Cyclic AMP Activation of the Extracellular Signal-regulated Kinases 1 and 2

IMPLICATIONS FOR INTESTINAL CELL SURVIVAL THROUGH THE TRANSIENT INHIBITION OF APOPTOSIS*

Jeffrey A. Rudolph†, Julia L. Poccia, and Mitchell B. Cohen

From the Division of Gastroenterology, Hepatology, and Nutrition, Cincinnati Children’s Hospital Medical Center and the University of Cincinnati College of Medicine, Cincinnati, Ohio 45229-3039

The proliferative compartment of the intestinal crypt is critical in the process of intestinal epithelial cell homeostasis. The ability of these progenitor crypt cells to resist apoptosis and ensure restitution during a potentially lethal insult, but retain the ability to remove damaged or altered cells afterward, is necessary for preservation of the crypt-villus unit. We have examined the ability of cAMP to transiently inhibit apoptosis via the extracellular signal-regulated kinases 1 and 2 (ERK1/2), in T84 cells, an intestinal crypt-like cell line, using the cAMP analog 8-bromo-cAMP and cholera toxin (CT), cAMP-mediated ERK1/2 activation was first measured by Western blot analysis of the phosphorylated (activated) and total (activated and inactivated) forms of ERK1/2. Cyclic AMP activated ERK1/2 in a time- and dose-dependent manner, and the effect was inhibited by PD098059, an inhibitor of the ERK1/2 signaling pathway. However, inhibition of protein kinase A (PKA) did not alter the activation of ERK1/2. CT transiently inhibited both staurosporine and Fas antibody mediated apoptosis as measured by a caspase-3 activation assay and the detection of nucleosomes in an apoptosis based enzyme-linked immunosorbent assay. This inhibitory effect was reversed by the simultaneous addition of PD098059. Our data suggest that in the T84 cell line, cAMP activates ERK1/2 in a PKA independent fashion and a physiological consequence of this activated pathway is the transient inhibition of apoptosis. These findings suggest a novel pathway that intestinal cells use to protect against injury while maintaining the overall ability to remove damaged cells and preserve intestinal homeostasis.

Intestinal crypt cells play a unique role in driving proliferation for the entire crypt-villus unit. Apoptosis is a critical process in the intestinal crypt that serves to maintain cellular homeostasis throughout the intestine. In the murine intestinal crypt, apoptosis plays a key regulatory role by the efficient removal of genetically damaged enterocytes, and by preventing the proliferation of aberrant, and possibly tumorigenic, phenotypes (1–3). Apoptosis also serves as an end point in a number of human pathophysiologic processes including inflammation (4, 5), chemotherapy-induced mucositis (6), and it is a key component in intestinal allograft transplant rejection (7). During a potentially damaging insult, exuberant crypt cell apoptosis has the potential to affect gut restitution by altering the proliferative compartment, which is responsible for replenishing damaged epithelium. Therefore, the ability to prevent apoptosis in normal enterocytes after an acute stress while conserving the ability to remove aberrant and potentially tumorigenic cells is central to the orderly maintenance of intestinal cell turnover.

Many of the signal transduction pathways involved in apoptosis are regulated, in part, by mitogen-activated protein kinase (MAPK) signaling cascades. The extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) are a superfamily of MAPKs that have been implicated in proliferation, differentiation, and apoptosis (8). ERK 1 and 2 share 90% sequence homology (9) and identical domains with respect to their phosphorylation sites (10) that are responsible for activation. The ability of ERK1/2 mice to remain viable and fertile, with only relatively minor phenotypic changes suggests functional redundancy between the ERK family members (11). In intestinal epithelial cells, ERK1/2 function has been most extensively studied in the process of differentiation (12–15). With regard to the regulation of apoptosis, ERK1/2 is also emerging as a pathway involved in intestinal epithelial cell survival (3, 14, 16).

