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Impaired CREB-1 Phosphorylation in Antifolate-Resistant Cell Lines with Down-Regulation of the Reduced Folate Carrier Gene

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[1] The Abbreviations used are: CRE, cyclic AMP-response element; CREB-1, CRE-binding protein 1; CREM, CRE modulator; EMSA, electrophoretic mobility shift assay; hRFC, human reduced folate carrier; AC, adenylyl cyclase; PKA, protein kinase A; IBMX, 3-isobuty-1-methylxanthine; CTX, cholera toxin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 2-DGE, 2-dimensional gel electrophoresis.

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Abstract

The human reduced folate carrier (hRFC) is the dominant transporter for the uptake of antifolates used in cancer chemotherapy. Recently we have shown that decreased CRE-dependent transcription contributes to the loss of hRFC gene expression in multiple antifolate-resistant cell lines. This was associated with markedly decreased levels of phosphorylated CREB-1 (pCREB-1) and CRE-binding. Consistent with the auto-regulation of CREB-1 gene expression by pCREB-1, prominently decreased CREB-1 mRNA levels were observed in antifolate-resistant cells. We therefore explored the possibility that these cells were defective in CREB-1 phosphorylation, thereby resulting in down-regulation of some cAMP-responsive genes. Two-dimensional gel electrophoresis revealed that CREB-1 and its phosphoisoforms were markedly decreased in these cells. Treatment with forskolin, an activator of adenylyl cyclase, restored both CREB-1 and pCREB-1 levels; this resulted in restoration of CRE-binding, CRE-reporter activity, as well as CREB-1 and RFC mRNA levels. Hence, the protein kinase A (PKA) pathway was examined using various agents that augment intracellular cAMP levels including cholera toxin, an upstream agonist that renders stimulatory G-proteins (Gαs) constitutively active. Treatment of antifolate-resistant cells with these agents resulted in restoration of pCREB-1 levels and CRE-reporter activity. Furthermore, transient transfection with a constitutively transcriptionally active VP16-CREB-1 that does not require phosphorylation for its activity resulted in restoration of CREB mRNA levels but not pCREB-1 levels. This is the first demonstration that resistance to various antifolates may potentially be associated with impaired activity of Gαs or their coupled receptors resulting in loss of CREB-1 phosphorylation and consequent down-regulation of cAMP responsive genes.

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Introduction

Cyclic AMP (cAMP) stimulates the expression of various target genes via a conserved cAMP-responsive element (CRE), which consists of an eight-base pair palindrome (TGACGTCA) (Montminy, 1997). Multimerization of the CRE strongly enhances cAMP inducibility as revealed by the cooperative actions of two tandem CREs located in the human α chorionic gonadotropin promoter (Delegeane et al., 1987). Following affinity purification with a double-stranded CRE oligonucleotide, the 43 kDa CRE-binding protein (CREB-1) was selectively isolated (Andrisani et al., 1988; Montminy and Bilezikjian 1987). Using an *in vitro* transcription assay, purified CREB-1 stimulated the expression of a CRE-containing somatostatin promoter template (Montminy and Bilezikjian 1987).

Several lines of evidence demonstrate that protein kinase A (PKA) is required for the cAMP-dependent transcription. First, mutant PC12 pheochromocytoma cells that are devoid of PKA activity failed to stimulate a CRE reporter gene expression (Montminy et al., 1986). Second, overexpression of the specific protein kinase A inhibitor (PKI) abolished cAMP-dependent transcription in transfected cells (Grove et al., 1987). Third, microinjection of purified PKA catalytic subunit into fibroblasts induced CRE-β-galactosidase reporter gene activity without the requirement of cAMP (Riabowol et al., 1988). Indeed, when incubated with the catalytic subunit of PKA *in vitro*, the affinity-purified 43 kDa CREB-1 protein was phosphorylated at a single serine (i.e. Ser133) (Montminy and Bilezikjian 1987; Yamamoto et al., 1988). Fourth, microinjection of anti-CREB-1 antibodies into NIH-3T3 fibroblasts abolished the cAMP-dependent induction of a CRE reporter gene (Meinkoth et al., 1991).

CREB-1 belongs to the CREB transcription factor family that, besides CREB-1, it consists of activating transcription factor 1 (ATF1) and cAMP modulating protein (CREM)

(Shaywitz and Greenberg, 1999). The CREB family of activators regulates a striking number of physiologic processes including intermediary metabolism, cell cycle control, cellular proliferation, cell survival-death decisions, and neuronal signaling by altering basic patterns of CRE-dependent gene expression (Montminy, 1997). CREB-1 binds to DNA as a homodimer via a carboxy-terminal basic region/leucine zipper (bZIP) motif (Dwarki et al., 1990) that is conserved in several nuclear factors including ATF-2, *jun*, *fos* and *myc* (Landschulz et al., 1988). CREB family members are activated by phosphorylation in response to a number of signaling pathways, including cAMP, calcium, stress and mitogenic stimuli (Montminy, 1997). As mentioned above, phosphorylation of CREB family proteins at Ser133 in response to a cAMP stimulus induces target gene expression. Phosphorylated (Ser133) CREB-1 binds directly to CBP and its paralogue p300. In turn, CBP and p300 mediate transcriptional induction via their physical association with RNA polymerase II holoenzyme complexes (Montminy, 1997).

Reduced folates are vitamins necessary for one-carbon transfer reactions resulting in the *de novo* biosynthesis of nucleotides and amino acids (Stockstad, 1990). However, as mammalian cells are devoid of tetrahydrofolate biosynthesis, these cofactors are taken up into mammalian cells primarily by the reduced folate carrier (RFC)[1] (Matherly and Goldman, 2003; Jansen and Pieters, 1998). Moreover, RFC is the dominant transporter for antifolates including methotrexate (MTX), raltitrexed (Tomudex) (Jackman et al., 1991) and pemetrexed (multitargeted antifolate, MTA) (Shih et al., 1997). MTX is used in the treatment of various human malignancies, whereas raltitrexed and pemetrexed have been recently approved for the treatment of advanced colorectal cancer and malignant pleural mesothelioma, respectively (reviewed in Van Custem et al., 2002; Manegold, 2003, respectively).

