Cyclooxygenase (COX)-2, a rate-limiting enzyme of prostaglandin (PG) production, is overexpressed in colorectal adenomas and adenocarcinomas, and its inhibition by nonsteroidal antiinflammatory drugs protects against colorectal cancer. Mechanisms of cancer promotion by COX-2 are not fully understood, but signaling through prostaglandin (PGE2) receptors is a contributing factor. The major PGE2 receptors on epithelial cells, EP2 and EP4, increase cAMP production, which promotes growth and inhibits apoptosis in some cell types. Here, we show that CAMP agonists, including PGE2, chola toxin, and a membrane-permeant CAMP analog, protect normal and transformed intestinal epithelial cells from apoptosis induced by diverse stimuli. This protection is associated with CAMP-mediated, rapid induction of cellular inhibitor of apoptosis protein (c-IAP)-2 and delayed induction of LIVIN, but not of six other members of the IAP family. Concurrently and characteristic of IAP functions, the activity, but not generation, of the cleaved form of the central executioner caspase 3 is inhibited. Induction of c-IAP2 expression by CAMP agonists is accompanied by phosphorylation of CAMP response element binding protein and CAMP response element-dependent activation of transcriptional reporters. Furthermore, inhibition of COX-2 in cells overexpressing the enzyme decreases c-IAP2 expression and promotes apoptosis, both of which are reversible by PGE2 addition, suggesting that COX-2-promoted antiapoptosis is mediated by release of PGE2 and subsequent CAMP-dependent c-IAP2 induction. These results help to explain the cancer chemoprotective effects of nonsteroidal antiinflammatory drugs by defining a mechanism through which CAMP signaling can promote the development of colorectal and possibly other epithelial cancers by means of disruption of normal apoptotic processes.

Colorectal cancer is among the most common cancers in the United States, causing >50,000 deaths each year. The development of colon cancer is a multistep process involving inactivation and ectopic activation of different genes, and a morphologic progression from superficial adenomatous polyps to frank, invasive adenocarcinoma (1, 2). One of the earliest events in the development of colon adenomas and cancers is the overexpression of cyclooxygenase (COX)-2, a key rate-limiting enzyme of prostaglandin (PG) production. Up to 80% of colon adenomas and cancers express increased levels of COX-2 (3–5), which is one of the strongest disease associations of any gene known to be involved in colon cancer formation. Increased COX-2 expression is found not only in the adenoma or cancer epithelium but also in interstitial cells such as macrophages, suggesting that paracrine pathways play a role in mediating the functions of COX-2 (6). The COX-2 gene is not mutated in patients with adenomatous polyposis coli, a premalignant condition with a high risk of developing colon cancer, indicating that it acts as a “modifier” gene (7). Clinical studies have provided unequivocal evidence that long-term use of COX inhibitors, i.e., nonsteroidal antiinflammatory drugs (NSAIDs) such as sulindac or aspirin, is associated with a 40–50% reduction in the incidence of colon adenomas and cancers (8–11). These data are supported by animal studies, which have shown that inhibition or genetic ablation of COX-2, as well as COX-1, suppresses the development of precancerous and cancerous intestinal lesions in experimentally induced models (12–14).

Different mechanisms have been proposed to account for the antitumor activity of NSAIDs; these mechanisms can be broadly divided into inhibition of proliferation and angiogenesis and promotion of cell death (8, 9, 15). NSAIDs induce apoptosis in colorectal cancer cell lines (16, 17), which is likely to be physiologically significant, as dysregulation of apoptosis is an important feature of the development of colorectal cancers. Thus, partial suppression of apoptosis occurs early in tumorigenesis in 85% of human colorectal adenomas and cancers attributable to critical genetic mutations (18). The inhibition of apoptosis allows the mutated cells to accumulate in adenomatous polyps. Apoptosis becomes progressively more inhibited as the cells acquire additional genetic mutations and phenotypic changes (19), indicating that suppression of apoptosis occurs throughout the adenoma/cancer progression, whereas other events, such as angiogenesis, occur only late in that sequence.