A key factor in the determination of ERK1/2 function is the length of time it is phosphorylated (17), which is in part determined by the intracellular signals responsible for the activation of its signaling cascade. 3’-5’ cyclic adenosine monophosphate (cAMP) can activate ERK1/2, which results in either the initiation or inhibition of apoptosis depending on cell type. ERK1/2 signaling through cAMP has been documented in a variety of cell types (18) and has been shown to inhibit apoptosis in thyroid follicular cells (19), the PC12 neuronal cell line (20), primary cultured hepatocytes (21), and most recently the T84 intestinal cell line (22).

In the intestinal epithelium, cAMP has been implicated indirectly in murine enterocyte survival through prostaglandin E2 (PGE2) activation of the EP2 receptor, which signals via cAMP (23). Transgenic mice lacking the EP2 receptor are more prone to irradiation induced intestinal apoptosis (24) and show...
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**EXPERIMENTAL PROCEDURES**

**Cell Culture and Stimulation of CAMP—**T84 cells (American Type Culture Collection, Manassas, VA) were grown in F-12/Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% newborn calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), ampicillin (8 μg/ml), and buffered with 44 mM sodium bicarbonate to a final pH of 7.2–7.4. Cells were subcultured into 48-well tissue culture plates at a density of 1 × 10^4 cells/cm^2, fed every other day, and incubated using the standard conditions of 37 °C and 5% CO₂. Cells were confluent at day 8 and used 10 days after subculture. Cells were incubated with either 200 ng/ml CT (List Biological Laboratories, Campbell CA), 8-bromo-cyclic AMP (8-Br-cAMP; Sigma), 100 μM heat-stable enterotoxin (ST) (gift of R. Giannella), or 30 μM H-89 (N-[2-bromo-3-cyclohexylaminolaminoethyl]-5-isouquinolinesulfonamide dihydrochloride) (Sigma) for the indicated times prior to apoptosis assays or the analysis of ERK1/2 activation.

**Detection of cAMP-stimulated ERK1/2 Activation—**After incubation with CT, 8-Br-cAMP, and/or H-89, cells were homogenized using TNN buffer (50 mM Tris, 150 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 25 μg/ml protease inhibitors) (12). Homogenates were centrifuged at 15,000 rpm on a table top centrifuge, and the protein in the suspension was quantified using the Bradford reagent-based assay (Bio-Rad). 10 μg protein was electrophoresed on pre-cast 4–12% Bis-Tris gels (Invitrogen) in MES/SDS running buffer (50 mM 2-(MES, sodium salt) and 25 mM SDS) and transferred to nitrocellulose membranes (Invitrogen) as described previously (30). Immunoblotting was performed using the Anti-ACTIVE MAPK® antibody, rabbit (Promega, Madison, WI) specific for phosphorylated/activated ERK1/2 at 1:5,000 dilution followed by peroxidase-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), and visualized by enhanced chemiluminescence (PerkinElmer Life Sciences Inc.). To assure equal loading, blots were stripped at 50 °C for 30 min in stripping solution (62.5 mM Tris, 2% SDS, 100 mM 2-mercaptoethanol) and probed with p44/42 MAP kinase antibody (Cell Signaling Technology, Inc., Beverly, MA) specific for both the phosphorylated and unphosphorylated (total) ERK1/2 at a 1:1,000 dilution. Results were quantified using a spot densitometer and expressed as a ratio of active:total ERK1/2 integrated density values.

**Induction of Apoptosis—**Apoptosis was induced in T84 cells using the protein kinase inhibitor, staurosporine (STS) (Sigma). In pilot experiments we determined that 4 μM STS added to confluent cells resulted in floating cells 4 h after exposure (data not shown). T84 cells were grown to confluence and stimulated with CT or 8-Br-cAMP. 4 μM STS was added, and cells were incubated for an additional 4 h. For experiments using the Fas ligand apoptotic pathway, cells were incubated with 500 ng/ml of the activating Fas-Ab clone CH11 (Upstate Biotechnology Inc., Lake Placid, NY) for 18 h to induce apoptosis.

