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Synergistic and coordinate expression of the genes encoding ribonucleotide reductase subunits in Swiss 3T3 cells: Effect of multiple signal-transduction pathways

(growth control/bombesin/vasopressin/cAMP/S phase)

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ABSTRACT **Ribonucleoside-diphosphate reductase (ribonucleotide reductase, EC 1.17.4.1) is the enzyme responsible for the *in vivo* production of deoxyribonucleotides for DNA synthesis and is essential for cell proliferation. We examined the signal transduction pathways leading to expression of the M1 and M2 subunits of this enzyme in Swiss 3T3 mouse fibroblasts by Northern blot analysis. Stimulation of quiescent cells resulted in coordinate expression of both subunits, beginning at 8 hr after serum addition, in late G_1 phase, and peaking at 18–24 hr. Serum increased M2 message to 30 to 50 times that of quiescent cells, in contrast with M1 message, which was increased 10 times. Agents that elevated cAMP, including forskolin, and the cAMP analogue 8-bromo-cAMP modestly stimulated gene expression. Each of these agents was synergistic with insulin, and these combinations induced expression equivalent to that induced by serum stimulation. Likewise, agents that activate protein kinase C such as phorbol 12,13-dibutyrate, bombesin, and vasopressin were also synergistic with insulin with respect to ribonucleotide reductase gene expression, as was epidermal growth factor, which stimulates receptor tyrosine kinase activity. The time course for induction of mRNA expression by each of these agents alone or in combination was identical to that for induction stimulated by serum. Finally, the synergistic effects apparent in Northern analysis of ribonucleotide reductase gene expression were mirrored in parallel determinations of DNA synthesis. Thus, the combinatorial nature of signal transduction pathways resulting in proliferation of Swiss 3T3 cells is expressed at the level of ribonucleotide reductase gene expression.**

Growth factors act through incompletely defined signal transduction pathways to elicit the mitogenic response. Swiss 3T3 cells have emerged as a useful model to explore the molecular features of these pathways. These mouse fibroblasts become synchronously arrested in a quiescent G_0/G_1 state and then can be stimulated to proliferate by a variety of growth factors, pharmacological agents, or mitogenic neuropeptides (1). These studies reveal the paradigm that growth factor-activated signaling pathways act synergistically to induce a proliferative response (1, 2). Two key pathways include one triggered by activation of protein kinase C (PKC) and one initiated by cAMP-dependent protein kinase. Other pathways, which are less clearly defined, mediate responses to polypeptide growth factors that activate receptor tyrosine kinases (1–3). It is unclear how the early events stimulated by the addition of mitogens are transduced into later events that are more directly related to cell replication. In this context, it would be of great advantage to study the effect of the various molecular pathways on the expression of specific mRNAs tightly related to DNA synthesis rather than on the complex S phase itself (4).

An essential requirement for DNA synthesis during S phase is a large expansion in the pools of deoxyribonucleoside triphosphates (5). Ribonucleoside-diphosphate reductase (ribonucleotide reductase; EC 1.17.4.1), which catalyzes the reduction of ribonucleoside diphosphates to deoxyribonucleoside diphosphates, is the only *in vivo* source of deoxyribonucleotides for DNA synthesis (5). The activity of this enzyme appears to be regulated by allosteric effector mechanisms, *de novo* synthesis (6), and possibly post-translational modification (7). Ribonucleotide reductase is composed of two subunits, M1 and M2. The M1 subunit contains the allosteric effector sites, whereas the M2 subunits contain the tyrosyl radicals necessary for the reduction reaction. The active enzyme is thought to be composed of pairs of M1 and M2 subunits. The genes encoding both subunits have been cloned from several organisms (8, 9) and localized to different chromosomes in human and mouse cells (10, 11). In quiescent cells, ribonucleotide reductase activity is very low to undetectable, but it increases dramatically after serum stimulation (12–14). It is not known, however, if the expression of the genes coding for the M1 and M2 subunits of ribonucleotide reductase is coordinately induced by activation of distinct signal transduction pathways that act synergistically to stimulate DNA synthesis. In the present study we sought to determine which mitogenic pathways lead to increased expression of the M1 and M2 genes in Swiss 3T3 cells.