Most studies have suggested that NSAIDs induce apoptosis in intestinal epithelial cells through COX inhibition, although some reports have proposed that COX-independent events might mediate NSAID effects (20). The latter are probably less relevant physiologically, as they require high NSAID concentrations that are difficult to achieve in humans without severe toxic side effects (9). Among the COX-dependent events, production of PGs is likely to be critical, although elevated levels of the PG precursor arachidonic acid after NSAID treatment may also contribute to apoptosis (21). Evidence for a key role of PGs in mediating the functions of COX-2 in tumorigenesis comes from studies in genetically engineered mutant mice. Mice that lack the PGE2 receptors, EP2 and EP4, have a marked decrease in colon cancer incidence in chemically induced or genetic models (22, 23). Furthermore, production of PGE2 is increased in human and experimental colon cancers (8, 24). The EP2 and EP4 receptors, the major PGE2 receptors on intestinal epithelial cells (25, 26), mediate their activities through increase in CAMP production. Together, these data are compatible with the hypothesis that CAMP-dependent cell functions contribute to the development of colon cancer through the inhibition of apoptosis. In the studies reported here, we have tested key aspects of this hypothesis in defined cell culture models and have identified an underlying mechanism for the antipapoptic effects of CAMP.

Materials and Methods

Antibodies and Other Reagents. The following antibodies were used: mouse IgM anti-Fas, clone CH-11 (Medical Biological...
Laboratories International, Watertown, MA); rabbit antibodies to cellular inhibitor of apoptosis protein (c-IAP)-2 (Chemicon); mouse anti-human c-IAP1 and mouse anti-poly(ADP-ribose) polymerase (PARP) (PharMingen); rabbit antibodies against the cAMP response element (CRE)-binding protein (CREB), phospho-CREB (phosphorylated on Ser-133), IκBα, phospho-IκBα (phosphorylated on Ser-32), and caspase 3 (Cell Signaling Technology); and mouse anti-actin (Santa Cruz Biotechnology). Cholera toxin (CTX; Vibrio cholerae Inaba 569B), 8-(4-chlorophenylthio)-cAMP (8-CPT-cAMP), SC-236, human recombinant tumor necrosis factor α (TNFα), and staurosporine were from Calbiochem. PGE2 was from Sigma.

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 t-glutamine, as described previously (27). HCA-7 human colon epithelial cells (28) and RIE-1 normal rat intestinal epithelial cells (29) were grown in DMEM with 10% bovine calf serum and 2 mM glutamine. Cells were seeded at 0.5–2 × 106 cells per well into six-well plates, and allowed to attach overnight. After 12 h of serum starvation, cells were incubated for 1–4 h with or without cAMP agonists (5 μM PGE2, 250 ng/ml CTX, or 100 μM 8-CPT-cAMP) and further incubated for 12 h with anti-Fas Ab (100 ng/ml) or staurosporine (100 μM), or no additional agonists. The concentrations of PGE2 and CTX used in the experiments were optimal for elevating cAMP levels and inducing cAMP-dependent functions in T84 and other intestinal epithelial cells, as determined by preliminary studies (data not shown) and prior work by others (30, 31).

Analysis of Apoptosis. Oligonucleosome release into the cytoplasm was assayed with the Cell Death Detection ELISAPLUS kit (Roche Diagnostics). For assessment of DNA laddering, cells were harvested by trypsinization and suspended in 100 μl 50% Ham’s F12 medium supplemented with 5% newborn calf serum and 2 mM glutamine. Cells were incubated for 1–4 h with or without cAMP agonists (5 μM PGE2, 250 ng/ml CTX, or 100 μM 8-CPT-cAMP) and further incubated for 12 h with anti-Fas Ab (100 ng/ml) or staurosporine (100 μM), or no additional agonists. The concentrations of PGE2 and CTX used in the experiments were optimal for elevating cAMP levels and inducing cAMP-dependent functions in T84 and other intestinal epithelial cells, as determined by preliminary studies (data not shown) and prior work by others (30, 31).

