Cyclic AMP promotes CREB-dependent induction of the cellular inhibitor of apoptosis protein-2 and suppresses apoptosis of colon cancer cells through ERK1/2 and p38 MAPK

Hiroshi Nishihara‡, Michael Hwang‡, Shinae Kizaka-Kondoh§, Lars Eckmann¶, and Paul A. Insel ‡¶||

Departments of ‡Pharmacology and ¶Medicine, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0636, §Department of Molecular Oncology, Kyoto University Graduate School of Medicine, Kyoto 606-8501, Japan

|| To whom correspondence should be addressed:

Paul A. Insel, MD
Tel: 858-534-2295
Fax: 858-822-1007
Email: pinsel@ucsd.edu
Summary

We recently reported that cyclic AMP (cAMP) suppresses apoptosis in colon cancer cells and induces cellular inhibitor of the apoptosis protein-2 (c-IAP2) via a cAMP-responsive element (CRE), which suggested a mechanism for chemoprevention of colon cancer by non-steroidal anti-inflammatory drugs. In this study, we used T84 human colon cancer cells to define the pathway by which increases in cAMP induce c-IAP2 expression. Treatment with several different cAMP agonists stimulated phosphorylation of CRE binding protein (CREB) and activated expression of c-IAP2 in a CREB-dependent manner. Studies with pharmacological inhibitors revealed that cAMP-dependent phosphorylation of CREB required activation of extracellular signal-regulated kinase (ERK) 1/2 and p38 mitogen-activated protein kinase (MAPK), but was largely independent of protein kinase A (PKA). Immunoblots and transcriptional reporter assays using specific inhibitors, as well as expression of constitutively active forms of MEK1 and M KK3, showed that c-IAP2 induction by cAMP is regulated predominantly through ERK1/2 and p38 MAPK and suggested involvement of p90 ribosomal protein S6 kinase (RSK) and mitogen and stress response kinase (MSK)-1 as well. Consistent with those results, we found that cAMP-dependent suppression of apoptosis was blocked by treatment with inhibitors of ERK1/2 and p38 MAPK. We conclude that cAMP can induce c-IAP2 expression in colon cancer cells through CREB phosphorylation and CRE-dependent transcription in a manner that involves activation of ERK1/2 and p38 MAPK. These results emphasize that activation of kinases other than PKA can mediate the actions of agents that increase cAMP, particularly in the regulation of CREB-dependent events.
Introduction

Suppression of normal apoptotic pathways contributes to tumor progression and confers resistance to cytotoxic anticancer drugs and radiation (1). The second messenger cAMP has anti-apoptotic actions (2-4) and its primary effector enzyme, PKA, is a target for cancer therapy (5-7). Prostaglandin E2 (PGE2), which is formed from arachidonic acid by cyclooxygenases (COX), binds to Gs-coupled receptors and increases intracellular cAMP concentration (8). COX levels are high in human colon cancers (9) and COX inhibition prevents cell proliferation and promotes apoptosis (10,11). Recently, we reported that increases in cAMP levels promoted by PGE2 or other agents that raise cAMP inhibit apoptosis in colon cancer cells through the induction of c-IAP2, therefore suggesting a novel mechanism of cancer chemoprevention by non-steroidal anti-inflammatory drugs (12).

Inhibitors of apoptosis proteins (IAPs) are characterized by a domain of about 70 amino acids, termed the baculoviral IAP repeat (BIR), based on the original discovery of these apoptosis suppressors in the baculoviral genome (13,14). IAP family proteins have potentially important roles in the regulation of apoptosis and tumorigenesis (15,16). The expression of survivin, one of the IAP family proteins, is significantly increased in several human cancers (17). Although eight human IAPs have been identified (18), c-IAP1/BIRC2 and c-IAP2/BIRC3 are the only IAPs that appear to be part of a signaling complex recruited to the cytoplasmic domain of the type-2 TNF receptor (20). In addition, c-IAP2 was also suggested to be a causative gene of mucosa-associated lymphoid tissue (MALT) lymphoma and to have a role in carcinogenesis and tumor progression (19). The active forms of caspase-3 and -7 are directly inhibited by c-IAP2 (20), which can also prevent the proteolytic processing of pro-caspase-3, -6 and -7 by blocking the cytochrome c-induced activation of pro-caspase-9 (21).
Expression of c-IAP2 is regulated through multiple regulatory elements in its promoter region. A NF-κB binding site is essential for induction by TNFα in Jurkat T cells (22) and radiation in HEK293 cells (23). Dexamethasone induces c-IAP2 expression through a putative glucocorticoid response element in A549 human lung cancer cells (24), while a CRE has an essential role in induction of c-IAP2 expression by cAMP in T84 colon cancer cells (12).