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However, inherent and acquired drug resistance are major obstacles towards curative cancer chemotherapy (Matherly and Goldman, 2003; Jansen and Pieters, 1998). One predominant mechanism of antifolate resistance *in vitro* and *in vivo* is impaired drug transport (reviewed in Jansen and Pieters, 1998; Zhao and Goldman, 2003). For example, inactivating mutations in the hRFC gene were detected in antifolate-resistant tumor cell lines (Gong et al., 1997; Jansen et al., 1998; Wong et al., 1999; Drori et al., 2000; Rothem et al., 2002), in acute lymphoblastic leukemia (ALL) (Kaufman et al., 2004), and in osteosarcoma patients (Yang et al., 2003). Recently we have identified a novel mechanism of impaired antifolate transport that is based on the loss of hRFC gene expression in antifolate-resistant cell lines; this was due to transcriptional silencing resulting from loss of expression and/or function of various transcription factors (Rothem et al., 2003; Rothem et al., 2004). Specifically, decreased binding (or loss) of various transcription factors to CRE, GC-box, Mzf-1, AP-1 and E-box consensus sites in the hRFC promoter was detected in 50-80% of the antifolate-resistant cell lines. This was primarily due to decreased expression and/or function of the cognate transcriptional activators. Furthermore, two thirds of the antifolate-resistant cell lines displayed a marked decrease in transcription factor binding to a single CRE site present in the minimal promoter A of the hRFC gene (Rothem et al., 2004); this was associated with a profound decrease in pCREB-1 levels. Here we explored the molecular basis for the loss of pCREB-1 and CRE-binding in these antifolate-resistant cells. We find a markedly impaired phosphorylation of CREB-1 in these drug-resistant variants. Remarkably, various agents that augment intracellular cAMP levels restored CREB-1 phosphorylation, CRE-binding and CRE-dependent reporter gene activity. This consequently resulted in restoration of CREB-1 and hRFC gene expression. Using various agonists along the cAMP signaling pathway we propose that the CREB-1 phosphorylation defect is possibly due to an impaired activity of Gαs subunits or their coupled

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receptors. However, further studies will be necessary to identify the specific lesion in this cAMP-PKA signaling pathway.

Materials and Methods

Drugs: MTX, aminopterin, dibutyryl cAMP, 3-isobuty-1-methylxanthine, forskolin and cholera toxin were from Sigma. Novel antifolate drugs were generous gifts from the following sources: AG2034, Dr. T. Boritzki (Agouron Pharmaceuticals, Inc.); PT523, Dr. W.T. McCulloch (Sparta Pharmaceuticals) and ZD9331, Dr. A. Jackman, (Institute of Cancer Research, Sutton, UK).

Cell Lines and Tissue Culture: CCRF-CEM, a human T-cell leukemia line and its antifolate-resistant sublines (Rothem et al., 2002; Rothem et al., 2004) were maintained in RPMI-1640 medium containing 2.3 μM folic acid (Biological Industries, Beth-Haemek, Israel) supplemented with 10% fetal calf serum (GIBCO), 2 mM glutamine, 100 units/ml penicillin G (Sigma) and 100 μg/ml streptomycin sulfate (Biological Industries, Beth-Haemek, Israel). The cell lines were established by single step or stepwise antifolate selection of parental CCRF-CEM cells as previously described (Rothem et al., 2002).

Electrophoretic Mobility Shift Assays: Nuclear extracts were prepared from exponentially growing cells (2 $x10^7$ cells) as previously described (Schreiber et al., 1989). DNA-protein complexes were formed by incubating nuclear extract proteins (6 μg) with [α-^{32}P]dCTP end-labeled CRE double-stranded oligonucleotides as detailed elsewhere (Ohlsson and Edlund, 1986). Protein concentration was determined by the colorimetric method of Bradford (1976).

Semi-quantitative RT-PCR Analysis of CREB-1 and RFC Expression: Cells ($1x10^7$) from the mid-log phase of growth were harvested by centrifugation, washed with PBS and total RNA was isolated using the Tri-Reagent kit according to the instructions of the manufacturer (Sigma). A portion of total RNA (20 μg in a total volume of 20 μl) was reverse transcribed

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using M-MLV (180 units, Promega) in a reaction buffer containing random hexamer primers, dNTPs and a ribonuclease inhibitor Rnasin (Promega). Portions of cDNA (~50 ng) synthesized from parental cells and their antifolate-resistant sublines were amplified using 10 pmols of each primer in 2xReddyMix PCR master mix reaction buffer according to the instructions of the manufacturer (ABgene, Surrey, UK). The PCR reaction was performed as follows: initial melting at 95^{o}C for 5 min, followed by 30 cycles each of 1 min at 95^{o}C, annealing at 60^{o}C for 45 seconds, elongation at 72^{o}C for 1 min, followed by 10 min extension at 72^{o}C. Then, the PCR products were resolved on 2% agarose gels containing ethidium bromide. The primers used for the semi-quantitative RT-PCR of RFC and GAPDH were previously described (Rothem et al., 2003). Semi-quantitative RT-PCR analysis of CREB-1 was performed as described above for RFC, using the forward primer: 5'-CCCAGCCATCAGTTATTCAG-3' and the reverse primer 5'-GAGTTGGCACCGTTACAGTG-3'.

Transient Transfections with Expression Constructs and Reporter Gene Activity: Exponentially growing suspension cells ($2x10^7$) were harvested by centrifugation and transiently transfected by electroporation (1000 μF, 234 V) with 10 μg of the following expression plasmids pVP16-CREB-1 (kindly provided by Dr. M. Montminy) and CRE-Lucifease (kindly provided by Dr. A. Aronheim). Cells were then seeded at $2x10^6$/ml in pre-warmed growth medium, incubated for 24 h at 37^{o}C and harvested for extraction of total RNA and nuclear proteins.

CRE-Luciferase Activity Assay: Twenty two hr after transient transfection with the p-VP16-CREB-1 and/or CRE-Lucifease expression vectors, cells were incubated for 2-3 hr hr in growth medium containing or lacking 20μM forskolin, 1mM 3-isobuty-1-methylxanthine, 1mM dibutyryl cAMP or 0.5 μg/ml cholera toxin. Cells were then harvested by centrifugation, washed with PBS, lysed and firefly luciferase activity was assayed using a luciferase kit

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(Promega) and a luminometer. Results presented were obtained from at least three independent transient transfections performed in duplicate cultures.

