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Expression of Q227L-Gα_s in MCF-7 human breast cancer cells inhibits tumorigenesis

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ABSTRACT **The effects of expression of mutant (Q227L)-activated Gα_s and elevation of cAMP on mitogen-activating protein kinase (MAPK) activity and the transformed phenotype were studied in the MCF-7 human mammary epithelial cell line. Elevation of cAMP partially inhibited the epidermal growth factor-stimulated DNA synthesis and the intrinsic MAPK (ERK-1 and ERK-2) of serum-starved MCF-7 cells. Addition of 8Br-cAMP or expression of mutant (Q227L)-activated Gα_s in MCF-7 cells blocked the ability of these cells to grow in an anchorage-independent manner, as assessed by colony formation in soft agar. 8Br-cAMP in the culture medium also blocked estrogen stimulation of MCF-7 cell proliferation *in vitro*. MCF-7 cells expressing Q227L-Gα_s grew very slowly *in vitro*, and when these cells were injected s.c. into athymic mice implanted with estrogen pellets, the frequency of tumor formation was reduced greatly and the sizes of the tumors formed were much smaller than those in mice injected with MCF-7 cells that had been transfected with the empty vector. These results indicate that the intracellular levels of cAMP in transformed mammary epithelial cells can be a crucial factor in determining the expression of the transformed phenotype. Interactions between the G_s/adenylyl cyclase and MAPK-1,2 signaling pathways could be one mechanism by which expression of the transformed phenotype in mammary epithelial cells are regulated.**

Interactions between signaling pathways regulate many cellular processes, including proliferation and transformation. It is now well established that many growth factors that bind receptor-tyrosine kinases use Ras as the signal transducer to activate a cascade of protein kinases, including Raf and MEK-1,2, resulting in the activation of mitogen-activated protein kinase (MAPK) 1 and 2 (1, 2). In fibroblast-derived cell lines such as NIH 3T3, Ras directly interacts with Raf (3) and Raf activates MEK, which activates MAPK 1 and 2 (1, 2). Constitutively activated MEK induces transformation of NIH 3T3 cells (4, 5). These observations have indicated that activation of the Raf/MEK/MAPK may be sufficient to trigger transformation in at least fibroblasts. A number of studies also have shown that elevation of cAMP blocks signaling through the Ras/Raf/MEK pathway (6–9) and blocks Ras-induced transformation through protein kinase A (9). Regulation at the level of Raf appears to be a major site where protein kinase A modulates signal transmission to MAPK-1 and -2 (7, 8, 10).

We sought to extend our observations that mutant-activated Gα_s and elevation of cAMP block Ras-induced MAPK activity and transformation (9) to other systems. The human breast cancer cell lines appeared to be a particularly attractive system because many studies indicated that signaling from receptor and nonreceptor tyrosine kinases play a crucial role in the transformation of mammary epithelia (11–13). If signaling through the MAPK pathway were involved in maintaining the transformed state in mammary epithelia, then expression of activated Gα_s could be useful in blocking the expression of the transformed phenotype *in vivo*. To examine this hypothesis, we developed stably transfected MCF-7 mammary epithelial cell lines expressing activated Gα_s and tested their capability to proliferate in soft agar and to form tumors in athymic mice.

MATERIALS AND METHODS

Materials. DMEM and phenol red-free DMEM were from GIBCO/BRL. Fetal bovine serum and charcoal-treated fetal bovine serum were from HyClone. Recombinant human insulin from Novo-Nordisk (Copenhagen) was obtained from the Mount Sinai Pharmacy. The 17β-estradiol used in tissue culture experiments was from Sigma. The 17β-estradiol pellets (0.72-mg pellet, 60-day release) was from Innovative Research of America. MAPK antibodies were from New England Biolabs. Sources of others reagents have been described previously (9).

Cell Culture. Human breast cancer MCF-7 cells were purchased from American Type Culture Collection. The cells were grown in DMEM supplemented with 2 μg/ml insulin, 1 mM sodium pyruvate, 1 mM nonessential amino acids, 4 mM glutamine, 10% fetal calf serum, and antibiotics (penicillin/streptomycin). For routine culture, cells were grown in phenol red containing medium. When required, 1 week before experiments cells were grown in phenol red-free medium supplemented with 5% dextran-coated, charcoal-stripped fetal calf serum.