**Nucleosome Detection as a Marker for Apoptosis—**After pretreatment and apoptosis induction, experimental and control wells of T84 cells were processed for the DNA Cell Death Detection ELISA (Roche Applied Science) to determine nucleosome formation, according to manufacturer’s protocol. Briefly, cells were lysed, and the homogenates were spun at 4 °C for 30 min to remove large genomic DNA. Supernatant was added to ELISA plates coated with anti-histone antibody and incubated with peroxidase conjugated anti-DNA antibody. Nucleosome detection was based on a colorimetric change in substrate added to the wells after conjugation. All samples were analyzed in duplicate, blank values were subtracted, and average values for each treated well were determined.

**Caspase-3 Activation Assay—**A second, independent assay for the determination of apoptosis was performed. Caspase-3 activation was determined using the Caspase-3 Assay kit (BD Biosciences) according to the multiwell plate protocol in the manufacturer’s instructions. Briefly, T84 cells were grown to confluence, and CAMP stimulation/apoptosis induction was performed as described above. Cells were washed once and lysed using lysis buffer (10 mM Tris-HCl, 10 mM NaHPO₄/NaPO₄, 130 mM NaCl, 1% Triton X-100, 10 mM sodium PIP) for 30 min at 4 °C. 50 μl of lysate was added to a reaction mixture containing 37.5 mM Ac-DEVD-7-amino-4-methylcoumarin, a fluorogenic substrate, in protease assay buffer (20 mM HEPES, 10% glycerol, 2 mM dithiothreitol) and incubated for 1 h at 37 °C. Previous experiments using recombinant caspase-3 showed maximum fluorogenic detection after 20–30 min incubation and no diminution of signal at greater than 60 min. The fluorescence of cleaved Ac-DEVD-7-amino-4-methylcoumarin was read on a SpectraMax Gemini II fluorometer with an excitation wavelength of 380 Å and emission wavelength of 440 Å and SoftPro 3.01 software. All samples were analyzed in triplicate, blank values were subtracted, and average values for each treated well were determined.

**Statistical Analysis—**For apoptosis assays, results are expressed in mean values ± S.E., and p values were determined using a two-tailed student’s t test for paired samples assuming normal distribution. Single factor analysis of variance was used where appropriate.

**RESULTS**

**Cyclic AMP Activation of ERK1/2 in T84 Cells—**To demonstrate the effect of increasing intracellular cAMP levels on CT-treated signaling, confluent T84 cells were incubated with CT, and ERK1/2 was detected using a phosphorylated antibody specific for ERK1/2 (Fig. 1A). Cells were harvested beginning at 15 min to account for the lag phase due to CT internalization and activation of adenylate cyclase in non-disrupted T84 cells (28). Hypertonic medium was used as a positive control. CT activated ERK1/2 in as little as 1 h, peaked at 3 h, and decreased by 6 h. Unlike CT, 8-Br-cAMP stimulates cAMP-mediated pathways in a more rapid fashion as evidenced by maximum chloride secretion in ~15 min in T84 cells (31). When cells were treated with 8-Br-cAMP (Fig. 1B), similar results were obtained, with a peak of activation at 15 min and a decrease in...
cAMP acts upon the ERK1/2 signaling cascade, prior to the phosphorylation of ERK1/2.

Cyclic AMP Activation of ERK1/2 Is Independent of PKA Activation—In many non-intestinal cell lines, cAMP-mediated ERK1/2 activation occurs via the cAMP-binding protein, protein kinase A (PKA) (18, 34). The role of PKA in cAMP-mediated activation of ERK1/2 in T84 cells was determined using a pharmacological inhibitor of PKA, H-89.

PKA activity was first measured in T84 cells using a commercial based assay (Promega) that utilizes the phosphorylation of Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide), a synthetic substrate specific for PKA. Addition of increasing doses of H-89 2 h prior to cell harvest showed a dose-dependent effect (data not shown). Incubation of T84 cells with 30 μM H-89 for 2 h suppressed PKA activity by 86% when compared with cells treated with media alone (Fig. 3A). 30 μM H-89 was then added to confluent T84 cells 2 h prior to the addition of 1000 μM 8-Br-cAMP. After 1 h further incubation, cell homogenates were immunostained for both phosphorylated ERK1/2 and total ERK1/2. A representative blot is shown in Fig. 3B. The addition of 30 μM H-89, a dose sufficient to markedly inhibit PKA activity, did not block 8-Br-cAMP mediated ERK1/2 signaling. This data suggest that the cAMP-mediated activation of ERK1/2 is not dependent upon PKA.

