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Cyclic AMP Mediated GSTP1 Gene Activation in Tumor Cells Involves the Interaction of Activated CREB-1 With the GSTP1 CRE: A Novel Mechanism of Cellular GSTP1 Gene Regulation

Hui-Wen Lo and Francis Ali-Osman*

Section of Molecular Therapeutics, Department of Neurosurgery and The Brain Tumor Center, University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030

Abstract The human GSTP1 gene is frequently over-expressed in many human cancers and the expression increases with tumor progression and is associated with a more aggressive biology, poor patient survival, and resistance to therapy. The molecular regulation of the human GSTP1 gene during malignancy is, however, still not well understood. Recently, we reported the presence of a cAMP response element (CRE) in the 5′-region of the human GSTP1 gene, raising the possibility that the cAMP signaling pathway, frequently aberrant in human cancers, may play an important role in the transcriptional activation of the GSTP1 gene in human tumors. In this study, we report that the GSTP1 gene is an early cAMP response gene. Treatment of cells of the human lung carcinoma cell line, Calu-6, with 25 μM forskolin to activate the cAMP pathway resulted in a rapid and significant (sevenfold after 30 min) increase in GSTP1 gene transcripts, which peaked at 12-fold after 4 h. The forskolin-activated GSTP1 transcription in Calu-6 cells was suppressed dose-dependently by a 2-h pre-treatment with 0.1, 1.0, and 10 μM of the adenylate cyclase inhibitor, 2′, 5′-dideoxyadenosine. Western blot analysis showed a rapid, fivefold increase, in GSTP1 protein levels after treatment with 25 μM forskolin, with a peak at 2 h post-treatment. The levels of phosphorylated CRE (Ser133) binding protein-1 (CREB-1) increased rapidly, sevenfold at 30 min, and reached 10-fold at 4 h following forskolin treatment. Intracellular cAMP levels also increased rapidly reaching 12-fold at 30 min. Gel mobility shift and supershift assays and *DNase I* footprinting analyses demonstrated that CREB-1 bZIP and CREB-containing nuclear extracts recognized the GSTP1 CRE with high affinity and specificity. Binding of CREB-1 bZIP to the GSTP1 CRE was abolished when the GSTP1 CRE sequence, 5′-CGTCA-3′, was mutated at the core nucleotides. Finally, transfection studies using luciferase plasmid constructs showed the GSTP1 CRE to be required for the cAMP-activated gene expression. Together, these findings describe a novel cAMP- and CREB-1-mediated mechanism of transcriptional regulation of the GSTP1 gene and suggest that this may be an important mechanism underlying the increased GSTP1 expression observed in tumors with an aberrant cAMP signaling pathway and in normal cells under conditions of stress, associated with increased intracellular cAMP. J. Cell. Biochem. 87: 103–116, 2002.
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The human glutathione S-transferase P1 (GSTP1) is a member of a superfamily of GSTs best-known for the central role they play in Phase II metabolism. In this function, GSTs catalyze the nucleophilic attack of electrophilic groups provided by a carbon, a nitrogen, or a sulfur atom of various substrates, via the sulfur atom of glutathione (γ-L-glutamyl-L-cysteinyl glycine), GSH [Hayes and Pulford, 1995]. The formation of a thioether bond between GSH and electrophiles yields a conjugate that is more water-soluble and can be metabolized further to mercapturic acids, and then excreted [Boyland and Chasseaud, 1969]. More recently, GSH

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*Correspondence to: Francis Ali-Osman, Section of Molecular Therapeutics, Department of Neurosurgery (Box 64), The Brain Tumor Center, University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030.
E-mail: faliosman@mdanderson.org

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conjugates have been shown to be transported from cells by ATP-dependent efflux pumps, including the multidrug resistance associated proteins, MRPs [Ishikawa and Ali-Osman, 1993; Jedlitschky et al., 1994; Muller et al., 1994]. As such, GSTs serve an important role in the metabolism and detoxification of endogenous compounds, such as, leukotriene A4, prostaglandin, and reactive oxygen species produced during oxidative stress [Mannervik, 1985; Chang et al., 1987; Nicolson et al., 1993]. Other electrophilic substrates of GSTs include compounds of xenobiotic origin, such as, natural toxins, various pesticides, mutagens, carcinogens, and anti-cancer drugs [Mannervik, 1985; Ishikawa and Ali-Osman, 1993; Goto et al., 1999; Srivastava et al., 1999; Ali-Osman and Ishimoto, 2001].