RT-PCR Analysis. RNA extractions, reverse transcription, and PCR amplification were performed as described before (27, 32). The primers, annealing temperatures, cycle numbers, and expected PCR product sizes were as follows: NAIP/BIRC1, 5′-CTTACGAAAGAATCACGCTGATC-3′ (sense) and 5′-GCCATTTCTCTTCAAACACCTCCCAC-3′ (antisense), 63°C, 33 cycles, 150 bp; IAP/BIRC2, 5′-CTCCAGGCTTTCTCACAACC-3′ (sense), 5′-CCAGTACTGAGCTTCCAC-3′ (antisense), 59°C, 28 cycles, 232 bp; IAP/BIRC3, 5′-TCCATCAAATCTGTGAAACTC-3′ (sense) and 5′-AGCAGAGGCTACTGGTCCAC-3′ (antisense), 56°C, 30 cycles, 194 bp; XIAP/BIRC4, 5′-AGACACCATAACCCGGGGAACC-3′ (sense), 5′-GTTTTCCCACCAAAACGAC-3′ (antisense), 61°C, 29 cycles, 170 bp; survivin/BIRC5, 5′-GACCACCGATCTTCTACATCGAGCA-3′ (sense), 5′-AAAGGAAAGGCCAGCCGAC-3′ (antisense), 61°C, 31 cycles, 216 bp; apolipoprotein B/BIRC6, 5′-GCTGAACCTCACCATCAAGTC-3′ (sense) and 5′-CAAGTACTGGTCCAGTCC-3′ (antisense), 61°C, 31 cycles, 216 bp; apolipoprotein B/BIRC6, 5′-GCTGAACCTCACCATCAAGTC-3′ (sense) and 5′-CAAGTACTGGTCCAGTCC-3′ (antisense), 61°C, 31 cycles, 216 bp; PAP, 5′-AGGTGCAGAGGATAAAGTACAG-3′ (sense), 53°C, 35 cycles, 254 bp. Primers and PCR conditions for the analysis of β-actin and COX-2 were as previously described (33).

Immunoblot Analysis. For detection of c-IAP1 and c-IAP2, cells were lysed in 1% Triton-X100/10 mM Tris·HCl, pH 7.4/5 mM EDTA/150 mM NaCl/0.5 mM Na2VO4/10 mM NaF/F/μl/ml aprotinin/1 mM PMSF. For detection of caspase 3, PARP, CREB, and IκB, cells were lysed in RIPA buffer (1% Triton X-100/0.5% deoxycholic acid/0.1% SDS/50 mM Tris·HCl, pH 7.4/150 mM NaCl) containing 0.5 mM Na2VO4, 10 mM NaF, 1 μg/ml aprotinin, and 1 mM PMSF and then sonicated. Lysates were centrifuged at 20,000 × g at 4°C for 15 min, and proteins in the supernatants were separated by SDS/PAGE and analyzed by immunoblotting. Specific binding was visualized by SuperSignal West Dura extended duration substrate (Pierce) and quantified by using an EpiChemII darkroom image analyzer (Ultraviolet Products).

Dual Luciferase Assay. To construct WT (−1,931 to −27), ΔCREI (−242 to −27), and CREII (−86 to −27) reporter plasmids, the 5′-flanking region of a 3.5-kb c-IAP2 promoter fragment was digested with exonuclease III, and differently sized fragments were inserted into the firefly luciferase reporter plasmid pGL3-basic (Promega). CREI was prepared by inserting a 531-bp-digested fragment (−1,931 to −1,440) of the WT promoter region into pGL3-basic. T84 cells in six-well plates were transfected with 1 μg of pGL3-Luc containing various promoter regions and 0.1 μg of RL-SV40-Luc, carrying Renilla luciferase under the control of a constitutively active simian virus 40 promoter, by using the FuGene6 transfection reagent (Roche). After 12 h, cells were stimulated with CTX or TNFα, followed by further incubation for 24 h. The dual-luciferase assay was conducted with a dual-luciferase reporter assay system (Promega).

Caspase 3 Assay. Caspase 3 activity was assayed with a colorimetric assay kit (R & D Systems).

