Cyclic AMP Responses Are Suppressed in Mammalian Cells Expressing the Yeast Low $K_m$ cAMP-Phosphodiesterase Gene*

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A genomic DNA fragment from *Saccharomyces cerevisiae* which contains the SRA5 (=PDE2) gene, coding for a low $K_m$ cAMP-phosphodiesterase, was transfected into Chinese hamster ovary cells. Clones carrying the cAMP-phosphodiesterase gene were capable of growth in the presence of cholera toxin, which slows the growth of untransfected cells by elevating their cAMP levels. The cholera toxin-resistant transfected cell lines expressed high levels of cAMP-phosphodiesterase mRNA and cAMP-phosphodiesterase activity. Basal intracellular cAMP levels were not significantly affected by the presence of the yeast cAMP-phosphodiesterase gene, but elevation of cAMP levels in response to cholera toxin or prostaglandin E1 was suppressed. Induction of the cAMP-responsive tyrosine aminotransferase promoter by cholera toxin was also blocked in cell lines carrying the yeast cAMP-phosphodiesterase gene. Cholera toxin-resistant transfected cell lines were sensitive to the growth inhibitory effects of $N^6$-$O^\prime$-dibutyryladenosine 3',5'-monophosphate, which can be used to bypass the effects of the yeast cAMP-phosphodiesterase.

Many hormones exert their intracellular effects by binding to cell surface receptors and, through the intermediary actions of GTP-binding proteins, cause an effector enzyme to increase synthesis of a second messenger molecule. In many cases, the effector enzyme is adenylyl cyclase and the intracellular second messenger is cAMP. The conclusion that a particular hormone requires an intracellular increase in cAMP to exert its effect is often based on the following correlaton: if cAMP concentrations rise after application of the hormone and if a variety of other agents, which include cholera toxin, pertussis toxin, forskolin, or phosphodiesterase inhibitors, cause an effect similar to that caused by the hormone, a case has been made that the intracellular second messenger is cAMP. There are several difficulties with this approach. Forskolin and phosphodiesterase inhibitors are nonspecific; the former affects ion channels (1, 2) and the latter, because they are often purine derivatives, may react with adenosine receptors or other intracellular targets. ADP-ribosylating toxins, such as cholera toxin, or prostaglandin E1 was suppressed. Induction of the cAMP-responsive tyrosine aminotransferase promoter by cholera toxin was also blocked in cell lines carrying the yeast cAMP-phosphodiesterase gene. Cholera toxin-resistant transfected cell lines were sensitive to the growth inhibitory effects of $N^6$-$O^\prime$-dibutyryladenosine 3',5'-monophosphate, which can be used to bypass the effects of the yeast cAMP-phosphodiesterase.

To control CAMP levels we have introduced an exogenous cAMP-phosphodiesterase gene into mammalian cells. The choice of genes was limited to the eukaryotic phosphodiesterase genes that have been cloned and sequenced. These include genes from *Drosophila melanogaster* (4), *Dicyostelium discoideum* (5), *Saccharomyces cerevisiae* (6-8), and recently mammalian cells (9-11). The yeast low $K_m$ cAMP-phosphodiesterase was the most suitable for introduction into mammalian cells because it has a low $K_m$, it is CAMP specific, and its activity is not modulated by calmodulin or cGMP (12, 13). The gene coding for the yeast cAMP-phosphodiesterase was cloned by complementation of a mutation that suppresses the effects of an activated RAS2 gene and was named PDE2 (6) or SRA5 (7) (suppressor of ras). We have inserted this gene into a retroviral vector that produces high expression of inserted genes in mammalian cells.

To test whether introduction of this construct into mammalian cells causes the production of active cAMP-phosphodiesterase, we chose Chinese hamster ovary (CHO) cells. CHO cells grow well even if they carry mutations in the regulatory or catalytic subunits of their cAMP-dependent protein kinase (14, 15), indicating that cAMP-dependent pathways in wild-type CHO cells are not essential for growth.

