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Coordinated induction of GST and MRP2 by cAMP in Caco-2 cells: Role of protein kinase A signaling pathway and toxicological relevance

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ABSTRACT

The cAMP pathway is a universal signaling pathway regulating many cellular processes including metabolic routes, growth and differentiation. However, its effects on xenobiotic biotransformation and transport systems are poorly characterized. The effect of cAMP on expression and activity of GST and MRP2 was evaluated in Caco-2 cells, a model of intestinal epithelium. Cells incubated with the cAMP permeable analog dibutyryl cyclic AMP (db-cAMP: 1,10,100 μM) for 48 h exhibited a dose–response increase in GST class α and MRP2 protein expression. Incubation with forskolin, an activator of adenylyl cyclase, confirmed the association between intracellular cAMP and upregulation of MRP2. Consistent with increased expression of GSTα and MRP2, db-cAMP enhanced their activities, as well as cytoprotection against the common substrate 1-chloro-2,4-dinitrobenzene. Pretreatment with protein kinase A (PKA) inhibitors totally abolished upregulation of MRP2 and GSTα induced by db-cAMP. *In silico* analysis together with experiments consisting of treatment with db-cAMP of Caco-2 cells transfected with a reporter construct containing CRE and AP-1 sites evidenced participation of these sites in MRP2 upregulation. Further studies involving the transcription factors CREB and AP-1 (c-JUN, c-FOS and ATF2) demonstrated increased levels of total c-JUN and phosphorylation of c-JUN and ATF2 by db-cAMP, which were suppressed by a PKA inhibitor. Co-immunoprecipitation and ChIP assay studies demonstrated that db-cAMP increased c-JUN/ATF2 interaction, with further recruitment to the region of the *MRP2* promoter containing CRE and AP-1 sites. We conclude that cAMP induces GSTα and MRP2 expression and activity in Caco-2 cells *via* the PKA pathway, thus regulating detoxification of specific xenobiotics.

Introduction

The gastrointestinal epithelium functions as a selective barrier to absorb nutrients, electrolytes and water but at the same time restricting the passage of potentially toxic compounds, such as drugs and food contaminants, into the systemic circulation. This restrictive function is dependent on the integrity of tight junctions and metabolic capability of the absorptive cells. The intestinal epithelium is able to metabolize and secrete orally incorporated xenobiotics back into the intestinal lumen through specific transporters located apically. Major transporters playing such a role are P-glycoprotein (P-gp: MDR1 and Abcb1), multidrug resistance-associated protein 2 (MRP2: Abcc2) and breast cancer resistance protein (BCRP: Abcg2) (Doyle and Ross, 2003; Mottino et al., 2000; Sun et al., 2004), all members of the ABC family of efflux pumps. Particularly, MRP2 is expressed in enterocytes from the duodenum and jejunum, and acts in tandem with the metabolizing enzymes glutathione-S-transferase (GST; EC 2.5.1.18) and UDP-glucuronosyltransferase (EC 2.4.1.17) to ameliorate intestinal absorption of xenobiotics and to protect the enterocytes themselves from potential toxic effects.

Intestinal barrier function can be modulated by a number of endogenous compounds, including growth factors and hormones (Schneeman, 2002). We recently demonstrated that glucagon-like peptide 2 (GLP-2), an important hormone with trophic properties directed to the gut (Drucker, 2002), positively modulates the expression (protein and mRNA) and activity of intestinal Mrp2 and of GST class α in rats, and that this regulation is mediated by activation of adenylyl cyclase (Villanueva et al., 2010). Mrp2 and GST expression and activity are also upregulated in lactating mother rats (Luquita et al., 1999; Mottino et al., 2001). Based on previous findings demonstrating increased levels of GLP-2 in lactating mother rats (Jacobs

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et al., 1981), we have postulated an association between upregulation of Mrp2 and increased secretion of GLP-2 in these animals. This could represent an adaptive strategy to deal with the significant increase in food consumption by lactating mother rats (up to 300% over control rats) and concomitantly, in the risk to exposure to food contaminants.

Adenosine 3′,5′-cyclic monophosphate (cAMP), the adenylyl cyclase product, is a ubiquitous intracellular messenger involved in the action of a wide range of molecules such as hormones, neurotransmitters, cytokines and growth factors. It is therefore implicated in different processes including molecular contraction, exocytosis, growth, cell differentiation, synthesis of steroids, transport of nutrients such as glucose, *etc.* (Beavo and Brunton, 2002; McKnight, 1991). Of additional relevance, adenylyl cyclase in association with G-protein-coupled receptors constitute universal therapeutic targets, and still offer scope for new and much improved drugs (Garland, 2013). Based on our previous findings demonstrating GLP-2-induced upregulation of Mrp2 and GST, we postulate activation of the cAMP pathway to be also of toxicological relevance. The intracellular participants mediating such regulations downstream of cAMP were not characterized yet. Acute exposure to cAMP regulates insertion of Mrp2 into the plasma membrane in rat liver (Schonhoff et al., 2010), whereas no information is available on long-term effects. Whether cAMP regulates expression and activity of GST and MRP2 in intestinal cells either of human or animal origin is less known.

The human colon carcinoma cell line, Caco-2, has been proven appropriate to study specific drug biotransformation enzymes and transporters. Despite its colonic origin, Caco-2 cells differentiate into polarized cells after reaching confluence (7–9 days onwards), acquiring morphological and biochemical characteristics that mimic those of the normal intestinal epithelium (Hidalgo et al., 1989). Cyclic-AMP is present at significant levels in the mammalian small intestinal mucosa (Debnam and Sharp, 1993; Steiner et al., 1972) as well as in Caco-2 cells (Mesonero et al., 1995; Pignata et al., 1994), and its signals have been linked to the regulation of a wide variety of functions. In the current study we evaluated the effect of regulation of expression and activity of GST and MRP2 by cAMP in this cell model and the potential intracellular candidates involved downstream of cAMP. The data demonstrate that this nucleotide induced an upregulation of expression of both MRP2 and GST class α with a concomitant increase in their activities, and that these modifications likely involved participation of protein kinase A (PKA). In the particular case of MRP2, regulation was at transcriptional level and likely involved participation of the specific transcription factors c-JUN and ATF2 (activating transcription factor-2), downstream of cAMP/PKA. These regulations resulted in increased cytoprotection of Caco-2 cells against chemical toxicity.

Materials and methods

Chemicals. Dibutyryl cyclic AMP (db-cAMP), glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), probenecid (Pro), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltretazolium bromide (MTT), phenylmethylsulfonyl fluoride (PMSF), pepstatin A, leupeptin, dihydrochloride hydrate (H89), forskolin and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma-Aldrich (St. Louis, MO, USA). KT 5720 was from Santa Cruz Biotechnology and actinomycin D (ActD) from Fluka, St. Louis. All other chemicals were of analytical grade purity.

Cell culture and treatments. The human Caco-2 cell line was purchased from the American Type Culture Collection (Manassas, USA) and used within a limited range of passages (30–35) to minimize the generation of heterogeneous subpopulations of cells. Cells were routinely maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (PAA, Pasching, Austria), 1% (v/v) nonessential amino acids (Cat number 11140050, Invitrogen, Carlsbad, CA, USA), 1% (v/v) L-glutamine, 1% (v/v) of a mixture of antibiotics (10,000 units/ml penicillin and 10,000 μg/ml streptomycin) and amphotericin B 6 ng/ml (PAA, Pasching, Austria). They were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

For studies involving quantification of protein and mRNA levels, cells were seeded in 6-well plates at a density of 2.5×10^5 cells/well and maintained in culture for 14 days. After that, the medium was replaced by fresh medium and db-cAMP was added at different concentrations (1, 10 or 100 μM). The treatment time was 48 h for MRP2 and GST western blot, activity, and cytotoxicity studies, or 3 h for MRP2 and GSTA isoforms real-time PCR studies. In the first case (48-h incubation protocol), the medium was replaced by a fresh one containing db-cAMP after the initial 24 h. In studies involving inhibition of protein kinase A (PKA), 1 μM KT 5720 or 10 μM H89 were incorporated to the incubation medium 30 min before addition of db-cAMP. In experiments performed to characterize mechanistically the effect of db-cAMP on MRP2 mRNA expression, cells were pretreated with 5 mg/ml Actinomycin D (ActD), an RNA polymerase inhibitor, for 30 min before db-cAMP incorporation. Experiments were directed to explore the role of cAMP generated endogenously, then the adenylyl cyclase activator forskolin was added at 0.1, 1 or 10 μM concentrations and cells were incubated for 48 h for protein analysis, or alternatively 20 min for quantification of cAMP levels. In the case of protein analysis, after the first 24 h the medium was replaced with a fresh one containing forskolin.

To explore the potential participation of transcription factors as mediators of cAMP effects, cells were seeded in 6-well plates (2.5×10^5 cells/well) and maintained in culture for 14 days. The cells were then treated with 10 μM db-cAMP for 60 min for the determination of transcription factors' protein levels and their mutual interaction (immunoprecipitation assays), or for 30 min for the assessment of their phosphorylation status. Experiments involving incorporation of the PKA inhibitor KT 5720 in these same cultures were performed as described above.

All reagents were dissolved in dimethyl sulfoxide (DMSO). Only DMSO was added to control cells and its final concentration in culture media was always below 0.1%.

Preparation of cell lysates and subcellular fractions. Total cell lysates were used in the assessment of content of total and phosphorylated transcription factors. Cells were cultured as described above except that the medium was replaced by serum-free medium 24 h before the experiments. Then the cells were harvested in 100 μl of ice-cold RIPA buffer (Thermo Scientific, Rockford, IL, USA) containing phosphatase inhibitor cocktail 1 (1/100) (Sigma-Aldrich, St. Louis, MO, USA), 1 mM sodium vanadate, and 1 mM sodium fluoride, and sonicated.