In this study, we analyzed the signal transduction pathways that mediate induction of c-IAP2 in response to increases in cAMP in T84 cells. We show that cAMP-promoted phosphorylation of CREB and transcriptional activation of c-IAP2 appear in large part to be mediated by activation of ERK1/2 and p38 MAPK, perhaps acting via p90RSK and MSK1.
Experimental Procedures

Antibodies and reagents—Anti-c-IAP2 polyclonal antibody (pAb) and anti-actin monoclonal antibody (mAb) were from Chemicon International (Temecula, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. pAbs against CREB, phospho-CREB, phospho-MSK1, phospho-p90RSK, p44/42 MAPK, phospho-p44/42 MAPK, p38 MAPK, and phospho-p38 MAPK, and the MEK1/2 inhibitors, PD98059 and U0126 were obtained from Cell Signaling Tech (San Diego, CA). mAb against Rap1 was from BD Bioscience Pharmingen (San Diego, CA). Cholera toxin (CTX), 8-CPT-cAMP, forskolin (Fsk), human recombinant TNFα, phorbol-12-myristate-12-acetate (PMA), H89, Rp-cAMPS, SB203580, and SB202190 were from Calbiochem (San Diego, CA). PGE2 was from Sigma-Aldrich Co. (St. Louis, MO). 8-(4-chlorophenylthio)-2′′-O-methyl-cAMP (8-CPT-2′′-O-Me-cAMP) was purchased from BIOLOG Life Science Institute (Bremen, Germany).

Plasmids—pCMV-HA (vector control), pCMV-CREB (wild-type CREB), pCMV-KCREB (dominant negative form), pCMV-CREB S133A (dominant negative form) were purchased from BD Clontech (Palo Alto, CA). pUSE-MEK1 (S218D/S222D) (dominant active form) was purchased from Upstate (Lake Placid, NY). pBabeHygro-MKK3A (S189E/T193E) (dominant active form) was a gift from Dr. Peiqing Sun (25). pGL3-c-IAP2-WT-Luc (-1931 to +27) and pGL3-c-IAP2-CREII-Luc (-87 to +27) were described previously (12).

Cell Culture—T84 human colon epithelial cells were cultured in 50% DMEM/50% Ham’s F12 medium supplemented with 5% newborn calf serum and 2 mM L-glutamine. CHO chinese
hamster ovarian cells were cultured in Ham’s F12 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine.

**Immunoblot analysis**—For detection of phospho-ERK1/2 and phospho-p38 MAPK, cells were lysed in 1% Triton-X100, 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 150 mM NaCl, 0.5 mM Na$_3$VO$_4$, 10 mM NaF, 1 µg/ml aprotinin, and 1 mM PMSF. For detection of other proteins, cells were lysed in 1 x SDS sample buffer, and sonicated twice for 15 sec. Proteins were separated by SDS-PAGE and analyzed by immunoblotting with the indicated Abs. Specific binding was visualized by Super Signal West Dura extended duration substrate (Pierce Biotechnology, Rockford, IL) and quantified by using an EpiChemi II darkroom image analyzer (Ultraviolet Products).

**Dual luciferase assay**—T84 cells in 24-well plates were transfected using the FuGene6 transfection reagent (Roche, Indianapolis, IN) with pGL3-c-IAP2 (-1931 to +27) or pGL3-c-IAP2-CREII-Luc (-87 to +27), pRL-SV40-Luc (Promega, Madison, WI), carrying Renilla luciferase under the control of a constitutively active SV40 promoter as a transfection control, and CREB, MEK1, or MKK3 expression vectors as indicated in the figure legends. The dual-luciferase assay was conducted with a Dual-Luciferase Reporter Assay System (Promega).

**PKA activity assay**—Kinase activity of PKA was assayed with the PepTag® Assay (Promega). Briefly, T84 cells, serum-starved for 6 h, were treated for 1 h with H89 (10 µM) or Rp-cAMPS (100 µM) and stimulated with Fsk or 8-CPT-cAMP for 3 min. Cells were washed with PBS twice and lysed in lysis buffer (1% NP40, 150 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM NaF,
cAMP-mediated induction of c-IAP2

1 mM PMSF). Lysates containing 8 µg of total protein were incubated with 2 µg of PepTag® A1 peptide in reaction buffer for 8 min. Phosphorylated peptide was separated by 0.8% agarose-gel electrophoresis and quantified by using an EpiChemi II darkroom image analyzer.

**ERK1/2 and p38 MAPK kinase assay**—Kinase activities of ERK1/2 and p38 MAPK were assayed with a p44/42 MAP Kinase Assay Kit (non-radioactive) and p38 MAP Kinase Assay Kit (non-radioactive) (Cell Signaling Tech.), respectively. Briefly, T84 cells were preincubated with inhibitors for 1 h, followed by incubation with 8-CPT-cAMP for 30 min. Cells were lysed in lysis buffer and centrifuged at 20,000 g for 10 min. The supernatants were incubated with immobilized anti-phospho p44/42 MAPK or anti-phospho p38 MAPK mAb for 4 to 12 h. Precipitated kinases were incubated with Elk or ATF-2 fusion protein for 30 min in kinase reaction buffer containing 200 µM ATP and phosphorylation of fusion proteins was detected by immunoblotting with phospho-specific Abs.