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1 The abbreviations used are: CHO, Chinese hamster ovary; kb, kilobase; PGE₂, prostaglandin E₂; Hepes, 4(2-hydroxyethyl)1-piperazinethanesulfonic acid; IBMX, isobutylmethylyxanthine; CAT, chloramphenicol acetyltransferase; TAT, tyrosine aminotransferase; UMS, upstream mouse sequence; GTP$\gamma$S, guanosine 5'-($\gamma$-thio)triphosphate; N$^6$-$O^\prime$-db-cAMP, N$^6$-$O^\prime$-dibutyryladenosine 3',5'-monophosphate; LTR, long terminal repeat.
The kinase mutations cause the cells to become resistant to growth inhibition by cholera toxin and non-hydrolyzable, membrane-permeable CAMP analogues. We predicted that expression of CAMP-phosphodiesterase would confer a similar resistance to the growth inhibitory effects of cholera toxin, but because the intracellular target of CAMP is not affected, the CAMP analogues that are not cleaved by the enzyme would still exert growth inhibitory and morphological effects on the cells. Pharmacological characterization of the enzyme and the uses to which this information can be put are described in the accompanying article (39).

MATERIALS AND METHODS

Cell Lines—The CHO cell lines 10011 and 10248 were grown in α-modified Eagle’s medium supplemented with 10% fetal bovine serum (GIBCO), 50 μg/ml penicillin, and 50 μg/ml streptomycin as described previously (16). Cell lines containing the yeast CAMP-phosphodiesterase gene were maintained in 100 μg/ml of G418 to prevent colony loss. Cell lines maintained in this way have proven stable for several months of serial passage. To ensure uniformity, the cells were replaced every month from frozen stocks. Before use in experiments, cell lines were passaged at least twice in the absence of cholera toxin. All experiments were performed with cells that were grown in the absence of G418 for at least 24 h.

Construction of pDOP-SRA5—A 2.2-kb Dral-BglII fragment, containing the entire coding sequence of the SRA5 gene, about 200 base pairs of 5' flanking sequence, and about 400 base pairs of 3' flanking sequence, including a polyadenylation consensus sequence, was excised from the plasmid pW4 (7, see Ref. 6 for a more detailed restriction map). The 5' BglII overhang of this fragment was filled using the Klenow fragment of DNA polymerase. BamHI linkers were ligated to the fragment, cut with BamHI to generate cohesive ends, and inserted into the BamHI cloning site of the retroviral expression vector pDOP (17). The vector containing the SRA5 gene in the correct orientation for transcription under the control of the murine sarcoma virus 5' long terminal repeat was named pDOP-SRA5 (Fig. 1).

Transfection and Selection of Clones—The pDOP-SRA5 construct (Fig. 1) was introduced into CHO cells by transfection rather than by retroviral infection because CHO cells are not hosts for retrovirus. Six million CHO cells were grown in 100-mm dishes at a density of 4 × 10^6 cells/dish. Twenty-four hours later, the dishes were washed twice with serum-free α-minimum essential medium. The cells were refed with serum-free α-minimum essential medium for 3 h in the presence or absence of cholera toxin at a final concentration of 100 μg/ml. Cell extracts were prepared as previously described (25) with the following modifications. Cell pellets were resuspended in buffer containing 0.1 M Hepes, pH 8.0, 1.0 mM MgCl2. Following homogenization, the extracts were centrifuged at 500 × g for 10 min at 4°C and 30 μg of supernatant protein, as determined by the Bradford assay (24), was used for the adenylate cyclase assay. Adenylate cyclase activity was determined by the method of Salomon et al. (26) with the following modification. The assay normally includes isobutylmethylxanthine (IBMX) to act as an inhibitor of the phosphodiesterase activity present in cell extracts. However, yeast CAMP-phosphodiesterase activity is not inhibited by IBMX, therefore we included a 100 μM unlabeled CAMP tracer in our assays. This concentration of CAMP was effective in blocking the breakdown of [3H]cAMP to [3H]5'-AMP as measured by preincubating with [3H]CAMP. Assays were carried out for 15–30 min and activity was expressed as pmol/min/mg protein.