MATERIALS AND METHODS

Cell Culture. Swiss 3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) in the presence of fetal bovine serum (10%), penicillin (100 units/ml), and streptomycin (100 μg/ml) in a humidified atmosphere containing 10% CO_2 at 37°C. Experimental cultures were plated at 6×10^5 cells per 90-mm dish (Nunc Petri) and used 6 days later when they were confluent and arrested in the G_0 phase of the cell cycle (i.e., quiescent) as judged by assays of DNA synthesis and cytofluorimetric analysis.

Assays of DNA Synthesis. DNA synthesis was determined by incubating cells, washed free of serum, in DMEM containing [^{3}H]thymidine [1 μCi/ml, 1 μM (1 Ci = 37 GBq)] and additions as indicated. The incorporation of radioactivity into trichloroacetic acid-precipitable material was measured as previously described (15).

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Abbreviations: PKC, protein kinase C; EGF, epidermal growth factor; PGE_1, prostaglandin E_1; NECA, 5′-(*N*-ethylcarboxamido)-adenosine; Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidine; IBMX, 3-isobutyl-1-methylxanthine; 8BrcAMP, 8-bromo-cAMP; PDB, phorbol 12,13-dibutyrate.

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Cytofluorimetric Analyses. Cells were detached by treatment with trypsin (0.025%) and 4 mM ethylenediaminetetraacetic acid, suspended in DMEM containing 10% fetal bovine serum, centrifuged at 167 × *g* for 5 min, washed with phosphate-buffered saline, then resuspended in staining solution containing propidium iodide (50 μg/ml), sodium citrate (1.0 mg/ml), and Triton X-100 (0.1%). Stained nuclei were analyzed on a fluorescence-activated cell sorter (FACStar 4; Becton Dickinson) after a 15-min incubation.

Northern Blot Analysis. Quiescent cultures of Swiss 3T3 cells stimulated with fetal bovine serum, peptide growth factors, or pharmacological agents were washed twice with phosphate-buffered saline at 4°C, then lysed with 4 M guanidine isothiocyanate. Total RNA was isolated by centrifugation through a cesium chloride gradient (16). RNA (20 μg per lane) was fractionated on a 1% agarose/6% formaldehyde gel by electrophoresis, transferred to a Hybond-N (or N plus, Amersham) nylon membrane by capillary action, then fixed by incubation at 80°C for 2 hr. Northern blots were prehybridized for 4–6 hr at 42°C in a solution containing 50% (vol/vol) formamide, 5× Denhardt's solution, 5× SSC, 0.5% SDS, and denatured salmon sperm DNA at 500 μg/ml (1× Denhardt's solution is 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone; 1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0). Hybridization was performed by the addition of 3.0–4.0 × 10^6 cpm/ml of ^{32}P-labeled DNA probe to the prehybridization mix and incubating for 18 hr at 42°C. Blots were washed free of unbound probe by sequential exposure to 2× SSC/0.1% SDS at 42°C for 30 min, then 0.1× SSC/0.1% SDS at 55–60°C for 30 min. After autoradiography at −70°C bound probes were removed by incubating the blots in 0.1× SSC/0.1% SDS at 100°C for 15 min. The blots were then rehybridized to a different radiolabeled probe. Autoradiographic bands were quantitated by scanning densitometry using an LKB Ultroscan XL densitometer.

Materials. Plasmid pERCD encodes a full-length cDNA for mouse ribonucleotide reductase M1 subunit and was a gift from Ingrid Caras (Genentech). Plasmid p10 encodes a full-length cDNA for mouse ribonucleotide reductase M2 subunit and was a gift from Lars Thelander (University of Umea, Sweden). The cDNA probe for mouse 18S ribosomal RNA was a gift from D. Edwards (University of Oxford). Probes were labeled with [^{32}P]dCTP by random priming (Amersham Multiprime Kit) (17). The remainder of the materials was commercially obtained (Sigma).