Cyclic AMP Activation of ERK1/2 Is Dependent upon Confluen—The regulation of MAPKs, such as ERK1/2, is both specific to cell type and dependent upon cellular context, such as the degree of differentiation, the cell cycle state, and/or the presence of cellular contacts. Although T84 cells remain poorly differentiated as they grow in culture (35), we sought to determine whether cAMP activation of ERK1/2 was dependent upon the degree of confluency. T84 cells were uniformly seeded onto 48-well tissue culture plates. Starting at day 1 (30% confluence) and continuing to day 8 (100% confluence), a subset of cells was exposed to media alone or 1000 μM 8-Br-cAMP for 1 h, and ERK1/2 phosphorylation was analyzed (Fig. 4). Day 1 lysates showed no increase in phosphorylated ERK1/2 signal with exposure to 8-Br-cAMP and less total ERK1/2 per 10 μg of total protein. By day 3, total ERK1/2 staining was similar to subsequent time points suggesting that total ERK1/2 remained at a constant level. The ability of cAMP to activate ERK1/2 increased as the cells became confluent (days 5 and 8), indicating that as T84 cells attain confluence, become more responsive to cAMP.

CT Inhibition of STS-mediated Apoptosis—To study the effect of CT on apoptosis, we examined two independent assays of the apoptotic cascade: nucleosome detection and caspase-3 activity. T84 cells were grown to confluence. CT was then added alone or 3 h prior to induction of apoptosis with STS. Fig. 5A demonstrates nucleosome detection in cells treated with media alone and under experimental conditions. Pretreatment with CT tended to decrease basal levels of nucleosome detection when compared with controls, but differences were not significant (p = 0.07). Addition of STS alone resulted in a large increase in nucleosome detection and served as a positive control. When CT was added 3 h prior to the addition of STS, nucleosome detection decreased ~20% (p = 0.001), suggesting that CT inhibited STS-mediated apoptosis. To verify the inhibitory effects of cAMP on STS-mediated apoptosis, a caspase-3 activity assay was performed (Fig. 5B). Similar to the ELISA analysis, addition of CT alone led to a small, but not significant, decrease in basal caspase-3 activity (p = 0.07). STS alone led to a large increase and served as a positive control. Addition of CT prior to STS inhibited caspase-3 activity by ~43% (p = 0.002) confirming the anti-apoptotic effect of CT on STS-mediated apoptosis.
T84 cells were incubated with media alone or 30 μM H-89 for 2 h and assayed using the SignaTect® cAMP-dependent protein kinase (PKA) assay system (Promega). PKA activity was measured as a function of mg soluble protein as quantified by the Bradford assay. When medium was added alone, the mean activity was 1.55 pmol of ATP/min/μg of protein (n = 7). Two-hour incubation with 30 μM H-89 decreased the activity by 86% (0.21 pmol of ATP/min/μg of protein) (n = 7). Error bars represent S.E. of the mean. *, p value ≤ 0.001. B, the effect of PKA inhibition on cAMP-mediated ERK1/2 activation. Confluent T84 cells were pretreated with medium alone or 30 μM H-89 for 2 h and then 1000 μM 8-Br-cAMP for 1 h. 10 μg of protein was immunoblotted for phosphorylated ERK1/2, stripped, and re-probed for total ERK1/2. Cells treated with medium alone or H-89 alone demonstrated no ERK1/2 activation. Cells treated with 8-Br-cAMP activated ERK1/2 irregardless of pretreatment with H-89. Duplicate results shown (n = 4 for each condition tested).