Of the eight human cytosolic GSTs, GSTP1 has been most frequently involved in both the formation and progression of tumors [Sato, 1989; Hara et al., 1990; Henderson et al., 1998; Nakae et al., 1998]. It is now well established that GSTP1 over-expression is a common feature of many human tumors, including, those of the brain, breast, ovary, esophagus, stomach, liver, pancreas, colon, skin, kidney, the lymphatic and hematopoietic systems, and the lung [Howie et al., 1990; Ramgamaltha and Tew, 1991; Tsuchida and Kato, 1992; Hayes and Pulford, 1995; Ali-Osman et al., 1997b]. The high GSTP1 expression is prognostic for poor patient survival and is a major determinant of tumor drug resistance [Gilbert et al., 1993; Hayes and Pulford, 1995; Ali-Osman et al., 1997b]. In normal cells, increased GSTP1 expression has been associated primarily with cells under physiological stress [Hayes and Pulford, 1995].

Despite the well-documented over-expression of the GSTP1 gene in tumors and in stressed normal cells, the molecular mechanisms underlying the regulation of the GSTP1 gene are still largely unclear. The GSTP1 gene, mapped to chromosome 11q13, is encoded in an allelopolymorphic gene locus, located in a 2.8 kb region, and consists of seven exons and six introns [Morrow et al., 1989; Ali-Osman et al., 1997a; Lo and Ali-Osman, 1997]. Functional studies have shown the TATAA box at −31 to −27, the two Sp1 sites at −46 to −41 and −57 to −51, and the AP-1 site at −69 to −63 to be critical for GSTP1 promoter activity of the GSTP1 gene [Morrow et al., 1990; Xia et al., 1991; Moffat et al., 1996; Jhaveri and Morrow, 1998; Borde-Chich'e et al., 2001]. Previous studies from our laboratory have demonstrated that seven repeats of retinoic acid response elements at +1,521 to +1,644 in intron 5 are responsible for retinoic acid-induced GSTP1 gene expression [Lo and Ali-Osman, 1997, 1998, 2000]. We subsequently showed that a p53 binding motif at +983 to +1,002 in intron 4 of the GSTP1 gene is required for wild-type p53-activated GSTP1 gene transcription [Lo et al., 1999].

cAMP is a second messenger that mediates many important cellular processes, including, cell growth and differentiation and the signaling cascades resulting from hormone, growth factors, stress, etc [Robison et al., 1968; Borrelli et al., 1992]. cAMP-mediated signaling pathways are essential for normal cell growth and differentiation, and many housekeeping genes are activated by transcription factors that are phosphorylated in the cAMP signaling pathway [Borrelli et al., 1992]. Intracellular cAMP levels are modulated by membrane-bound adenylate cyclases, the activation of which requires interactions with the seven transmembrane domain receptor-family, such as, β2-adrenergic and rhodopsin receptors [Dixon et al., 1986; Borrelli et al., 1992]. cAMP exerts its functions, in part, by activating protein kinases, notably, protein kinase A (PKA) [Taylor et al., 1990]. cAMP response element (CRE) binding proteins, CREBs, are substrates of PKA that bind to CREs present in cAMP-responsive genes, leading to their transactivation [Andrisani, 1999]. A consensus CRE, an eight-nucleotide palindromic sequence, 5′-TGACGTCA-3′, present in cAMP responsive genes is recognized by dimers consisting of proteins in the CREB/ATF and/or jun/fos (AP-1) families [Sassone-Corsi et al., 1990; Andrisani, 1999]. The consensus CRE is highly similar to the consensus AP-1 site (5′-TGACTCA-3′), the DNA binding site for the AP-1 oncoproteins jun/fos [Borrelli et al., 1992].

Recently, we reported that a functional CRE, located in the 5′-regulatory region at −512 to −505, proximal to the transcription start site of the GSTP1 gene, is a high-affinity binding site for CREB-1 bZIP [Lo and Ali-Osman, 2001]. In this study, we examined whether the GSTP1 gene is transcriptionally activated by the cAMP signaling pathway in tumor cells. We investigated the mechanism of such cAMP-dependent activation of the GSTP1 gene,

including, involvement of the activated CREB-1 interaction with the CRE in the 5′-regulatory region of the GSTP1 gene and examined the structural requirements of the GSTP1 CRE for the CREB-1 binding.