Western Blot Analysis: For one-dimensional gels, nuclear proteins (30 μg) were resolved by electrophoresis on 10% polyacrylamide gels containing SDS, electroblotted onto Protran BA83 cellulose nitrate membranes (Schleicher & Schuell, Dassel, Germany), reacted at a 1:1,000 dilution with anti-pCREB-1 (New England Biolabs), CREB-1 and PKA (RIIα) according to the instructions of the manufacturer (Calbiochem). Following three 10-min washes in TBS at room temperature, blots were reacted with second antibodies (Jackson Immunoreserach Labs, Baltimore, PA), rewashed and enhanced chemiluminescence (ECL) detection was performed according to the manufacturer's instructions (Biological Industries, Beth-Haemek, Israel). ECL was recorded on X-ray films using several exposure times.

High-Resolution Two-dimensional Gel Electrophoresis: Aliquots of nuclear proteins (120 μg) from the various cell lines were concentrated and desalted using a disposable Microcon YM3 centrifugal filter device (Milipore, Bedford, MA). Proteins were then precipitated with 80% acetone at –20^{o}C for 20 min. Proteins (~100 μg) were then subjected to high-resolution two-dimensional polyacrylamide gel electrophorsis (2-DGE); this was performed in a Bio-Rad system using 110 mm-long pH 3-10 immobilized pH gradients (IPG) strips and pre-casted Criterion 4-20% polyacrylamide gels (Bio-Rad, Hercules, CA, USA). Samples were dissolved in a buffer containing: 7 M urea, 2 M thiourea, 65 nM dithiothreitol, 0.125% (v/v) Biolytes 3-10, 2% CHAPS and 0.1% bromophenol blue. For the first-dimension, ~100 μg of protein were applied to a dehydrated IPG strip, and isoelectric focusing (IEF) was carried out at room temperature. Prior to the separation of the proteins by SDS gel electrophoresis and in order to achieve disulfide reduction, the IEF gel strips were equilibrated for 15 min in a buffer consisting of 37.5 mM Tris-HCl at pH 8.8, 6 M urea, 2% (w/v) sodium dodecyl sulfate, 30%

(w/v) glycerol, 0.5% dithiothreitol and 0.1% bromophenol blue. Then, in order to achieve carbamoylmethylation, the gel strips were re-equilibrated for 15 min in the same buffer containing 2% iodacetamide in place of dithiothreitol. The second-dimension separation was carried out by placing the strips on Criterion 4-20% polyacrylamide gels, following which gels were electroblotted onto Protran BA83-cellulose nitrate membranes (Schleicher and Schuell) and reacted with antibodies as described above.

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Results

Decreased CREB-1 mRNA levels and restoration by forskolin in antifolate-resistant cell lines: Recently we have shown that multiple antifolate-resistant tumor cell lines with impaired drug transport display markedly decreased pCREB-1 levels and a poor CRE-binding (Rothem et al., 2003; Rothem et al., 2004). As pCREB-1 is the transcriptionally active species, we here studied the mechanism underlying the marked decrease in pCREB-1 levels in these cell lines. Since CREB-1 gene expression is a subject for auto-regulation by pCREB-1 itself (Montminy, 1997), CREB-1 mRNA levels were first determined by semi-quantitative RT-PCR analysis (**Fig 1**). The antifolate-resistant cell lines displayed barely detectable levels of CREB-1 mRNA when compared to their parental cells. Remarkably, 2 hr treatment of these cell lines with 20 μM forskolin, a potent activator of adenylyl cyclase (AC) that induces an increase in intracellular cAMP levels, resulted in restoration of CREB-1 mRNA levels (**Fig 2**). Furthermore, transient transfection of these antifolate-resistant cell lines with an expression construct harboring a constitutively transcriptionally active CREB-1 derivative (i.e. VP16-CREB-1) also resulted in high CREB-1 mRNA levels (**Fig 2**).

Two-dimensional Western analysis of pCREB-1 species before and after treatment with forskolin: As mentioned above, since pCREB-1 is the transcriptionally active form, we determined the relative distribution of the non-phosphorylated CREB-1 and its phosphoisotypes using Western analysis after high-resolution 2-dimensional gel electrophoresis (2-DGE; **Fig 3**). Equal amounts of nuclear proteins were first separated by isoelectric focusing on an immobilized pH 3-10 gradient. Resolved proteins were subsequently separated by SDS-PAGE in the second dimension, transferred to nylon membranes and analyzed by Western analysis using an anti-CREB-1 antibody. Parental cells expressed prominent levels of the 43 kDa non-phosphorylated CREB-1 protein (**Fig 3A**, *arrowhead*) along with several pCREB-1

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species that showed both increasing acidity and a slightly increasing molecular mass (**Fig 3A**, *arrows*). In contrast, antifolate-resistant cells (shown is an example of $AG2034^{R2}$ cells), expressed poor levels of both CREB-1 (**Fig 3B**, *arrowhead*) and its more acidic pCREB-1 species (**Fig 3B**, *arrows*). Consistent with the RT-PCR results, treatment of $AG2034^{R2}$ cells for 2 hr with 20 μM forskolin, an activator of AC that increases intracellular cAMP levels, resulted in a complete restoration of both parental CREB-1 levels (**Fig 3C**, *arrowhead*) as well as of the various phosphoisotypes of CREB-1 (**Fig 3C**, *arrows*).

