Multiple transcription factors regulating the IL-6 gene are activated by cAMP in cultured Caco-2 cells

DAN D. HERSHKO, BRUCE W. ROBB, GUANGJU LUO, AND PER-OLOF HASSELGREN
Department of Surgery, University of Cincinnati, Cincinnati, Ohio 45267; and the Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215

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Hershko, Dan D., Bruce W. Robb, Guangju Luo, and Per-Olof Hasselgren. Multiple transcription factors regulating the IL-6 gene are activated by cAMP in cultured Caco-2 cells. Am J Physiol Regul Integr Comp Physiol 283: R1140–R1148, 2002; 10.1152/ajpregu.00161.2002.—Mucosal and enterocyte IL-6 production is increased during sepsis and endotoxemia. Recent studies suggest that cAMP potentiates IL-6 production in endotoxin- or IL-1β-stimulated enterocytes, but the molecular mechanisms are not known. We examined the role of the transcription factors NF-κB, activator protein (AP)-1, CCAAT/enhancer binding protein (C/EBP), and cAMP response element-binding protein (CREB) in cAMP-induced IL-6 production in cultured Caco-2 cells, a human intestinal epithelial cell line. In addition, the role of the protein kinase A (PKA), protein kinase C (PKC), and mitogen-activated protein (MAP) kinase signaling pathways was examined. Treatment of the cells with IL-1β increased IL-6 production and activated the IL-6 promoter in cells transfected with a luciferase reporter plasmid containing a wild-type IL-6 promoter. These effects of IL-1β were significantly potentiated by cAMP. When the binding sites for the individual transcription factors in the IL-6 promoter were mutated, results indicated that all four transcription factors may be involved in the cAMP-induced activation of the IL-6 gene. Treatment of the Caco-2 cells with cAMP increased the DNA binding activity of CREB, C/EBP, and AP-1, but not NF-κB. By using specific blockers, evidence was found that both PKA and p38 MAP kinase (but not PKC or p42/44 MAP kinase) may be involved in the cAMP-induced potentiation of IL-6 production. The present results suggest that cAMP activates multiple transcription factors involved in the regulation of the IL-6 gene and that the activation of these transcription factors may at least in part explain why cAMP potentiates IL-6 production in stimulated enterocytes.

Mucosal and enterocyte IL-6 production is increased in various conditions characterized by inflammation such as sepsis, endotoxemia, and treatment with proinflammatory cytokines, including IL-1β (12, 15, 19, 29). IL-6 is a pleiotropic cytokine that can have both pro- and anti-inflammatory properties (2, 17, 30). Recent studies from our laboratory suggest that IL-6 produced by enterocytes may have anti-inflammatory and cell-protective effects and that increased IL-6 levels in gut mucosa may counteract some of the injurious effects of sepsis and endotoxemia (18, 28).

The transcriptional regulation of the IL-6 gene is complex and involves at least four different transcription factors, i.e., NF-κB, activator protein (AP)-1, CCAAT/enhancer binding protein (C/EBP), and cAMP response element (CRE)-binding protein (CREB) (1, 26). The role of these transcription factors varies between different cell types and may also vary within the same cell depending on stimulus (25). Previous studies suggest that cAMP can regulate the IL-6 gene, but the influence of cAMP on IL-6 production seems to be cell specific. For example, cAMP inhibited IL-6 production in human lung fibroblasts (32) and endotoxin-treated Kupffer cells (6). In contrast, cAMP potentiated IL-6 gene transcription in a human astrocytoma cell line (10), in mesangial cells (7, 21), and in a murine monocyte-macrophage cell line (4).

Two previous reports suggest that enterocyte IL-6 production may be upregulated by cAMP. In one of those reports, treatment of cultured IEC-6 cells, a rat intestinal epithelial cell line, with cAMP augmented IL-1β-induced IL-6 production (13). In another study, we found that treatment of IEC-6 cells with PGE2, a natural adenylate cyclase agonist that increases intracellular cAMP levels, potentiated endotoxin-induced IL-6 production (14). Although both of these reports suggest that cAMP activates the IL-6 gene in stimulated enterocytes, the molecular mechanisms of this effect of cAMP were not examined. In particular, the involvement of different signaling pathways and the role of the various transcription factors that may regulate the IL-6 gene in cAMP-treated enterocytes are not known.