MATERIALS AND METHODS

Biochemicals and Molecular Biology Reagents

The activator and inhibitor of adenylate cyclases, forskolin and 2′, 5′-dideoxyadenosine, respectively, and peroxidase-conjugated secondary antibodies were purchased from Sigma (St. Louis, MO). The immunoassay kit, Correlate-EIA, for determination of intracellular cAMP levels was obtained from Assay Designs, Inc. (Ann Arbor, MI). ECL reagents were from Amersham Pharmacia Biotech (Piscataway, NJ). The polyclonal GSTP1 antibody was purchased from Biotrin (Ireland), polyclonal anti-phospho-CREB (Ser133) antibody from Cell Signaling (Beverly, MA), and monoclonal anti-CREB-1, polyclonal anti-β-actin from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant proteins, CREB-1 bZIP and c-jun, were obtained from Santa Cruz Biotechnology and Promega Corp. (Madison, WI), respectively. Restriction endonucleases, DNase I, DNA ligase were from Roche Biochemicals (Indianapolis, IN).

Cell Line

The Calu-6 human lung carcinoma cell line was obtained from the American Type Culture Collection (Gaithersburg, MD) and was maintained routinely as monolayers in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 0.1 mM non-essential amino acids, and 1 mM sodium pyruvate. This cell line has been shown in previous studies to have a functional cAMP signaling pathway and to respond to activators of adenylate cyclases [Ying et al., 1997].

DNA Probes

A full-length GSTP1 cDNA probe was amplified by PCR from a phagemid vector containing the GSTP1 cDNA, as previously described [Ali-Osman et al., 1997a] and used in the analyses of GSTP1 gene transcripts. The GAPDH cDNA probe was purchased from CLONTECH Laboratories (Palo Alto, CA) and used as a control in these analyses. The GSTP1 CRE used in *DNase I* analyses and chemical sequencing spanned nucleotide positions −549 to −501 of the GSTP1 gene and was custom-synthesized by Sigma-Genosys (Woodlands, Texas). The reverse strand of the GSTP1 CRE footprinting probe was end-labeled, as described by Sambrook et al. [1989], and was annealed to the forward strand prior to use.

DNA Mobility Shift and Supershift Analysis of CREB Binding to GSTP1 CRE

The binding of CREB-1 proteins to the CRE in the GSTP1 gene was examined by gel mobility shift and supershift assays using the GSTP1 CRE and the somatostatin CRE with the respective sequences, 5′-CGTGAGACTACGTCATAA-AA-3′ and 5′-CTTGGCTGACGTCAGAGAGA-3′. Complementary oligonucleotides were denatured, reannealed, and [γ-^{32}P]-endlabeled, as described previously [Lo and Ali-Osman, 1998]. Recombinant human CREB-1 bZIP protein containing the 10–14 kDa DNA binding and dimerization domain of the CREB-1 protein, corresponding to amino acids 254–327, was incubated at 0.25 and 0.5 μg in a 20 μl reaction mixture containing 2 ng [γ-^{32}P]-endlabeled probes, 100 mM Tris-HCl (pH 7.4), 100 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, and 1 μg poly (dI-dC) (Amershan Pharmacia Biotech, Piscataway, NJ). For binding studies using nuclear extract, 5 μg of extract protein from untreated or forskolin-treated Calu-6 cells replaced CREB-1 bZIP. After 15 min at room temperature, the mixture was electrophoresed in 8% non-denaturing polyacrylamide gel with 0.33 × Tris-Borate-EDTA (TBE). The gel was dried on Whatman filter paper and exposed to X-ray films. In competition experiments to determine binding specificity, CREB-1 bZIP was pre-incubated with 25-, 50-, and 100-fold excess of cold CRE probes for 15 min prior to the addition of ^{32}P-labeled probes. In the supershift assays, 0.5 μg CREB-1 bZIP was incubated with 1 μg of monoclonal anti-CREB-1 antibody, X-12 for 15 min on ice prior to, or after the binding reactions and subjected to electrophoresis as described above. Similar supershift reactions were set up for nuclear extracts except that 2 μg of monoclonal anti-CREB-1 antibody or polyclonal anti-phospho-CREB-1 antibody were added to preformed complex of 5 μg of nuclear extracts isolated from forskolin-treated Calu-6 and the ^{32}P-labeled GSTP1 CRE.