Western analysis of pCREB-1 levels before and after treatment with various cAMP reactive agents: The above findings with forskolin-treated $AG2034^{R2}$ cells upon 2-DGE were corroborated with the various antifolate-resistant cell lines using an antibody to pCREB-1. First, pCREB-1 levels were determined in the various antifolate-resistant cell lines by Western analysis with an antibody to pCREB-1 before and after treatment with forskolin (**Fig 4**). In concordance with the 2-DGE results, pCREB-1 levels were nearly undetectable in the various antifolate-resistant cell lines when compared to parental cells. However, 2 hr treatment of these drug-resistant cell lines with forskolin resulted in a dramatic restoration of pCREB-1 levels, sometimes exceeding parental levels (**Fig 4A**). Consistent with the effect of forskolin, we used various agents along the PKA pathway that augment intracellular cAMP including dibutyryl cAMP (Bt_2cAMP), a cell-permeable cAMP analogue; 3-isobutyl-1-methylxanthine (IBMX), an effective inhibitor of cAMP hydrolysis; cholera toxin (CTX), a most upstream agonist which renders the Gαs subunit constitutively active. Three hr treatment of antifolate-resistant cells with 1mM Bt_2cAMP, 0.5 μg/ml CTX (**Fig 4B,** *lower panel*) as well as 1mM IBMX (data not shown) resulted in restoration of parental pCREB-1 levels. Furthermore, transient transfection of antifolate-resistant cells with a constitutively transcriptionally active VP16-CREB-1 construct (i.e. that does not require CREB-1 phosphorylation for its activity) resulted in

restoration of parental CREB mRNA levels (**Fig 2**) but failed to restore wild type pCREB-1 levels (**Fig 4B**, compare lanes 6,10,14 and 18 with lanes 1,2). Reprobing with antibodies to AP2α, a transcription factor that was retained at normal levels in all antifolate-resistant cell lines (Rothem et al., 2004), confirmed that identical amounts of nuclear proteins were being analyzed (**Fig 4B,** *upper panel*). Moreover, total cell proteins were extracted from non-treated parental and antifolate-resistant cell lines, resolved by electrophoresis, transferred to nylon membranes and reacted with an antibody to PKA (RIIα), the cAMP-dependent kinase responsible for CREB-1 phosphorylation; this revealed identical PKA levels in the various antifolate-resistant cell lines (**Fig 4C**).

Restoration of CRE-binding and CRE-dependent reporter gene activity by cAMP-augmenting agents: Since treatment of antifolate-resistant cells with various cAMP-augmenting agents resulted in restoration of both CREB and pCREB-1 levels (**Fig 3** and **Fig 4A,B**), we examined whether CRE-binding and CRE-luciferase activity was also restored. Indeed, treatment with forskolin resulted in a marked restoration of CRE-binding (**Fig 5**). Furthermore, the poor pCREB-1 levels (**Fig 3** and **Fig 4A,B**) and the profoundly decreased CRE-binding (**Fig 5**) in the various antifolate-resistant cell lines were consistently reflected in a markedly diminished CRE-dependent luciferase reporter activity, thus displaying levels as low as 7.7% of parental cells' activity (**Fig 6**). Remarkably, 2-3 hr treatment of these drug-resistant cell lines with the various cAMP-augmenting agents, 21-22 hr after transient transfection with a CRE-luciferase reporter construct, resulted in a dramatic restoration of reporter gene activities (**Fig 6**); for example, after treatment with forskolin, antifolate-resistant cell lines displayed up to 295% of parental luciferase activity. Consistently, transient transfection of parental cells with an expression construct harboring a constitutively transcriptionally active CREB-1 form (VP16-CREB-1) did not result in any increase in CRE-driven luciferase activity.

In contrast, antifolate-resistant cell lines transiently transfected with this construct displayed a 9-53-fold increase in reporter gene activity since VP16-CREB-1 is constitutively active in transcription even in the absence of phosphorylation (**Fig 6**). These results establish that agents that augment cellular cAMP levels induce restoration of CRE-dependent gene expression in the various antifolate-resistant cell lines.

Restoration of hRFC gene expression in antifolate-resistant cell lines by treatment with forskolin and transfection with VP-16-CREB-1: Previously we have shown that various antifolate-resistant cell lines were impaired in antifolate transport (Rothem et al., 2002) due to a prominent decrease or complete loss of hRFC gene expression (Rothem et al., 2003; Rothem et al., 2004). Consistently here, semi-quantitative RT-PCR analysis corroborated these findings of the decrease or loss of RFC mRNA levels in these antifolate-resistant cell lines (**Fig 7**). Furthermore, treatment with forskolin which restored pCREB-1 levels (**Fig 3** and **Fig 4A,B**) resulted in a partial or complete restoration of hRFC gene expression in the various antifolate-resistant cell lines. Consistently, introduction of the constitutively transcriptionally active VP16-CREB-1 construct into these antifolate-resistant cells also resulted in a complete restoration parental RFC mRNA levels.

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Discussion

There is increasing evidence to suggest that binding of pCREB-1 to CRE in the hRFC promoter is an important contributor to the induction of hRFC gene expression. The minimal promoter A of the hRFC gene contains a single CRE-binding site; deletion of this inducible element resulted in a marked loss of reporter gene expression in transiently transfected HT1080 and HepG2 cells (Whetstine and Matherly, 2001). Consistently, disruption of this consensus CRE site in the minimal hRFC promoter A by site-directed mutagenesis resulted in a 60% loss of reporter gene activity in these cell lines (Whetstine and Matherly, 2001). Antibody-mediated supershift analysis identified CREB-1 as a major mediator of CRE-dependent hRFC gene expression (Rothem et al., 2003; Rothem et al., 2004; Whetstine and Matherly, 2001). Recently we have shown that a markedly decreased CRE-binding occurred in thirteen of seventeen antifolate-resistant cell lines that expressed poor RFC levels; this was associated with markedly decreased pCREB-1 levels (Rothem et al., 2003; Rothem et al., 2004). Based on these findings we here explored the hypothesis that the various drug-resistant cell lines with defective antifolate transport share a common impairment in CREB-1 phosphorylation resulting in decreased CRE-dependent hRFC gene expression. The present study provides several lines of evidence supporting the conclusion that these drug-resistant cell lines are apparently impaired in cAMP-dependent phosphorylation of CREB-1. First, Western analysis after 1- and 2-DGE revealed a marked decrease in both CREB-1 and pCREB-1 levels in these antifolate-resistant cell lines, relative to their parental cells. Second, various agents that induce PKA activity through expansion of intracellular cAMP levels led to parental pCREB-1 levels presumably via the following scenario: CREB-1 protein present at poor levels in antifolate-resistant cells underwent efficient PKA-dependent phosphorylation upon treatment with agents that augment cellular cAMP levels. This in turn led to transactivation of CREB-1 gene expression via its

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auto-regulatory mechanism, thereby resulting in restoration of newly synthesized CREB-1 and pCREB-1 levels. Third, transient transfection of the various antifolate-resistant cell lines with VP16-CREB-1 had only a minor effect (i.e. increase) on pCREB-1 levels. The ectopically overexpressed VP16-CREB-1 could readily bind to CRE consensus sequences and thereby restore CRE-luciferase reporter activity in the various antifolate-resistant cell lines. This consequently resulted in resumption of wild type CREB-1 mRNA levels. However, as these drug-resistant cell lines were defective in PKA-dependent phosphorylation, VP16-CREB-1 overproduction failed to restore substantial pCREB-1 levels.