RESULTS

Stimulation of Ribonucleotide Reductase Subunit Expression by Serum. Initially, we examined the effect of serum stimulation on the expression of the M1 and M2 subunits of ribonucleotide reductase in Swiss 3T3 fibroblasts. Quiescent cultures were transferred to medium containing 10% fetal bovine serum and assayed for M1 and M2 mRNA levels by Northern hybridization at various times after stimulation. Parallel cultures were used to determine DNA synthesis by [^{3}H]thymidine incorporation and cytofluorimetry. The results presented in Fig. 1 show that there was little expression of the M2 gene in quiescent cells in contrast to M1, which had low but detectable expression. An increase in the expression of both transcripts occurred after 8 hr of serum stimulation. After 18 hr of serum stimulation the increase in the level of M2 mRNA at peak ranged from 20- to 50-fold, whereas the induction of M1 is less pronounced (5- to 10-fold). The induction of message for M1 and M2 precedes entry into S phase, which begins about 16 hr after addition of serum as judged by either cytofluorimetry (Fig. 1) or [^{3}H]thymidine incorporation (data not shown). The results shown in Fig. 1 indicate that the genes encoding for the M1 and M2 subunits

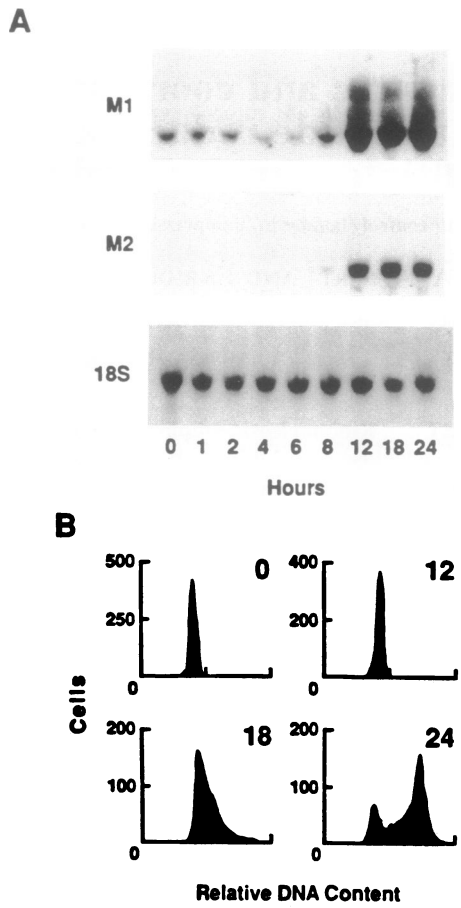

FIG. 1. Stimulation of ribonucleotide reductase gene expression by serum. (*A*) Northern blot analysis of mRNA concentration for the M1 [at 3.1 kilobases (kb)] and M2 (at 2.1 kb) subunits of ribonucleotide reductase was performed as described in the text. Each lane contained 20 μg of total cellular RNA from cells incubated with 10% fetal bovine serum for the hours indicated. For this experiment the specific activity of the M1 and M2 probes, the stringency of washing, and the time of autoradiography were similar. Densitometric analysis of the autoradiogram of the 18S ribosomal RNA indicates approximately equal loading of RNA from lane to lane and even transfer from the gel to the nylon membrane. (*B*) Cytofluorimetry was performed as described in the text on cell cultures stimulated with serum for the times shown. For the 0-hr culture the percentage of cells in S plus G_2/M phase was 2.5%. For the 12-hr culture this aggregate was 4.1%. For the 18-hr culture it was 54% and for the 24-hr culture it was 82.4%. For each time point 10^5 cells were analyzed. Parallel cultures assayed for DNA synthesis by [^{3}H]thymidine incorporation confirmed the cytofluorimetric analysis. Comparison of serum-stimulated and unstimulated cells accompanied each subsequent Northern analysis and yielded identical results (n = 18).

of ribonucleotide reductase are expressed in the late G_1 phase of the cell cycle and that the increase in their expression occurs in a coordinate fashion.