Transfections and Establishment of Clonal Cell Lines. For establishment of clonal cell lines, 20 μg of pRc/cytomegalovirus (CMV) or 20 μg of pRc/CMV-α_s* (9) were diluted into 220 μl of 0.1× TE (10 mM Tris·HCl, pH 8.0/1 mM EDTA) and mixed with 250 μl of 2× Hepes-buffered saline in a sterile 15-ml tube to which 31 μl of 2 M $CaCl_2$ was added slowly. The transfection mixtures then were distributed evenly over subconfluent MCF-7 cells for 24 h. The transfection medium was removed, and the cells were then fed with fresh medium (DMEM supplemented with 2 μg/ml insulin and 10% fetal calf serum). One day later, medium containing 0.8 g/liter G-418 was added to the cells, and neomycin-resistant cells were selected for 10 days. The individual neomycin-resistant cells were allowed to grow for another 2 weeks to form colonies. The colonies were picked by placing cloning cylinders around clearly separated colonies. A cAMP accumulation assay was used to select the α_s*-expressing MCF-7

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Abbreviations: MAPK, mitogen-activated protein kinase; CMV, cytomegalovirus; EGF, epidermal growth factor.

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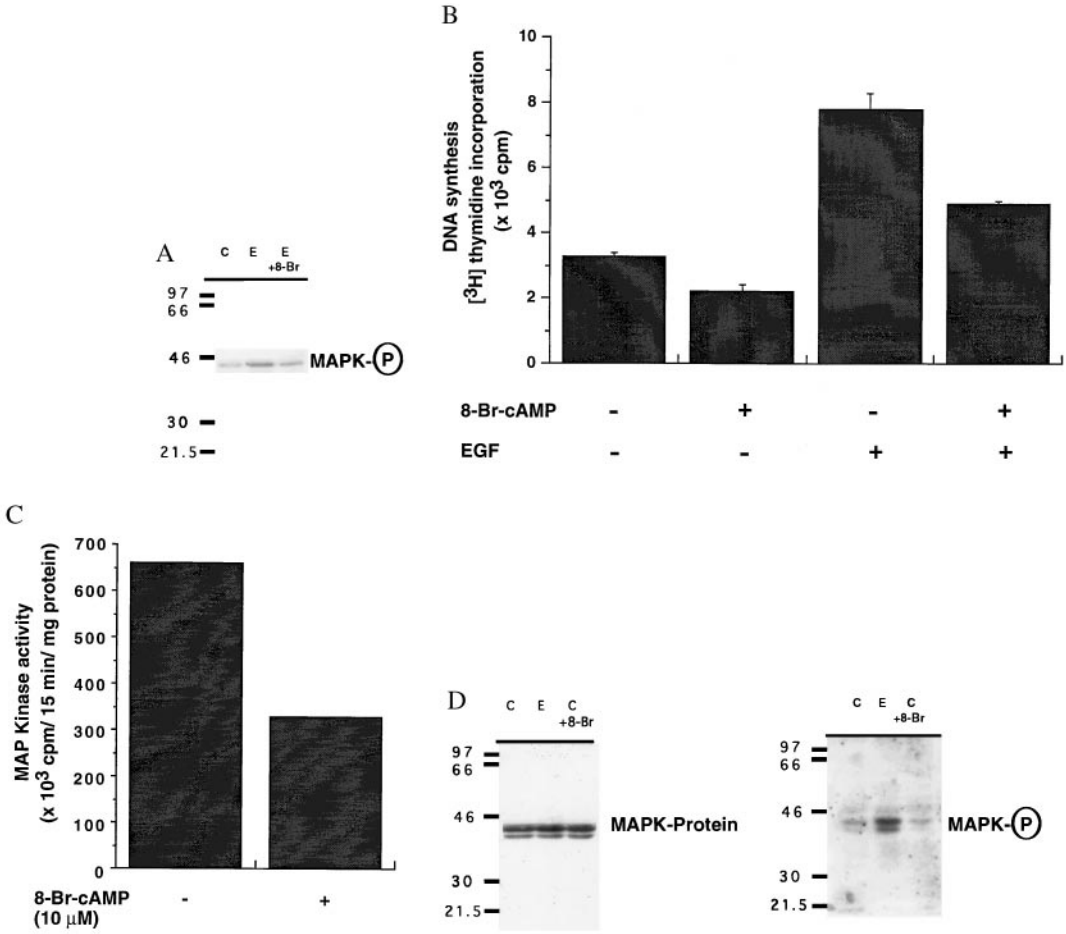