Fig. 3. CAMP-dependent ERK1/2 activation as a function of PKA. A, pharmacological inhibition of PKA in T84 cells by H-89. Confluent T84 cells were incubated with media alone or 30 μM H-89 for 2 h and assayed using the SignaTect® cAMP-dependent protein kinase (PKA) assay system (Promega). PKA activity was measured as a function of mg soluble protein as quantified by the Bradford assay. When medium was added alone, the mean activity was 1.55 pmol of ATP/min/μg of protein (n = 7). Two-hour incubation with 30 μM H-89 decreased the activity by 86% (0.21 pmol of ATP/min/μg of protein) (n = 7). Error bars represent S.E. of the mean. *, p value ≤ 0.001. B, the effect of PKA inhibition on cAMP-mediated ERK1/2 activation. Confluent T84 cells were pretreated with medium alone or 30 μM H-89 for 2 h and then 1000 μM 8-Br-cAMP for 1 h. 10 μg of protein was immunoblotted for phosphorylated ERK1/2, stripped, and re-probed for total ERK1/2. Cells treated with medium alone or H-89 alone demonstrated no ERK1/2 activation. Cells treated with 8-Br-cAMP activated ERK1/2 irregardless of pretreatment with H-89. Duplicate results shown (n = 4 for each condition tested).

CT Inhibition of Fas-mediated Apoptosis—To confirm that cAMP protects cells from apoptosis and that cAMP is not interacting directly with STS, Fas-Ab was used as a different proapoptotic agent. Confluent T84 cells were incubated with Fas-Ab for 18 h to induce apoptosis (29). To adjust for the longer induction period of Fas-mediated apoptosis, CT was added 6 h after the addition of Fas-Ab but prior to the activation of caspase-3. Fas-Ab increased both nucleosome formation (p = 0.0001) and caspase-3 activation (p = 0.0001). CT effectively inhibited Fas-Ab-mediated nucleosome formation by 27% (p = 0.0001), confirming that cAMP inhibits apoptosis and that this is not limited to the STS pathway (Fig. 6A). Similar results were achieved using the caspase-3 activation assay (Fig. 6B) (p = 0.0001). The inhibition of both STS- and Fas-Ab-mediated apoptosis demonstrates that cAMP interferes with apoptosis prior to the activation of caspase-3 but after the cell is committed to the apoptotic process.

CT Inhibition of STS-mediated Apoptosis Is Transient—To determine whether the length of incubation of CT played a significant role in the inhibition of apoptosis, T84 cells were preincubated with CT for varying lengths of time and nucleosome formation was measured (Fig. 7). Consistent with the lag phase of cAMP production, addition of CT at the same time as STS led to no detectable change in nucleosome formation. The inhibitory effect of CT was maximal at 1–6 h preincubation. Thereafter, the inhibitory effect decreased until no effect was seen when CT was preincubated for 24 h. Despite the transient nature of the inhibitory effect on nucleosome formation, incubation with CT produced a sustained increase in cAMP (Fig. 7, top). These results demonstrate that the effect of CT on apoptosis is transient in nature and that the timing of the inhibitory effect is coordinate with ERK1/2 activation.

CT Inhibition of Apoptosis Is Reversed by Inhibition of ERK1/2—Previous studies in non-intestinal cell lines have implicated cAMP-mediated ERK1/2 activation as a survival pathway. In addition, the transient response of CT coordinate with the timing of ERK1/2 activation in T84 cells suggested that ERK1/2 was a potential survival pathway. For this reason, the role of ERK1/2 in CT inhibition of apoptosis was examined. Cells were co-incubated with CT and/or 50 μM PD098059, prior to the induction of apoptosis with STS (Fig. 8). Cells incubated with PD098059 prior to STS showed an increase in nucleosome detection, even greater than the effect of STS alone. Cells co-incubated with CT and PD098059 demonstrated reversal of the anti-apoptotic effect of CT when added alone. Lower doses of PD098059 (10 and 20 μM) showed a dose-dependent effect on the reversal of CT-mediated effects (data not shown). These data implicate ERK1/2 as a survival pathway involved in CT-mediated inhibition of apoptosis.