Results
Inhibition of Apoptosis in Intestinal Epithelial Cells by cAMP Agonists. To explore the hypothesis that PGE2-stimulated cAMP is a key intracellular mediator of COX-2-dependent antiapoptosis and define the underlying mechanisms, we used the T84 human colon epithelial cell line, which exhibits inducible apoptosis and is responsive to PGE2 stimulation (31, 34, 35). Treatment of T84 cells with PGE2 significantly inhibited apoptosis, as assessed by quantitative analysis of DNA fragmentation (Fig. 1A) promoted by two different agents, anti-Fas Ab and staurosporine, which activate the two major cellular pathways of apoptosis, the extrinsic and intrinsic pathways. Anti-Fas Ab, through the Fas-associated death domain, directly activates caspase 8, which then cleaves and activates the common executioner caspase 3 (extrinsic pathway) (36, 37). Staurosporine induces the release of mitochondrial cytochrome c, which leads to activation of caspase 9 and caspase 3 (intrinsic pathway) (38).

The major PGE2 receptors on colon epithelial cells are EP2 and EP4 (25), a finding we confirmed by RT-PCR of T84 cells (data not shown). Both EP2 and EP4 receptors activate adenylyl cyclase and increase cAMP as a second messenger, suggesting that cAMP mediates antiapoptotic functions in epithelial cells. Consistent with this idea, we found that a membrane-permeant cAMP analog, 8-CPT-cAMP, and CTX, which catalyzes the ADP-ribosylation of Gs, regulatory protein, leading to the activation of adenylyl cyclase, also inhibited Fas- and staurosporine-promoted apoptosis (Fig. 1A). These findings were confirmed by evidence that CTX and 8-CPT-cAMP also inhibited the formation of apoptosis-associated DNA laddering in T84 cells (Fig. 1B and data not shown). We obtained similar results with the normal rat intestinal epithelial cell line, RIE-1 (Fig. 1C). Thus,
the antiapoptotic response to increases in cAMP in intestinal epithelial cells is independent of their state of transformation and conserved across at least two species.

**Induction of IAP Family Members by cAMP Agonists.** 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 apoptotic pathways, a stimulation of antiapoptotic pathways, or a combination thereof. Previous work (39–42) showed that BAD, a proapoptotic member of the Bcl-2 family of apoptosis regulators, is phosphorylated and inactivated by protein kinase A, the effector kinase of cAMP. Consistent with those findings, we suggested that PGE2 can induce expression of the apoptosis inhibitor BAD (43). Although such mechanisms may contribute to the cAMP-mediated inhibition of the mitochondrial pathway of apoptosis, they cannot account for the ability of cAMP to inhibit both pathways of apoptosis, death domain-mediated (promoted by Fas activation) and mitochondria-mediated (induced by staurosporine) in T84 cells. Therefore, we turned our attention to antiapoptotic regulators with the capacity to inhibit apoptosis caused by both pathways.

The earliest common step in the two pathways is cleavage and activation of the executor caspase 3 (38, 44). The activity of cleaved caspase 3 can be prominently inhibited by members of the IAP family (45). To determine whether IAPs are involved in the antiapoptotic actions of cAMP, we analyzed by gel electrophoresis to assess apoptotic DNA laddering. Lane M, 100-bp DNA ladder marker.

**Fig. 1.** Suppression of Fas- and staurosporine-promoted apoptosis by cAMP agonists in intestinal epithelial cells. T84 (A and B) and RIE-1 (C) cells, treated for 1 h with PGE2, CTX, or 8-CPT-cAMP (CPT), or left untreated (−), were incubated for 12 h with anti-Fas Ab or staurosporine, or no additional agonists (−). (A and C) Oligonucleosome release into the cytoplasm was assayed by ELISA and is presented as relative increase compared with untreated controls. Results are mean ± SEM (n = 4, **P < 0.01** versus respective control not treated with cAMP agonists). (B) Cytosolic DNA was extracted from T84 cells and analyzed by gel electrophoresis to assess apoptotic DNA laddering. Lane M, 100-bp DNA ladder marker.