Total cellular membrane fractions were used in the assessment of MRP2 protein content. After treatments, the medium was removed and the cells washed twice with ice-cold phosphate-buffered saline (PBS). They were then harvested in 1 ml of ice-cold 0.3 M sucrose supplemented with phenylmethylsulfonyl fluoride (1 mM), leupeptin (5 mg/ml) and pepstatin A (5 μg/ml) as protease inhibitors, and lysed by sonication on ice. Total cellular membranes were prepared by differential centrifugation as described (García et al., 2001) and resuspended in 100 μl of 0.3 M sucrose (with protease inhibitors). In order to evaluate GST protein content and activity, cytosolic fractions from Caco-2 cells were obtained by ultracentrifugation as described previously (Faist et al., 2002).

Protein concentration in total lysates and subcellular fractions was measured using bovine serum albumin as standard (Lowry et al., 1951).

Immunoprecipitation assays. After treatments, the medium was removed and cells were washed with PBS and harvested. Cell lysis and immunoprecipitation were performed using Protein A agarose (Roche Applied Science, Germany), following the manufacturer's protocol. Specific protein precipitation was achieved by using anti-c-JUN, anti-activating transcription factor-2 (ATF2), or normal rabbit IgG as a

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negative control (SC-1694, SC-187, and SC-2027, respectively, Santa Cruz Biotechnology, Inc.).

Western blot studies. Immunodetection of α, μ and π classes of GST in cytosolic fractions, and MRP2 in total cellular membranes were performed as previously described (Mottino et al., 2000; Villanueva et al., 2010). Western blotting of total cell lysates for the analysis of transcription factors was carried out using anti-ATF2, anti-c-JUN, anti-c-FOS or anti-cyclic AMP response element binding protein (CREB) antibodies (SC-187, SC-1694, SC-54, and SC-186, Santa Cruz Biotechnology, Santa Cruz, USA, respectively) for detection of their total protein content, or with anti-phospho-ATF2, anti-phospho-c-JUN, anti-phospho-c-FOS or anti-phospho-CREB antibodies (A4095, Sigma-Aldrich, St. Louis, MO, USA; SC-7981, SC-130181, and SC-7972, Santa Cruz Biotechnology, Santa Cruz, USA, respectively) for detection of their phosphorylated forms. Equal loading and transference of protein was systematically checked by both detection of β-actin using a monoclonal antibody to human β-actin (Sigma-Aldrich, St. Louis, MO, USA), and staining of the membranes with Ponceau S. Western blot studies of immunoprecipitated proteins were carried out using anti-ATF2 or anti-c-JUN antibodies. In these studies, the precipitated protein itself was systematically detected to control for immunoprecipitation efficiency. In all cases, immunoreactive bands were quantified with the Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD, USA).

RNA isolation and quantitative real-time PCR. At the end of incubations, cells were washed twice with cold PBS and scraped using TRIzol® reagent (Invitrogen Carlsbad, CA, USA), and total RNA was isolated according to the manufacturer's instructions. cDNA was produced using the Superscript Preamplification System for the first strand cDNA synthesis using random hexamers, according to the manufacturer's instructions (Invitrogen Carlsbad, CA, USA). Real-time PCR was performed on cDNA samples using the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen Carlsbad, CA, USA) and reactions were carried out on a Stratagene Mx3000P (Stratagene, La Jolla, CA, USA). Results were normalized to 18S rRNA as housekeeping gene. Sequences of primer pairs for human GSTA isoforms, MRP2 and 18S are summarized in Table 1. Relative levels of mRNA normalized to 18S rRNA were calculated based on the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001).

Confocal studies. Caco-2 cells were grown on 20-mm × 20-mm cover slips inserted in six-well culture plates for 14 days and treated as described above. At the end of the treatments, cover slips were washed twice with PBS, and fixed in 4% paraformaldehyde at room temperature for 10 min. Cells were permeabilized and non-specific binding sites were blocked by incubation with 0.1% Triton X-100 in 3% albumin in PBS, at room temperature, for 20 min. Cover slips were then incubated with anti-MRP2 (ALX-801-016, Alexis) and anti-zonula occludens 1 (ZO-1, a tight junction protein) (Zymed Laboratories, South San Francisco, CA) for 120 min. Detection was carried out by incubation with appropriate Cy2- or Cy3-conjugated donkey anti-IgGs (Jackson ImmunoResearch Laboratory, Inc., West Grove, PA) for 120 min. Fluorescence detection of these proteins was performed with a Nikon C1 Plus microscope (Tokyo, Japan). The nuclei were detected with DAPI resulting in a blue signal. To ensure comparable staining and image capture performance for control and treated cells, both groups were processed in parallel.

Table 1
Nucleotide sequences of human RT-PCR primers.

Gene	Forward	Reverse
MRP2	5′-CCAAAGACAACAGCTGAAA-3′	5′-TACTTGGTGGCACATAAAC-3′
GSTA1	5′-TACTACGTCGAGGAGCTTGA-3′	5′-GCCTCCATGACTGCGTTATT-3′
GSTA2	5′-GCCCAAGCTCCACTACTCC-3′	5′-GCTTGGCATCTTGTTCCTCA-3′
GSTA3	5′-CTGGTGGAACTTCTCTACTAT-3′	5′-CATGTTCTTAGCCTCCATGG-3′
GSTA4	5′-TCCGTGAGATGGGTTTTAGC-3′	5′-GGTGGTTACCATCCTGCAAC-3′
18S	5′-CGCCGCTAGAGGTGAAATTC-3′	5′-TTGGCAAATGCTTTCGCTC-3′

GST and MRP2 activities. The activity of MRP2 was determined as previously reported by Zhang et al. (2001) through determination of the amount of dinitrophenyl-S-glutathione (DNP-SG) extruded by Caco-2 cells into the culture medium. This methodology was previously used to evaluate MRP2 activity in this same model (Arias et al., 2014). Briefly, cells were cultured in 96-well plates at a density of 8.5×10^3 cells/well for 14 days and then treated with 10 μM db-cAMP, as described for detection of MRP2 protein levels. Treatment medium was then replaced by fresh medium containing 0.5 mM CDNB and cells were incubated at 10 °C for 30 min to allow CDNB to passively diffuse into the cytosol. In this condition, most of CDNB conversion to DNP-SG is spontaneous, *i.e.*, independent of GST activity (Townsend et al., 1998). The possibility that CDNB exerts cytotoxic effects under such incubation conditions was evaluated by measuring the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltretazolium bromide (MTT) to its formazan, as described (Rigalli et al., 2011). The results indicated that neither addition of CDNB to control or to db-cAMP-treated cells affected MTT conversion (data not shown). After that incubation, the medium was rinsed and cells were promptly washed twice with cold PBS. To evaluate the rate of DNP-SG secretion, cells were incubated with Hank's balanced salt solution at 37 °C for 60 min. At the end of incubations, medium samples were centrifuged (3 min, 300 *g*, 4 °C), the supernatants were treated with 10% perchloric acid, centrifuged (2 min, 14,000 *g*, 4 °C), and the supernatants used in DNP-SG detection by HPLC (Waters 600; Waters, Milford, MA) as described (Mottino et al., 2001). This assay was performed in the presence or absence of probenecid (Pro, 1 mM), to confirm MRP2 participation (Bakos et al., 2000).

Total GST activity was measured according to the method of Habig et al. (1974), which is based on the enzymatic conjugation of CDNB with GSH, thus rendering DNP-SG. After treatment with 10 μM db-cAMP, cells were harvested, lysed by sonication, centrifuged (20 min, 10,000 *g*, 4 °C), and the supernatants were used in the assays. The reaction mixture contained PBS pH 6.50, 1 mM CDNB and 1 mM GSH. Reaction was initiated by addition of cell supernatants. Formation of DNP-SG was determined spectrophotometrically at 340 nm.

Cytotoxicity assay. To test the potential protective role of db-cAMP treatment against exposure to CDNB, a highly cytotoxic drug and well-established MRP2 substrate precursor (Diah et al., 1999), cells were seeded in 96-well plates and treated with db-cAMP, as described above. After treatment, cells were incubated with fresh medium containing DMSO or increasing concentrations of CDNB (0; 10; 20; 30; 40; 50; 60; 70; 80; 90; 100; 200; 300; 400; 500 or 1000 μM), for an additional 24 h. Probenecid (1 mM) was used to confirm MRP participation. The viability of Caco-2 cells was assessed measuring the conversion of MTT to its formazan, as described (Rigalli et al., 2011). The data were best adjusted to a sigmoid curve using the GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). The goodness of adjustments was confirmed with R^2 values, which were 0.990 or higher. The LC_{50} value, which represents the concentration of CDNB resulting in 50% viability, was compared between control and treated cells.

Assessment of intracellular cAMP levels. Cells were cultured as described above and 24 h before the experiments, the medium was replaced by serum-free medium. Subsequently, cells were preincubated with the phosphodiesterase inhibitor IBMX (0.8 mM) for 5 min and then incubated with DMSO (control), or Forskolin (10 μM) for 20 min at 37 °C. Reaction was stopped by the addition of ice-cold ethanol for 1 h. Then ethanol was evaporated, and the residue was resuspended in ethanol for cAMP determination, as previously described (Davio et al., 1995).