The following evidence suggest that the markedly decreased phosphorylation of CREB-1 in the various antifolate-resistant cell lines is due to an upstream signaling defect in the cAMP-PKA pathway presumably at the level of the Gαs subunits and/or or their G-protein-coupled receptors. In order to explore the mechanism underlying the defect of CREB-1 phosphorylation, we undertook a series of experiments that examined various steps in the PKA signaling pathway. To this end, we used several agents that target various enzymes in the PKA pathway and thereby directly or indirectly result in augmentation of intracellular cAMP levels (summarized in **Fig 8**). These include Bt_2cAMP, a cell-permeable cAMP analogue that mimics the action of endogenous cAMP and also has a greater resistance to hydrolysis by cAMP phosphodiesterases; IBMX, an effective inhibitor of phosphodisterases with subsequent inhibition of cyclic nucleotide hydrolysis resulting in increased intracellular cAMP pools; forskolin, a potent activator of AC that brings about a marked synthesis of cAMP; and finally cholera toxin, an upstream agonist that catalyzes the ADP-ribosylation of Gαs subunits of G-proteins that are coupled to various receptors, and by doing so prevents the hydrolysis of GTP to GDP, thereby resulting in constitutively active Gαs subunits. Two-three hr treatment of the various antifolate-resistant cells with these agents restored CRE-binding, CREB-1 mRNA,

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CREB-1 and pCREB-1 protein levels, as well as CRE-luciferase activity. Since cholera toxin is the most upstream agent used here, we conclude that the defect in the PKA pathway is presumably at the level of Gαs or even more upstream at the level of the G-protein coupled receptors. The fact that antifolate-resistant cell lines maintained normal levels of PKA, the enzyme that directly phosphorylates CREB-1 along with the finding that fosrkolin, a potent activator of AC resulted in restoration of CREB-1 phosphorylation strongly attest that the CREB-1 phosphorylation defect is indeed upstream to PKA and AC.

Previous studies have clearly established that phosphorylation of Ser133 is a prerequisite for the conversion of CREB-1 to a transcriptional transcativator (Montminy, 1997). In this respect, induction of PKA activity in cultured glioma cells with the β-adrenoreceptor agonist isoproterenol resulted in increased phosphorylation of CREB-1; high-resolution 2-DGE revealed that pCREB-1 had a pI of 4.6 (Storm and Khawaja, 1999). Using high-resolution 2-DGE we consistently find here that forskolin-treatment of antifolate-resistant cells resulted in increased CREB-1 phosphorylation with an initial pI of ~ 4.5. However, we note that parental CCRF-CEM cells as well as forskolin-treated antifolate-resistant cells contained several phosphoisotypes of CREB-1 that became more acidic upon further phosphorylation after forskolin activation. This phenomenon that hyperphosphorylation renders proteins more acidic has been recently observed with various proteins including for example the p21-activated kinase 1 (Pak1) (Kissil et al., 2003); in this study, the effect of merlin, the neurofibromatosis 1 tumor suppressor gene product, on inhibition of Pak1 phosphorylation, was also examined by 2-DGE, thereby revealing multiple phosphoisotypes of Pak1 with increasing acidity. Examination of the current literature reveals several possibilities for multiple phosphoisotypes of CREB-1 and other members of the CRE-binding family including the CRE modulator (CREM) τ: **a)** Casein kinase II (CKII) phosphorylates CREM τ and

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presumably other family members including CREB-1 at multiple serine and threonine sites other than Ser133 (DeGroot et al., 1993). Furthermore, stimulation by forskolin of various transduction pathways including the cAMP-PKA pathway results in enhanced phosphorylation of Ser117, concomitant with an increase in the transactivation potential. **b)** Ser129, a consensus phosphoacceptor site for glycogen synthase kinase-3 (GSK-3) has been proposed to regulate CREB-1 activity in conjunction with Ser133 following cAMP induction (Fiol et al., 1994). Indeed, PKA-mediated phosphorylation of CREB-1 at ser 133 appears to promote GSK-3-mediated phosphorylation of Ser129. Importantly, site-directed substitution of Ser129 to Ala resulted in impairment of CREB-1 activity in PC12 cells, suggesting that Ser129 may contribute to CREB-1 activity. These authors therefore proposed that the hierarchical phosphorylation of CREB-1 at the PKA (i.e. Ser133) and GSK-3 (i.e. Ser129) sites are essential for the cAMP control of CREB-1 activity. **c)** Consistent with the above results Enslen et al. (1994) reported that CREB-1 undergoes phosphorylation at Ser133 as well as at a site independent of Ser133 and Ser 98 by a Ca^{2+}/Calmodulin-dependent protein kinase IV. Hence, CREB-1 and other transactivators of the CRE family can undergo phosphorylation at several sites other than Ser133.

In summary, in the current paper we have shown that transport-impaired, antifolate-resistant cell lines exhibit a cAMP-dependent phosphorylation defect. Following dissection of the PKA signaling pathway with various downstream and upstream agents that augment intracellular cAMP levels we conclude that the phosphorylation defect is possibly at the level of Gαs subunits and/or their G-protein coupled receptors. Clearly, further studies will be necessary in order to identify the specific lesion in this cAMP-PKA signaling pathway.

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Figure Legends

Fig 1: CREB-1 gene expression in parental cells and their antifolate-resistant cell lines. CREB-1 mRNA levels in parental and antifolate-resistant cells were determined by semi-quantitative RT-PCR analysis as detailed in Materials and Methods. A simultaneous PCR of GAPDH as a housekeeping gene control was used.