Mutational Analysis of GSTP1 CRE and its Binding to Activated CREB-1

These studies were designed to determine the core nucleotide sequence of the GSTP1 CRE and to investigate the specific structural requirements for functionality of this CRE. For this, we created a series of GSTP1 CRE mutants, containing single substitutions of each of the nucleotides in the GSTP1 CRE. The sequences of the eight mutated GSTP1 CRE oligonucleotides are shown in Table I. Each of the CRE mutants was examined for its ability to bind to CREB-1 bZIP by DNA gel mobility shift assays, as described earlier.

DNase I Footprinting Analysis of CREB-1/GSTP1 CRE Interaction

DNase I footprinting analysis was used to characterize and map the binding of CREB-1 bZIP to the CRE in the GSTP1 gene and was performed, as we described previously [Lo and Ali-Osman, 1998]. Reaction mixtures of 200 μl total volume contained 1.25 μg CREB-1 bZIP or five footprint units of c-jun, 1 μg poly (dI-dC), and ~10,000 cpm of ^{32}P-endlabeled GSTP1 CRE fragment in 10 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM DTT, 100 mM KCl, and 50 μg/ml bovine serum albumin (pH 8.0). The mixtures were pre-incubated at room temperature for 45 min and then digested with four units of *DNase I* for an additional 90 s. The digestion was stopped by adding 28 μl of 7 M ammonium acetate containing 125 mM EDTA (pH 8.0) and 1 μg glycogen. The DNA was extracted with phenol:chloroform:isoamyl alcohol, precipitated with 100% cold ethanol and washed twice with 70% ethanol. An aliquot of the precipitated DNA was subjected to adenine- and guanine-specific cleavage in a Maxam and Gilbert DNA sequencing reaction [Sambrook et al., 1989]. The DNA from the footprinting and chemical sequencing reactions were electrophoresed in 10% polyacrylamide gels containing 7 M urea in 0.5 × TBE running buffer, the gels were dried and autoradiographed on Kodak X-ray film at −80°C with intensifying screens.

TABLE I. Nucleotide Sequences of GSTP1 CRE Mutant Oligonucleotides

GSTP1 CRE mutants	Nucleotide sequences
Unmutated GSTP1 CRE	5′-CGTGAGA<u>CTACGTCA</u>TAAAA-3′
Mutant 1	5′-CGTGAGA<u>CTTCGTCA</u>TAAAA-3′
Mutant 2	5′-CGTGAGA<u>CTAGGTCA</u>TAAAA-3′
Mutant 3	5′-CGTGAGA<u>CTACATCA</u>TAAAA-3′
Mutant 4	5′-CGTGAGA<u>CTACGCCA</u>TAAAA-3′
Mutant 5	5′-CGTGAGA<u>ATACGTCA</u>TAAAA-3′
Mutant 6	5′-CGTGAGA<u>CAACGTCA</u>TAAAA-3′
Mutant 7	5′-CGTGAGA<u>CTACGTGA</u>TAAAA-3′
Mutant 8	5′-CGTGAGA<u>CTACGTCT</u>TAAAA-3′

The CRE sequences are underlined and the mutated nucleotides are in boldface.

Analysis of Functionality of the GSTP1 CRE in a Luciferase Plasmid Reporter System

The functionality of the GSTP1 CRE was elucidated by examining its ability to activate a TATAA box-containing luciferase reporter gene in cells following activation of the cAMP pathway. A DNA fragment containing four repeats of the GSTP1 CRE was synthesized and ligated into the *Sac I* site of the parental firefly luciferase plasmid, *pFR-Luc* (Stratagene, La Jolla, CA). Ligated plasmids were transfected into competent XL-1 Blue cells and positive clones were selected. The *pRL-CMV*, a *Renilla* luciferase expression plasmid, was used as a control for transfection efficiency, whereas, *pCRE-Luc*, containing four repeats of the somatostatin CRE sequences, was used as a positive control. The nucleotide sequence and direction of the CRE fragment in the reporter expression vectors was determined by a PCR-based dideoxynucleotide chain termination method using an AmpliCycle sequencing kit (Perkin Elmer, Foster City, CA). A modified calcium phosphate method, MBS Mammalian Transfection Kit (Stratagene), was used to transfect the plasmid vectors into Calu-6 cells. Twenty-four hours following transfection, the cells were treated with 25 μM forskolin or vehicle (0.025% DMSO) for an additional 24 h, harvested, and analyzed for luciferase activity using the commercial Dual-Luciferase Reporter Assay System (Promega) in a TD-20/20 Luminometer (Promega), according to the manufacturer's protocol.