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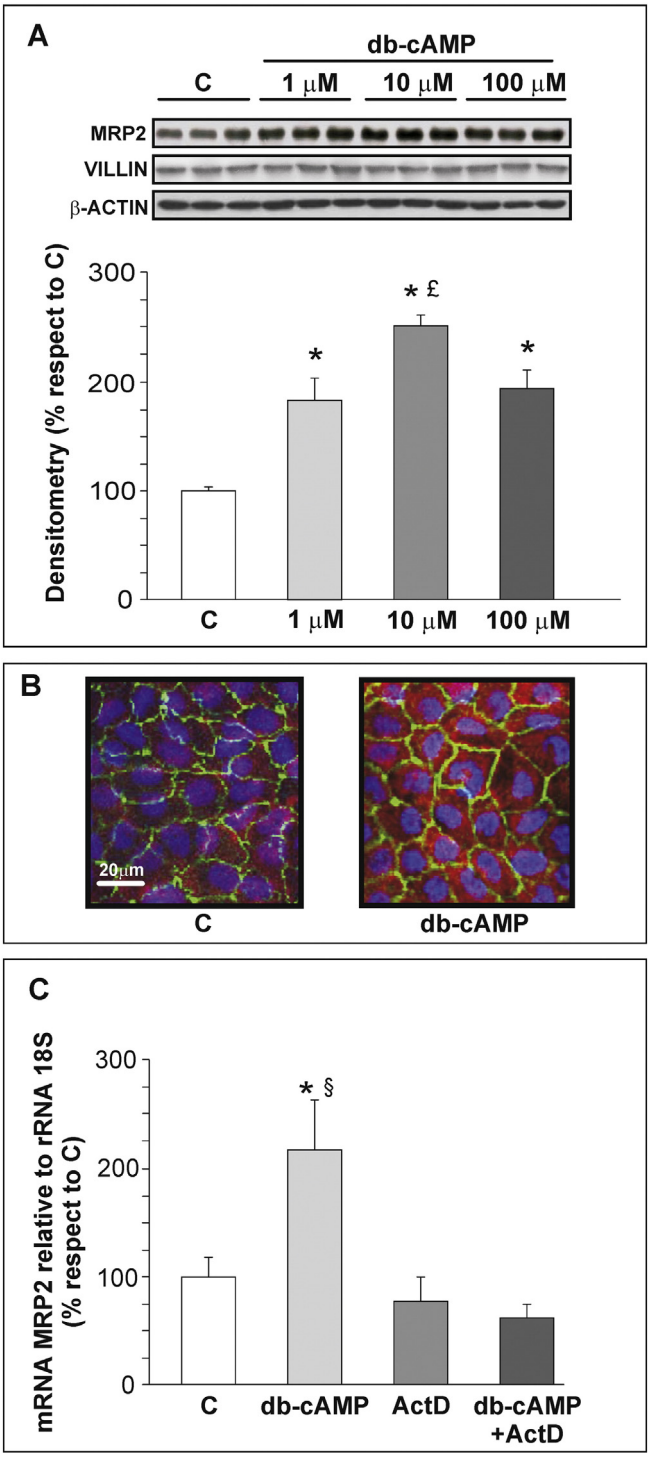

Fig. 1. Effect of db-cAMP on MRP2 expression. A — Western blot detection of MRP2 and villin in total cell membranes from Caco-2 cells treated with different concentrations of db-cAMP. Uniformity of protein loading and transfer from gel to nitrocellulose membrane were controlled with Ponceau S and detection of β-actin. Densitometry data were related to β-actin and presented as percentage of control cells (C). B — Confocal detection of MRP2. Top view of the monolayer culture, with the tight junction protein ZO-1 detected as green fluorescence and MRP2 as red fluorescence. Cell limit is enclosed by ZO-1. Treatment with db-cAMP resulted in increased detection of MRP2, without changes in ZO-1 signal. Nuclei were detected with DAPI (blue fluorescence). C — RT-PCR assessment of MRP2 mRNA levels in Caco-2 cells treated with db-cAMP (10 μM) and/or actinomycin D (ActD) (5 μg/mL), an inhibitor of transcription. Results were referred to 18s rRNA and expressed as percentage of control cells (C). Data are expressed as means ± S.D. of six individual experiments per group. (*) significantly different from C ($p < 0.05$), (£) significantly different from 1 and 100 μM ($p < 0.05$), and (§) significantly different from ActD and db-cAMP + ActD ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Transfection and MRP2 gene promoter assays. The region (−789/−603) from the MRP2 promoter was amplified by PCR using 5′-tgttggcatg tgcctgtagt-3′ and 5′-ctgtcatcgacccaaccttt-3′ primers (Fig. 5), and ligated inside SmaI digested pGL3 promoter vector (Promega). Orientation of the insert was evaluated by DNA sequencing (Maine University). Caco-2 cells were cultured for 14 days and then transfected with MRP2 promoter-luciferase reporter constructs (1 μg) using a cationic liposome method (Lipofectamine 3000, Invitrogen). Transfected cells were cultured as described above and treated with 10 μM db-cAMP for 24 h. Luciferase assay was performed using the Promega assay systems, as recommended by the manufacturer, and luminometric measurements were made using Synergy 2 Multi-Mode Microplate Reader (BioTek). Protein concentration was measured by Sedmak and Grossberg's (1977) method. Luciferase activity was normalized to protein concentration.

Chromatin immunoprecipitation (ChIP) assay. Caco-2 cells (10^6) were cultured for 14 days and then treated with 10 μM db-cAMP for 24 h, or with vehicle, and cross-linked with 1% formaldehyde (37 °C, 10 min). ChIP assay was performed following manufacturer protocol (Upstate Biotech, NY) using rabbit antibodies to c-JUN, ATF2, or normal rabbit IgG as a negative control (SC-1694, SC-187, and SC-2027, respectively, Santa Cruz Biotechnology, Inc). An aliquot of lysates (20 μl) was taken out as input control. DNA was purified by phenol/chloroform extraction and ethanol precipitation. For PCR analysis, we used primers that amplify 187 bp fragments of the human MRP2 promoter region from −789 to −603 bp, relative to the transcription start site, which include the putative activator protein-1 (AP-1) and CREB binding sites: 5′-tgttggcatgtgcctgtagt-3′ and 5′-ctgtcatcgacccaaccttt-3′ (Fig. 5). The thermocycling regime was 35 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min.

c-JUN overexpression assay. A plasmid (0.5 μg) designed to overexpress c-JUN was transfected into Caco-2 cells as described (Ruiz et al., 2013). Briefly, dishes received 0.5 μg of a plasmid carrying GFP protein (phr-GFP) as a control for transfection efficiency (Domizi et al., 2014). Plasmid DNA was purified by using a Wizard Miniprep kit (Promega). Cells were grown as described above and transfections were performed by using Lipofectamine 3000 (Invitrogen, Carlsbad, CA) following manufacturer's instructions. Incubation with db-cAMP was performed at a 10 μM concentration, for 24 h. At the end of incubations, c-JUN was detected by western blotting of total cell lysates and MRP2 mRNA by real-time PCR of total RNA.

Statistical analysis. Data are presented as means ± S.D. Statistical analysis was performed using Student's *t* test or one-way analysis of variance, followed by Bonferroni's test (when more than two groups were compared). Values of $p < 0.05$ were considered to be statistically significant.

Results

Effect of db-cAMP on MRP2 expression

We first examined the dose–response effect of db-cAMP (a cAMP membrane permeable analog) on MRP2 expression. Caco-2 cells were incubated with 1, 10 or 100 μM db-cAMP for 48 h and the levels of MRP2 were assessed by western blotting of total cellular membranes. As shown in Fig. 1A, db-cAMP produced an increase in MRP2 expression at all concentrations studied, with a maximal effect at 10 μM. Thus, the 10 μM dose was chosen to perform all subsequent experiments. In contrast, expression of villin, a major structural protein of cellular microvilli was not affected at any concentration of db-cAMP tested (Fig. 1A, densitometry not shown). Consistent with western blot studies, confocal microscopy detection of MRP2 (in red) showed a stronger signal in db-cAMP treated cells when compared to control cells, whereas detection of the tight junction protein ZO-1 (in green)

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exhibited similar signal intensity and localization between groups (Fig. 1B). The blue signal corresponds to the nuclei.

To establish whether upregulation of MRP2 protein is associated with modulation of MRP2 mRNA levels, we performed real-time PCR studies. Fig. 1C shows that db-cAMP treatment increased the expression of MRP2 mRNA by 124% relative to control cells, and that this increase was prevented by the RNA polymerase II inhibitor ActD, supporting a role for a transcriptional regulation of MRP2 by db-cAMP.

Effect of forskolin on MRP2 expression and cAMP levels

We investigated whether cAMP generated endogenously by activation of adenylyl cyclase, is also capable of regulating the expression of MRP2 as observed under db-cAMP conditions. Fig. 2 shows that the enzyme activator forskolin (0.1, 1 and 10 μM) induced a dose-dependent increase in intracellular cAMP levels (panel A), and concomitant increase in MRP2 protein level (panel B), with statistical significance reached at the 1 and 10 μM doses.