**Fig. 2.** Induction of epithelial c-IAP2 expression by cAMP agonists. T84 cells were treated with CTX, 8-CPT-cAMP (CPT), or PGE2, or left untreated (−), for the indicated times or for 4 h in C. Levels of the mRNAs of the indicated genes were analyzed by qualitative RT-PCR (A) and real-time RT-PCR (B), and protein levels were analyzed by immunoblot analysis (C and D). The bottom graphs in C and D show densitometric results of the respective blots, normalized against actin levels and expressed relative to unstimulated controls.

Induction of IAP Family Members by cAMP Agonists. 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 apoptotic pathways, a stimulation of antiapoptotic pathways, or a combination thereof. Previous work (39–42) showed that BAD, a proapoptotic member of the Bcl-2 family of apoptosis regulators, is phosphorylated and inactivated by protein kinase A, the effector kinase of cAMP. Consistent with those findings, we suggested that PGE2 can induce expression of the apoptosis inhibitor BAD (43). Although such mechanisms may contribute to the cAMP-mediated inhibition of the mitochondrial pathway of apoptosis, they cannot account for the ability of cAMP to inhibit both pathways of apoptosis, death domain-mediated (promoted by Fas activation) and mitochondria-mediated (induced by staurosporine) in T84 cells. Therefore, we turned our attention to antiapoptotic regulators with the capacity to inhibit apoptosis caused by both pathways.

The earliest common step in the two pathways is cleavage and activation of the executor caspase 3 (38, 44). The activity of cleaved caspase 3 can be prominently inhibited by members of the IAP family (45). To determine whether IAPs are involved in the antiapoptotic actions of cAMP, we analyzed by RT-PCR the expression in T84 cells of all eight known human IAP family members. Stimulation with CTX increased expression of c-IAP2 (also termed BIRC3) mRNA within 3 h and that of LIVIN (also termed BIRC7) within 24 h (Fig. 2A), although the latter seemed to be at lower levels than the former. Expression of mRNA for five other IAP family members was constitutive and not affected by CTX, whereas BIRC3 was not expressed by the cells (Fig. 2A). Real-time RT-PCR confirmed that CTX increased c-IAP2 mRNA levels by 3- to 5-fold compared with unstimulated cells, whereas c-IAP1 (also termed BIRC2) mRNA expression, used as a control, was unchanged (Fig. 2B). Furthermore, PGE2, CTX, and 8-CPT-cAMP increased c-IAP2 protein expression by 4 h after stimulation, with maximal levels (4- to 5-fold increase) by 8 h, as determined by immunoblot analysis of total cell lysates (Fig. 2C and D). The cAMP agonists did not affect protein expression of c-IAP1 (Fig. 2C) or XIAP (also termed BIRC4) (not shown).

We next used transcriptional reporter assays to define the mechanism of c-IAP2 mRNA induction by cAMP. CTX stimulated the activity of a reporter vector carrying 2 kb of the c-IAP2 promoter region (Fig. 3A, WT). TNFα, a known inducer of c-IAP2 expression (46), also stimulated this reporter, suggesting that both agonists induced transcriptional activation of the c-IAP2 gene. The promoter region of the c-IAP2 gene...
Fig. 3. CREB dependence of cAMP-induced c-IAP2 expression. (A) T84 cells, transfected with the reporter plasmids depicted on the left of A, were stimulated for 24 h with CTX or TNFα, or left unstimulated, and extracts were assayed by a dual luciferase assay. Results represent relative increases compared with untreated controls and are mean ± SEM (n = 4; *, P < 0.01 versus untreated controls; **, P < 0.01 versus CREI). (B) T84 cells were stimulated with CTX or TNFα for 4 h, or left unstimulated (−). Cell extracts were analyzed by immunoblots stained with the indicated antibodies. Similar data were obtained 1 h after agonist stimulation (data not shown).