FIG. 1. Effects of 8Br-cAMP on the activities of MAPK-1,2 and DNA synthesis in MCF-7 cells. (*A*) Immunoblot of phospho-MAPK-1,2 in extracts from cells treated with or without (*C*) EGF (10 ng/ml) after incubation with 8Br-cAMP (10 mM). (*B*) EGF stimulated DNA synthesis in cells incubated with and without 8Br-cAMP. Serum-starved cells were incubated with or without 8Br-cAMP (10 mM) for 12 h and then with 1 mCi [^{3}H]-thymidine, without or with EGF (10 ng/ml), for another 16 h. Cells then were washed and extracted with trichloroacetic acid, and the trichloroacetic acid precipitate was counted (9). Values are mean ± SD of triplicate determinations. (*C*) Effect of 8Br-cAMP on the basal MAPK activity of serum-starved MCF-7 cells. Cell extracts were resolved on Mono-Q columns by using fast protein liquid chromatography. Aliquots of column fractions were assayed. Total counts under the peak are plotted as a bar graph. For controls, extracts that had been heat inactivated were resolved on Mono-Q columns by fast protein liquid chromatography and assayed. Control activity was 50–75 per column aliquot. (*D*, left) Immunoblot of total ERK1 and ERK2 from serum-starved cells treated without any additives (*C*) or with EGF (10 ng/ml) or 8Br-cAMP (10 mM). (*D*, right) Immunoblot of phospho-MAPK-1,2 in extracts from serum-starved cells treated without any additives (*C*) or with EGF (10 ng/ml) or 8Br-cAMP (10 mM). Equal aliquots were used in both assays.

clonal cell lines. For this, cells were labeled with [^{3}H]-adenine and cAMP accumulation was measured in the presence of 0.1 mM 3-isobutylmethylxanthine. Clones displaying 50–100% increases in basal cAMP levels were used for further studies.

MAPK Assays. The subconfluent MCF-7, vector CMV, or the α_s*-expressing MCF-7 clonal cell lines were maintained in serum-free DMEM (without phenol red). After 24 h, various concentrations of 8Br-cAMP, as indicated, were added to the MCF-7 cells. Twelve hours later, the cells were extracted and the extract was resolved on Mono-Q columns by using fast protein liquid chromatography. MAPK activity in column fractions was measured by using peptide substrate $EGFR_{662-681}$ (9).

Phosphorylation of MAPK-1,2. MCF-7 cells were serum-starved for 24 h. Then, 12 h before the start of the experiments, 8Br-cAMP was added to the required plates. When needed, cells were stimulated with epidermal growth factor (EGF) for 15 min. After treatment, cells were extracted in RIPA buffer (0.15 mM NaCl/0.05 mM Tris·HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% SDS) containing protease and phosphatase inhibitors and lysed by sonication. Aliquots of cell extracts were resolved electrophoretically and transferred to Immobilon-P (Millipore) membrane. The membranes were blotted with antibodies to phospho-ERK-1,2 or antibodies that recognize ERK1,2 proteins irrespective of their phosphorylation state. Bands were visualized by chemiluminescence by using alkaline phosphatase-coupled secondary antibody.

Colony Formation in Soft Agar. MCF-7 cells were incubated with various concentrations of 8Br-cAMP for 1 week, and then aliquots were plated onto soft agar plates. Wild-type, empty vector pRc/CMV or the α_s*-expressing clonal cells were assayed for colony formation in soft agar. Cells (5×10^4) were suspended in 3 ml of medium (DMEM supplemented with 2 μg/ml insulin and 10% fetal calf serum) containing 0.3% agar. The mixture was added over a layer of 0.5% agar in DMEM on a 60-mm plate. Plates were fed weekly with 2 ml of DMEM with 10% fetal calf serum, 0.3% agar, 2 μg/ml insulin, and 8Br-cAMP if needed. Three weeks later, plates were stained with the vital stain 2-(*p*-isodophenyl)-3-(*p*-nitrophenyl)-5-phenyltertazolium chloride hydrate for 2 days. Colonies larger then 0.15 mm in diameter were scored.