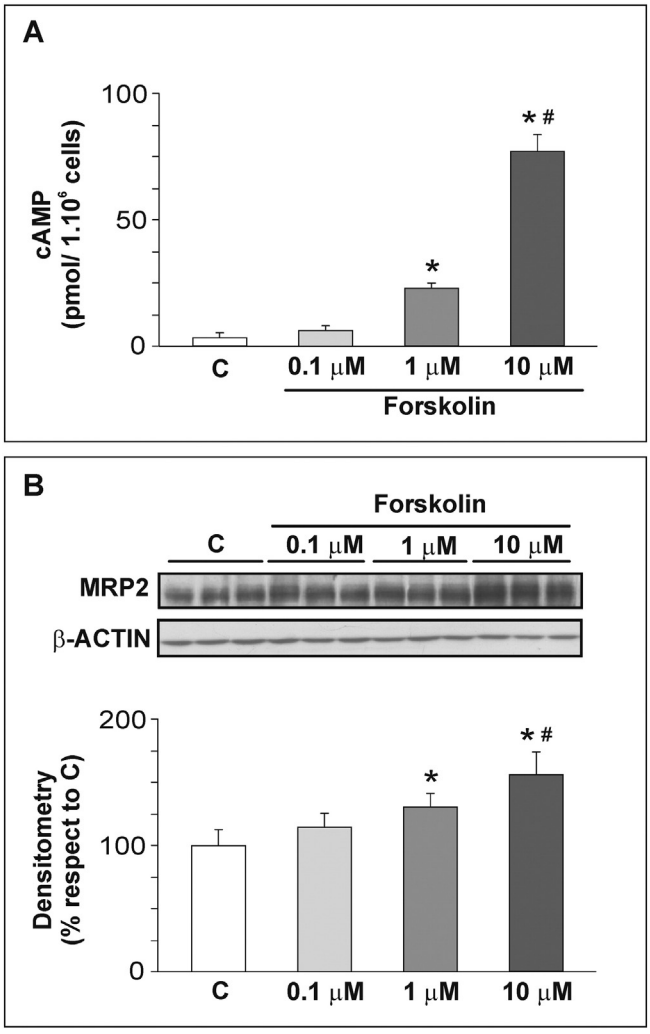

Fig. 2. Effect of adenylyl cyclase activation on MRP2 expression.
A — Effect of forskolin treatments on intracellular cAMP levels. B — Western blot analysis of MRP2 levels in total cell membranes from Caco-2 cells treated with different concentrations of forskolin (adenylyl cyclase activator). Uniformity of protein loading and transfer from gel to nitrocellulose membrane were controlled with Ponceau S and detection of β-actin. Densitometry data were related to β-actin.
Results are presented as percentage of control (C) and are expressed as means ± S.D. of six individual experiments per group.
(*) significantly different from control ($p < 0.05$), and (#) significantly different from 0.1 and 1 μM ($p < 0.05$).

Effect of db-cAMP on MRP2 activity and CDNB cytotoxicity

To determine whether MRP2 upregulation results in increased transport activity, we assessed the ability of Caco-2 cells to extrude DNP-SG, a model substrate, to the incubation medium. Fig. 3A shows that the efflux of DNP-SG into the medium was significantly higher (+51.6%) in db-cAMP than in control cells. Probenecid decreased DNP-SG efflux in both control and treated cells to the extent that former differences between groups were abolished, consistent with participation of MRP2. Because the intracellular amounts of DNP-SG detected at the beginning of the incubations were similar between db-cAMP and control groups (data not shown), we exclude a differential rate of synthesis to condition the excretion phase.

The protective effect of upregulation of MRP2 against CDNB toxicity was evaluated through determination of cell survival (Schinkel and Jonker, 2003). The data on LC_{50} (expressed as μM concentration of CDNB) are shown in Fig. 3B. The results demonstrate a protective role for db-cAMP as LC_{50} was higher in cells treated with this nucleotide than in control cells (+55%). As expected, the MRP inhibitor abolished the effect of db-cAMP on LC_{50} leading to similar values between

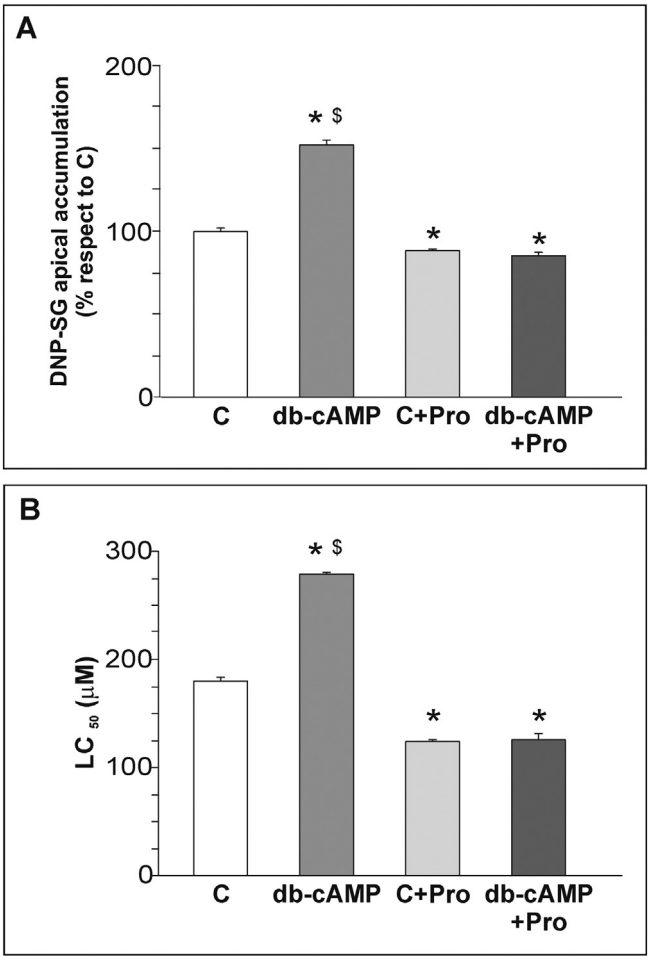

Fig. 3. Effect of db-cAMP on MRP2 activity and CDNB cytotoxicity.
Caco-2 cells were treated with 10 μM db-cAMP for 48 h before assessment of activity or cytotoxicity. A — DNP-SG accumulation in the cell medium after 60 min of incubation with its precursor CDNB (0.5 mM). Results are presented as percentage of control cells (C). B — Cell survival after incubation with concentrations of CDNB varying between 0 and 1 mM. Results are presented as the CDNB concentration that allows 50% of the cell population to survive.
The assays were performed with or without addition of the MRP inhibitor Probenecid (Pro). Data are expressed as means ± S.D. of six individual experiments per group.
(*) significantly different from control ($p < 0.05$), and ($) significantly different from C + Pro and db-cAMP + Pro ($p < 0.05$).

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control and treated groups. Interestingly, and as was observed for MRP2 activity determinations, probenecid decreased the LC_{50} value also in control cells, suggesting a role for constitutively expressed MRP2 in cytoprotection.

Participation of the PKA pathway in MRP2 upregulation

To identify the signaling events involved in MRP2 upregulation and considering that db-cAMP is a potent inducer of PKA, we evaluated its potential mediation. Caco-2 cells were pretreated with the specific PKA inhibitors KT 5720 and H89 for 30 min followed by incubation with db-cAMP for 48 h. Western blot studies revealed that both KT 5720 and H89, incorporated independently to the incubation media, were able to prevent MRP2 induction by db-cAMP (Figs. 4A and B, respectively). Mediation of PKA was also evaluated by real-time PCR. Fig. 4C shows that the increase in MRP2 mRNA levels induced by db-cAMP was also abolished by KT 5720 pretreatment. Taken together, these results indicate that the PKA pathway is involved in MRP2 transcriptional regulation, downstream of cAMP.

Role of CREB and AP-1 transcription factors in MRP2 upregulation by db-cAMP

Transcription factors can mediate the reported transcriptional upregulation of MRP2 downstream of PKA. CREB, c-JUN, c-FOS and ATF2 are potential candidates; the latter three belong to AP-1 family. To identify the existence of cAMP response element (CRE) and AP-1 binding sites in the 5′-flanking region of human *MRP2* gene we performed an *in silico* analysis using the TFSearch database (http://www.cbrc.jp/research/db/TFSEARCH.html). Using a 90% homology cutoff the software revealed a consensus sequence for AP-1, whereas restriction to 85% homology led to identification of a CRE binding site (Fig. 5A). To determine whether these CRE and AP-1 binding sites are functionally important in *MRP2* transcription under current experimental conditions, we generated a reporter construct including a short promoter segment (−789/−603) upstream the transcription start site, which contains the CRE and AP-1 binding sites identified previously, additionally fused to a promoter-luciferase reporter plasmid (pGL3-promoter). After treating transfected cells with db-cAMP for 24 h, luciferase activity increased by 47% when compared with untreated cells (Fig. 5B). This result clearly confirms that the region between (−789/−603) contains elements that can regulate *MRP2* transcription in response to db-cAMP treatment. Moreover, KT 5720 pretreatment decreased luciferase activity to the extent that the former difference between control and db-cAMP groups was abolished, consistent with the participation of PKA signaling pathway.

It is known that transcription factor activity can be regulated through changes in expression and/or posttranslational modifications such as phosphorylation of specific amino acid residues. We evaluated the effect of db-cAMP on expression and phosphorylation status of CREB, c-JUN, c-FOS and ATF2. We found that c-JUN but not ATF2 protein level was upregulated after treating Caco-2 cells with 10 μM db-cAMP for 60 min (Fig. 6A). Western blot studies using antibodies directed against phosphorylated forms revealed increased detection for both p-c-JUN and p-ATF2 in response to db-cAMP (+38% and +113%, respectively, Fig. 6B). As result of these regulations, the ratio p-ATF2/ATF2 was significantly increased in treated cells, whereas that of p-c-JUN/c-JUN remained unchanged (Fig. 6C). Regarding c-FOS and CREB, neither their expression nor their phosphorylation status was affected by db-cAMP treatment (data not shown). In order to establish a causal relationship between modifications in expression/phosphorylation of c-JUN and ATF2 and PKA activation, we assessed the effect of KT 5720 pretreatment on these same modifications.

The data on Figs. 7A and B confirm that PKA activation was indeed involved in the modulations of c-JUN and ATF2 by db-cAMP reported above.