Synergistic Stimulation of M1 and M2 Expression by cAMP-Increasing Agents and Insulin. To determine the effect of an increase in cellular cAMP on the expression of M1 and M2, quiescent cultures of Swiss 3T3 cells were treated with the diterpene forskolin, a direct activator of adenylate cyclase (18); prostaglandin E_1 (PGE_1) and 5'-(*N*-ethylcarboxamido)-adenosine (NECA), which increase cAMP synthesis through a receptor-mediated pathway (19, 20); or the cell-permeant cAMP analogue 8-bromo-cAMP (8BrcAMP) (21). NECA, PGE_1, and forskolin were also tested in the presence of inhibitors of cAMP degradation [e.g., 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidine (Ro 20-1724) or 3-isobutyl-1-methylxanthine (IBMX)]. Since cAMP acts as a potent

mitogenic signal for Swiss 3T3 cells in the presence of insulin (1, 3, 19–22), the cAMP-increasing agents were added either without or with insulin, and the treated cells were compared with serum-treated and control untreated cells. Previously, we verified that insulin does not affect the rapid and sustained increase in cAMP that these agents induce (18, 19, 23). Total cellular RNA was extracted and hybridized sequentially with probes specific for mRNAs encoding the M1 and M2 subunits of ribonucleotide reductase and with a probe for mouse 18S ribosomal RNA to verify equivalent loading of sample in each lane. In cells treated with the cAMP-increasing agents forskolin plus Ro 20-1724, PGE_1 plus Ro 20-1724, or 8BrcAMP alone, the levels of either M1 or M2 mRNA were increased but markedly lower than those induced by serum (Fig. 2). Addition of insulin at 1 μg/ml also caused an increase in M1 and M2 expression that was approximately the same as that induced by these cAMP-increasing agents. NECA and the phosphodiesterase inhibitor Ro 20-1724 neither increased M1 or M2 message when used alone nor induced a sustained elevation of cAMP at the concentrations tested (19, 20, 23). However, the addition of cAMP-increasing agents together with insulin caused a dramatic increase in the levels of both M1 or M2 mRNA, comparable to that achieved by the addition of serum (Fig. 2). Scanning densitometry of the autoradiograms indicated that cAMP-increasing agents, insulin, or their combination increased M2 mRNA by 6-, 8-, and 26-fold, respectively. The combination of cAMP-increasing agents and insulin synergistically induced stimulation of [^{3}H]thymidine incorporation into DNA that paralleled the M1 and M2 gene expression (results not shown).

Forskolin alone (1–25 μM) caused a small increase in M1 and M2 expression, whereas forskolin and insulin strikingly enhanced the M1 and M2 mRNA levels to a level comparable to that induced by serum (Fig. 3). Similar results were observed when insulin was added in the presence of various concentrations of 8BrcAMP (0.1–2.5 mM). The time course of the low expression of M1 and M2 in cells stimulated by insulin at 1 μg/ml, forskolin at 25 μM, or 8BrcAMP is not different from that promoted by serum (results not shown).