Fig 2: Effect of forskolin and transient transfection with a pcDNA3/VP16-CREB-1 construct on CREB-1 mRNA levels in parental cells and their antifolate-resistant cell lines. CREB-1 gene expression in parental and antifolate-resistant cells was determined by semi-quantitative RT-PCR analysis as detailed in Materials and Methods. A simultaneous PCR of GAPDH as a control of a housekeeping gene was used. The H_2O group represents a negative PCR control in which no cDNA was present.

Fig 3: Western blot analysis after high-resolution 2-DGE of CREB-1 and its phosphorylated species before and after treatment of parental and antifolate-resistant cells with forskolin. Nuclear proteins (100 μg) isolated from untreated parental (A) and antifolate-resistant AG2034^{R2} cells (B) as well as forskolin-treated AG2034^{R2} cells (C) were subjected to high-resolution 2-DGE. Then, the blots were reacted with antibodies to CREB-1 in order to detect non-phosphorylated (*arrowhead*) as well as the phoshorylated species of CREB-1 (*arrows*). Note that the non-phosphorylated CREB-1 had a molecular mass of ~43 kDa and an apparent pI of ~ 4.5, whereas the increasing phosphorylation levels of CREB-1 resulted in a consistent increase in the molecular mass along with an increasing acidity (i.e. decreasing pI).

Fig 4: Western blot analysis of pCREB-1 and PKA RIIα expression in parental and antifolate-resistant cell lines before and after treatment with forskolin (A) or Bt_2cAMP, CTX or after transient transfection with pcDNA3/VP-16-CREB-1 (B). Parental CCRF-CEM cells and their antifolate-resistant sublines were treated for either 2 hr with 20 μM forskolin (A), for 3 hr with

1mM Bt_2cAMP, 0.5 μg/ml CTX, or transiently transfected for 24 hr with the expression vector pcDNA3/VP-16-CREB-1 (B). Nuclear proteins (30 μg) were then resolved by 10 % polyacrylamide gels containing SDS, transferred to Protran BA83 cellulose nitrate membranes, and reacted with antibodies to human pCREB-1 (A, B). Western blots were then reprobed with an antibody to AP2α (B). Total cell proteins were extracted from the various cell lines before treatment, resolved by electrophoresis, transferred to nylon membranes and reacted with an antibody to PKA RIIα (C) as detailed in Materials and Methods.

Fig 5: Effect of forskolin on [^{32}P]CRE-binding of nuclear proteins isolated from parental and antifolate-resistant cells. Electrophoretic mobility shift assay (EMSA) with [^{32}P]-labeled CRE oligonucleotides was performed as follows: nuclear proteins (6 μg) from parental CCRF-CEM cells and their antifolate-resistant sublines before and after treatment with forskolin were first incubated with [^{32}P]-labeled CRE oligonucleotide, resolved by electrophoresis on polyacrylamide gels and analyzed by a Phosphorimager.

Fig 6: CRE-luciferase reporter gene activities following transient transfection into parental and antifolate-resistant cells in the presence or absence of forskolin, Bt_2cAMP, IBMX and CTX as well after transient transfection with pcDNA3/VP-16-CREB-1. A CRE-Luciferase construct was transfected by electroporation into parental and antifolate-resistant cells after which a portion of cells was treated with the various agents for 2 or 3 hr. Cells were then lysed and reporter gene activities were determined as detailed in Materials and Methods. Results presented are mean reporter activity (relative to untreated parental cells) ± S.D. obtained from three independent experiments.

Fig 7: RFC gene expression in parental and antifolate-resistant cells before and after treatment with forskolin or transient transfection with pcDNA3/VP16-CREB-1. The effect of forskolin and transient transfection with pcDNA3/VP16-CREB-1 on RFC mRNA levels in parental and

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antifolate-resistant cells was determined by semi-quantitative RT-PCR as detailed in Materials and Methods.

Fig 8: A schematic model summarizing the various agents used in the PKA signaling pathway that augment cellular cAMP levels.