DISCUSSION

Our data extends previous findings that cAMP inhibits apoptosis in T84 cells (22) by providing an alternative/additional mechanism, i.e. ERK1/2 activation, a known survival pathway in intestinal cells. The susceptibility of a cell to apoptosis is dependent upon a balance of pro- and anti-apoptotic factors. When a cell is exposed to an apoptotic stimulus and survival is the outcome, it is likely a complex network of responses that involves the initiation of cell survival signal transduction pathways and the production of numerous anti-apoptotic factors. For example, ERK1/2 acts in concert with NF-κB in the survival response of YAMC cells exposed to tumor necrosis factor-α (TNF-α) (14). Thus the initiation of multiple anti-apoptotic signaling pathways provide redundancy and, at times, synergy to preserve cell survival (14, 36). In T84 cells, cAMP induces cAMP-responsive element (CRE)-mediated transcriptional up-regulation of cellular inhibitor of apoptosis protein-2 (cIAP-2) (22), an inhibitor of caspase-3 activation (37), and known anti-apoptotic factor. Our data demonstrate the reversal of the cAMP survival effect with the addition of the MEK1/2
inhibitor, PD098059, and strongly suggests that ERK1/2 is a necessary component that may be acting in concert with cIAP-2 and potentially other anti-apoptotic factors that result in cell survival. A second feature of this survival response is that in T84 cells, STS-mediated apoptosis is only transiently inhibited by CT. This effect occurs despite the fact that CT induced cAMP levels continue to rise for 24 h after the initial exposure. The timing is coordinate with the activation of ERK1/2 and provides further evidence to support the role of ERK1/2 in cAMP-mediated cell survival. Therefore, we conclude that the inhibition of apoptosis by cAMP phosphorylation by cAMP is closely linked to the transient nature of ERK1/2 activation, and we propose that it is tightly controlled by ERK1/2 activation. A transient response of the intestine to an acute stress would lead to the preservation of the intestinal proliferative unit while allowing the removal of damaged cells afterward to maintain intestinal homeostasis. This would be crucial in the small intestine, an organ where rapid turnover, yet low cancer rates, exist (2) and would confer a distinct advantage over a conversion to a more permanent anti-apoptotic, and potentially tumorigenic, phenotype.

MAPK signaling pathways are often regulated in a cell specific manner and the effect of cAMP on ERK1/2 signaling is no exception. Activation of ERK1/2 through cAMP has been demonstrated in other transformed cell lines (18–20) as well as in primarily derived non-intestinal epithelial cell lines (38, 39), while in other cell lines cAMP can inhibit ERK1/2 (18). Our data clearly indicate that in confluent T84 intestinal cells, cAMP phosphorylates ERK1/2. The specificity of this signaling pathway in T84 cells was determined on the basis of a dose response, an inhibition of activation through the pharmacologic

**Fig. 5. Cholera toxin inhibition of STS-mediated apoptosis.** Confluent T84 cells were pretreated for 3 h with either medium alone or 200 ng/ml CT. A subset of each group of cells was then incubated for 4 h with 4 μM STS to induce apoptosis. A, nucleosome detection by ELISA as a marker for apoptosis. CT alone resulted in a small, but not significant, decrease in nucleosome formation. STS alone led to a large increase in nucleosome detection and served as a positive control. Pretreatment with CT prior to the addition of STS led to a 20% reduction in nucleosome detection, suggesting an inhibitory effect on apoptosis. B, caspase-3 activation as a marker for apoptosis. Addition of CT alone resulted in a small but not significant decrease in caspase-3 activation, while STS alone led to a large increase in caspase-3 activation. Pretreatment with CT before the addition of STS led to a 43% decrease in detectable caspase-3 activation, suggesting the inhibition of apoptosis. *, p value ≤ 0.002 CT pretreatment with STS compared with STS alone. n = 6 for each condition tested.

