent and quiescent cultures of Swiss 3T3 cells were treated for 5 min in DMEM with factors as in A above, lysed in lysis buffer, immunoprecipitated with anti-p42MAPK polyclonal antibody, and the immune complexes analyzed in an immune complex kinase assay using MBP peptide as a substrate (see "Experimental Procedures"). Results are expressed as cpm/1.5 × 10^6 cells, and the data are shown as the mean ± S.E. for three independent experiments each performed in duplicate. The specific activity of γ-32P]ATP used was 900-1200
cpm/pmol.

vancing evidence that cAMP-induced mitogenesis does not re-

quire the activation of MAP kinase.

Mitogenic Combinations of cAMP Do Not Activate MEK-1 and

MEK-2 or p74raf-1. The finding of a striking dissociation

between the mitogenic effects of CAMP and the activation

of p42MAPK and p44MAPK compared to other mitogens such PDGF

and bombesin led us to explore the effect of CAMP upon up-
stream components of the MAP kinase cascade. This was

achieved using highly sensitive assays in which the activation of

immunoprecipitated kinases from stimulated cells was

measured by their ability to activate, in vitro, GST fusion

proteins of downstream kinases. Forskolin did not stimulate

MEK-1 and MEK-2 activity whereas PDGF and bombesin both

caus ed marked activation of these kinases (Fig. 6A). Next we
cultures of untransfected and Swiss 3T3 cells overexpressing WT, 21416
examined the kinetics of p74
mental Procedures”). Results are expressed as percent of maximum complex kinase assay using MBP peptide as a substrate (see “Experimental Procedures.” Results are expressed as percent of maximum PDGF-stimulated activation (19000–25000 cpm/1.5 × 10⁶ cells at 5 min), and the data are shown as the mean ± S.E. for two independent experiments each performed in triplicate. The specific activity of ([γ-32P]ATP used was 900-1200 cpm/pmol. C, confluent and quiescent cultures of untransfected and Swiss 3T3 cells overexpressing WT, Ala217, and Ala221 MEK-1 were washed and treated for 5 min at 37 °C with 10 nmol PDGF, lysed in lysis buffer, immunoprecipitated with anti-p42 MAPK polyclonal antibody, and the immune complexes analyzed in an immune complex kinase assay using MBP peptide as a substrate (see “Experimental Procedures”). Results are expressed as percent of maximum PDGF-stimulated activation (2000–2500 cpm/1.5 × 10⁶ cells at 5 min), and the data are shown as the mean ± S.E. for three independent experiments each performed in duplicate. The specific activity of ([γ-32P]ATP used was 900-1200 cpm/pmol. C, confluent and quiescent cultures of untransfected and Swiss 3T3 cells overexpressing WT, Ala217, and Ala221 MEK-1 were washed and incubated at 37 °C in 2 ml of DMEM/Waymouth’s medium containing 1 μCi/ml of [3H]thymidine and 10 μM forskolin with 50 μM IBMX and 1 μg/ml of insulin (FSK). After 40 h, DNA synthesis was assayed by measuring the [3H]thymidine incorporated into acid-precipitable material. Results are expressed as a percentage of the incorporation induced by 10% FBS, and data are shown as mean ± S.E. (n = 8).

examined the kinetics of p74⁰⁷⁴ Raf-1 activity in response to PDGF and forskolin with insulin. As shown in Fig. 6B, PDGF caused a marked stimulation of p74⁰⁷⁴ Raf-1 activity peaking at 5 min. In contrast, p74⁰⁷⁴ Raf-1 activity in forskolin-treated cells did not differ from that of control cells.

cAMP Pretreatment Attenuates PDGF-stimulated Activation of p74⁰⁷⁴ Raf-1 and p42MAPK but cAMP Enhances Its Mitogenic Effects—It has recently been demonstrated in Rat-1 fibroblasts and in other cell types that elevation of intracellular cAMP can inhibit PDGF and EGF-stimulated p42MAPK and p44MAPK activation (35–41). Although the mechanisms involved are not fully understood, it is clear that cAMP does not inhibit GTP loading of Ras (37, 40, 41) but interferes with Ras/Raf activation probably via inhibition of p74⁰⁷⁴ Raf-1 (35–37).