Fig. 4. cAMP inhibits caspase 3 activity but not cleavage. T84 cells were treated for 1 h with CTX or PGE2, or left untreated (−), and further incubated for 12 h with anti-Fas Ab or no additional stimulator (−). (A) Cell extracts were assayed enzymatically for caspase 3 activity. Results are expressed relative to untreated controls and are mean ± SEM (n = 4; *, P < 0.01 versus anti-Fas Ab-stimulated control not treated with cAMP agonists). (B and C) Cell extracts were prepared and analyzed by SDS/PAGE, followed by immunoblotting (IB) and staining with the indicated antibodies. The graphs below the immunoblots show densitometric results of a representative experiment (B) or several independent experiments (C) (mean ± SEM, n = 3). Data are presented as ratio of cleaved to total (i.e., cleaved and full-length) product relative to the ratio in cells treated with anti-Fas Ab but not CTX.

The studies above showed that cAMP agonists induce expression of c-IAP2 in intestinal epithelial cells but did not directly address the role of COX-2 in this event. To determine the functions of COX-2, we used HCA-7 human colon epithelial cells, which express high levels of COX-2 (Fig. 5D) and release PGE2 constitutively (28). Treatment of these cells with the specific COX-2 inhibitor SC-236 promoted basal and staurosporine-induced apoptosis (Fig. 5A), indicating that COX-2 exerted antiapoptotic functions under these conditions, which is consistent with prior findings under resting conditions (43). Addition of PGE2 to staurosporine-treated cells reversed the stimulatory effects of SC-236 (Fig. 5A), suggesting that this PG is responsible, at least in part, for the COX-2-dependent antiapoptosis. The promotion of apoptosis in HCA-7 cells by COX-2 inhibition with SC-236 was accompanied by a decrease in the expression of c-IAP2, which, like the effects on apoptosis, could be reversed by addition of PGE2 to SC-236-treated cells (Fig. 5B). Neither COX-2 inhibition nor PGE2 stimulation had any effect on the levels of c-IAP1 in HCA-7 cells. In contrast to results with HCA-7 cells, SC-236 treatment did not affect basal apoptosis and decreased (albeit not significantly, P = 0.07), rather than increased, staurosporine-promoted apoptosis in T84 cells (Fig. 5C), which lack COX-2 expression (Fig. 5D). Further-
more, SC-236 did not alter c-IAP2 levels in T84 cells (data not shown). Taken together, these results are consistent with the conclusion that COX-2-promoted antiapoptosis is mediated by the release of PGE2 and subsequent cAMP-dependent induction of c-IAP2 expression in intestinal epithelial cells.

Discussion

These studies show that cAMP mediates antiapoptotic functions in normal and transformed intestinal epithelial cells. This finding has implications for understanding the regulation of normal turnover of the intestinal epithelium and the dysregulation of cell death in the development of colorectal cancer. In the normal intestine, the proliferating, undifferentiated epithelial cells reside at the base of the crypts. Cells then become postmitotic, differentiate, and migrate to the surface or villus, where they undergo apoptotic cell death (52). This differentiatior process is accompanied by a decrease in the capacity to generate cAMP after agonist stimulation (53), but the physiologic significance of this finding has remained unclear. Our data suggest the concept that the attenuation of cAMP generation is a contributory, if not necessary, step to overcome the antiapoptotic functions of cAMP, thereby facilitating normal programmed cell death of differentiated epithelial cells. Thus, cAMP may act as a physiological regulator of cell removal by apoptosis in the normal gastrointestinal tract.

The observation that cAMP suppresses apoptosis in transformed colon epithelial cells suggests that this second messenger might play a role in the formation of colorectal adenomas and cancers (54). Impairment of apoptosis occurs early and progresses throughout the colon adenoma–cancer sequence (55), although it is not known which proapoptotic stimuli and signaling pathways are physiologically relevant for the apoptotic cell death of intestinal epithelial cells at different stages of transformation. Prior studies suggested that COX-2-dependent events can inhibit mitochondria-mediated and spontaneous, but poorly defined, apoptosis in intestinal epithelial cells (56, 57). Our studies indicate that the antiapoptotic functions of cAMP agonists, including PGE2, extend to both major pathways of apoptosis and are thus likely to apply to a wide range of physiologically relevant stimuli. These data support the notion that the functions of COX-2 in colon cancer development are mediated by the release of PGE2, which activates cAMP-dependent suppression of apoptosis induced by different proapoptotic stimuli in intestinal epithelial cells. This survival advantage allows the affected cells to accumulate genetic mutations leading to the ultimate loss of proliferative control.