Tumorigenesis in Athymic Mice. 17-β-estradiol pellets (0.72 mg) were implanted s.c. in athymic mice. One day later, 10^6 control (neo^r) or α_s*-expressing MCF-7 clonal MCF-7 cells

were injected. Mice showing tumors were counted, and the tumor sizes were measured initially after 1 month and subsequently on a weekly basis for another 4 weeks. Final measurements after 8 weeks are given. All experiments were repeated at least twice, most three or more times. Qualitatively similar results were obtained. Typical experiments are shown.

RESULTS

MCF-7 cells have EGF receptors (14) that are known to signal through Ras and Raf to MAPK-1,2 (1, 2). MAPK-1,2 are activated by phosphorylation (15), and phospho-MAPK-1,2 immunoblots are widely used as an indicator of their state of activation. (16). Elevation of cAMP and activation of protein kinase A block signal transmission at the level of Raf (8, 10). Hence, we determined whether EGF stimulated the phosphorylation of MAPK-1,2 and whether this stimulation was blocked by cAMP. For this, serum-starved MCF-7 cells were incubated with and without 8Br-cAMP and then stimulated with EGF. Cell extracts then were resolved electrophoretically on SDS/polyacrylamide gels, transferred to Immobilon membranes, and blotted with phospho-MAPK-1,2-specific antibodies. EGF stimulated the phosphorylation of MAPK-1,2, and 8Br-cAMP greatly reduced this stimulation (Fig. 1*A*). We then checked whether 8Br-cAMP also inhibited EGF-stimulated DNA synthesis. When serum-starved MCF-7 cells were incubated with 8Br-cAMP and then tested for EGF-stimulated [^{3}H]-thymidine incorporation, it was found that 8Br-cAMP significantly inhibited EGF-stimulated [^{3}H]-thymidine incorporation (Fig. 1*B*). In this experiment, we also noticed that treatment with 8Br-cAMP significantly inhibited basal [^{3}H]-thymidine incorporation. Hence, we checked whether there was any effect of 8Br-cAMP on the basal MAPK activity of serum-starved MCF-7 cells. In initial experiments, MAPK activities were measured in fast protein liquid chromatography fractions by using $EGFR_{662–681}$ peptide as a substrate. It was found that incubation of serum-starved MCF-7 cells with 8Br-cAMP resulted in a 50% inhibition of the basal MAPK activity (Fig. 1*C*). This result was unusual in comparison with our data from NIH 3T3 fibroblasts in which no measurable basal MAPK activity was seen in serum-starved cells. To further verify this effect of 8Br-cAMP, we checked the phosphorylation state of MAPK-1 and -2 in MCF-7 cells. Treatment did not result in any change in total ERK-1,2 protein levels (Fig. 1*D*, left). Measurable levels of phosphorylated MAPK-1,2 were observed in serum-starved MCF-7 cells. (Fig. 1*D*, right), and as expected, EGF stimulated the phosphorylation of MAPK-1,2. 8Br-cAMP decreased basal phosphorylation in serum-starved MCF-7 cells (Fig. 1*D*, right).

In NIH 3T3 cells, we had observed that a partial suppression of Ras-stimulated MAPK activity and DNA synthesis resulted in a full inhibition of Ras-induced transformation (9). Hence, we decided to check whether 8Br-cAMP blocked expression of the transformed phenotype in MCF-7 cells as well. For this, we assessed the anchorage-independent growth of MCF-7 cells by measurement of colony formation in soft agar in the presence of varying concentrations of 8Br-cAMP. Increasing concentrations of cAMP suppressed colony formation by MCF-7 cells (Fig. 2). Although in long term experiments such as this a full effect was observed with 1 mM 8Br-cAMP, complete inhibition was not seen reproducibly at this concentration in acute MAPK experiments. Therefore, we used 10 mM 8Br-cAMP for the acute experiments.