**Fig. 6. Cholera toxin inhibition of Fas-Ab-induced apoptosis.** Confluent T84 cells were incubated with 500 ng/ml Fas-Ab or medium alone for 18 h. After 6 h, 200 ng/ml CT was added to a subset of cells in the Fas-Ab treatment group. A, nucleosome detection by ELISA as a marker for apoptosis. Incubation with Fas-Ab led to a large increase in nucleosome detection. Addition of CT during Fas-Ab incubation decreased nucleosome detection by 27%. B, caspase-3 activation as a marker for apoptosis. Caspase-3 activation increased as a consequence of incubation with Fas-Ab. CT treatment led to a 36% decrease in caspase-3 activation, suggesting an inhibition of apoptosis. RFU, relative fluorescence unit. *, p value ≤ 0.0001 CT-treated cells during Fas-Ab incubation compared with Fas-Ab incubation alone. n = 6 for each condition tested.
blockade of the ERK1/2 signaling cascade, and the lack of cross-activation by cGMP.

Cyclic AMP signal transduction relies upon its binding to and activation of a cAMP-binding protein. The binding protein that is most often responsible for ERK1/2 activation is PKA (18, 34, 38). For this reason, pharmacological inhibition of PKA was used to determine PKA dependence of cAMP on ERK1/2 activation. Despite using a dose of H-89 that sufficiently inhibited PKA activity, there was no diminution of ERK1/2 activity when stimulated with 8-Br-cAMP. This strongly suggests that PKA is not involved in the activation of ERK1/2 in T84 cells. Other notable exceptions of PKA-independent cAMP mediated activation of ERK1/2 are in rat thyroid follicular cells (40) and in human renal cortical collecting duct cells (39). In T84 cells, cAMP-mediated chloride secretion occurs through a PKA-dependent mechanism (41). Dephosphorylation of the specific pathways involving cAMP-mediated survival and secretion and the point of divergence will be paramount in designing strategies that will support intestinal cell survival without the consequence of a secretory diarrhea in conditions of acute intestinal injury due to apoptosis. Further work is currently ongoing to determine the signaling pathway that links cAMP to the ERK1/2 signaling pathway and whether it is independent of intestinal secretion.

In addition to PKA independence, another aspect of cAMP activation of ERK1/2 was explored by examining T84 cells at various stages of confluency. Only confluent or near confluent T84 cells can activate ERK1/2 via cAMP, implying that the formation of cell-cell contacts is important. ERK1/2 signaling has been previously linked to cell adhesion in fibroblasts (42) and in a model of cyclic strain in the intestinal Caco-2 cell line through focal adhesion kinase (Fak) (43). Cyclic AMP signaling through Fak or a similar integrin-associated pathway was not evaluated in this study. An alternative interpretation of the data is that the state of differentiation plays a role in this signaling pathway. T84 cells remain poorly differentiated when grown in culture (35). In addition, ERK1/2 activation is inversely correlated with differentiation in various other intestinal cell lines (12, 15) and in vivo, ERK2 appears to be active primarily in the undifferentiated cells of the intestinal crypt (44). Cyclic AMP production in response to various agonists is also greater in the undifferentiated cells of the rabbit intestinal crypt when compared with the villus epithelium (45). Together, these data suggest that while the undifferentiated state of T84 cells is likely to permit cAMP-mediated ERK1/2 activation, other factors that are defined by cell context, such as the ability to form cell-cell contacts, may also be involved in the regulation of this signaling pathway.

Taken together, these data demonstrate that in the T84 intestinal cell line, cAMP activates ERK1/2 via a PKA independent mechanism, and leads to the transient inhibition of apoptosis. This effect implies a potential mechanism for the inhibition of intestinal crypt cell apoptosis in response to an acute stress. Determination of the pathways utilized by cAMP to activate ERK1/2 and/or other potential survival factors, how they differ from cAMP-mediated chloride secretion, and demonstration of how the various survival mechanisms interact separately and in concert will be paramount in designing therapies to induce epithelial restitution in time of acute injury (e.g., radiation injury, chemotherapeutic mucositis, and ischemia) without enhancing a long term, tumorigenic effect in the intestine.
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