Assay of Intracellular cAMP Levels Following Adenylate Cyclase Activation

Calu-6 cells in exponential growth were treated with 25 μM of the adenylate cyclase activator, forskolin, for 0, 30, and 60 min and lysed in 0.1 M HCl for 10 min. Following centrifugation at 1,200*g* for 5 min at 4°C, supernatants were collected and the cAMP concentrations were determined by immunoassay, using a commercial kit, Correlate-EIA, according to the

manufacturer's instructions in a microtiter plate reader with wavelength of 405 nm against correction at 570 nm.

Transcriptional Activity of GSTP1 Gene Following Activation of the cAMP Signaling

These studies were designed to determine whether the GSTP1 gene undergoes transcriptional activation following activation of the cAMP signaling pathway. Calu-6 cells in exponential growth were treated with forskolin (25 μM) and over a period of 30 min to 24 h, the cells were harvested, and the levels of GSTP1 gene transcripts were determined by Northern analyses, as previously described [Chomczynski and Sacchi, 1987; Lo and Ali-Osman, 1997]. Briefly, total RNA was isolated by the acid guanidinium thiocyanate–phenol–chloroform method, fractionated by electrophoresis in formaldehyde-containing agarose gels, and capillary transferred to nylon membranes. The membranes were baked, blocked in prehybridization solution for 2 h at 42°C, and hybridized with [α-32P]-labeled GSTP1 probe ($10–20 \times 106$ cpm/ml) for additional 16–24 h at 42°C, washed, and autoradiographed.

Analysis of the GSTP1 Gene Transcriptional Activity Following Suppression of cAMP Signaling

These studies were performed to further establish that the GSTP1 gene is transcriptionally regulated by the cAMP signaling pathway. Calu-6 cells were pre-treated with 0.1, 1.0, 10 μM of the specific adenylate cyclase inhibitor, DDA, for 2 h, followed by 25 μM forskolin for an additional 2 h. The level of GSTP1 gene transcripts was then determined by Northern analyses, as described earlier.

Analysis of CREB-1 Ser133 Phosphorylation Following Activation of cAMP Signaling

The cAMP cascade leading to gene activation involves phosphorylation of CREB-1 at Ser133 [Andrisani, 1999]. Consequently, we examined changes in the levels of phospho-CREB-1 (Ser133) under conditions of activation of adenylate cyclases that we had previously shown to result in GSTP1 gene activation. Calu-6 cells were treated with 25 μM forskolin for 0–4 h, lysed by ultrasonication and centrifuged at 10,000*g* for 20 min. Protein concentrations in the resulting supernatants were determined by the Bradford method [Bradford, 1976] and the levels of GSTP1, total CREB-1, phospho-CREB-1 (Ser133) and β-actin proteins were determined by Western blot analyses, as previously described [Ali-Osman et al., 1997a]. Briefly, following electrophoresis, proteins were transferred from SDS–polyacrylamide gels onto polyvinylidene fluoride membranes using a semi-dry blotting system (Hoefer Semiphor, CA). The membranes were hybridized with a polyclonal GSTP1 antibody, a polyclonal phospho-CREB (Ser133) antibody, a monoclonal anti-CREB-1 antibody, or a polyclonal β-actin antibody, each at the effective pre-determined dilutions, washed and reacted with peroxidase-conjugated secondary antibodies. Reactive bands were visualized by the ECL, chemiluminisence method using a commercial kit (Amershan Pharmacia Biotech). Protein loading was monitored by Coomassie Blue staining and by probing of replicate gels with polyclonal β-actin antibody.