Because of the multiple biological effects of IL-6, a better understanding of the molecular regulation of enterocyte IL-6 production has important clinical implications. The role of cAMP and mechanisms by which cAMP regulates enterocyte IL-6 production are particularly significant, considering the role of cAMP in the regulation of multiple other functions of the intestinal mucosa, including chloride secretion and production of...
cholceystokinin and other gastrointestinal hormones. The purpose of this study was to test the hypothesis that cAMP potentiates IL-6 production in IL-1β-stimulated cultured Caco-2 cells, a human intestinal epithelial cell line, by activating the mitogen-activated protein (MAP) kinase signaling pathway and by increasing the activity of multiple transcription factors.

MATERIALS AND METHODS

Cell culture. Caco-2 cells were from American Type Culture Collection (Rockville, MD). The cells were grown under conditions described in detail recently (8, 9, 20). Cells, between passages 5 and 25, were seeded at a density of 100,000 cells/cm² onto six-well culture plates (for determination of IL-6 levels by ELISA and luciferase assays) or onto 10-cm tissue culture plates (both from Fallon-Becton Dickinson, Franklin Lakes, NJ) for EMSA and Western blot analysis. The cells were grown for 72 h to 90% confluence before use. It should be noted that cells at this stage are not completely differentiated. In previous studies, however, we found that the regulation of IL-6 production by IL-1β was almost identical in Caco-2 cells grown to 90% confluence and in Caco-2 cells grown for 3 wk to full differentiation on Transwell filters (16).

Before experiments, cells were washed three times with serum-free DMEM and then treated with human recombinant IL-1β, 8-bromo-cAMP, PGE₂, 8-bromo-cGMP, or PMA at concentrations and for time periods described in RESULTS. In other experiments, the cells were treated with the protein kinase A (PKA) inhibitor N-(2-bromo-3-cinnamylamino)-ethyl-5-isoquinolinesulfonamide (H-89) or the protein kinase C (PKC) inhibitor GF-109203. After the different treatments, nuclear and cytoplasmic extracts were prepared as described previously (8, 9, 20). Culture medium was harvested and stored at −70°C until determination of IL-6 levels. All experiments were performed at least three times to ensure reproducibility.

Determination of cell viability. Cell viability was determined by measuring mitochondrial respiration, assessed by the mitochondrial-dependent reduction of 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) to formazan as described previously (19). Cell viability was not influenced by any of the experimental conditions in the present study (data not shown).

Western blot analysis. Western blot analysis was performed as described in detail previously (20). To prevent dephosphorylation, SDS buffer (62.5 mM Tris, pH 6.8, 1% SDS, 10% glycerol, 5% 2-mercaptoethanol) was used for the preparation of cell extracts. Aliquots of 20 µl of the whole cell lysates were boiled for 3 min and then separated by electrophoresis on 8–16% Tris-glycine gradient gel (Invitrogen, San Diego, CA). A protein ladder (See-Blue; Invitrogen) was included as molecular weight marker. The proteins were transferred to nitrocellulose membranes (Xcell II Blot Module; Novex), which were blocked with 5% nonfat dried milk in Tris-buffered saline (pH 7.6) containing 0.05% Tween 20 (TTBS) for 1 h. The membranes were then incubated with rabbit antibodies specific for phospho-CREB (PhosphoPlus CREB(Ser133); pCREB), p38 MAP kinase, phospho-p38 MAP kinase, p44 MAP kinase, and phospho-p44 (New England BioLabs, Beverly, MA) for 1 h and then washed three times with TTBS before incubation with peroxidase-conjugated secondary antibody for 45 min. After successive washes, the membranes were incubated in enhanced chemiluminescence reagents and exposed on radiographic film (Eastman-Kodak, Rochester, NY).