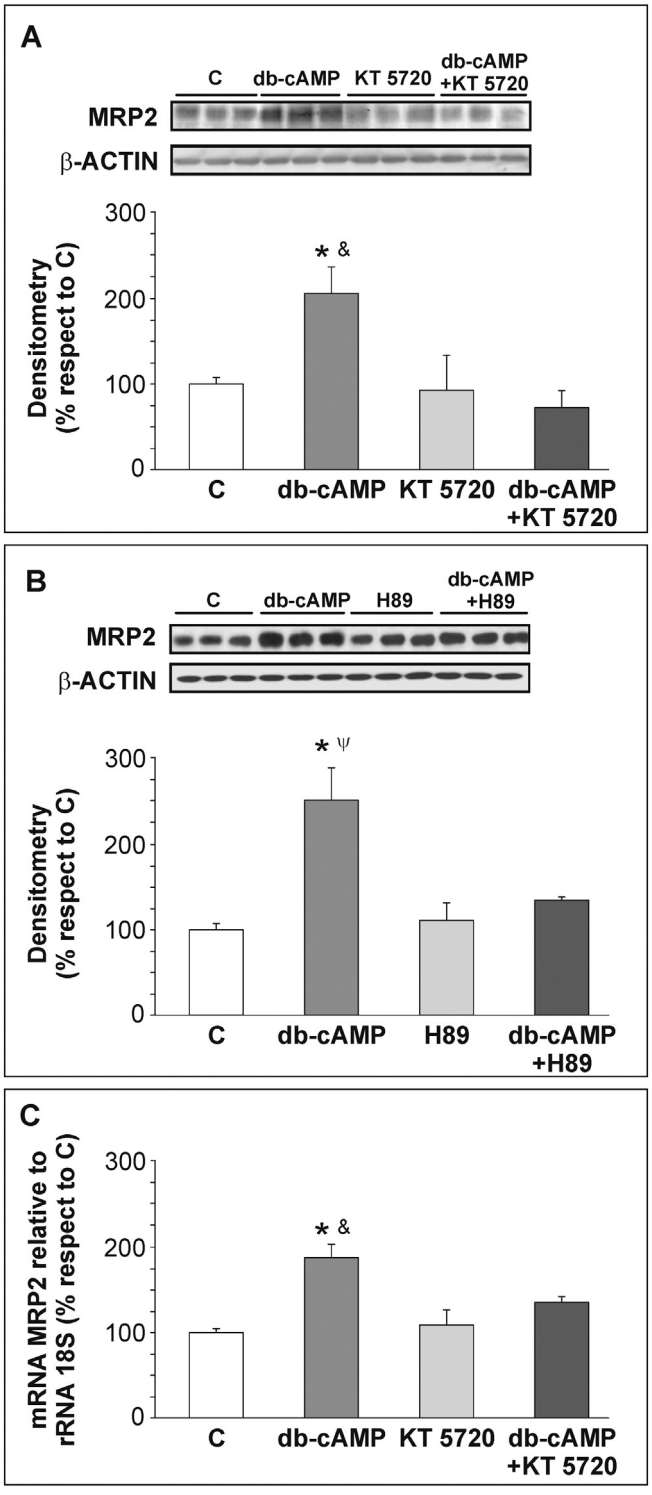

Fig. 4. PKA mediation of MRP2 induction by db-cAMP.
A and B — Western blot detection of MRP2 in total cell membranes from Caco-2 cells treated with db-cAMP (10 μM), in the presence or absence of the PKA inhibitors KT 5270 or H89, respectively. Uniformity of protein loading and transfer from gel to nitrocellulose membrane were controlled with Ponceau S and detection of β-actin. Densitometry data were related to β-actin. C — RT-PCR assessment of MRP2 mRNA levels in Caco-2 cells treated with db-cAMP (10 μM), with and without addition of the PKA inhibitor KT 5270. The results were referred to 18s rRNA.
Data are presented as percentage of control cells (C) and expressed as means ± S.D. of six individual experiments per group.
(*) significantly different from control ($p < 0.05$), (&) significantly different from KT 5720 and db-cAMP + KT5720 ($p < 0.05$), and (Ψ) significantly different from H89 and db-cAMP + H89 ($p < 0.05$).

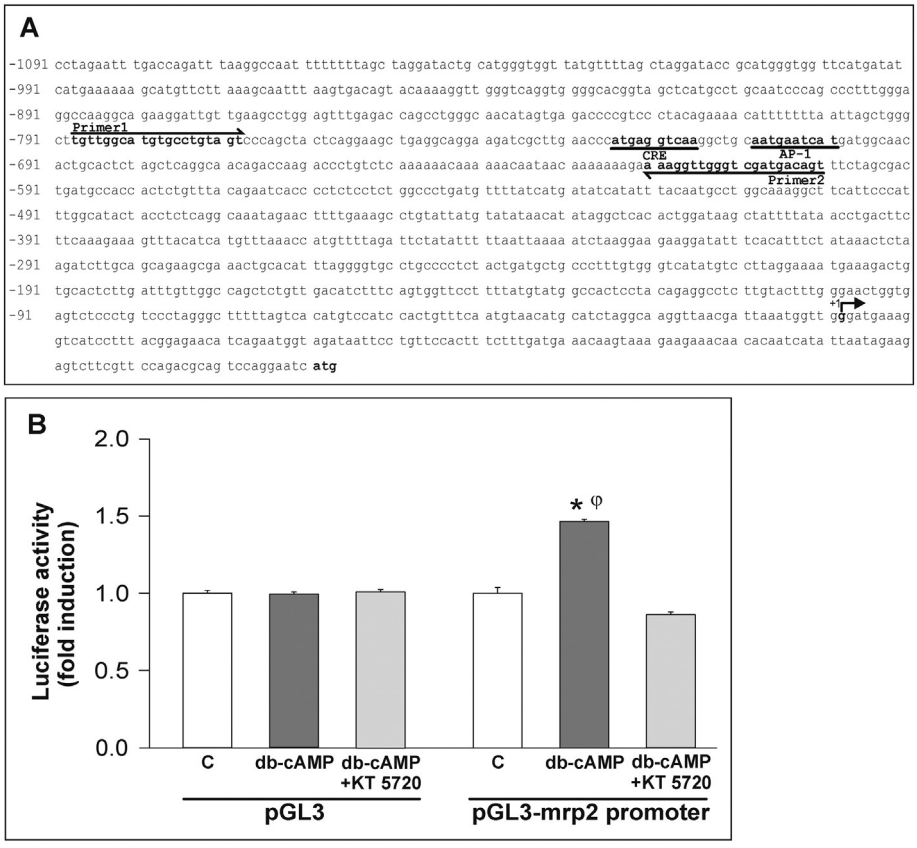

Fig. 5. Analysis of MRP2 proximal promoter region.
A — Consensus elements for AP-1 and CREB nuclear factors are underlined. The analysis was performed by TFSearch database (http://www.cbrc.jp/research/db/TFSEARCH.html). Numbers represent the positions of nucleotides with relation to the transcription initiation site +1 indicated by an arrow. Primer 1 and 2 sequences, indicated by arrows, are the primer sequences used for cloning the proximal promoter region into pGL3-promoter plasmid and for the PCR of the ChIP assay. B — MRP2-promoter reporter plasmid or pGL3-basic empty (1 μg) vector (Promega), was transfected in Caco-2 cells. Luciferase activity was measured 24 h after db-cAMP (10 μM) treatment and is given relative to protein concentration.
Results represent the mean fold induction ± S.D. obtained from four independent experiments.
(*) significantly different from control ($p < 0.05$), and (φ) significantly different from db-cAMP + KT 5720 ($p < 0.05$).

Effect of db-cAMP on c-JUN/ATF2 interaction and subsequent binding to the MRP2 promoter

The increases in ATF2 phosphorylation and c-JUN expression and phosphorylation suggest that they are more active in cells treated with db-cAMP than in control cells. This could in turn result in higher mutual interaction, since heterodimer formation is known to be a direct effector of gene promoter activation (Hayakawa et al., 2004; Liu et al., 2009; Song et al., 2011). We performed co-immunoprecipitation studies and confirmed a greater fraction of c-JUN co-precipitated with ATF2 in the db-cAMP group than in controls, which was confirmed by reverse co-immunoprecipitation (Fig. 8A). Immunoprecipitations performed with normal rabbit IgG (negative controls) showed no signal for either c-JUN or ATF2 (data not shown). To evaluate whether the c-JUN/ATF2 heterodimer is recruited to the promoter region of *MRP2* after db-cAMP treatment, we performed ChIP assays using a pair of primers that include the putative CRE and AP-1 binding sites previously identified in the *MRP2* promoter region (Fig. 5A). The data show that specific anti-c-JUN and anti-ATF2 antibodies successfully co-immunoprecipitated c-JUN or ATF2 with the *MRP2* promoter (−789/−603) in cells treated with db-cAMP, whereas no bands were detected in control cells (Fig. 8B). Treatment with non-immune IgG failed to immunoprecipitate the *MRP2* promoter, either in control or db-cAMP treated cells.

Considering that ATF2 is constitutively expressed (Karin et al., 1997) and that db-cAMP only affected the expression of the transcription factor c-JUN in our experimental conditions, we further explored if c-JUN overexpression itself could mimic MRP2 induction as observed in db-cAMP treated cells. These studies demonstrate that overexpression of c-JUN resulted in upregulation of MRP2 mRNA to a similar extent reported under db-cAMP treatment conditions (see Fig. 8C *vs.* Fig. 1C).