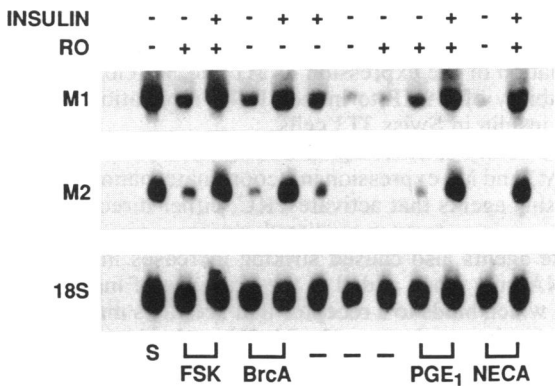

FIG. 2. Stimulation of ribonucleotide reductase gene expression by cAMP in the absence or presence of insulin. Northern blot analysis of mRNA concentration for the M1 and M2 subunits of ribonucleotide reductase was performed. Cells were assayed 18 hr after the addition of the cAMP-elevating agents alone or with insulin as indicated. Concentrations of each agent used were as follows: fetal bovine serum (S), 10%; forskolin (FSK), 25 μM; Ro 20-1724 (RO), 10 μM; 8BrcAMP (BrcA), 2.5 mM; insulin, 1 μg/ml; PGE_1, 200 ng/ml; and NECA, 10 μM. Control, Ro 20-1724, forskolin, PGE_1, NECA, and NECA plus Ro 20-1724 all resulted in [^{3}H]thymidine incorporation of less than 5×10^3 cpm per culture. Forskolin plus Ro 20-1724, 8BrcAMP, insulin, and PGE_1 plus Ro 20-1724 yielded [^{3}H]thymidine incorporation of 2–2.2 $\times 10^4$ cpm per culture. Serum, forskolin plus Ro 20-1724 plus insulin, 8BrcAMP plus insulin, PGE_1 plus Ro 20-1724 plus insulin, or NECA plus Ro 20-1724 plus insulin resulted in [^{3}H]thymidine incorporation of 1.3–2.2 $\times 10^5$ cpm per culture.

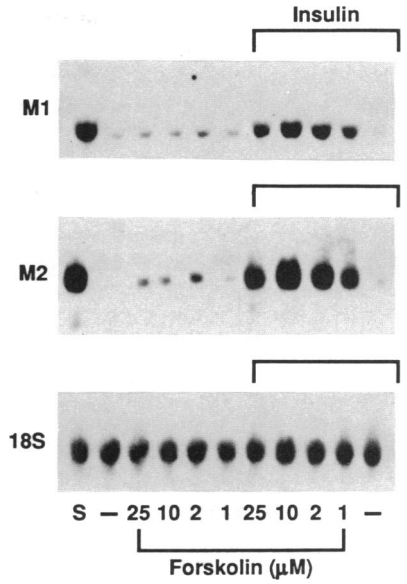

FIG. 3. Stimulation of ribonucleotide reductase gene expression by forskolin in the absence or presence of insulin. Forskolin-induced expression at 18 hr of the mRNA for M1 and M2 subunits of ribonucleotide reductase is shown by Northern blot analysis; insulin at 1 μg/ml was present as indicated by the bracket. The [^{3}H]thymidine incorporations (cpm per culture) in parallel cultures induced by 25 μM forskolin, 1 μg/ml insulin, forskolin plus insulin, and serum (S) were 5×10^3, 2.5×10^4, 1.7×10^5, and 2.2×10^5, respectively.

These experiments excluded the possibility that the effects shown in Figs. 2 and 3 resulted from changes in the time course of expression rather than in the level of M1 and M2 expression.

Effect of Phorbol 12,13-Dibutyrate (PDB), Bombesin, Vasopressin, Epidermal Growth Factor (EGF), and Insulin on M1 and M2 mRNA Expression. In Swiss 3T3 cells PKC activation stimulates DNA synthesis in synergy with insulin (1–3). To test whether these synergistic effects can be detected at the level of the expression of the ribonucleotide reductase subunits, quiescent cells were treated with PDB, a direct activator of PKC either in the absence or in the presence of insulin. PDB, at concentrations known to activate PKC in 3T3 cells (24), caused a small increase in the expression of both M1 and M2, comparable to the increase induced by forskolin (Fig. 4). Addition of various concentrations of PDB together with insulin resulted in dramatic enhancement of M1 and M2 expression in a dose-dependent manner. The levels achieved (50-fold) were similar to the induction by serum in parallel cultures. The time course of stimulation of M1 and M2 expression by PDB alone is identical to that induced by serum, albeit to a much lower level (results not shown).

In Swiss 3T3 cells the mitogenic neuropeptides bombesin and vasopressin are known to stimulate inositolphospholipid breakdown, Ca^{2+} mobilization, PKC activation (1, 3), and tyrosine phosphorylation (25). However, bombesin, but not vasopressin, stimulates arachidonic acid release and metabolism (26) and induces significant DNA synthesis in the absence of any other exogenously added factor (27). Vasopressin induces DNA synthesis only in the presence of insulin or other factors (28). We tested the effects of bombesin and vasopressin on M1 and M2 mRNA expression either in the absence or in the presence of insulin. Bombesin increased the expression of M1 and M2 approximately 50% more than insulin alone, and the two together were synergistic, exceeding the levels achieved by serum stimulation. In contrast, vasopressin caused a much smaller stimulation of M1 and M2 mRNA expression, comparable to forskolin or 8BrcAMP alone, but caused a dramatic increase in expression when