Transient Transfections and CAT Assays—The plasmid pBTLT-CAT was a gift from Dr. G. Schutz (Institute of Cell & Tumor Biology, German Cancer Research Center, Heidelberg, Federal Republic of Germany) and contains 3.0 kb of upstream promoter sequence of the CAMP-responsive rat liver tyrosine aminotransferase gene (27, 28). Single-copy CAT reporter site (CAT) that expresses a bacterial chloramphenicol acetyltransferase gene (CAT) which acts as a reporter gene to assess transcriptional activity of the promoter (29). The 3.0-kb TAT promoter fragment was cloned into the plasmid pSV0UMS-CAT in which all the SV40 early promoter sequences have been deleted and an upstream mouse sequence (UMS) from the mouse c-mos gene has been introduced upstream of the CAT gene segment. The UMS sequence is thought to act as a transcription termination sequence, thus further reducing the background CAT activity (30). Two plasmids were constructed, the details of which will be published elsewhere. The first, pTAT-CAT contains 3.0-kb upstream TAT promoter sequence cloned immediately downstream from the UMS segment. The second construct, pTATCAT contains 2.5 kb of upstream TAT promoter sequence cloned in the reverse orientation immediately downstream from the UMS sequence and upstream from the CAT gene in pSV0UMS-CAT.

CHO cells were seeded in 10-cm^2 dishes at a density of 4 × 10^5 cells/dish (no cholera toxin treatment) or 6 × 10^5/dish (cholera toxin; 100 μg/ml). The following day the cells were transiently transfected with the plasmids pTAT-CAT or pTAT-CAT by the CaPO4 precipitation method. Briefly, the plasmid DNA (10 μg/dish) was mixed in a solution containing 0.05 volumes of 2.5 M CaCl2. This solution was added drop by drop to each dish. Four h later the dishes were washed twice with serum-free α-minimum essential medium for 3 h in the presence or absence of cholera toxin at a final concentration of 100 μg/ml. Cell extracts were prepared as previously described (25) with the following modifications. Cell pellets were resuspended in buffer containing 0.1 M Hepes, pH 8.0, 1.0 mM MgCl2. Following homogenization, the extracts were centrifuged at 500 × g for 10 min at 4°C and 30 μg of supernatant protein, as determined by the Bradford assay (24), was used for the adenylate cyclase assay. Adenylate cyclase activity was determined by the method of Salomon et al. (26) with the following modification. The assay normally includes isobutylmethylxanthine (IBMX) to act as an inhibitor of the phosphodiesterase activity present in cell extracts. However, yeast CAMP-phosphodiesterase activity is not inhibited by IBMX, therefore we included a 100 μM unlabeled CAMP tracer in our assays. This concentration of CAMP was effective in blocking the breakdown of [3H]cAMP to [3H]5'-AMP as measured by preincubating with [3H]CAMP. Assays were carried out for 15–30 min and activity was expressed as pmol/min/mg protein.

Cat Assays—CHO cells were harvested by scraping from Petri dishes and disrupted by two rounds of freezing and thawing. Homogenates were assayed for CAMP-phosphodiesterase activity with 10

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2 R. Wilson, personal communication.

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unpublished results.
The cell lines used to produce the growth curves presented in Fig. 1, the CAMP-resistant cell line 10248 (panel B), and the CAMP-resistant cell line CHO 10001, a cell line transfected with the pSV2neo control plasmid (CHO-C1), and the three cell lines containing the yeast CAMP-phosphodiesterase gene (CHO-PDE4, CHO-PDE9, and CHO-PDE10). The plot was probed with the 0.5-kb BamHI-EcoRI fragment from the yeast SRA5 gene in pDOP-SRA5. Only the CHO-PDE cell lines carrying the SRA5 gene produced detectable yeast CAMP-phosphodiesterase transcripts of the predicted size. One transcript extended from an initiation site in the 5' LTR to the polyadenylation site of the SRA5 gene (approximately 3.4 kb), the other extended from the 5' LTR to the polyadenylation site in the 3' LTR (approximately 6.7 kb). CHO-PDE cell lines 11-16 were also tested and contained the SRA5 transcripts (data not shown).

Detection of the Yeast Enzyme by Kinetic Analysis—The yeast CAMP-phosphodiesterase is easily detected in enzyme assays because of its 170 nM Km. Mammalian phosphodiesterases have a range of Km values between 2-fold and 500-fold higher (32). Extracts of CHO cells were assayed at low CAMP concentrations (below the Km of the yeast enzyme) where the yeast enzyme is relatively more efficient than the endogenous enzyme.