Effect of db-cAMP on GST expression and activity

GST is an important biotransformation system which generates substrates for MRP2. Both systems act in tandem to conjugate and eliminate potentially harmful xenobiotics. Western blot studies shown in Fig. 9A demonstrate that treatment of Caco-2 cells with db-cAMP resulted in a significant increase in GSTα protein level at all concentrations studied, reaching a maximal effect at 10 μM, whereas the π classes remained unchanged (data not shown). GST class μ was not detectable, either in controls or db-cAMP treated cells. Additional experiments, performed to determine the mediation of PKA, show that pretreatment with KT 5720 totally prevented upregulation of GSTα induced by the 10 μM concentration of db-cAMP (Fig. 9B). At this same concentration, db-cAMP produced an increase in mRNA levels of GSTA1, A2, A3 and A4, isoforms belonging to the α class (Fig. 9C). Consistent with western blot findings, preincubation with KT 5720 completely blocked these changes. We further evaluated GST conjugating activity toward CDNB in cytosol of Caco-2 cells and found a significant increase for db-cAMP cells (8.6 ± 0.7 μmol/min/mg of protein) when compared to control cells (4.2 ± 0.3 μmol/min/mg of protein; $p < 0.05$, $N = 6$).

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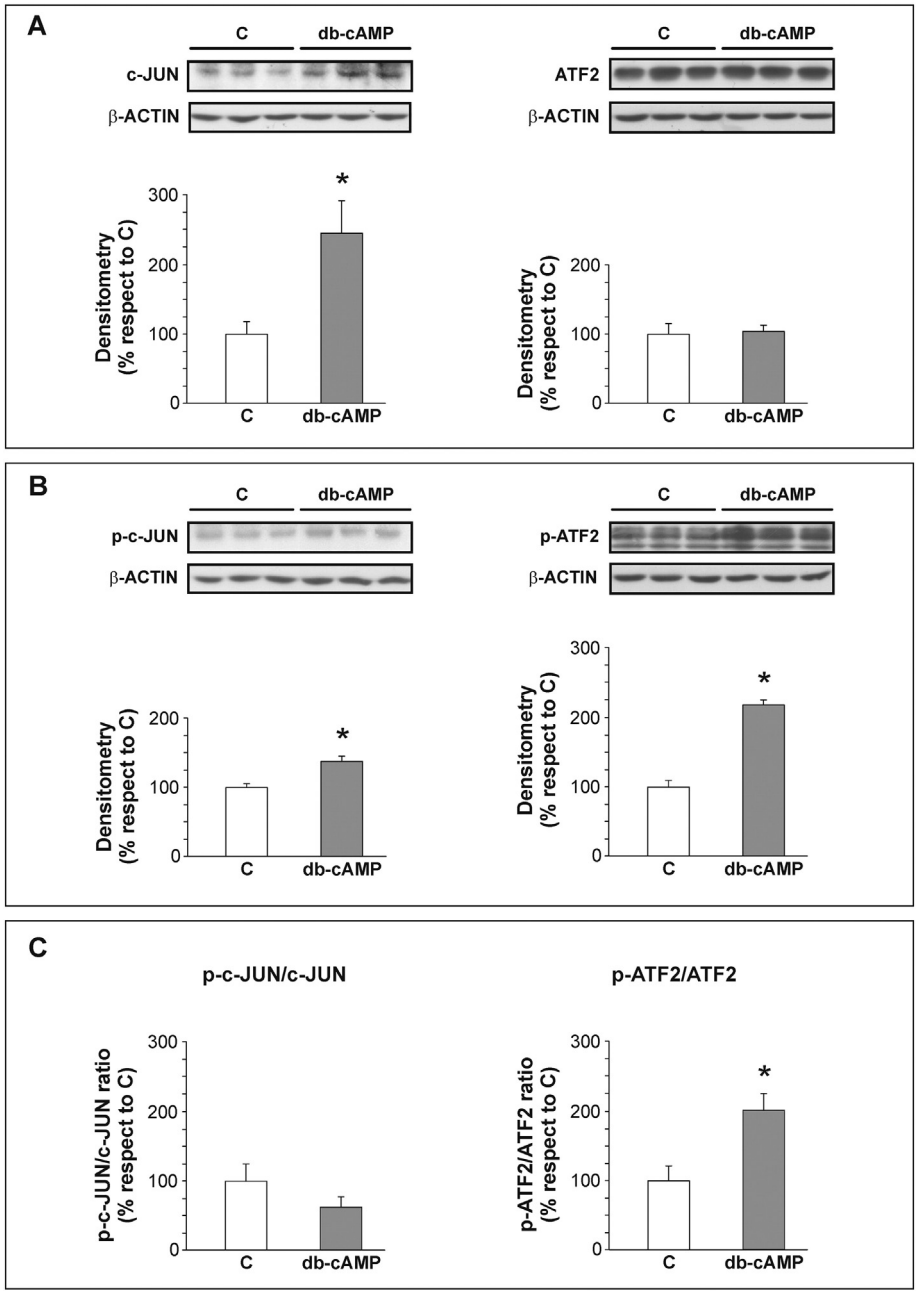

Fig. 6. Effect of db-cAMP on expression and phosphorylation of transcription factors.
Caco-2 cells were treated with db-cAMP (10 μM) and total cell lysates were analyzed by western blotting. A — Expression levels of total c-JUN and ATF2. B — Expression levels of phosphorylated c-JUN and ATF2. In both cases, uniformity of protein loading and transfer from gel to nitrocellulose membrane were controlled with Ponceau S and detection of β-actin. C — c-JUN and ATF2 phosphorylated/total ratio.
Data on densitometry were related to β-actin and presented as percentage of control cells (C) and are expressed as means ± S.D. of six individual experiments per group.
(*) significantly different from control ($p < 0.05$).

Discussion

We here report for the first time that 48 h exposure to cAMP upregulates expression of human GSTα and MRP2, at protein and mRNA levels (Figs. 1 and 9). Initial experiments performed in Caco-2 cells incubated with db-cAMP, a cAMP permeable analog, were further confirmed by activation of adenylyl cyclase leading to increased endogenous production of cAMP (Fig. 2), and subsequent induction of MRP2 expression (protein and mRNA). Consistent with these findings, GST and MRP2 activities were also increased after db-cAMP treatment, resulting in increased protection against toxicity exerted by the common substrate CDNB (Fig. 3).

PKA is considered an essential mediator of a wide range of physiological effects initiated by increased intracellular cAMP levels. To investigate the involvement of PKA-dependent pathways in MRP2 induction, we examined MRP2 protein and mRNA expression in the presence of the specific PKA inhibitors H89 and KT 5720, and found suppression of the induction of both mRNA and protein observed in cells treated with db-cAMP alone (Figs. 4A, B and C). Further experiments using ActD suggest transcriptional regulation of MRP2. These observations collectively indicate that cAMP likely stimulates transcription of *MRP2* gene in Caco-2 cells in a PKA-dependent manner.

Active subunits of PKA are capable of phosphorylating target proteins on specific amino acid residues (Montminy, 1997). In view of

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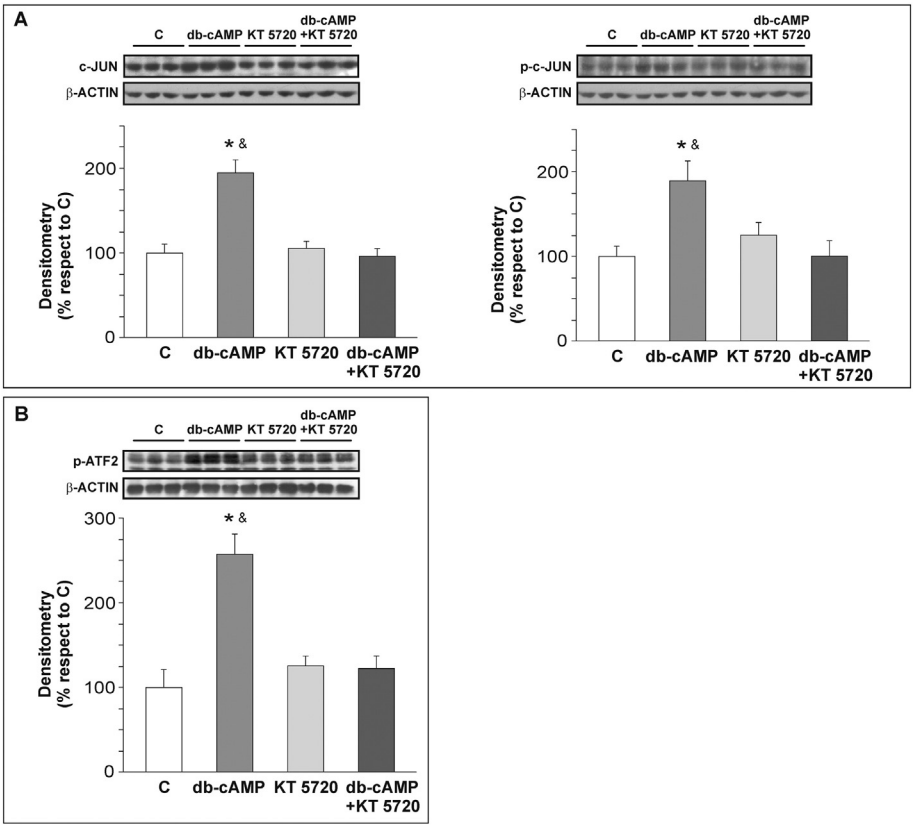

Fig. 7. PKA mediation on expression/phosphorylation of c-JUN and ATF2.
Caco-2 cells were treated with db-cAMP (10 μM), in the presence and absence of the PKA inhibitor KT 5270. Total lysates were prepared and analyzed by western blotting for detection of c-JUN or phospho-c-JUN (panel A), or phospho-ATF2 (panel B). Uniformity of protein loading and transfer from gel to nitrocellulose membrane were controlled with Ponceau S and detection of β-actin.
Data on densitometry were related to β-actin and presented as percentage of control cells (C) and are expressed as means ± S.D. of six individual experiments per group.
(*) significantly different from control ($p < 0.05$), and (&) significantly different from KT 5720 and db-cAMP + KT5720 ($p < 0.05$).

the current results, we considered the possibility of PKA-mediated activation of transcription factors, ultimately leading to increased interaction with the *MRP2* promoter. Initially we performed an *in silico* analysis of the proximal promoter region, comprising the region 1091 bp upstream of the transcriptional start point. As shown in Fig. 5A we identified one CRE and one AP-1 binding site, which could be implicated in the transcriptional regulation of MRP2 by cAMP. We confirmed their participation in our experimental conditions, as db-cAMP treatment resulted in increased luciferase activity in cells transfected with a reporter construct containing the (−789/−603) bp fragment (Fig. 5B). Notably, pretreatment of the cells with the KT 5720 inhibitor abolished the db-cAMP-induced increase in luciferase activity, once more confirming PKA mediation (Fig. 5B).