**Rap1-pull down assay**—Cells were serum-starved overnight and incubated with inhibitors for 1 h, followed by further stimulation as indicated in the figure legends. Cells were lysed in lysis buffer [1% Nonidet P-40, 25 mM HEPES (pH 7.4), 150 mM NaCl, 10% glycerol, 1 mM EDTA, 10 mM MgCl2, 0.5 mM Na3VO4, 10 mM NaF, 1 µg/ml aprotinin, and 1 mM PMSF], and centrifuged at 20,000 g for 1 min. The supernatants were incubated with 15 µg of Ral GDS-RBD agarose (Upstate) for 45 min, and the beads were washed with lysis buffer three times. The precipitates were separated by SDS-PAGE and analyzed by immunoblotting with anti-Rap1 mAb.
Analysis of apoptosis—T84 cells were incubated with inhibitors for 1 h, followed by further incubation with CTX or 8-CPT-cAMP for 1 h. Cells were stimulated with staurosporine for 12 h and lysed in lysis buffer. After centrifugation of cells at 250 g for 5 min, supernatants were isolated and assayed with the Cell Death Detection ELISA PLUS kit (Roche).

Caspase-3 Assay—Caspase-3 activity was assayed with a colorimetric assay kit (R & D Systems, Minneapolis, MN).

Data presentation and analysis—Results are shown as mean ± SEM of at least 3 – 4 experiments as indicated in figure legends. Comparisons for change in response were by paired or unpaired T test as appropriate, with $P < 0.05$ considered significant.
Results

cAMP induces c-IAP2 expression predominantly through PKA-independent pathways in T84 colon epithelial cells—We have previously shown that increases in cellular cAMP levels can transcriptionally induce c-IAP2 expression in T84 cells (12). To characterize the underlying mechanisms, we investigated the effects of multiple agents that raise cellular cAMP concentrations to regulate the activity of a full-length transcriptional reporter of c-IAP2. PGE$_2$ binds to $G_s$-coupled EP2 and EP4 receptors on colon epithelial cells and increases intracellular cAMP concentration (26,27). Forskolin (Fsk) directly stimulates adenyl cyclase activity and increases cAMP production. We also used cholera toxin (CTX), which catalyzes the ADP-ribosylation of $G_s$ leading to the activation of adenyl cyclase, and the membrane-permeant cAMP analog, 8-CPT-cAMP. Stimulation of T84 cells with all four agonists that raise intracellular cAMP increased c-IAP2 promoter activity in a concentration-dependent manner (Fig. 1A).

To begin to define the downstream signaling mechanism for the cAMP-promoted increase in c-IAP2 promoter activity, we first focused on the role of PKA, the major effector kinase that mediates the action of cAMP. Surprisingly, two different pharmacological inhibitors of PKA, H89 (10 µM) and Rp-cAMPS (100 µM), showed only limited inhibition (< 25%) of cAMP-dependent increases in c-IAP2 promoter activity (Fig. 1B) and protein expression (Fig. 1C), although increases in PKA activity after Fsk and 8-CPT-cAMP stimulation were effectively inhibited by the same concentrations of these agents (Fig. 1D). These data suggested that cAMP-induced c-IAP2 expression is predominantly regulated by PKA-independent pathways in colon epithelial cells. Subsequent studies were designed to examine this possibility.
cAMP-mediated induction of c-IAP2

cAMP stimulates c-IAP2 promoter activity through phosphorylation of CREB—Our previous data indicated that a CRE in the c-IAP2 promoter region is necessary for induction by cAMP (12), suggesting that CREB may be a prominent factor in controlling cAMP-dependent c-IAP2 transcription. To test this idea directly, we performed co-transfections with a minimal c-IAP2 transcriptional reporter that contains the proximal CRE region and is fully responsive to cAMP stimulation (12) and an expression vector encoding one of two dominant-negative mutants of CREB, KCREB or CREB S133A. KCREB forms an inactive dimer with endogenous CREB and blocks its ability to bind to CRE (28). CREB S133A contains a serine to alanine mutation at position 133, which blocks phosphorylation of the mutant CREB and thus its ability to initiate CRE-dependent transcription (29). Both dominant-negative forms of CREB abrogated increased c-IAP2 promoter activity induced by 8-CPT-cAMP (Fig. 2A) and CTX (data not shown), whereas co-transfection with wild-type CREB showed a slight, albeit not significant, increase in c-IAP2 promoter activity compared to vector-transfected control. Furthermore, consistent with its functional importance, endogenous CREB was phosphorylated in response to treatment of T84 cells with Fsk or 8-CPT-cAMP, whereas activating transcription factor-2 (ATF-2) and CCAAT/enhancer-binding protein (C/EBP), other transcriptional factors that bind to CRE, were only minimally phosphorylated under these conditions (Fig. 2B and data not shown). Together, these data indicate that phosphorylated CREB plays an essential role for c-IAP2 gene transcription in response to increases in cAMP.