As cAMP is a mitogen in Swiss 3T3 cells, rather than growth inhibitory as it is in Rat-1 cells, we examined the effect of forskolin upon PDGF stimulated p74⁰⁷⁴ Raf-1 and p42MAPK activation. Preincubation with forskolin abolished PDGF-stimulated p42MAPK activation as shown in the mobility shift assay (Fig. 7A). This effect appeared to be mediated at the level of p74⁰⁷⁴ Raf-1, as PDGF-stimulated activation of this kinase was markedly inhibited by cAMP (Fig. 7B). However, PDGF-stimulated DNA synthesis in the presence of cAMP elevating agents was significantly enhanced (p < 0.001) (Fig. 7C). In other experiments, we found that forskolin augmented [3H]thymidine incorporation induced by PDGF at 2.5 and 5 ng/ml by 178 and 205%, respectively (data not shown). These findings again illustrate dissociation of activation of the MAP kinase cascade from cAMP-induced mitogenesis.

Mitogenic Combinations of cAMP Induce Phosphorylation and Activation of p70⁰⁷⁰ S6K and Rapamycin Inhibits cAMP-stimulated Mitogenesis—The 90-kDa ribosomal S6 kinase (p90S6K) is phosphorylated and activated by p42MAPK and p44MAPK and thus lies on the MAP kinase cascade (5). Indeed, we could not demonstrate any activation of this enzyme by cAMP providing further corroborative evidence that the MAP kinase pathway was not activated (data not shown). A distinct S6 kinase family, p70/85kDaS6K, has been shown to be activated by a number of growth factors (42). Selective inhibition of the phosphorylation and activation of this kinase by the immunosuppressant rapamycin has suggested that p70S6K plays a role in serum-stimulated DNA synthesis in Swiss 3T3 cells (33).
CAMP Stimulates Mitogenesis but Not MAP Kinase Cascade

**DISCUSSION**

The reincarnation of DNA synthesis in G0-arrested cells can be induced by multiple signaling pathways that act in a combinatorial and synergistic fashion (21, 43, 44). In the present study we utilized PDGF, bombesin, and cAMP in combination with insulin to promote maximum levels of DNA synthesis in Swiss 3T3 cells. Although these mitogenic signals must converge prior to DNA replication, the precise point of convergence in G1 remains unknown. The redundancy in signaling pathways prior to the point of convergence implies that some events may be sufficient to stimulate DNA synthesis but not necessarily obligatory for the action of all mitogens. p42MAPK and p44MAPK are activated by a number of mitogenic signaling pathways linked to both tyrosine kinase and G-protein-linked receptors (1–3). These include the Raf and MEK kinase pathways which transduce the signals from such effectors as p21ras (4), the proto-oncogenic products c-myc (7) and c-jun (8), and the transcription factor TCF 6 (6). Sustained activation of p42MAPK and p44MAPK, therefore, may be an obligatory step in the action of all mitogens leading to DNA synthesis as, in fact, has been proposed (45). However, several recent observations suggest that activation of p42MAPK or p44MAPK may not be an obligatory point of convergence in the action of all mitogenic signals. Interleukin-4 stimulates proliferation of two cell lines of T-lymphocyte and myeloid origin but does not activate MAP