Stimulation of intestinal epithelial cells with cAMP agonists induces the expression of c-IAP2, most likely through CREB-dependent transcriptional activation. This observation suggests a molecular basis to account for the ability of cAMP agonists to suppress apoptosis mediated by both major proapoptotic pathways, as c-IAP2 directly inhibits the activity of the central executioner caspase 3 (50). Genetic ablation experiments will be required to determine the exact importance of this antiapoptotic regulator in mediating cAMP-dependent antiapoptosis. However, the involvement of c-IAP2 in this process is suggested by the finding that only the activity of cleaved caspase 3, not its generation from the inactive precursor, was inhibited in cells treated with cAMP agonists, as this reflects the high affinity of c-IAP2 for the cleaved form of caspase 3 but not its precursor (50, 51). The molar ratio of c-IAP2 to activated caspase 3 critically determines the degree of apoptosis inhibition (58) and thus the potential to contribute to colorectal carcinogenesis. These data do not rule out a role for other mechanisms in mediating cAMP-dependent antiapoptosis, such as the induction of the antiapoptotic regulator Bcl-2 (43) or the protein kinase A-dependent inactivation of the proapoptotic regulator BAD (39–41). Rather, these different mechanisms may act synergistically to inhibit apoptosis in response to proapoptotic signals encountered by the intestinal epithelium within its microenvironment.

The in vitro results presented here provide compelling evidence for the importance of PGE2, COX-2, and c-IAP2 in regulation of apoptosis of colon cancer cell lines, but ultimately such ideas must be tested in the in vivo setting. The findings are consistent with data from certain in vivo models, such as gene knockout of EP2 receptors in Apc<sup>−/−</sup> mice, which suggested a key role for cAMP induced by PGE2, a product of overexpressed COX-2, in intestinal tumorigenesis (23). In addition, c-IAP2 has been reported to be increased in many human cancer cell lines, including colon cancer (59). Furthermore, our data have potential implications for understanding the pathogenesis of nonintestinal cancers where COX-2 seems to play a role similar to that in colorectal cancer. PG production and COX-2 expression are increased in non-small-cell lung cancers, prostate adenocarcinomas, head and neck squa-

Fig. 5. Inhibition of COX-2 promotes apoptosis and suppresses c-IAP2 expression. HCA-7 (A) and T84 cells (C) were treated for 24 h with or without SC-236 (25 nM) and PGE2 (5 μM), as indicated, and further incubated for 6 h (HCA-7) or 12 h (T84) with staurosporine (100 nM) or no additional stimulator (–). Oligonucleosome release into the cytoplasm was assayed by ELISA and is presented as relative increase compared with untreated controls. Results are mean ± SEM (n = 3; *, P < 0.05; N.S., not significant). (B) Extracts of HCA-7 cells, treated for 24 h with or without SC-236 and PGE2, were analyzed by SDS/PAGE, followed by immunoblotting with the indicated antibodies. The histogram shows densitometric results of the respective blots expressed relative to unstimulated controls. (D) Expression of COX-2 and β-actin mRNA were analyzed in HCA-7 and T84 cells by RT-PCR, using 38 and 28 cycles of amplification, respectively.
mous cell carcinomas, and some breast cancers (60–63). Both clinical and animal studies have suggested that treatment with COX-2 inhibitors can reduce the incidence of nonintestinal cancers (61–63). The mechanisms by which COX-2 inhibition prevents the development of these cancers are poorly understood, but our results suggest that PG- and cAMP-dependent up-regulation of c-IAP2 in the affected epithelial cells is one potential mechanism for mediating the cancer-promoting functions of COX-2.

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