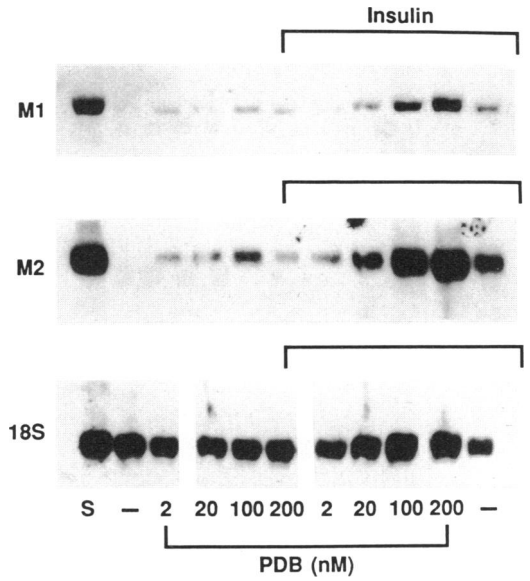

FIG. 4. Stimulation of ribonucleotide reductase gene expression by PDB in the absence or presence of insulin. PDB was added at various concentrations for 18 hr either without or with insulin at 1 μg/ml. Expression of the mRNA for M1 and M2 subunits of ribonucleotide reductase was monitored by Northern blot analysis. The incorporations of [^{3}H]thymidine (cpm per culture) in parallel cultures induced by 200 nM PDB, PDB plus insulin, or serum (S) were 3×10^3, 6×10^4, and 10^5, respectively.

added in the presence of insulin to the levels achieved by serum stimulation (Fig. 5). These results demonstrate that PKC activation acts synergistically with insulin to induce M1 and M2 mRNA expression in 3T3 cells.

EGF stimulates DNA synthesis in Swiss 3T3 cells when added with insulin. Insulin, at the concentrations used in this study, does not stimulate inositol phosphate production, Ca^{2+} mobilization, or PKC activation in Swiss 3T3 cells or in

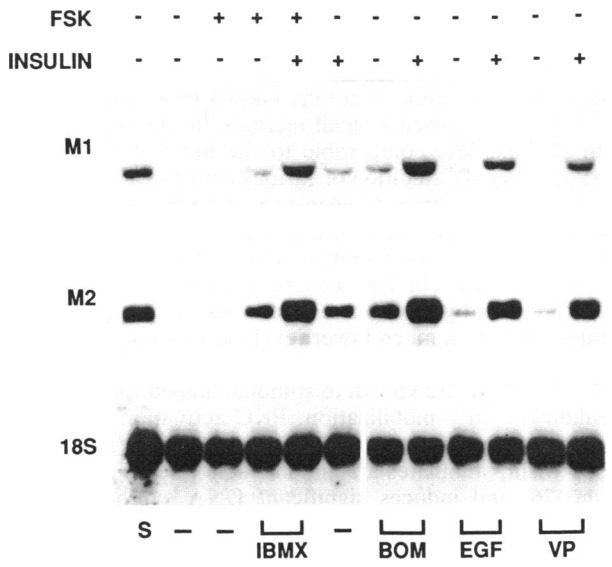

FIG. 5. Stimulation of ribonucleotide reductase gene expression by multiple mitogenic agents. Cells treated with bombesin (BOM) (10 ng/ml), EGF (5 ng/ml), or vasopressin (VP) (20 ng/ml) were evaluated by Northern analysis to determine induction of expression (at 18 hr) of the genes coding for the M1 and M2 subunits of ribonucleotide reductase. Shown for comparison is the effect of forskolin (FSK) (25 μM), forskolin (25 μM) plus IBMX (50 μM), and the two in conjunction with insulin (1 μg/ml) as well as serum (S) (10%) and untreated control cells.

many other target cells (29–31). EGF induces tyrosine phosphorylation and activation of the γ-1 isoform of phospholipase C, especially in cell lines that overexpress this receptor (32, 33). In Swiss 3T3 cells, however, it has been well documented that EGF does not stimulate inositolphospholipid hydrolysis, Ca^{2+} mobilization, or PKC activation (1). Neither EGF nor insulin increases cellular cAMP. Hence, EGF and insulin stimulate DNA synthesis by pathways that do not involve either inositolphospholipid breakdown or enhanced cellular cAMP. EGF alone caused a small but detectable increase in M1 and M2 mRNA expression similar to forskolin alone. This effect was dramatically enhanced to the levels achieved by serum stimulation when this peptide growth factor was added in the presence of insulin (Fig. 5).