EMSA. EMSAs were performed as described in detail recently (8, 9, 20). NF-κB gel shift oligonucleotide 5’ ACT TGA GGG ACC TTG CCAAG C 3’, C/EBP gel shift oligonucleotide 5’ TGC AGA TTG CGC AAT CTG CA3’, AP-1 gel shift oligonucleotide 5’ CCG TTG ATG ACT CAG CCG GA3’, and CRE gel shift oligonucleotide 5’ AGA GAT TGC CTG AGC TGA ACG AGC TAG 3’ were from Santa Cruz Laboratories (Santa Cruz, CA). Probes were end-labeled with [γ-32P]ATP using polynucleotide kinase T4 (GIBCO BRL, Grand Island, NY). End-labeled probe was purified from unincorporated [γ-32P]ATP using a purification column (Bio-Rad Laboratories) and recovered in Tris-EDTA buffer, pH 7.4. Labeled probe was added to nuclear extracts, and the samples were incubated for 30 min on ice. Samples were then subjected to electrophoretic separation on a nondenaturing 5% polyacrylamide gel at 30 mA using Tris borate-EDTA buffer (0.45 M Tris borate, 0.001 M EDTA, pH 8.3). Blots were dried at 80°C for 3 h and analyzed by exposure to PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA). For supershift analysis, antibodies to p60, p52, p50, Rel B, C/EBP-β, C/EBP-δ, Jun B, c-Fos, Jun D, CREB, CREM, and ATF 1 were added 30 min before addition of the radio-labeled probe.

Cell transfections and luciferase assays. Wild-type and different mutant IL-6 promoter luciferase reporter plasmid constructs were kindly provided by Dr. O. Eickelberg (Yale University, New Haven, CT). The IL-6 promoter had been point-mutated at the binding sites for NF-κB, AP-1, CREB, or C/EBP before the plasmids were provided to us (5). Caco-2 cells (10³ cell/cm²) were seeded onto six-well culture dishes and grown to 50% confluence before transfection. The Lipofectin (GIBCO-BRL) transfection method was used. Briefly, LipofectAmine Plus was incubated with serum-free OPTIMEM and 1 µg of plasmid at room temperature for 15 min. The Caco-2 cells were washed three times with serum-free medium, and the 4:1 lipid-DNA complexes were added to the cells. After incubation at 37°C for 4 h, the culture medium was changed to DMEM supplemented with 10% fetal bovine serum and incubated for an additional 24 h at 37°C. After the cells had been washed with serum-free DMEM, they were treated with IL-1β (0.5 mg/ml) in serum-free medium for 8 h. For luciferase assays, cells were washed twice with PBS, pH 7.4, and 250 µl of Luciferase Cell Culture Lysis Reagent (Promega, Madison, WI) were added to each well for 15 min, after which the cells were harvested and stored at −70°C. The next day the samples were thawed and centrifuged at 14,000 g for 2 min. Supernatant (30 µl) was combined with 100 µl of Luciferase Assay Substrate (Promega) in Sarstedt 12 × 75-mm tubes in duplicate and read for 10 s on a Berthold AutoLumat LB953 luminometer. An expression vector containing the pCMV-SPORT-BGal plasmid was used as a control for transfection efficiency.

Determination of IL-6. IL-6 was determined by a commercially available human-specific ELISA (Endogen, Cambridge, MA) according to the manufacturer’s guidelines. The limit of detection was 1 pg/ml.

Determination of intracellular cAMP levels. Cells were lysed and intracellular cAMP levels were determined by a commercially available cAMP enzyme immunoassay (ELA) system (Amersham Biosciences, Piscataway, NJ). The limit of detection was 62.5 fmol/ml.

Statistical analysis. Results are expressed as means ± SE. Student’s t-test or ANOVA followed by Tukey’s test was used for statistical analysis. Significance was set at P < 0.05.
RESULTS

Although previous studies suggest that cAMP may potentiate IL-6 production in cultured rat intestinal epithelial cells (13, 14), it is not known if cAMP regulates the IL-6 gene in human enterocytes as well. In initial experiments we therefore tested the effect of cAMP on IL-6 production in cultured Caco-2 cells. Treatment of the cells with the membrane-permeable cAMP analog 8-bromo-cAMP alone did not influence IL-6 production (Fig. 1A). Similar to previous reports (9, 18–20), IL-1β increased IL-6 production. In this and most of the subsequent experiments, an IL-1β concentration of 0.5 ng/ml was used because this concentration resulted in a maximal IL-6 production in cultured Caco-2 cells in previous studies (19). Importantly, addition of cAMP to IL-1β-treated cells resulted in a substantial, dose-dependent potentiation of IL-6 production with a maximal effect of cAMP noticed at a concentration of 0.5 mM.