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**FIG. 7.** CAMP pretreatment attenuates PDGF-stimulated activation of p74^{rd} and p42^{MAPK} but enhances its mitogenic effects. A, confluent and quiescent cultures of Swiss 3T3 cells were washed and treated for 5 min with 100 ng/ml PDGF (+) or without (-) 5 min of pretreatment with 50 εM forskolin and 50 εM IBMX (FSK) or with vehicle (-), lysed in sample buffer, and analyzed by Western blotting with anti-p42^{MAPK} polyclonal antibody. The positions of non-phosphorylated p42^{MAPK} and the slower migrating phosphorylated form pp42^{MAPK} are indicated. B, confluent and quiescent cultures of Swiss 3T3 cells were washed and treated for 5 min at 37 

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**FIG. 8.** Mitogenic combinations of cAMP stimulate the phosphorylation of p70^{s6k} and inhibition of p70^{s6k} by rapamycin inhibits CAMP-induced mitogenesis. Upper panel, confluent and quiescent cultures of Swiss 3T3 cells were washed and incubated at 37 

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**TABLE II**

| Addition   | p70^{s6k} activity |
|------------|--------------------|
| FSK        | 2.49 ± 0.07        |
| Ins        | 6.29 ± 0.56        |
| FSK + Ins  | 8.55 ± 0.66        |
| FSK + Ins + rap | 0.98 ± 0.09       |

Confluent, quiescent cultures of Swiss 3T3 cells were washed and treated for 15 min with various factors: 10 εM forskolin with 50 εM IBMX (FSK), 1 εM of insulin (Ins), 10 εM forskolin with 50 εM IBMX, and 1 εM of insulin (FSK + Ins), or with 30 min of pretreatment with 20 ng/ml rapamycin (+ rap). Lysates of the treated cells were immunoprecipitated with anti-p70^{s6k} antibody and the immune complexes analyzed in an immune complex kinase assay as described under “Experimental Procedures.” Results shown as -fold stimulation of vehicle-treated cells and are the means ± S.E. of three experiments performed in duplicate.
kinase (18). Additionally, thyroid-stimulating hormone stimulates mitogenesis in thyrocytes but does not stimulate tyrosine phosphorylation of MAP kinase (17, but see also 46). In Swiss 3T3 cells, activin, a member of the transforming growth factor β family of cytokines, is mitogenic but appears not to stimulate MAP kinase activation (19). These findings suggest but do not prove that p42MAPK and p44MAPK activation is not obligatory for DNA synthesis because the sensitivity of the assays used has been questioned (47) and none of the studies examined the effects of interfering mutants that block MAP kinase activation in vivo.

The results presented in this study demonstrate that the potent mitogenic effects of cAMP are not mediated via the MAP kinase cascade in Swiss 3T3 cells. Agents that elevate intracellular cAMP by distinct mechanisms are able to stimulate DNA synthesis to a level comparable to that seen with PDGF and bombesin. Additionally, cAMP-elevating agents either alone or in combination with insulin induce a significant increase in cell number demonstrating that these agents also stimulate progression through later stages of the cell cycle. However, using three separate assays we have shown that cAMP fails to detectably activate either p42MAPK or p44MAPK. This is in marked contrast to the effects of PDGF and bombesin which gave prolonged activation of these kinases. Interfering mutants of MEK-1 stably transfected into Swiss 3T3 also provide further convincing evidence that MAP kinase activation is not involved in cAMP-induced mitogenesis. In these cells, PDGF-stimulated MAP kinase activation was significantly attenuated but the mitogenic effect of forskolin was uninhibited. In line with this conclusion, cAMP does not cause a significant induction of c-fos (27), the expression of which is regulated by the MAP kinase cascade through phosphorylation of TCF 62 (48). Swiss 3T3 cells stably transfected with a constitutively activated Gsα subunit are highly sensitive to the mitogenic effects of forskolin, and yet in this distinct cellular model again we did not detect p42MAPK activation in response to this cAMP elevating agent. Furthermore, we have shown that cAMP fails to stimulate the upstream kinases in the cascade namely p74raf-1 (see below) and MEK-1/MEK-2. In contrast, under identical experimental conditions we have confirmed that PDGF and bombesin potently stimulate p42MAPK and p44MAPK together with the upstream kinase MEK-1/2 in Swiss 3T3 cells. Thus in this study we have demonstrated that cAMP does not utilize proteins at three levels of the MAP kinase cascade dissociating its mitogenic effects from a persistent activation of this pathway.