DISCUSSION

Quiescent cultures of Swiss 3T3 cells can be stimulated to recommence DNA synthesis by distinct and interactive signal transduction pathways (1–3). These signaling pathways trigger a cascade of molecular events including rapid transcriptional activation of a set of genes (34). The mechanism(s) by which these early events are transduced into later events in G_1 remains poorly understood. In this study, we determined whether multiple signaling pathways leading to DNA synthesis also induced the expression of mRNA encoding the M1 and M2 subunits of ribonucleotide reductase, the key enzyme that catalyzes the reduction of ribonucleotides to the corresponding deoxyribonucleotides required for DNA synthesis (5).

The results presented here demonstrate that the expression of the M1 and M2 subunits is enhanced in a coordinated manner by many proliferative stimuli that act via different signal-transduction pathways. Thus, we analyzed the cAMP pathway by utilizing multiple agents that increase cellular cAMP, including activators of cAMP synthesis, inhibitors of cAMP degradation, and cell-permeant analogues of cAMP (19–23). Regardless of the agent used to elevate cAMP, we found that neither M1 nor M2 expression was markedly enhanced but, in every case, a striking increase in these mRNA levels could be elicited by the concomitant addition of any of these agents together with insulin. Thus, the regulation of the expression of M1 and M2 closely parallels the ability of cAMP to induce DNA replication in synergy with insulin in Swiss 3T3 cells.

The ability of other signal-transduction pathways to regulate M1 and M2 expression in a coordinate manner was tested by using agents that activate PKC, either directly—e.g., by PDB—or in a receptor-mediated manner—e.g., vasopressin. These agents also caused striking increases in M1 and M2 mRNA only when added in the presence of insulin. Bombesin, which binds to a receptor that activates multiple signal-transduction pathways (3, 24–26), stimulates substantial DNA synthesis in the absence of other factors, and its mitogenic effect is further potentiated by insulin (27). We found that bombesin, in the absence of any other factor, induces a marked increase in M1 and M2 expression and that this effect is further enhanced by insulin. These effects are mirrored by DNA synthesis activity. Finally, EGF, which together with insulin stimulates DNA synthesis in the absence of PKC activation or cAMP accumulation in Swiss 3T3 cells (1, 2), also caused synergistic stimulation of M1 and M2 expression. Collectively, these experiments show that multiple signaling pathways leading to DNA synthesis caused a striking increase in the expression of the genes encoding the M1 and M2 subunits of ribonucleotide reductase. Although our studies demonstrate that the expression of these genes is strictly coordinated in cells stimulated by multiple signal-transduction pathways, it is not clear whether they are regulated by identical mechanisms.

The entry into DNA synthesis is a complex process that requires changes in the level and activity of many enzymes and proteins involved in the replication and conformational changes of the DNA and in the supply of deoxyribonucleotide precursors (4, 5). In theory, the synergistic effects leading to DNA synthesis may result from regulation of each molecular component of the DNA synthetic machinery by a different signaling pathway. Alternatively, the synergistic effects of several pathways could be exerted at the level of each component. The striking synergistic effects presented here clearly support the second possibility. Whether multiple signaling pathways directly regulate the expression of the M1 and M2 genes or converge into an earlier point in G_1 resulting in the accumulation and/or modification of a protein(s) that, in turn, regulates their expression in a coordinate manner is an important question that requires further experimental work. Studies of the effect of different signal transduction pathways on the expression of these genes in Swiss 3T3 cells provide an approach that should assist in resolving these questions.

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