To test whether cAMP could potentiate the effect of lower IL-1β concentrations, we treated cells with IL-1β at concentrations from 0.1 to 0.5 ng/ml in the absence or presence of 0.5 mM cAMP. A potentiating effect of cAMP was seen also at the lower IL-1β concentrations (Fig. 1B).

When cells were treated with PGE2, a similar potentiation of the IL-1β-induced IL-6 production was noticed (Fig. 1C), lending further support to the concept.

Fig. 1. A: IL-6 production in cultured Caco-2 cells treated with 0.5 ng/ml of IL-1β alone or in combination with different concentrations of 8-bromo-cAMP for 24 h. Results are means ± SE; n = 3 for each condition. Almost identical results were seen in 3 separate experiments. *P < 0.05 vs. untreated cells; †P < 0.05 vs. IL-1β by ANOVA. B: IL-6 production in cultured Caco-2 cells treated for 24 h with different concentrations of IL-1β (0.1–0.5 ng/ml) alone or in combination with 8-bromo-cAMP (0.5 mM). Results are means ± SE; n = 3 for each condition. *P < 0.05 vs. corresponding concentration of IL-1β alone. C: IL-6 production in cultured Caco-2 cells treated for 24 h with 0.5 ng/ml of IL-1β alone or in combination with PGE2 (2 μM) or 8-bromo-cGMP (0.5 mM). Results are means ± SE; n = 3 for each condition. Almost identical results were observed in 3 separate experiments. *P < 0.05 vs. untreated cells; †P < 0.05 vs. IL-1β by ANOVA.
that cAMP activates the IL-6 gene in stimulated enterocytes. To test whether the potentiating effect of cAMP on IL-6 production was specific or may be elicited by other cyclic nucleotides as well, cells were treated with the membrane-permeable 8-bromo-cGMP. This substance did not influence the expression of IL-6 under basal conditions or in IL-1β-treated cells (Fig. 1C).

Because the results described above strongly suggest that cAMP can influence IL-6 production in IL-1β-stimulated Caco-2 cells, it was important to test whether IL-1β alone alters intracellular cAMP levels. Treatment of cells with IL-1β (0.5 ng/ml) for up to 24 h did not increase cellular cAMP levels (Fig. 2). In contrast, in cells treated with 8-bromo-cAMP, intracellular cAMP levels rose sharply.

cAMP typically exerts its biological effects by activating PKA. The involvement of PKA in cAMP-induced IL-6 production in the enterocyte, however, is not known. To test the role of PKA in the present experimental model, we treated the Caco-2 cells with the PKA inhibitor H-89. This treatment resulted in a dose-dependent inhibition of IL-6 production in cells treated with IL-1β and cAMP with a maximal effect of H-89 noticed at a concentration of 10 μM (Fig. 3A). In contrast, the PKC inhibitor GF-109203 did not influence IL-6 production, not even at a high (4 μg/ml) concentration (Fig. 3B). To test whether the lack of effect of GF-109203 in this experiment reflected an inability of the drug to act under the present experimental conditions, an experiment was performed to provide a “positive control.” When cells were treated with PMA, which typically activates PKC, IL-6 production was increased and this increase was blocked by GF-109203 (Fig. 3B). Thus the lack of effect of GF-109203 on IL-6 production in cells treated with IL-1β and cAMP did not reflect an inactive drug but suggests that cAMP-stimulated IL-6 production in IL-1β-treated cells is not mediated by PKC activity.

Because recent studies provided evidence for crosstalk between the cAMP and MAP kinase signaling pathways (22), we tested the role of the MAP kinase pathway by treating cells with the p38 inhibitor SB-203580 or the p42/p44 inhibitor PD-98059. SB-203580, but not PD-98059, inhibited IL-6 production in stimulated Caco-2 cells (Fig. 4A). To test the effectiveness of
PD-98059 under the present experimental conditions, the influence of the drug on p42/44 phosphorylation was examined. When cells were treated with PD-98059, the IL-1β-induced phosphorylation of p42/44 was prevented (Fig. 4B), suggesting that the lack of effect of PD-98059 on IL-6 production in cells treated with IL-1β and cAMP did not reflect inability of the drug to block kinase activity under the present experimental conditions. Taken together with the effects of SB-203580 on IL-6 production and phosphorylation of p38 (Fig. 4B), the results shown in Fig. 4 suggest that the p38 but not the p42/44 MAP kinase signaling pathway is involved in cAMP-induced potentiation of IL-6 production in IL-1β-treated Caco-2 cells. It should be noted that the experimental groups were not identical in Fig. 4, A and B. Thus, in the experiment depicted in Fig. 4B, the effects of SB-203580 and PD-98059 were tested in cells treated with IL-1β only, whereas in Fig. 4A, the inhibitors were tested in cells treated with IL-1β + cAMP. The reason to perform the experiment in Fig. 4B was to make certain the inhibitors were effective in cultured Caco-2 cells.