CREB phosphorylation is mediated by ERK1/2 and p38 MAPK—Phosphorylation of CREB is required for its transcriptional activation (30). Although PKA is an important kinase for CREB phosphorylation, we found that in T84 cells, the PKA inhibitors, H89 and Rp-cAMPS only
slightly suppressed its cAMP-dependent phosphorylation (Fig. 2B). Therefore, we hypothesized that in this system other kinases, such as p90RSK, a kinase downstream of ERK1/2 (31), and MSK1, a kinase downstream of ERK1/2 and p38 MAPK (32), both of which can phosphorylate CREB (33), might participate in the phosphorylation of CREB in response to increases in cAMP. We found that both p90RSK and MSK1 were phosphorylated after stimulation with 8-CPT-cAMP (Fig. 3) and Fsk (data not shown). Phosphorylation of p90RSK promoted by 8-CPT-cAMP was inhibited by the MEK1/2 inhibitor, U0126 (10 µM), while it was slightly, but not significantly, increased by the p38 MAPK inhibitor, SB202190. 8-CPT-cAMP-promoted phosphorylation of MSK1 was inhibited moderately by both U0126 and SB202190, and more strongly by the combination of these agents (Fig. 3). The PKA inhibitors, Rp-cAMPS (Fig. 3) and H89 (data not shown), did not affect Fsk- or 8-CPT-cAMP-stimulated phosphorylation of p90RSK or MSK1. These results suggest that cAMP activates p90RSK through ERK1/2, and MSK1 through ERK1/2 and p38 MAPK in T84 cells and that activation of these kinases occurs independently of PKA.

Importantly, CREB phosphorylation promoted by 8-CPT-cAMP (Fig. 3) or Fsk (data not shown) was significantly suppressed by U0126 or SB202190 alone, and this suppression was even more pronounced after addition of both inhibitors. Addition of Rp-cAMPS, which by itself only minimally decreased CREB phosphorylation (Fig. 2B and 3, lane b), together with U0126 and SB202190 caused only modest and not significant further inhibition beyond that produced by the latter agents (Fig. 3, lane e and f). Similar results were obtained by using other inhibitors for ERK1/2 and p38 MAPK, i.e., PD98059 and SB203850, respectively (data not shown). These data suggest that ERK1/2 and p38 MAPK are involved in the cAMP-dependent phosphorylation of CREB.
cAMP activates ERK1/2 and p38 MAPK in T84 cells—Because the results in Fig. 3 suggested the involvement of ERK1/2 and p38 MAPK in the phosphorylation of p90 RSK and MSK1, we directly examined the activation of ERK1/2 and p38 MAPK in response to agents that raise cAMP levels. Treatment of T84 cells with PGE2, Fsk or 8-CPT-cAMP enhanced phosphorylation of ERK1/2 and p38 MAPK within 30 min, as did TNFα as a positive control (Fig. 4A). Furthermore, in vitro kinase assays demonstrated that increased phosphorylation of ERK1/2 and p38 MAPK after 8-CPT-cAMP stimulation was accompanied by activation of these kinases. Thus, activated-ERK1/2 or p38 MAPK immunoprecipitated from 8-CPT-cAMP-stimulated cell lysates showed an increased ability, relative to control cells, to phosphorylate their respective substrates, Elk or ATF-2 fusion proteins (Fig. 4B and C). Increased ERK1/2 kinase activity was completely inhibited by treatment with U0126 but was insensitive to PKA inhibitors (Fig. 4B), documenting further that ERK1/2 activation is PKA-independent in T84 cells. In contrast, in CHO cells ERK1/2 activation by cAMP is a PKA-dependent event, because H89 inhibited ERK1/2 phosphorylation induced by 8-CPT-cAMP stimulation (Fig. 4E), a finding consistent with prior reports (34). The PKA inhibitors also failed to inhibit the cAMP-dependent increase in p38 MAPK activity (Fig. 4C). Taken together, these results support the conclusion that increases in cAMP activate both ERK1/2 and p38 MAPK in T84 cells and these activations appear to occur independent of PKA.

In addition to PKA, another recently recognized effector of cAMP action is Epac, a guanine nucleotide-exchange factor for the small GTPase Rap, which is able to regulate ERK1/2 activity in some cells (35,36). Because our results showed that cAMP-promoted ERK1/2 activation is a PKA-independent event, we examined the ability of 8-CPT-2’-O-Me-cAMP, a specific activator...
of Epac that activates Rap1 in CHO, PC12, and HEK293T cells (34), to stimulate ERK1/2 activation in T84 cells. We found that 8-CPT-2'O-Me-cAMP failed to activate ERK1/2 and p38 MAPK in these cells (Fig. 4D and E), although as a positive control, this analog was able to activate Rap1 (Fig. 4F), consistent with the conclusion that cAMP-promoted ERK1/2 activation in T84 cells occurs via an Epac-independent pathway.

**cAMP induces c-IAP2 expression through the activation of ERK1/2 and p38 MAPK**—We next examined the involvement of ERK1/2 and p38 MAPK in cAMP-dependent transcriptional activation of the c-IAP2 gene. Stimulation of c-IAP2 promoter activity by CTX and 8-CPT-cAMP (see Fig. 1A) was significantly diminished by adding the MEK1/2 inhibitor, U0126, and the p38 MAPK inhibitor, SB202190, and this inhibition was even greater by combined treatment with both inhibitors (Fig. 5A). Consistent with this, increased c-IAP2 protein expression after stimulation with CTX and 8-CPT-cAMP was also markedly inhibited by the combination of inhibitors of MEK1/2 and p38 MAPK (Fig. 5B). Addition of Rp-cAMPS yielded no significant further inhibition of c-IAP2 expression, providing additional support for the conclusion that PKA has a limited, if any, role in mediating cAMP-dependent c-IAP2 induction.