RESULTS

Growth Properties of Transformants—Chinese hamster ovary cells were transfected with the plasmid pDOP-SRA5 which has the yeast SRA5 (=PDE2) gene inserted into the unique BamHI site of pDOP, as shown in Fig. 1, or with the pDOP plasmid alone. In some experiments pSV2neo, which confers only G418 resistance, was used. The yeast CAMP-phosphodiesterase gene contains its own ATG initiation codon, termination, and polyadenylation signals. The plasmid pDOP-SRA5 can also be used for making recombinant retrovirus after transfection into a packaging cell line. Although we have successfully used recombinant retrovirus-mediated gene transfer in other cell types,2 this retrovirus does not infect CHO cells efficiently and therefore transfection was used instead. Cells transfected with pDOP, pDOP-SRA5, or pSV2neo were selected for resistance to G418. The pool of G418-resistant colonies transfected with pDOP-SRA5 was selected a second time with 100 ng/ml cholera toxin. Approximately 5-10% of the G418-resistant transfected clones were completely resistant to the growth inhibitory and morphological effects of cholera toxin. The remaining colonies had an intermediate phenotype; they grew better than control cells but not as fast as the 5% that were studied further. A number of cholera toxin and G418-resistant colonies were grown clonally and examined for their growth characteristics. Fig. 2 shows the effect of cholera toxin on the growth of the parental cells (panel A), the CAMP-resistant cell line 10248 (panel B), and two independent CHO-PDE clones (panels C and D). The cell lines used to produce the growth curves presented in panels C and D were shown by DNA slot blot analysis to contain the yeast CAMP-phosphodiesterase gene (data not shown). Cholera toxin slows the growth of CHO cells enough to distinguish resistant colonies growing on plastic or in soft agar. Growth of strain 10248, which has an altered regulatory subunit of the CAMP-dependent protein kinase was unaffected by cholera toxin. As predicted, the growth of CAMP-phosphodiesterase containing clones was unaffected by cholera toxin. The morphological effect of cholera toxin, which causes the cells to become spindle shaped and clustered, is also blocked in the CHO-PDE cell lines as shown in Fig. 3E.

Transcription of the Yeast PDE2/SRA5 Gene—The yeast CAMP-phosphodiesterase gene is transcribed in these cholera toxin resistant clones. Fig. 4 shows a Northern blot of 20 μg of total RNA extracted from the untransfected parental cell line CHO 10001, a cell line transfected with the pSV2neo control plasmid (CHO-C1), and the three cell lines containing the yeast CAMP-phosphodiesterase gene (CHO-PDE4, CHO-PDE9, and CHO-PDE10). The blot was probed with the 0.5-kb BamHI-EcoRI fragment from the yeast SRA5 gene in pDOP-SRA5. Only the CHO-PDE cell lines carrying the SRA5 gene produced detectable yeast CAMP-phosphodiesterase transcripts of the predicted size. One transcript extended from an initiation site in the 5' LTR to the polyadenylation site of the SRA5 gene (approximately 3.4 kb), the other extended from the 5' LTR to the polyadenylation site in the 3' LTR (approximately 6.7 kb). CHO-PDE cell lines 11-16 were also tested and contained the SRA5 transcripts (data not shown).

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FIG. 3. Effect of cholera toxin and N\textsuperscript{6},O\textsuperscript{2}-dibutyryladenosine 3',5'-monophosphate on the morphology of CHO-C3 and CHO-PDE12 cells. CHO-C3 (panels A-C) and CHO-PDE12 (panels D-F) cells were plated at 5 x 10\(^4\) cells/cm\(^2\) with no additions (panels A and D), with 100 ng/ml cholera toxin (panels B and E), or with 1 mM N\textsuperscript{6},O\textsuperscript{2}-dibutyryladenosine 3',5'-monophosphate (panels C and F). Photographs were taken after 3 days of growth.