Recruitment of the CREB family of transcription factors has been classically implicated in the regulation of gene promoters by cAMP (Meinkoth et al., 1993). Recently, Sampurno et al. (2013) demonstrated that in mouse colorectal cancer (CRC) cells and adenomas, pCREB is elevated and is required for the increased expression of Mrp2. This was tentatively associated with increased chemoresistance to frontline drugs such as oxaliplatin, 5-fluorouracil, and camptothecin. Using CREB KO mice, these same authors observed that CREB is not critical for the regulation of major gastrointestinal homeostatic functions such as cell differentiation, contrasting with its role under malignancy conditions. In the current study we found that neither total expression of CREB nor its phosphorylation status was modified by db-cAMP treatment, suggesting a minor participation in MRP2 upregulation. The differential role of p-CREB in mouse colorectal cancer cells or adenomas *vs.* Caco-2 cells may result from intrinsic differences between cell models or more likely from the different stages of the cultures. Our Caco-2 cells are fully differentiated as demonstrated by stable villin expression and alkaline phosphatase activity (unpublished results), key markers of cellular differentiation, observed from 10 days of culture onwards. It is possible that CREB participation in MRP2 regulation depends on the prevalence of a proliferative *vs.* differentiated stage of the cultures, which could also explain the lack of participation of this transcription factor in the regulation of major homeostatic functions of the mouse gastrointestinal epithelium under normal, differentiated conditions, as reported by Sampurno et al. (2013).

AP-1 belongs to the basic leucine zipper (bZIP) group of DNA binding proteins and collectively refers to homo- or heterodimeric transcription factors, composed of JUN, FOS or ATF subunits. The AP-1 complexes could bind to both AP-1 and CRE consensus sequences (Karin et al., 1997; Vesely et al., 2009), and participate in diverse biological processes that include cell proliferation, survival, and differentiation (Karin et al., 1997). As a result of the evaluation of the expression of AP-1 subunits, we observed that only c-JUN was upregulated after treatment with db-cAMP (see Fig. 6A). Since phosphorylation correlates with the increased transcriptional activity of AP-1 subunits (Karin et al., 1997; Vesely et al., 2009), we also evaluated their phosphorylated status. The data indicate induced phosphorylation of c-JUN and ATF2 (see Figs. 6B, C and 7) but not of c-FOS by db-cAMP. ATF2 is constitutively expressed and requires phosphorylation by protein kinases to become active (Bhoumik et al., 2008). While c-JUN activity is also regulated by changes in its phosphorylation status, in contrast to ATF2, changes in protein levels of c-JUN represents an additional way to modulate transcriptional activity (Karin et al., 1997). AP-1 family members are frequent mediators of the cAMP and PKA pathways to activate target genes (Hayakawa et al., 2004; Liu et al., 2009; Song et al., 2011;

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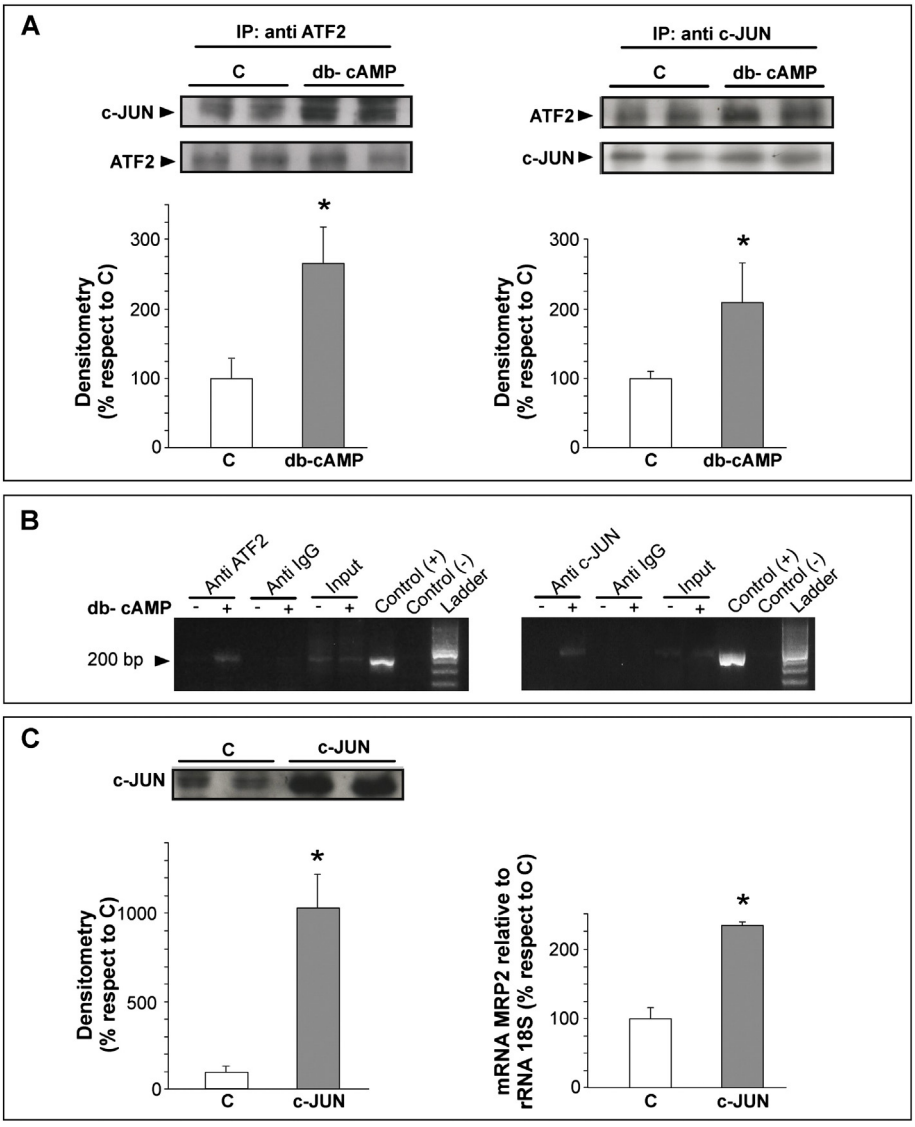

Fig. 8. Effect of db-cAMP on c-JUN/ATF2 dimer formation and further recruitment of this complex to *MRP2* promoter. Effect of c-JUN overexpression on MRP2 mRNA levels.
A — Co-immunoprecipitation of c-JUN and ATF2 transcription factors. Total cell lysates from Caco-2 control and treated cells with db-cAMP (10 μM) were incubated with anti-c-JUN or anti-ATF2 antibodies (as indicated at the top of the Western blot images), followed by Western blot detection with the opposite antibody and densitometry (upper), or with same IP antibodies (lower) to control for IP efficiency. B — ChIP assay with anti-c-JUN or anti-ATF2 antibodies followed by PCR using Primers 1 and 2. Control (+) and Control (−) represent the positive and negative controls respectively. C — Caco-2 cells were transfected with a c-JUN overexpression plasmid (or empty vector) and subjected to Western blot detection of c-JUN to confirm its upregulation, and of RT-PCR to detect MRP2 mRNA levels. Data on MRP2 mRNA were referred to 18s rRNA.
Data are presented as percentage of control cells (C) and are expressed as means ± S.D. of six individual experiments per group.
(*) significantly different from control ($p < 0.05$).

Szabo-Fresnais et al., 2008). Experiments performed in the presence of the PKA inhibitor KT 5720 confirmed participation of PKA in c-JUN and ATF2 activation (Figs. 7A and B). While c-JUN or ATF2 direct phosphorylation by PKA has not been demonstrated yet, additional kinases have been identified as mediators between these transcription factors and PKA; JNK1, p38, and PI3K are typical examples (Chen et al., 2007; Liao et al., 2010). In unpublished western blot studies, we observed that db-cAMP increased the intracellular levels of phosphorylated p38, suggesting but not directly proving, that this kinase could be implicated. More direct experiments are necessary to confirm participation of p38 or other mediators, or alternatively to demonstrate a direct action of PKA, in c-JUN/ATF2 activation.