Although previous reports of increased IL-6 mRNA levels in cAMP- and PGF2α-treated enterocytes (13, 14) suggest that IL-6 is regulated at the transcriptional level, the influence of cAMP on IL-6 gene activity and transcription factors regulating the IL-6 gene in the enterocyte has not been reported. To test the effect of cAMP on IL-6 gene activation, Caco-2 cells were transfected with a plasmid containing a wild-type IL-6 promoter cloned upstream from a luciferase reporter gene. When these cells were treated with IL-1β, luciferase activity was doubled (Fig. 5A). cAMP alone did not influence luciferase activity but potentiated the effect of IL-1β.

To test the role of different transcription factors in IL-1β/cAMP-induced activation of the IL-6 gene, cells were transfected with luciferase reporter plasmids containing IL-6 promoter constructs in which the binding sites for the various transcription factors had been altered by site-directed point mutations (5). When these cells were treated with IL-1β and cAMP, results suggested that all four transcription factors under study here (NF-κB, AP-1, C/EBP, and CREB) participated in the regulation of the IL-6 promoter in IL-1β-stimulated Caco-2 cells treated with cAMP (Fig. 5B). The most pronounced inhibition of promoter activation was seen in cells transfected with a plasmid containing a CRE mutated binding site. None of the binding site deletions resulted in a complete inhibition of IL-6 promoter activation, suggesting that multiple transcription factors regulate the IL-6 gene under the present experimental conditions.

To further examine the influence of cAMP on transcription factor activity, EMSAs were performed. The classical example of a cAMP-activated transcription factor is CREB (3). Treatment of the Caco-2 cells with IL-1β or cAMP increased CREB DNA binding activity with an additive effect noticed in cells treated with both IL-1β and cAMP (Fig. 6A). Supershift analysis suggested that the different CREB family members examined here (CREB, CREM, and ATF-1) were similarly affected by cAMP in IL-1β-treated cells (Fig. 6B). CREB is activated by PKA-regulated phosphorylation of the protein at serine residue 133 (31). Cellular levels of pCREB were next determined by Western blotting using a specific anti-pCREB antibody. Treatment of the Caco-2 cells with IL-1β resulted in a modest increase in pCREB (Fig. 6C). cAMP and the combined treatment with cAMP and IL-1β resulted in a pronounced increase in pCREB levels that was almost completely abolished by H-89.

Finally, we examined the effect of cAMP on the DNA binding activity of other transcription factors involved in the activation of the IL-6 promoter. C/EBP and AP-1 DNA binding activities were increased by IL-1β and cAMP, similar to the pattern observed for CREB (Fig. 7). In contrast, NF-κB activity was increased by IL-1β but was not influenced by cAMP. Supershift analysis
revealed that the addition of cAMP to IL-1β-stimulated cells activated the individual members of the C/EBP and AP-1 families to a similar extent (Fig. 8).

DISCUSSION

The results reported here suggest that cAMP potentiates IL-1β-induced IL-6 production in human intestinal epithelial cells by activating the IL-6 gene. The data also support the concept that the PKA and p38 MAP kinase signaling pathways are involved in this response to cAMP and that the transcription factors CREB, AP-1, NF-κB, and C/EBP may, at least in part, regulate the IL-6 gene under these experimental conditions. Because increased enterocyte and mucosal IL-6 levels may have anti-inflammatory and cell-protective effects (2, 18, 28, 30), methods to increase enterocyte IL-6 production may have important clinical implications.

The present result of cAMP-induced potentiation of IL-6 production in stimulated Caco-2 cells is similar to results reported previously in cultured rat intestinal epithelial cells (13, 14). The present study expanded previous reports by defining some of the molecular mechanisms involved in cAMP-regulated IL-6 production in the enterocyte, in particular the role of different signaling pathways and transcription factors.