FIG. 4. Expression of yeast low K\textsubscript{m} cAMP-phosphodiesterase mRNA. Twenty \(\mu\)g size-fractionated total RNA from the indicated cell lines was analyzed by Northern blot, using a \(^{32}\)P-labeled fragment of the SRA5 gene as a probe (see "Materials and Methods"). pDOP-SRA5 transfected cell lines express two messenger RNAs: one extending from the 5'-LTR to the 3'-LTR (6.7 kb) and the other from the 5'-LTR to the polyadenylation site of the SRA5 gene (3.4 kb).

enzymes. The SRA5 containing strains CHO-PDE4, CHO-PDE9, CHO-PDE10, CHO-PDE12, CHO-PDE13, CHO-PDE14, CHO-PDE15, and CHO-PDE16 had respectively 8.2-, 7.8-, 5.3-, 10.6-, 7.9-, 8.8-, 10.7-, and 9.9-fold more cAMP-phosphodiesterase activity than control CHO-C3 or CHO-C1 cells when measured at a low substrate concentration (45 nM). Furthermore, kinetic analysis using different cAMP concentrations showed that the component of the cAMP-phosphodiesterase activity unique to the extracts from the pDOP-SRA5 transfected cells had an apparent \(K_m\) of 100-300 nM (Fig. 5). This is close to the 170 nM value reported for the yeast low K\textsubscript{m} cAMP-phosphodiesterase (12). As the cAMP levels are increased while the amount of enzyme is kept constant, the fraction of total cAMP hydrolyzed decreases, as shown in Fig. 5. However, even in the substrate range of 1-10 \(\mu\)M, where the contribution of endogenous phosphodiesterases to the cAMP-hydrolyzing activity is increased (due to their higher \(K_m\)), the majority of the hydrolytic activity is due to the yeast cAMP-phosphodiesterase.

Control of Cholera Toxin-stimulated cAMP Levels—The
constitutive activation of adenylate cyclase activity by cholera toxin results in elevated cAMP levels in pDOP control transfected (CHO-C3) cells. Cells incubated for 6 h in the presence of 100 ng/ml cholera toxin had a 36-fold increase in cAMP levels (Table I). The CHO-PDE12 cell line showed no increase in cAMP levels when grown in the presence of cholera toxin for 6 h (Table I). The reduction of cholera toxin-stimulated cAMP levels is consistent with the cholera toxin-resistant phenotype of the CHO-PDE cell lines and presumably results from the rapid conversion of cAMP to 5'-AMP because of the presence of the yeast low K₅ₐ CAMP-phosphodiesterase activity. Elevation of cAMP levels by prostaglandin E₂ were also diminished in the CHO-PDE cell lines (see below).

Despite the presence of the yeast low K₅ₐ cAMP-phosphodiesterase activity in CHO-PDE cell lines, the basal cAMP levels of these cell lines were not significantly different from the control cell lines (Table I). In yeast, cAMP levels are regulated over a large concentration range by a negative feedback loop which includes the catalytic subunit of the cAMP-dependent protein kinase, the CDC25, RAS1, and RAS2 gene products, and adenylate cyclase (33). The maintenance of basal cAMP levels in CHO cells expressing large amounts of cAMP-phosphodiesterase might be caused by a similar compensatory mechanism which activates the endogenous adenylate cyclase. To assay adenylate cyclase activity in mammalian cell extracts it is necessary to include a phosphodiesterase inhibitor such as IBMX in the reaction mixture. The yeast enzyme is relatively insensitive to the phosphodiesterase inhibitors IBMX and RO 20-1724 (IC₅₀ > 300 μM at 25 mM CAMP; data not shown). Therefore, an excess of non-radioactive cAMP is required in the reaction mixture for the CHO-PDE cell lines to quench the yeast CAMP-phosphodiesterase activity and prevent hydrolysis of the radioactive reaction product. Basal levels of adenylate cyclase activity or activation of the membrane adenylate cyclase by pretreatment of cells with cholera toxin in vivo, or activation in vitro by NaF, GTP, or GTP-γS showed no significant difference between control and SRA5 expressing cell lines (data not shown), indicating that the CHO-PDE cells do not have an activated adenylate cyclase to compensate for the high phosphodiesterase activity.