It was suggested that heterodimeric association of AP-1 subunits like c-JUN and ATF2 is of frequent occurrence and constitutes an intrinsic property of these particular subunits to acquire an efficient transcriptional activity (Carrillo et al., 2010). Our co-immunoprecipitation results are consistent with this postulate as increased interaction is detected in the db-cAMP group (Fig. 8A). More importantly, the ChIP analysis shows that, after treatment with db-cAMP, the complex c-JUN/ATF2 binds the MRP2 promoter fragment containing CRE and AP-1 binding sites (see Fig. 8B). It is well known that AP-1 is a converging point not only to regulate expression of several genes but also to autoregulate expression of AP-1 subunits, thereby increasing their abundance to amplify the signals to external stimuli. Autoregulation represents an additional strategy to modulate composition and biological function of AP-1 dimers (Shaulian and Karin, 2002). On this regard, it was observed that the c-JUN/ATF2 heterodimer induces c-JUN expression through transcriptional activation of the c-JUN promoter (Liu et al., 2006). To establish more directly the role of upregulation of c-JUN in increased expression of MRP2, we overexpressed this transcription factor in Caco-2 cells and found induction of MRP2 mRNA levels to a similar extent observed for db-cAMP treatment (compare Fig. 1C with Fig. 8C),

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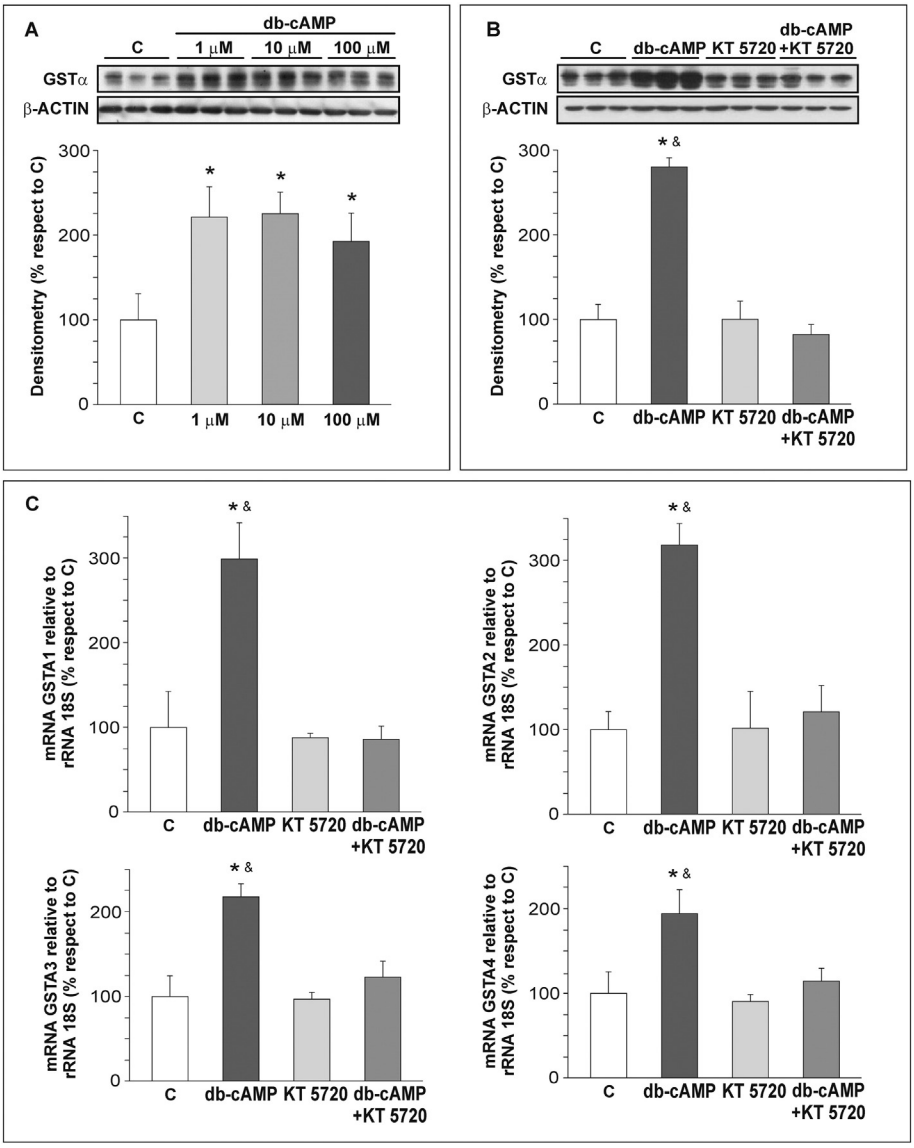

Fig. 9. Effect of db-cAMP on expression of GSTα.
Western blot analysis of GSTα in cytosolic fractions from Caco-2 cells treated with different concentrations of db-cAMP (panel A), or with db-cAMP (10 μM) in the presence and absence of the PKA inhibitor KT5720 (panel B). Uniformity of protein loading and transfer from gel to nitrocellulose membrane were controlled with Ponceau S and detection of β-actin. Densitometry data were related to β-actin and presented as percentage of control cells (C). C — RT-PCR analysis of major GSTA isoforms of Caco-2 cells treated with db-cAMP (10 μM) in the presence or absence of the PKA inhibitor KT 5720. Data on GSTA isoforms mRNA were referred to 18s rRNA.
Data are presented as percentage of control cells (C) and are expressed as means ± S.D. of six individual experiments per group.
(*) significantly different from control ($p < 0.05$), and (&) significantly different from KT 5720 and db-cAMP + KT 5720 ($p < 0.05$).

suggesting c-JUN induction to be a key participant downstream of PKA. Taken together the data support our postulate that c-JUN and ATF2, members of the AP-1 family, are necessary participants in MRP2 transcriptional regulation by cAMP/PKA.

Another contribution of the current study is the observation that db-cAMP positively regulates expression and activity of GSTα (Fig. 9). This is consistent with previous findings about upregulation of this same class of GST in rat intestine by GLP-2 administered *in vivo*, in which increases in intracellular cAMP levels were postulated to be implicated (Villanueva et al., 2010). The data obtained in Caco-2 cells provide direct demonstration on the regulation of GSTα by cAMP, and are consistent with a major role for this GSTα class in differentiated Caco-2 cells (Peters and Roelofs, 1989). As a further contribution, we identified PKA as a mediator of regulation of GSTα downstream of cAMP (Fig. 9). More direct evidence is necessary to probe that AP-1 is the final effector in the cAMP–PKA pathway, as demonstrated for MRP2. However, previous evidence indicated that a single *cis*-regulatory element in rat and mouse GSTα promoter containing two adjacent AP-1-like binding sites is responsible for GST basal and xenobiotic-inducible activity (Daniel, 1993), raising the possibility of a similar regulation for human GST.

Whether the current data on MRP2 and GSTα regulation by cAMP can be extrapolated to the human intestine is uncertain. However, the following evidence suggests such possibility. The cAMP–PKA pathway is fully functional in the Caco-2 cell model under differentiated conditions (Pignata et al., 1994). In line with this, PKA regulation of carbohydrate transport by TNF-α occurs in these cells in a similar fashion as seen *in vivo* (Amador et al., 2007; Barrenetxe et al., 2013). Also, cAMP/PKA signaling mediates the trafficking of CFTR and V-ATPase to the apical membrane of Caco-2 cells as well as to the brush border membrane in the intestine (Collaco et al., 2013). Regarding AP-1 components, they are also functionally expressed in differentiated Caco-2 cells and can be regulated through changes in both expression and binding capabilities (Ding et al., 1999). Finally, AP-1 plays significant physiological

roles in the intestinal epithelium, and additionally, its expression can be modulated by enteral nutrients (Sato et al., 2005).

Conclusion

We demonstrated for the first time that 48 h exposure of Caco-2 cells to cAMP positively modulates the expression and activity of GST and MRP2, ultimately resulting in a reduction of xenobiotic cytotoxicity. Details on the pathways involved downstream of cAMP, including PKA activation, are presented schematically in Fig. 10. In view of the universal role of cAMP as a mediator of the action of endogenous molecules such as hormones, neurotransmitters, cytokines and growth factors or exogenous compounds such as therapeutic drugs, regulation of intestinal membrane barrier function associated to GST and MRP2 activity would be expected under specific physiological, pathophysiological, or drug therapy conditions.

Conflict of interest

The authors declare that they have no conflict of interest.

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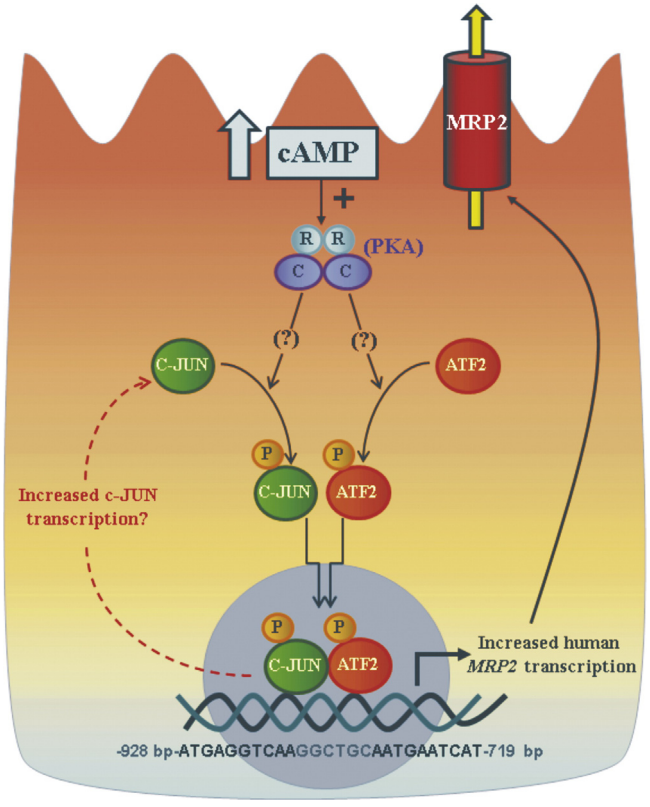

Fig. 10. c-JUN and ATF2 mediate human MRP2 upregulation by cAMP.
Graphical representation of the sequence of events starting with increased intracellular cAMP levels, ultimately leading to induction of the expression of human MRP2 protein. As a first step of this sequence, cAMP activates PKA and subsequently, PKA directly or indirectly promotes phosphorylation and activation of the transcription factors c-JUN and ATF2. Secondly, active c-JUN and ATF2 interact to each other and the resulting complex is recruited to a specific promoter region of the human *MRP2* gene, thus inducing transcription. On this regard, two binding sites (AP-1 and CRE) proved to be putative targets of the complex. The possibility of autoregulation of the expression of c-JUN protein by the c-JUN/ATF2 complex is also indicated.

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