RESULTS

Homology Between the GSTP1 CRE and Other Functional CREs

We recently identified a single consensus CRE motif, 5′-CTACGTCA-3′, at −505 to −512 in the 5′-regulatory region of the human GSTP1 gene [Lo and Ali-Osman, 2001]. Table II shows that this CRE is highly homologous to and shares a six-nucleotide match to the palindromic consensus CRE (5′-TGACGTCA-3′) present in the somatostatin gene [Montminy et al., 1986; Andrisani et al., 1987]. The CRE sequence in the GSTP1 gene contains the minimal core sequence, 5′-CGTCA-3′, required for CRE functionality which is shared by consensus CREs in other cAMP-responsive genes [Fink et al., 1988]. The GSTP1 CRE motif belongs to the class of "imperfect" CREs present in a number of cAMP-responsive genes, including, those of β2-adrenergic receptor [Collins et al., 1990], phosphoenolpyruvate carboxykinase [Short et al., 1996], renin [Borensztein et al., 1994], c-fos [Sassone-Corsi et al., 1988], and insulin [Philippe and Missotten, 1990], tyrosine aminotransferase [Boshart et al., 1990], proenkephalin [Hyman et al., 1988], and lysyl oxidase [Ravid et al., 1999]. Sequence variations within CREs, as in the "imperfect" CREs, have, in some cases, been shown to result in alterations in binding affinity of the CRE to the CREB-1/ATF family of proteins [Benbrook and Jones, 1994]. Thus, the

TABLE II. Comparison of CRE Sequences Within the Human GSTP1 Gene and Other cAMP-Responsive Genes

GENE	Sequence	Location	Reference
hGSTP1 CRE	CTACGTCA	−512/−505	Lo and Ali-Osman [2001]
Somatostatin	TGACGTCA	−48/−41	Montminy et al. [1986]
Fibronectin	TGACGTCA	−173/−166	Dean et al. [1989]
β2-adrenergic receptor	GTACGTCA	−57/−50	Collins et al. [1990]
Phosphoenolpyruvate carboxykinase	TTACGTCA	−90/−83	Short et al. [1996]
Renin	TAGCGTCA	−225/−218	Borensztein et al. [1994]
c-fos	TGACGTTT	−66/−59	Sassone-Corsi et al. [1988]
	TGACGCAG	−297/−290	Berkowitz et al. [1989]
Insulin	TGACGTCC	−185/−178	Philippe and Missotten [1990]
Tyrosine aminotransferase	CTGCGTCA	−3651/−3644	Boshart et al. [1990]
Proenkephalin	CTGCGTCA	−93/−86	Hyman et al. [1988]
Lysyl oxidase	TTACGTGA	−100/−93	Ravid et al. [1999]

binding and functional studies described below were essential to better characterize and establish the functionality of the CRE in the GSTP1 gene.

CREB-1 bZIP Binds to the GSTP1 CRE with High Specificity and Affinity

The results of the analysis of binding of CREB-1 to the CRE in the human GSTP1 gene determined by DNA gel mobility shift and supershift assays using purified recombinant human CREB-1 polypeptide, CREB-1 bZIP (254–327), that contains the DNA binding and dimerization domains of CREB-1, and binds constitutively to CRE motifs, are summarized in Figure 1A. No shifted bands were observed in the absence of CREB-1 bZIP, or when CREB-1 antibody alone was incubated with the GSTP1 CRE motif. Two shifted bands, the lower one more prominent, were observed in the reactions containing CREB-1 bZIP and both bands showed increased binding intensity with increasing CREB-1 bZIP. The lower band was significantly out-competed by 25-, 50-, and 100-fold excess unlabeled somatostatin CRE, suggesting a higher binding specificity of this band relative to the upper band.

In the supershift analysis (Fig. 1A), two supershifted bands were also observed, corresponding to the complexes of CREB-1 antibody and the two bands observed in the gel shift studies. There was no difference in the supershift patterns of mixtures in which CREB-1 antibody was pre-incubated with CREB-1 bZIP and those in which antibody was added to a preformed CRE:CREB-1 bZIP complex.

The nucleotide sequence of the GSTP1 CRE differs only minimally from the "perfect" eight-nucleotide palindromic CRE in the somatostatin gene recognized by the CREB/ATF family of transcription factors [Sassone-Corsi et al., 1990; Andrisani, 1999]. Our results, Figure 1A, show that this minimal nucleotide variation did not affect the binding affinity or specificity of the GSTP1 CRE to the bZIP domain of CREB-1 protein. In contrast to controls with no CREB-1 bZIP, Figure 1B shows that CREB-1 bZIP bound strongly and concentration-dependently to the somatostatin CRE and the resulting complexes migrated similarly to the GSTP1 CRE/CREB-1 bZIP complex (Fig. 1A), when normalized for the shifted band intensity. The bound somatostatin CRE in the DNA:protein complex was effectively competed out with increasing amounts of unlabeled GSTP1 CRE. As with the GSTP1 CRE, identical supershift patterns were observed when the CREB-1 antibody was added to CREB-1 bZIP, both before and after incubation of the somatostatin CRE.