As an alternative approach to defining the role of ERK1/2 and p38MAPK in the regulation of c-IAP2 expression, we performed co-transfections with a c-IAP2 promoter-reporter and expression vectors for dominant active forms of MEK1 and/or MKK3, which are potent activators of ERK1/2 and p38 MAPK, respectively (25,37). Forced expression of active forms of MEK1 or MKK3 induced c-IAP2 promoter activity, which was further enhanced by co-transfection with both expression plasmids (Fig. 5C). Based on these data, we conclude that
ERK1/2 and p38 MAPK play a pivotal role in the cAMP-promoted induction of c-IAP2 via increased phosphorylation of CREB.

**cAMP regulates apoptosis in colon epithelial cells through ERK1/2 and p38 MAPK**—In order to assess the biological significance of the involvement of ERK1/2 and p38 MAPK in the c-IAP2 induction stimulated by cAMP, we assayed T84 cells for apoptosis. Treatment of T84 cells with CTX or 8-CPT-cAMP inhibited apoptosis induced by staurosporine, as assessed by quantitative analysis of DNA fragmentation. This inhibition was abrogated by adding a combination of inhibitors of ERK1/2 and p38 MAPK (Fig. 6A). When we examined these inhibitors individually, U0126 and SB202190, as well as H89 but not Rp-cAMPS, each significantly blunted the 8-CPT-cAMP-promoted inhibition of staurosporine-induced apoptosis (Fig. 6B). The difference between the responses to H89 and Rp-cAMPS might relate to the ability of H89 to not only inhibit PKA but also MSK1 (38). U0126 and SB202190 also blocked the inhibition of caspase-3 activity produced by CTX or 8-CPT-cAMP treatment of T84 cells (Fig. 6C); this effect was not further enhanced by addition of Rp-cAMPS. These results are consistent with the action of c-IAP2 in binding to the active form of caspase-3 and thereby diminishing its activity (39), and with results of experiments shown above for the role of kinases other than PKA in regulation of c-IAP2 expression. Figure 7 shows a model that summarizes these pathways.
Discussion

Apoptosis is a complex cellular process that is regulated by a balance of stimulatory and inhibitory pathways. The ability of cAMP to inhibit apoptosis might result from a blockade of pro-apoptotic pathways, a stimulation of anti-apoptotic pathways, or a combination thereof. While some of pro-apoptotic members of the Bcl-2 family, such as BAD, have been reported to be phosphorylated and inactivated by PKA, thereby resulting in inhibition of apoptosis (40-43), we recently identified an alternative mechanism for cAMP-mediated anti-apoptosis: an inhibition of apoptosis in intestinal epithelial cells by agonists that increase cAMP and induce c-IAP2 via a cAMP-responsive element (CRE) in the c-IAP2 promoter (12). We showed that c-IAP2 induction by increases in cAMP levels inhibits apoptosis that occurs in response to anti-Fas Ab (extrinsic apoptotic pathway) or staurosporine (intrinsic apoptotic pathway). The results thus suggested a novel mechanism for the cancer-chemopreventive effect of non-steroidal anti-inflammatory drugs, which inhibit COX and decrease PGE2 synthesis (12).

In the current studies, we have identified new aspects of the signal transduction pathway by which increases in cAMP induce c-IAP2 expression in colon cancer cells (Fig. 7). The data show that CREB phosphorylation is a key step but that the phosphorylation appears to occur by kinases other than the expected involvement of PKA. Activation of p90RSK and MSK1, which follow ERK1/2 and p38 MAPK activation, appears to be involved in both CREB phosphorylation and c-IAP2 induction. The current data also suggest that agents that raise intracellular cAMP and induce c-IAP2 expression in T84 cells do not appear to act via ATF-2 and CCAAT/enhancer-binding protein (C/EBP), other transcriptional factors that bind to CRE, because we found minimal phosphorylation of ATF-2 and C/EBP after cAMP treatment.
Conventional ideas emphasize that cAMP-mediated transcriptional responses involve the ability of PKA to phosphorylate CREB (30). However, the data shown here provide evidence for an alternative mechanism whereby cAMP promotes phosphorylation of CREB through ERK1/2 and p38 MAPK and perhaps other protein kinases as well. Previous data have implicated a role for p90RSK in the phosphorylation of CREB promoted by certain growth factors (44,45). Several of our results imply a limited involvement of PKA: 1) two different PKA inhibitors (H89 and Rp-cAMPS) used at concentrations that block PKA activity yielded only minimal inhibition of CREB phosphorylation, c-IAP2 promoter activity and c-IAP2 protein levels, and 2) addition of Rp-cAMPS along with inhibitors of ERK1/2 and p38 MAPK did not cause greater inhibition of cAMP-induced c-IAP2 induction nor abrogation of inhibition of apoptosis or caspase activation by cAMP in spite of inhibition of PKA activity. Thus, the current results emphasize a role for ERK1/2 and p38 MAPK in cAMP-mediated induction of c-IAP2. However, because H89 and Rp-cAMPS had some ability to decrease cAMP-promoted CREB phosphorylation and c-IAP2 promoter activity, PKA might contribute in a limited way to CRE-dependent transcription through CREB phosphorylation.