The serine/threonine protein kinase p74raf-1 has been shown to play a central role in the mitogenic response of cells to growth factors and many oncogenes (49). It associates with activated Ras and stimulates the downstream elements of the MAP kinase cascade (50). However, our results also provide evidence that p74raf-1 activation is not an obligatory step in cAMP mitogenic signal transduction. Mitogenic combinations of cAMP do not induce a significant increase in p74raf-1 activity. Indeed, an increase in cAMP strikingly inhibited the activation of p74raf-1 and p44MAPK by PDGF but significantly increased the mitogenic effect of PDGF. Our results therefore demonstrate, for the first time, that cAMP can stimulate cell proliferation and inhibit the MAP kinase cascade in the same cell type providing further evidence for a dissociation between mitogenesis and activation of this pathway.

It is now clear that cAMP mitogenic signaling is not confined to Swiss 3T3 cells. Agents which elevate intracellular cAMP are mitogenic for number of cell types including mammary, keratinocyte, and kidney epithelial cells (51). Additionally, growth hormone-releasing factor, which stimulates cAMP accumulation, is mitogenic for the rat anterior pituitary somatotroph (51). Further insight into the importance of the cAMP pathway in cell proliferation has come with the identification of constitutively activated Gsα subunits in a variety of tumors (52). These mutations, which result in a persistently elevated intracellular cAMP, are potentially oncogenic and demonstrate that cAMP may play a role in cellular transformation. Interestingly, our results with Swiss 3T3 cells stably transfected with a constitutively activated Gsα subunit indicate that cAMP can initiate a mitogenic response that is not mediated via the MAP kinase cascade in these cells.

Many growth factors activate a parallel but distinct signaling pathway leading to the phosphorylation and activation of p70s6k which rapidly phosphorylates the S6 protein of 40 S ribosomal subunit (43). Inhibition of the activation of this enzyme with the immunosuppressant rapamycin or with neutralizing antibodies demonstrates that p70s6k plays a role in serum-stimulated mitogenesis (33, 53). Interestingly, it has been recently reported that p70s6k phosphorylates CREM, which is also a target for cAMP-protein kinase, in response to mitogenic factors leading to a strong increase in transcriptional activation (54). This identifies a point of convergence between the p70s6k and cAMP-protein kinase pathways that is distinct from the nuclear targets of the MAP kinase cascade. Our results show that cAMP in combination with insulin stimulates the activity and phosphorylation of p70s6k as demonstrated by immune complex kinase and mobility shift assays. Rapamycin completely prevented the increase in activity and phosphorylation induced by cAMP. Crucially, we show here for the first time that rapamycin markedly attenuated the mitogenic effect of cAMP in combination with insulin. Thus our results identify p70s6k, as opposed to MAP kinase, MEK, and Raf-1, as an important element in cAMP-induced mitogenic signaling pathways.

In conclusion, our results demonstrate that cAMP, a potent mitogen for Swiss 3T3 cells, does not induce a significant and persistent activation of p42MAPK and p44MAPK and fails to stimulate the upstream kinases of the cascade namely p74raf-1 and MEK1/2. These findings, dissociating the mitogenic effects of cAMP from activation of the MAP kinase cascade, support the hypothesis that this kinase cascade is one of the parallel pathways that can lead to DNA synthesis rather than an obligatory point of convergence in mitogenic signaling.

Acknowledgments—We thank Professor C. Marshall for the GST-MEK-1 and GST-p42MAPK expression vectors, F. Obermuller and Dr. H. Land for the retrovirus producer cell line GP + E transfected with MEK-1 mutants in pBABE puro, and Dr. J. Van Lint for the anti-p42MAPK antibody.
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