Prostaglandin E₂, Effects—One potential use of the technique developed here is to block the cAMP-mediated effects of hormones. PGE₁ causes a rapid rise in cAMP levels in CHO cells (Fig. 6). If the yeast cAMP-phosphodiesterase activity and the rise in cAMP caused by PGE₁ occur in the same cellular compartment, we expect that the rise should be diminished by the presence of the enzyme. The results in Fig. 6A show that this is the case. In the CHO-C3 cell line, cAMP levels were stimulated 7–15-fold by 10 μM PGE₁, depending on the experiment, peaking around 3 min (Fig. 6A). In the CHO-PDE12 cell line, cAMP levels were stimulated only 2–4-fold by 10 μM PGE₁, peaking at about 1 min (Fig. 6B). Integration of the two curves in Fig. 6A revealed that approximately 70% of the cAMP response of the CHO-C3 control cell line was eliminated in the CHO-PDE12 cell line. When cAMP levels were measured 1 min after stimulation with different PGE₁ concentrations, CHO C3 cells and CHO-PDE12 cells required equivalent amounts of PGE₁ (about 0.3 μM) to induce half-maximal activation of cAMP levels (Fig. 6B). This is what one would expect to occur in the presence of large amounts of CAMP-phosphodiesterase, when receptor binding, coupling to G-proteins, and activation of the adenylate cyclase are unaffected.

Reversibility of the Effects of the SRA5 Gene—The method used here blocks the cAMP signal transduction system at a step before cAMP can activate the cAMP-dependent protein kinase. Because this molecule is left intact, it is possible to activate the cAMP-dependent protein kinase in cells that contain the yeast cAMP-phosphodiesterase by adding cell-permeant cAMP analogues that are not hydrolyzed but which bind to and activate the cAMP-dependent protein kinase. N⁶,O⁶-dibutyrlyadenosine 3',5'-monophosphate (N⁶,O⁶-db-CAMP) is one such analogue. At 1 mM, N⁶,O⁶-db-CAMP inhibits growth of both control CHO-C3 and CHO-PDE12 cells by about 60%. Furthermore, the cell shape changes that are induced by cholera toxin in CHO-C3 cells but not in CHO-PDE12 cells, can be seen in both cell lines after treatment with 1 mM N⁶,O⁶-db-CAMP, as shown in Fig. 3 (compare panels C and E). A detailed pharmacological analysis of the yeast low K₅ₐ cAMP-phosphodiesterase is described in the accompanying paper (39).

Effects on a CAMP Inducible Promoter—The 3.0-kb upstream segment of the cAMP-inducible TAT promoter (27, 28) was cloned in the correct and reverse orientations into the pSV,UMS-CAT reporter plasmid (pTAT-CAT and

| Cell line | No cholera toxin | 100 ng/ml cholera toxin |
|-----------|-----------------|------------------------|
| CHO-C3    | 9 ± 1           | 320 ± 37 (36.0X)       |
| CHO-PDE12 | 7 ± 1           | 5 ± 1 (0.7X)           |

* M. M. Gottesman and G. Johnson, unpublished observations.
pTAT-CAT, respectively) and transiently transfected into CHO C1, CHO PDE10, and CHO PDE12 cell lines. Induction of TAT promoter activity by cholera toxin (100 ng/ml) was seen as a 2.0–2.5-fold increase in CAT activity in the control cell line CHO-C1, which does not express the yeast cAMP-phosphodiesterase activity (Fig. 7). In cell lines CHO-PDE10 and CHO-PDE12, in which the cholera toxin-induced increase in cAMP levels is ablated due to expression of the yeast cAMP-phosphodiesterase, there was no increase in TAT promoter activity as assessed by CAT activity (Fig. 7). Transfection of the pTAT-CAT plasmid containing the TAT promoter in the reverse orientation resulted in no detectable CAT activity in any of the cell lines. Thus, the inability of the cells expressing the yeast cAMP-phosphodiesterase to increase cAMP levels affects gene induction as well as growth and morphology.