We were interested in determining whether the effects of cAMP elevation that were observed in these *in vitro* experiments also occurred *in vivo*. Because it is not possible to selectively elevate cAMP levels in MCF-7 cells for prolonged periods *in vivo*, we decided to express stably the mutant-activated $G\alpha_s$ in MCF-7 cells. For this, MCF-7 cells were transfected with the vector pRc/CMV without any insert or

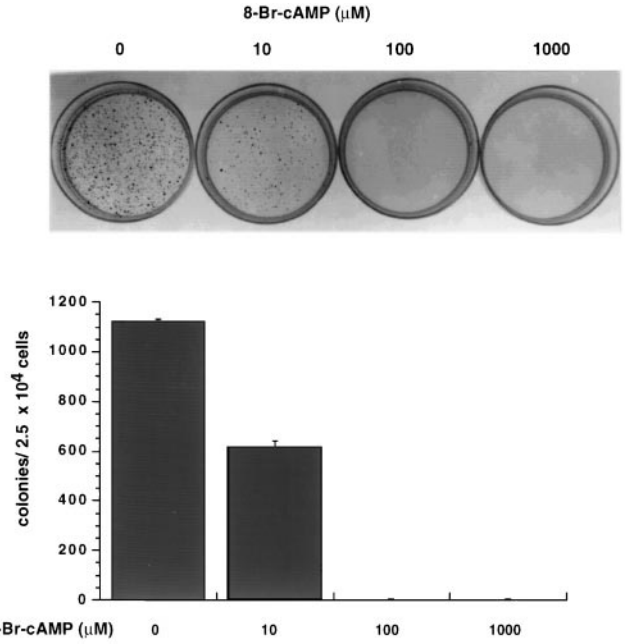

FIG. 2. Effect of varying concentrations of 8Br-cAMP on soft agar colony formation by MCF-7 cells. Cells were seeded and colony formation was assayed as described in *Material and Methods*. Four plates per condition were counted for each 8Br-cAMP concentration. Values are mean ± SD. A picture of a typical plate at each concentration of 8Br-cAMP also is shown.

with Q227L-$G\alpha_s$ (α_s*). Neomycin-resistant stable clonal lines were selected. The α_s*-expressing lines were identified by their elevation of basal cAMP levels. Under standard culture conditions, the α_s*-expressing lines grew slower (Fig. 3*A*). Under conditions in which the vector-transfected cell line formed over 500 colonies, the α_s*-expressing line formed under 50 colonies (Fig. 3*B*). To verify that the observed effect of α_s* was not caused by clonal variation, we compared three control (neo^r) and three α_s*-expressing lines. All three α_s*-expressing lines showed markedly reduced capability to form colonies in soft agar (Fig. 3*C*).

Because the α_s*-expressing MCF-7 cells did not show a transforming phenotype *in vitro*, we next determined whether the expression of α_s* reduced the capability of MCF-7 cells to form tumors in athymic mice. For MCF-7 cells to grow and form tumors in athymic mice, estrogen is required (17), and hence, we first checked the effect of 8Br-cAMP on estrogen regulation of MCF-7 cell proliferation in culture. For this, MCF-7 cells were cultured in phenol red-free medium-supplemented charcoal-stripped serum. Under these conditions, estrogen (10 nM) stimulated the proliferation of MCF-7 cells. Inclusion of 8Br-cAMP in the medium inhibited the estrogen effect (Fig. 4).

Athymic mice were implanted with estrogen pellets and 24 h later injected with either control or α_s*-expressing lines. Animals were scored for tumor formation initially after 1 month and then weekly for another 4 weeks. In three separate experiments, we found that the α_s*-expressing MCF-7 cell lines had a greatly reduced capability of forming tumors in athymic mice and the tumors that formed were much smaller in size after 8 weeks (Table 1). Two mice, one from the control (CMV-1) cell group and one from the CMV-α_s*-2 cell-injected group, are shown in Fig. 5.

DISCUSSION

The role of cAMP in regulating mammary carcinoma proliferation has been studied for some time (18–20). Initial studies