CAMP is involved in multiple cellular processes that are thought to be mediated by PKA and Epac (46). Increases in cAMP activate ERK1/2 through PKA in CHO, PC12, and HEK293T cells (34), or through Epac-Rap1 in primary kidney cells (35), while ERK1/2 is inhibited by increases in cAMP in NIH 3T3 cells (34) and C6 rat glioma cells (47). Moreover, an increase in cAMP levels promoted by thyroid-stimulating hormone (TSH) stimulation activates p38 MAPK through PKA in TSH receptor-overexpressing CHO cells (48), while cAMP activates p38 MAPK in a PKA-independent manner in Th2 effector T cells (49). Here we show that increases in cAMP induce ERK1/2 and p38 MAPK activation in colon epithelial cells through PKA-
Epac-independent pathways, suggesting involvement of alternative mechanisms of MAPK activation.

MAPKs regulate cellular activities ranging from gene expression, mitosis, motility, metabolism, to apoptosis. The ability of ERK1/2 to regulate cell proliferation has led to the exploration of inhibitors of ERK1/2 as possible anticancer agents (50,51). The potential role of p38 MAPK in cancer chemotherapy has also been considered, although p38 MAPK inhibitors have primarily been tested for inflammatory disorders (52,53). In fact, inhibition of p38 MAPK promotes cell death of UV-treated melanoma cells (54) and TNFα-treated myelomonocytic cells (55). Our results emphasize the function of ERK1/2 and p38 MAPK in inhibition of apoptosis through c-IAP2 induction and suggest a mechanism that may contribute to actions of those kinases in cell death.

CREB has been reported to regulate a number of target genes related to tumorigenesis and to cell survival (56,57), including cyclin D1 or BRCA1 (30). Our findings emphasize the biological significance of CREB phosphorylation as a key event in the induction of c-IAP2 via multiple protein kinases in addition to PKA. The data also suggest that kinase inhibitors, other than inhibitors of PKA, might be able to blunt cAMP-promoted anti-apoptosis. As an alternative approach, one might consider a CRE-decoy system, in which a decoy oligodeoxynucleotide carrying a CRE can be used to inhibit CRE-directed gene transcription and tumor growth without affecting normal cell growth (58).
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References


Figure legends

Fig. 1. cAMP induces c-IAP2 expression primarily through PKA-independent pathways.

A. T84 cells, transfected with pGL3-c-IAP2-WT-Luc and pSV40-RL-Luc, were stimulated for 6 h with PGE₂, Fsk, or 8-CPT-cAMP (CPT), or for 12 h with CTX at the indicated concentrations. Cell extracts were assayed by a dual luciferase assay. Results represent relative increases compared to untreated controls and are mean ± SEM (n=4; *, P < 0.05 versus controls, respectively). B. T84 cells, transfected with pGL3-c-IAP2-WT-Luc and pSV40-RL-Luc, were treated with H89 (10 µM) or Rp-cAMPS (Rp, 100 µM) for 1 h, and stimulated for 6 h with 8-CPT-cAMP (CPT, 100 µM) or 12 h with CTX (250 ng/ml). CTX and CPT both significantly increased c-IAP2 promoter activity in the absence or presence of inhibitors (P < 0.05). Results shown represent relative promoter activity compared to CTX- or CPT-treated cells without inhibitors as 100% and are mean ± SEM (n=3; *, P < 0.05; N.S., not significant). C. T84 cells were treated for 1 h with H89 (10 µM) or Rp-cAMPS (Rp, 100 µM) and further stimulated for 6 h with 8-CPT-cAMP (CPT, 100 µM) or 12 h with CTX (250 ng/ml). Cells were lysed in SDS-sample buffer and proteins were analyzed by immunoblotting with the indicated antibodies. The bottom graphs show densitometric results of the respective blots, normalized against actin levels and expressed relative to unstimulated controls and are mean ± SEM (n=3; *, P < 0.05; N.S., not significant). CTX and CPT both significantly increased c-IAP2 protein expression in the absence or presence of inhibitors (P < 0.05). D. T84 cells, serum-starved for 12 h, were treated with H89 (10 µM) or Rp-cAMPS (Rp, 100 µM) for 1 h, and stimulated with Fsk (1 µM) or 8-CPT-cAMP (CPT, 100 µM) for 3 min. Cell extracts were assayed for PKA activity using the PepTag® Assay. Results represent relative changes in PKA activity compared to untreated.
controls and are mean ± SEM (n=3; *, P < 0.05 versus Fsk- or CPT-treated cells without inhibitors, respectively).