CREB-1 in Nuclear Extracts of Cells with Activated cAMP Signaling Pathway Binds to the GSTP1 CRE

Figure 2 shows the results of the studies on the binding affinity and specificity of CREB-1 to the GSTP1 CRE examined by DNA gel mobility shift and supershift assays using nuclear extracts from Calu-6 cells treated with 25 μM forskolin for 2 h. These extracts contain high levels of Ser133-phosphorylated CREB-1 (see Fig. 7). The gel shift results, Figure 2A, show two shifted bands in the reactions containing the GSTP1 CRE and nuclear extract, with the intensity of both bands increasing with increasing amounts of nuclear extract protein. In Figure 2B, the lower band was significantly out-competed by 100-fold excess unlabeled GSTP1 and somatostatin CREs, whereas, the higher band was competed out by only the somatostatin CRE. Figure 2B also shows that

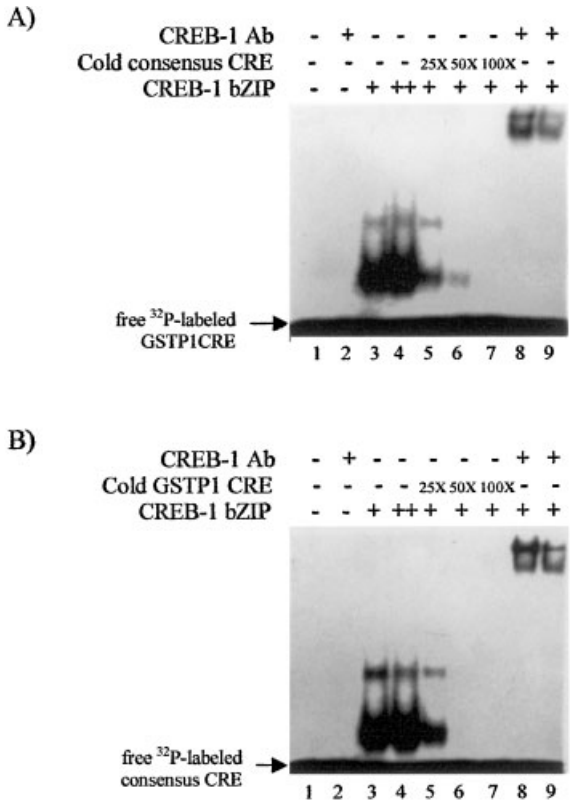

Fig. 1. CREB-1 bZIP binds to the GSTP1 and somatostatin CREs with high affinity. DNA gel mobility shift and supershift assays were performed using [γ-^{32}P]-ATP-endlabeled GSTP1 and somatostatin CREs. (**A**) Two nanograms of ^{32}P-labeled GSTP1 CRE was used in each reaction. The reaction mixtures in the lanes contained: **lane 1**, control; **lane 2**, 1 μg CREB-1 antibody; **lane 3**, 0.25 μg CREB-1 bZIP; **lane 4**, 0.5 μg CREB-1 bZIP. For **lanes 5, 6,** and **7**, 2 ng ^{32}P-labeled GSTP1 CRE was added after pre-incubation of 0.5 μg CREB-1 bZIP with 25-, 50-, and 100-fold excess of unlabeled somatostatin CRE. In **lane 8**, CREB-1 antibody was added to the pre-formed mixture of ^{32}P-labeled GSTP1 CRE and CREB-1 bZIP, while for **lane 9**, ^{32}P-labeled GSTP1 CRE was added following pre-incubation of CREB-1 bZIP with the CREB-1 antibody. (**B**) The reactions in **panel B** were performed similar to those in **panel A**, except that ^{32}P-labeled somatostatin CRE replaced the GSTP1 CRE. For lanes 5, 6, and 7, 25-, 50-, and 100-fold excess of unlabeled GSTP1 CRE was used. All mixtures were electrophoresed in an 8% non-denaturing polyacrylamide gel with 0.33 × TBE. The gel was dried on Whatman filter paper and exposed to X-ray films. Unbound ^{32}P-labeled CRE is indicated by the arrows.