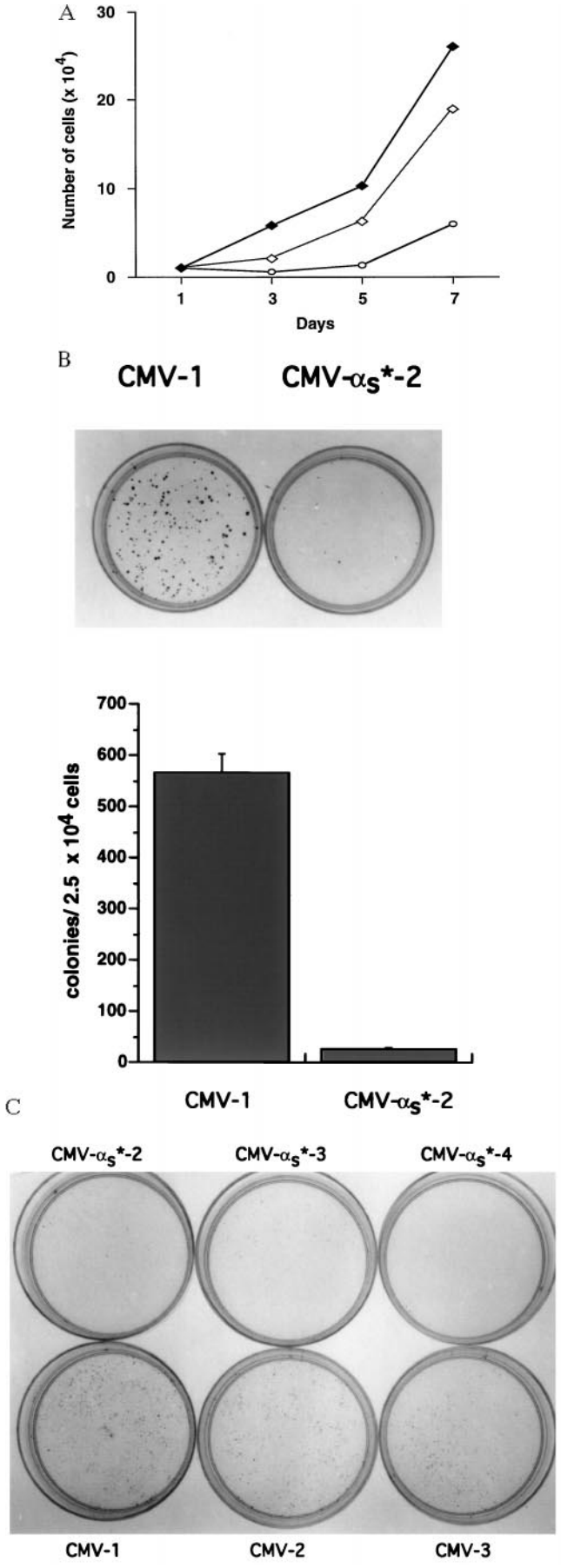

FIG. 3. Effect of α_s* expression on proliferation and soft agar colony formation by MCF-7 cells. (*A*) Wild-type (◆), vector-transfected (◇), and α_s*-expressing cells (○) were grown for 7 days. Cells were counted in duplicate every other day. Values are average of duplicate determinations, which were within 10%. (*B*) Cells were seeded and colony formation was assayed as described in *Material and Methods*. Four plates for the vector-transfected cell line and the α_s*-expressing cell line were counted. Values are mean ± SD. Pictures of a typical vector-transfected cell line and α_s*-expressing cell line are shown. (*C*) Three different vector-transfected and α_s*-expressing cell lines were compared for ability to form colonies in soft agar. Plating was carried out in triplicate. A typical plate for each condition is shown.

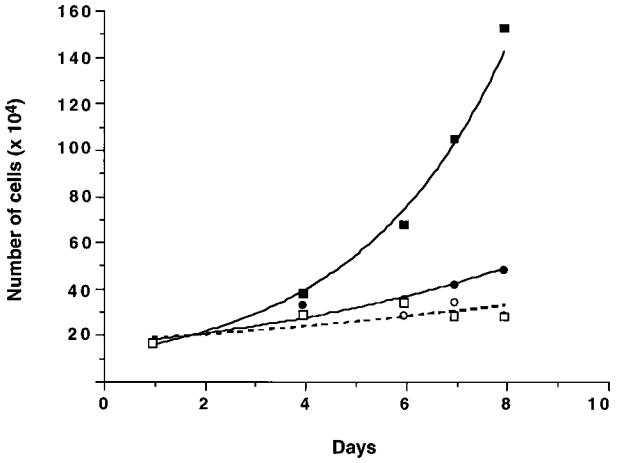

FIG. 4. Effect of 8Br-cAMP or α_s* expression on estrogen-stimulated proliferation of MCF-7 cells. Cells were incubated without (closed symbols) and with (open symbols) 8Br-cAMP (1 mM) in the absence (○, ●) and presence (□, ■) of 10 nM estrogen. Values are mean of triplicate determinations. Coefficient of variance was <10%.