**Fig. 2.** cAMP promotes c-IAP2 promoter activity through phosphorylation of CREB.  A. T84 cells, transfected with pCMV-HA (Vector), pCMV-CREB WT (WT), pCMV-KCREB (K), or pCMV-CREB S133A (S133A), and pGL3-c-IAP2-CREII-Luc and pSV40-RL-Luc, were stimulated for 6 h with 8-CPT-cAMP (CPT, 100 µM). Cell extracts were assayed by a dual luciferase assay. Results represent relative increases compared to vector-transfected, untreated controls, and are mean ± SEM (n=3; *, P < 0.05; N.S., not significant, respectively).  B. T84 cells were serum-starved for 12 h, and incubated for 1 h with or without H89 (10 µM), Rp-cAMPS (Rp, 100 µM) as indicated. After 30 min further incubation with Fsk (1 µM) or 8-CPT-cAMP (CPT, 100 µM), cells were lysed in SDS-sample buffer and proteins were analyzed by immunoblotting with the indicated antibodies. The right graph shows densitometric results of the blots for phospho-CREB normalized to total CREB expression. Data are expressed relative to unstimulated cells without inhibitors, and are mean ± SEM (n=3; *, P < 0.05 versus Fsk- or CPT-treated cells without inhibitors, respectively).

**Fig. 3.** CREB phosphorylation is mediated by ERK1/2 and p38 MAPK. T84 cells were serum-starved for 12 h, and incubated for 1 h with SB202190 (SB, 20 µM), U0126 (U, 10 µM), Rp-cAMPS (Rp, 100 µM), alone or in combination as indicated. After 30 min further incubation with 8-CPT-cAMP (CPT, 100 µM), cells were lysed in SDS-sample buffer and proteins were analyzed by immunoblotting with the indicated antibodies. The lower graph shows densitometric results of the blots for phospho-CREB normalized to total CREB expression. Data
are expressed relative to unstimulated controls without inhibitors, and are mean ± SEM (n=3; *, P < 0.05; N.S., not significant). Inhibitors, with the exception of Rp alone (lane b), significantly decreased CREB-phosphorylation (lane c to f, P < 0.05).

**Fig. 4. cAMP activates ERK1/2 and p38 MAPK in a PKA-independent manner.** A. T84 cells were serum-starved for 12 h, and treated with PGE$_2$ (1 µM), Fsk (10 µM), 8-CPT-cAMP (100 µM), or TNFα (100 ng/ml) for 30 min. Cells were lysed and analyzed by immunoblotting with indicated antibodies. B, C. Cells, serum-starved for 12 h, were treated with H89 (10 µM), Rp-cAMPS (Rp, 100 µM), or U0126 (U, 10 µM) for 1 h, and incubated with 8-CPT-cAMP (CPT, 100 µM) for 30 min. Cell lysates were prepared and analyzed by non-radioactive kinase assay (KA). Immunoprecipitated phospho-ERK1/2 or phospho-p38 MAPK were incubated with Elk or ATF-2 fusion protein for 30 min at 30 °C in the presence of ATP (200 µM) and phosphorylated fusion proteins were analyzed by immunoblotting with the indicated antibodies (KA, top panel). The total amount of fusion protein present in each reaction was visualized by Coomassie blue staining (KA, second panel). Total cell lysates were analyzed in parallel by immunoblotting (IB) with the indicated antibodies (third and fourth panel). D, E. T84 cells (D), serum-starved for 12 h, or CHO cells (E) were treated with H89 (10 µM) for 1 h, and incubated with 8-CPT-cAMP (CPT, 100 µM) or 8-CPT-2’-O-Me-cAMP (Me, 100 µM) for indicated time (D) or 15 min (E). Cells were lysed and analyzed by immunoblotting with indicated antibodies. F. T84 cells, serum-starved for 12 h, were incubated with 8-CPT-cAMP (CPT, 100 µM), 8-CPT-2’-O-Me-cAMP (Me, 100 µM) or PMA (1 µM) for 2 min. Cells were lysed and assayed for Rap1 activation with a pull-down assay. Precipitates (top panel) and total cell lysates (bottom panel) were analyzed by immunoblotting with anti-Rap1 mAb.
Fig. 5. cAMP induces c-IAP2 protein expression through ERK1/2 and p38 MAPK activation. A. T84 cells, transfected with pGL3-c-IAP2-Luc and pSV40-RL-Luc, were treated for 1 h with Rp-cAMPS (Rp, 100 µM), SB202910 (SB, 20 µM), U0126 (U, 10 µM), alone or in combination, and stimulated for 12 h with CTX (250 ng/ml) or 6 h with 8-CPT-cAMP (CPT, 100 µM). Cell extracts were assayed by a dual luciferase assay. Results represent relative promoter activity compared to CTX- or CPT-treated cells without inhibitors as 100% and are mean ± SEM (n=3; *, P < 0.05 versus CTX- or CPT-treated cells without inhibitors; #, P < 0.05 relative to cells treated with either of the inhibitors alone; N.S., not significant). B. T84 cells were treated for 1 h with Rp-cAMPS (Rp, 100 µM), SB202910 (SB, 20 µM), and/or U0126 (U, 10 µM) as indicated, and incubated for 12 h with CTX (250 ng/ml) or 6 h with 8-CPT-cAMP (CPT, 100 µM). Cells were lysed in SDS-sample buffer and proteins were analyzed by immunoblotting with the indicated antibodies. The lower graphs show densitometric results of the respective blots, normalized against actin levels and expressed relative to unstimulated controls without inhibitors, and are mean ± SEM (n=3; *, P < 0.05 versus untreated controls; #, P < 0.05; N.S., not significant). C. T84 cells were transfected with pUSE-MEK1 (S218D/S222D) (MEK1-active) and/or pBabeHygro-MKK3A (MKK3-active), and pGL3-c-IAP2-Luc and pSV40-RL-Luc, and incubated for 12 h. Cell extracts were assayed by a dual luciferase assay. Results represent relative increases compared to vector-transfected controls and are mean ± SEM (n=3; *, P < 0.05 versus vector-transfected cells; #, P < 0.05).