Although the present report is the first to examine the influence of cAMP on IL-6-related transcription...
phorylation of CREB occurring during the preparation of nuclear extracts for EMSA (although no evidence for that interpretation was provided). The diverse regulation of different IL-6-related transcription factors in different cell types illustrates the need to define the regulation of the transcription factors in individual cell types when the influence of cAMP on IL-6 is examined.

An interesting finding in the present study was the apparent discrepancy between the experiments with mutated IL-6 promoter plasmids and EMSA with regard to NF-κB. Thus, whereas EMSA suggested that cAMP did not influence NF-κB, results from cells transfected with an NF-κB mutated IL-6 promoter suggested that the NF-κB binding site was important for the IL-1β/cAMP-induced activation of IL-6. Although we do not have an explanation for these results at present, it is possible that they reflect an overlap between different transcription factors with regard to their binding sites. Thus, it is possible that the absence of a functional NF-κB binding site reduces the binding of other transcription factors as well, in addition to NF-κB. Evidence for overlapping binding of transcription factors to more than one binding site has been reported previously (23). Interaction (cross talk) between the different transcription factors may be another reason why simple mutation of individual binding sites may not always give rise to results that adequately reflect the role of individual transcription factors. It is also possible that other mechanisms, not studied here, are involved in the activation of the transcription factors in the IL-1β-stimulated cells. For example, IL-1β can generate oxygen radicals, and the transcription factors examined here are redox sensitive. The potential role of oxygen radicals in the cAMP-induced increase in transcription factor activity in IL-1β-treated enterocytes remains to be determined.

Another noteworthy observation in the present study was the finding that although cAMP by itself increased the DNA binding activity of CREB, AP-1, and C/EBP, cAMP alone did not activate the IL-6 gene or increase IL-6 production. This result illustrates the fact that DNA binding determined by EMSA does not always reflect the complex mechanisms involved in gene activation such as interaction between transcription factors, nuclear cofactors, and chromatin. The result that cAMP by itself was insufficient to induce IL-6 gene transcription and IL-6 production suggests that cAMP may be important for the modulation of IL-6 production during inflammation when primary regulators of IL-6, such as IL-1β, are abundant.

Although the biological role of IL-6 produced by the enterocyte is not completely understood, the present results taken together with previous reports from our and other laboratories support the concept that mucosal and enterocyte IL-6 may have anti-inflammatory and protective effects. In recent studies we found that induction of the heat shock response was associated with increased mucosal IL-6 levels in vivo (28) and increased enterocyte IL-6 production in vitro (18, 20). Increased mucosal IL-6 production in mice expressing
the heat shock response was associated with reduced endotoxin-induced permeability and mucosal injury (27, 28). In other experiments, we have found evidence that IL-6 can confer thermotolerance to enterocytes (unpublished observations). In light of the present study, it is interesting to note that the heat shock response results in increased cAMP levels in different cell types (11, 31). Finally, elevation of cellular cAMP levels has anti-inflammatory effects as reflected by decreased NF-κB activity and inhibited production of various proinflammatory cytokines, including TNF-α (24). It has therefore been hypothesized that cAMP directs gene expression toward an inflammation-repressing pattern (7). Increased IL-6 production by cAMP would therefore support the concept that IL-6 may exert anti-inflammatory and protective effects in the enterocyte and mucosa.

Several limitations of the present study need to be taken into account when the results are interpreted. First, although Caco-2 cells are frequently used to study enterocyte-related metabolic and inflammatory responses, it is important to remember that the cell line originates from a colon adenocarcinoma. It will be important in future experiments to examine whether similar mechanisms of cAMP-induced IL-6 production as found here regulate IL-6 production in other enterocyte cell lines as well. Second, cells were studied at 90% confluence rather than after full differentiation. We reported recently that the response to IL-1 with regard to IL-6 production was similar in 90% confluent and fully differentiated Caco-2 cells (16), but it remains to be determined if cAMP influences IL-6 production similarly in 90% confluent and fully differentiated Caco-2 cells. Finally, because the present experiments were performed in vitro, additional studies will be needed to test whether similar mechanisms as observed here are involved in cAMP-mediated IL-6 production in vivo.
REFERENCES