**DISCUSSION**

CHO cells transfected with the vector pDOP-SRA5 produce a substantial amount of active yeast cAMP-phosphodiesterase, which prevents cAMP levels from accumulating upon stimulation of the adenylate cyclase. Expression of the yeast cAMP-phosphodiesterase activity in the CHO-PDE cell lines permitted normal growth and morphology in the presence of cholera toxin. The rise in intracellular cAMP levels that occurs after stimulation with PGE1 was also limited. Activation of the cAMP-stimulatable tyrosine aminotransferase promoter by cholera toxin was prevented in CHO-PDE cell lines. The effects of the enzyme can be overcome with cAMP analogues that are not hydrolyzed but which activate the cAMP-dependent protein kinase, creating an experimental situation similar to that provided by a conditional mutant.

It is not clear why basal cAMP levels are unaffected by expression of yeast cAMP-phosphodiesterase in CHO cells. CHO cells expressing the yeast cAMP-phosphodiesterase do not seem to compensate for this activity by activating adenylate cyclase. One possibility is that there is a cAMP pool that is sequestered in a compartment not accessible to the yeast cAMP-phosphodiesterase (such as a pool bound to the regulatory subunit of the cAMP-dependent protein kinase). The cholera toxin or PGE1-sensitive pool of cAMP is apparently accessible to the yeast cAMP-phosphodiesterase. These results imply that basal cAMP levels do not reflect phosphodiesterase activity in CHO cells.

The ability to elevate cAMP levels in eukaryotic cells by pharmacological means has aided in understanding the elements of the second messenger cascade and has led to clinical applications, ranging from grafting of cholera toxin-treated skin allografts (34) to the treatment of cardiac failure with phosphodiesterase inhibitors (35). There are experimental situations in which it would be useful to be able to reduce cAMP levels. However, few strategies to reduce cAMP levels or to antagonize the effects of cAMP on the cAMP-dependent protein kinase have been developed. One method that has been used is the introduction of genes coding for mutant regulatory subunits of the cAMP-dependent protein kinase (18, 36). The resultant regulatory subunits bind cAMP with greatly reduced affinity but still bind the catalytic subunit. In another approach, sequences of the inhibitor protein of the catalytic subunit of the cAMP-dependent protein kinase have been introduced transiently into cells and the induction of cotransfected cAMP-responsive reporter genes by cAMP analogues has been reduced (37, 38). It has not yet been possible to create permanent cell lines which express an excess of protein kinase inhibitor, the effects of which are irreversible (38).

The elevation in cAMP levels by cholera toxin is chronic and is known to block cell growth by activation of the cAMP-dependent protein kinase. In those clones that we have analyzed, there is enough phosphodiesterase activity to reduce cAMP levels so that the cells can grow. A mutated cAMP-dependent protein kinase would give the same phenotype (as shown in Fig. 2). However, in these transfected lines we know that this enzyme is intact. First, the frequency of the transfectants is too high to be accounted for by mutagenesis. Second, N6-O"-db-CAMP, which is a poor substrate for the yeast enzyme, causes the CHO-PDE cells to assume a morphology that is identical to CHO-C control cells treated with cholera toxin or cAMP analogues, which could only happen if the cAMP-dependent protein kinase can be activated.

The cAMP response to PGE1 is fast and transient. Even under these conditions the yeast cAMP-phosphodiesterase expressed in the CHO-PDE12 cells is able to substantially reduce the cAMP response to PGE1. About 70% of the normal cAMP increase of control cells is eliminated. Whether this is sufficient to completely block the activation of the cAMP-dependent protein kinase by PGE1, is not known. In CHO cells, PGE1 produces no effects on growth or morphology, so that these events cannot be monitored at a phenotypic level as they can be with cholera toxin.

The pDOP-SRA5 vector provides a transferable genetic tool to suppress the effects of adenylate cyclase stimulation in eukaryotic cells in a pharmacologically reversible manner. Cells expressing high amounts of cAMP-phosphodiesterase can be used for studying the role of cAMP in the control of cell growth, differentiation, or metastasis in vitro. The gene should continue to function in vivo if cells are placed into syngeneic or immunologically incompetent animals, offering experimental possibilities currently unavailable with a strictly pharmacological approach. One possibility is that the expression of the yeast cAMP-phosphodiesterase gene can be placed under the control of specific promoters and limited to particular target tissues in transgenic animals.

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