Fig. 6. cAMP regulates apoptosis through ERK1/2 and p38 MAPK. T84 cells were incubated for 1 h with H89 (10 µM), Rp-cAMPS (Rp, 100 µM), SB202910 (SB, 20 µM), and/or
U0126 (U, 10 µM) as indicated, followed by further incubation for 1 h with CTX (250 ng/ml) or 8-CPT-cAMP (CPT, 100 µM). Cells were stimulated with staurosporine (200 nM) for 12 h, and oligonucleosome release into the cytoplasm was assayed by ELISA (A, B), or for 6 h and caspase-3 activity was assayed by ELISA (C). A, C. Results represent relative increases compared to untreated cells without inhibitors, respectively, and are mean ± SEM (n=3; *, P < 0.05 versus staurosporine-treated cells without CTX or CPT stimulation; #, P < 0.05). B. Results represent relative amounts of 8-CPT-cAMP-mediated inhibition of DNA fragmentation induced by staurosporine compared to CPT- and staurosporine-treated cells without inhibitors as 100%, and are shown as mean ± SEM (n=3; *, P < 0.05 versus CPT- and staurosporine-treated cells without inhibitors).

**Fig. 7. Model of cAMP-mediated c-IAP2 induction.** PGE₂, CTX and Fsk-stimulated increases in cAMP activate PKA, ERK1/2 and p38 MAPK followed by p90RSK and MSK1 activation. The actions of cAMP on ERK1/2 and p38 MAPK activation are likely to be indirect as indicated in the figure with 2 arrows. CREB, phosphorylated in response to activation of p90RSK and MSK1, and to a lesser degree PKA, then induces c-IAP2 expression through CRE within the proximal c-IAP2 promoter region. c-IAP2 protein inhibits activated caspase-3 and apoptosis.
Fig. 2

A

Relative c-IAP2 promoter activity (Stimulated/Control)

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* * N.S. N.S.

B

Phospho-CREB/Total CREB

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IB: α-Actin mAb

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<tr>
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**IB:** α-Phospho-p90 RSK pAb

**IB:** α-Phospho-MSK1 pAb

**IB:** α-Phospho-CREB pAb

**IB:** α-CREB pAb

**IB:** α-Actin mAb

**Phospho-CREB/Total CREB**

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Fig. 4

A. IB: α-phospho-p44/42 pAb
IB: α-p44/42 MAPK pAb
IB: α-phospho-p38 pAb
IB: α-p38 MAPK pAb

B. CPT
IB: α-phospho-Elk1 pAb
IB: α-phospho-p44/42 pAb
IB: α-p44/42 MAPK pAb
IB: α-phospho-ERK1/2 pAb
IB: α-p44/42 MAPK pAb
Phospho-Elk1
Total Elk1
Phospho-ERK1/2
Total ERK1/2

C. CPT
IB: α-phospho-ATF-2 pAb
IB: α-phospho-p38 MAPK pAb
IB: α-p38 MAPK pAb
Phospho-ATF-2
Total ATF-2

D. CPT
0  5  15  30
IB: α-phospho-p44/42 MAPK pAb
IB: α-p44/42 MAPK pAb
IB: α-phospho-p38 MAPK pAb

E. CPT
H89
IB: α-phospho-p44/42 pAb
IB: α-p44/42 MAPK pAb

T84
IB: α-phospho-p44/42 pAb
IB: α-p44/42 MAPK pAb

CHO
IB: α-phospho-p44/42 pAb
IB: α-p44/42 MAPK pAb

F. CPT
Me
IB: α-Rap1 mAb
GTP-Rap1
Total Rap1
Fig. 7

The diagram illustrates the signaling pathways involving EP2, EP4, PGE2, CTX, Fsk, and their interactions with G_α, AC, cAMP, PKA, ERK, p38, MSK1, p90 RSK, CREB, CRE, c-IAP2, and apoptosis. The diagram shows the activation of these pathways and their inhibitors, such as H89, U0126, and SB202190.
Cyclic AMP promotes CREB-dependent induction of the cellular inhibitor of apoptosis protein-2 and suppresses apoptosis of colon cancer cells through ERK1/2 and p38 MAPK

Hiroshi Nishihara, Michael Hwang, Shinae Kizaka-Kondoh, Lars Eckmann and Paul A. Insel

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