Fig 1

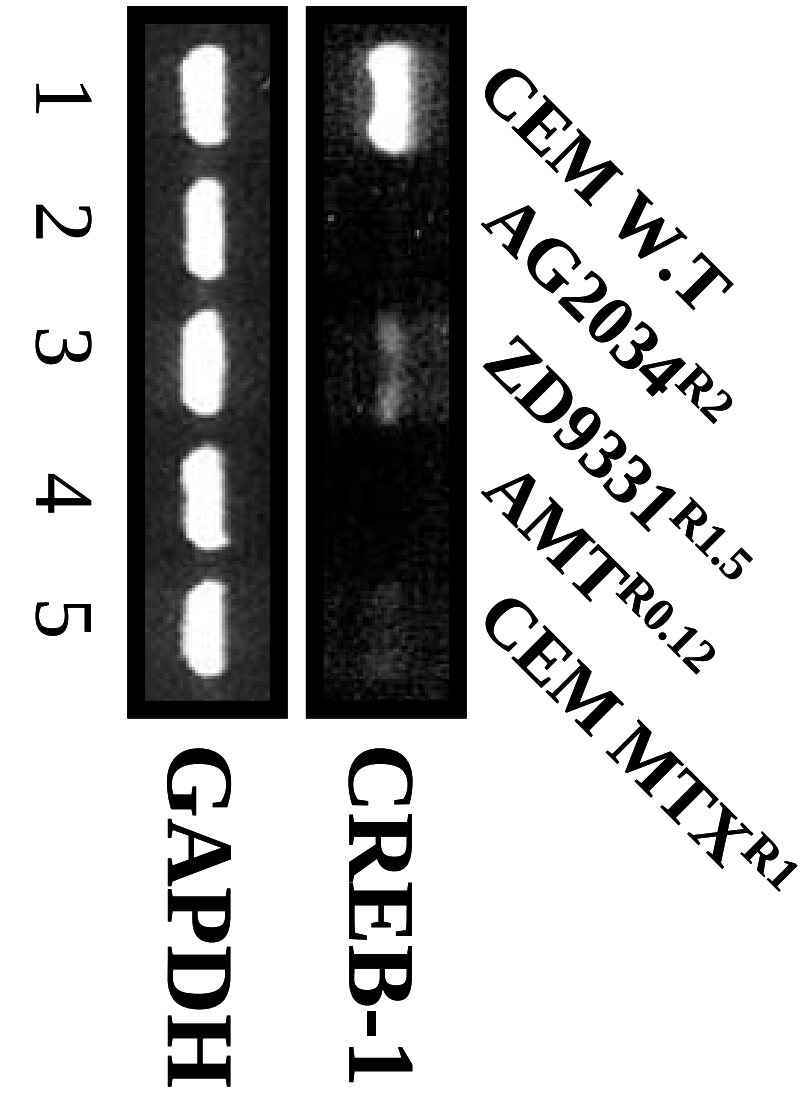

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Fig 2

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Fig 3

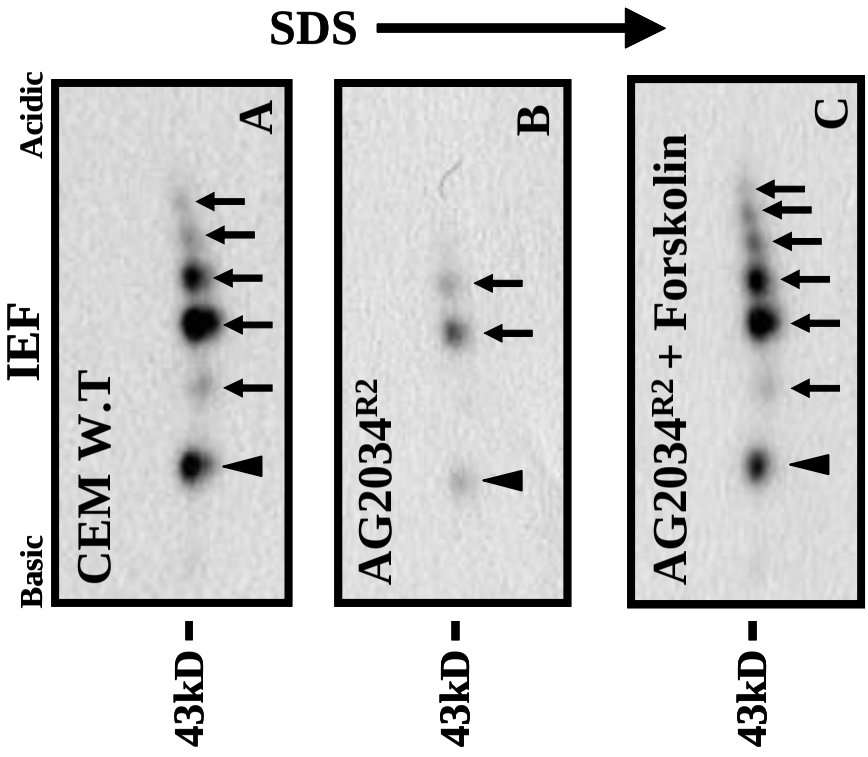

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Fig 4A

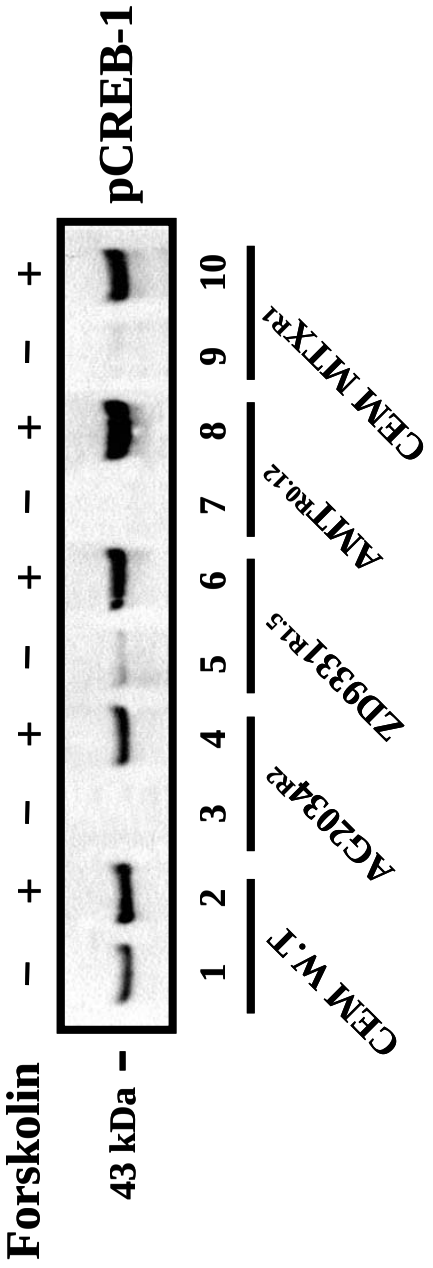

Fig 4B