indicated that dibutryl-cAMP in conjunction with arginine suppressed the proliferation of MCF-7 cells (18). Subsequently chloro-derivatives of adenosine were reported to be potent inhibitors of MCF-7 cell proliferation (19). A recent evaluation of the chloro-derivatives of cAMP indicated that their growth inhibitory effects were not related to their ability to activate protein kinase A. Their effects appear rather to be caused by toxicity of their breakdown products (20). Nevertheless, even in that study, elevation of cAMP by forskolin was found to inhibit proliferation of MCF-7 cells. In the results presented here, we show that elevation of cAMP levels has substantial effects on expression of the transformed phenotype of MCF-7 cells. 8Br-cAMP also inhibited EGF-stimulated MAPK-1,2 in MCF-7 cells and more interestingly inhibited the "basal" MAPK activity in serum-starved MCF-7 cells. This elevated MAPK activity may be caused by autocrine factors secreted by MCF-7 cells that stimulate this pathway. Such factors have long been postulated to sustain the proliferation of MCF-7 cells (21). It is noteworthy that our observations in MCF-7 cells are similar to those we had with NIH 3T3 cells (9), suggesting that the Raf to MAPK-1,2 signal transmission may be important for the expression of the transformed phenotype in mammary epithelia. Of course, activation of this pathway alone may not be sufficient for transformation as has been demonstrated for rat intestinal epithelial cells (22).

For the *in vitro* experiments, we were able to use 8Br-cAMP. However, this approach would not be feasible for *in vivo* experiments such as tumor formation in athymic mice. To elevate cAMP constitutively in MCF-7 cells, we expressed α_s*. Expression of α_s* in MCF-7 cells such that ambient cAMP levels were raised 50–100% did not kill the cells; rather, under standard culture conditions, these cells grew slower than normal MCF-7 cells. The growth rate of the α_s*-expressing cells was similar to that seen with wild-type cells in the

Table 1. Effect of α_s* expression on MCF-7-induced tumors in Na/Na mice

Experiment	MCF-7 clone	Animals showing tumors, *n*	Mean tumor diameter, mm
1	CMV-1	4/5	5.6 ± 0.8
	CMV-α_s-2	0/5	
	CMV-α_s-3	1/5	0.4
2	CMV-1	9/10	6.9 ± 0.5
	CMV-α_s-2	0/10	
	CMV-α_s-4	2/10	3.8, 4.5
3	CMV-1	4/4	6.8 ± 0.3
	CMV-α_s-2	2/5	1.6, 2.5
	CMV-α_s-3	2/5	1.9, 3.1

Indicated MCF cell clonal lines were injected into mice that had been implanted with estrogen pellets. Tumor diameter was measured with calipers. Both length and width were measured, and diameter for each tumor was calculated as length + width/2.

presence of 8Br-cAMP. Addition of 8Br-cAMP or expression of α_s* caused a partial inhibition of MAPK-1,2 and DNA synthesis. However, the α_s*-expressing cells have a greatly diminished capability to display a transformed phenotype. This was visible both in *in vitro* assays such as soft agar colony formation and in *in vivo* assays such as tumor formation in athymic mice. The tumorigenesis assays are important because they show for the first time that interactions between signaling pathways may be useful in inhibiting tumor formation *in vivo*. Up to now, it had not been certain that the interactions between the cAMP and MAPK signaling pathways that regulate proliferation *in vitro* would work *in vivo* or whether there would be compensatory mechanisms *in vivo* that would negate the anti-proliferative and anti-transforming effects of the cAMP pathway. The experiments presented here show that targeted continuous elevation of cAMP can be used to block tumor formation. In these experiments, we introduced the α_s* cDNA by transfection; however it may be possible to deliver this gene by the use of viral vectors by using approaches that currently are used in gene therapy. Further experiments are needed to ascertain whether interactions between signaling pathways can be used as a basis for therapeutic intervention in proliferative disorders.

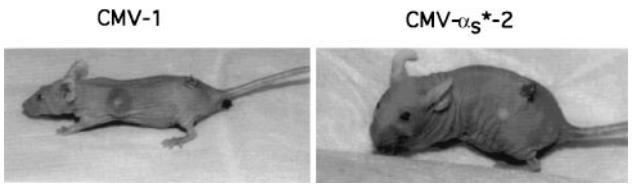

FIG. 5. Pictures of mice injected with either vector-transfected or α_s*-expressing MCF-7 cells. Animals were implanted with the estrogen pellet and injected with the indicated cell line. Tumor after 8 weeks is shown.

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