Fig 4C

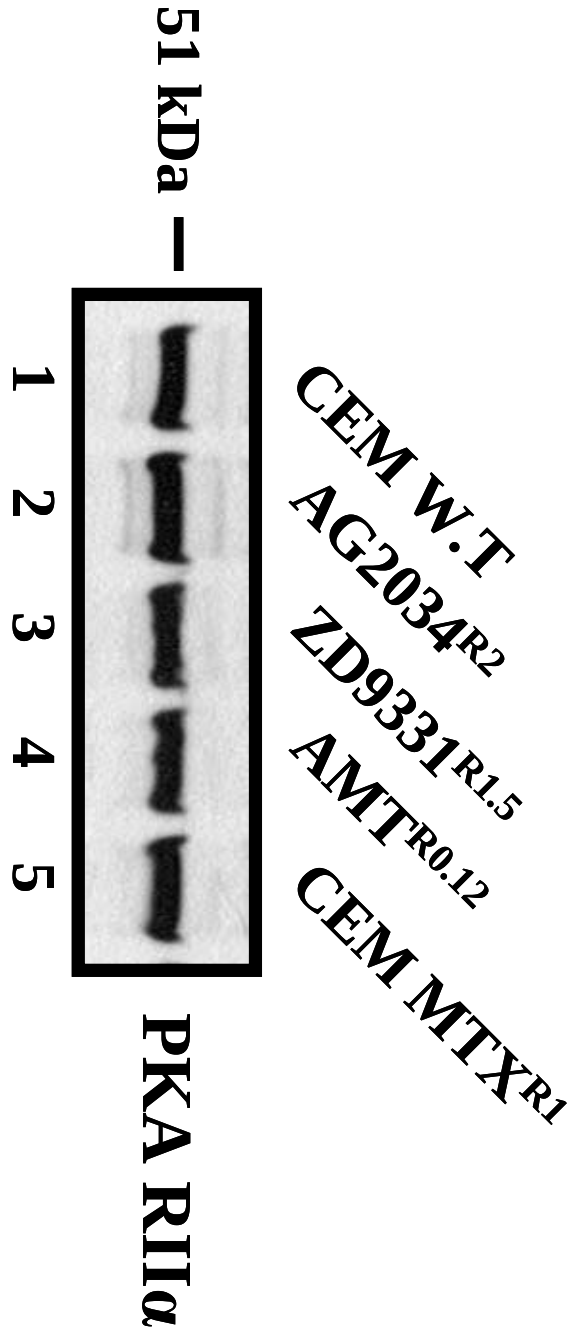

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Fig 5

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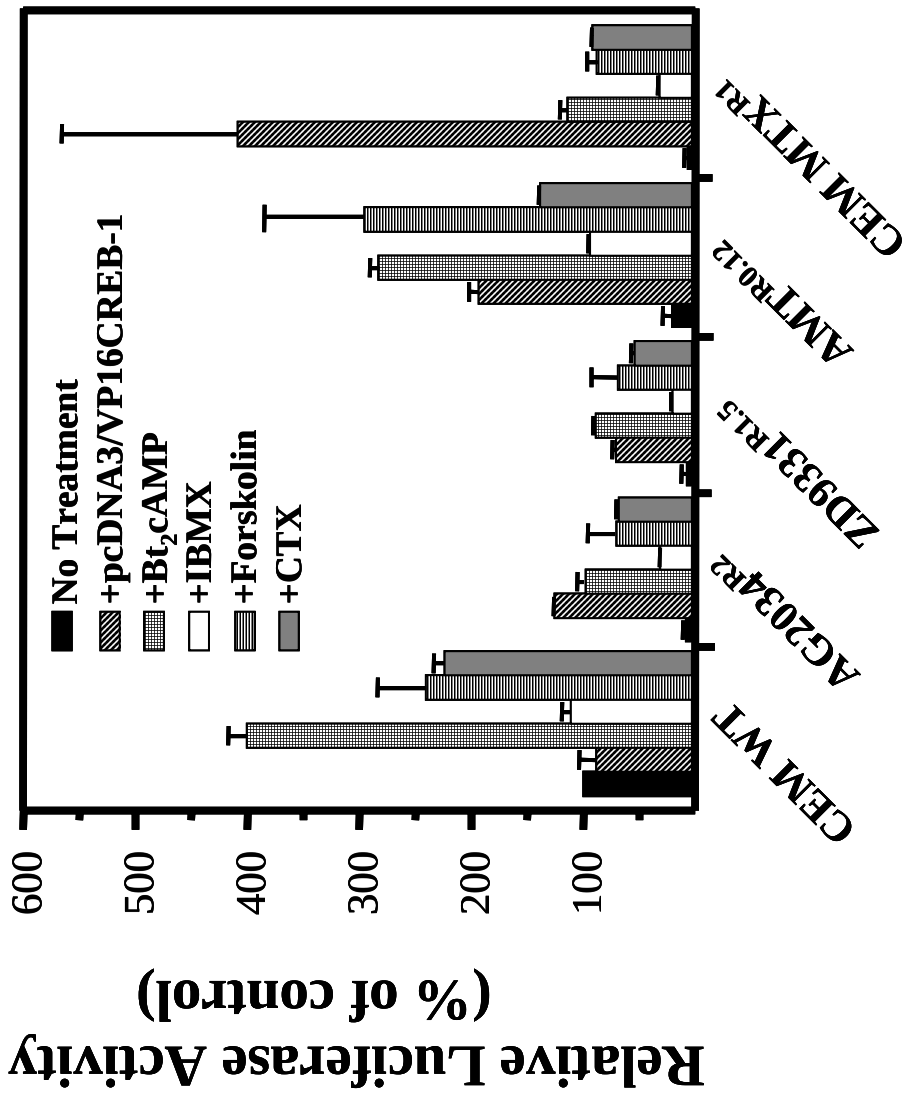

Fig 6

Fig 7

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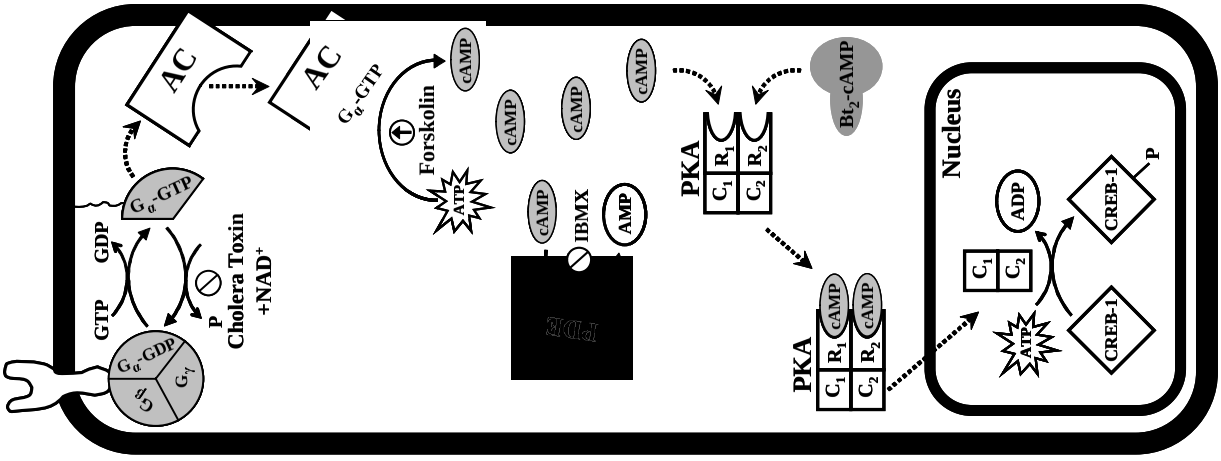

Fig 8