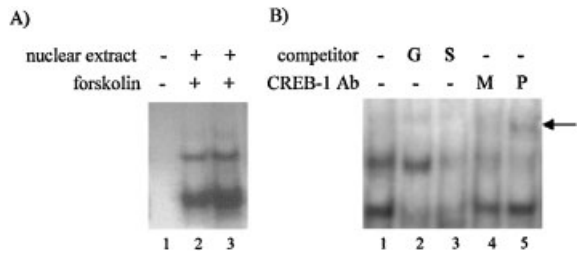

Fig. 2. CREB-1-containing nuclear extract binds to the GSTP1 CRE. DNA gel mobility shift and supershift assays were performed using [γ-^{32}P]-ATP-endlabeled GSTP1 CRE. Two nanograms of ^{32}P-labeled GSTP1 CRE was used in each reaction. (**A**) The reaction mixtures in the lanes contained: **lane 1**, control; **lane 2**, 2.5 μg nuclear extract from untreated Calu-6 cells; **lane 3**, 2.5 μg nuclear extract from forskolin-treated Calu-6 cells. (**B**) For the competition and supershift experiments, 2 ng ^{32}P-labeled GSTP1 CRE was added after pre-incubation of 2.5 μg nuclear extract isolated from forskolin-treated Calu-6 cells without (lane 1) and with 100-fold excess of unlabeled GSTP1 CRE (G, lane 2) and somatostatin CRE (S, lane 3). In **lanes 4** and **5**, 2 μg monoclonal CREB-1 antibody (M) and polyclonal CREB-1 antibody (P) were added to the pre-formed mixture of ^{32}P-labeled GSTP1 CRE and CREB-1. All mixtures were electrophoresed in an 8% non-denaturing polyacrylamide gel with 0.33 × TBE. The gel was dried on Whatman filter paper and exposed to X-ray films. The supershifted band containing the CREB-1:antibody:CRE complex is indicated by an arrow.

both CREB-1 antibodies supershifted the higher band, however, the polyclonal antibody was significantly more efficient than the monoclonal antibody in recognizing the complex between phospho-CREB-1 and the GSTP1 CRE.

CREB-1 bZIP Binds to the CRE in the GSTP1 Gene With High Specificity: Footprinting Analysis

The results of the *DNase I* footprinting analyses (Fig. 3) in which the reverse strand of the GSTP1 CRE-containing fragment, corresponding to nucleotide positions −549 to −501, was annealed to the forward strand and incubated with CREB-1 bZIP, or c-jun showed specific binding of CREB-1bZIP to the GSTP1 CRE. Lanes 1 and 2 in Figure 3 show the Maxam and Gilbert sequencing (C+T) of the GSTP1 CRE-containing fragment and the footprint control reaction without CREB-1 bZIP, respectively. CREB-1 bZIP (lane 3) strongly protected a core DNA region that corresponded to the CRE motif, whereas c-jun (lane 4) failed to protect the CRE motif, indicating binding specificity.

Mutations in the Core GSTP1 CRE Sequence Abolish CREB-1 bZIP Binding

Although, the binding affinity and specificity of CREB-1 to CREs is dependent on the structure of the CREs, the specific nucleotide sequence requirements for binding are still unclear. The results of the mutational analyses (Fig. 4) of the GSTP1 CRE performed with eight successive CRE mutants, each containing a single nucleotide substitution, showed that binding of CREB-1 bZIP was abolished in each of the CREs that was mutated within the core sequence, 5′-CGTCA-3′. In contrast, mutations in non-core

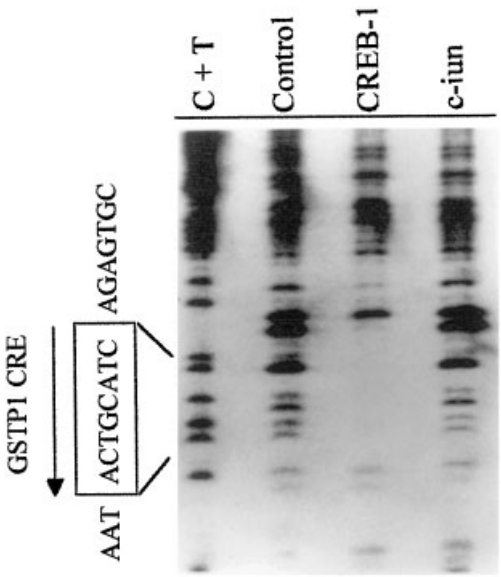

Fig. 3. CREB-1 binds to the CRE in the GSTP1 5′-regulatory region. *DNase I* footprinting assays were performed to examine the binding of CREB-1 bZIP to the GSTP1 CRE. The reverse strand of the GSTP1 CRE probe was ^{32}P-labeled, annealed to the forward strand, and chemically sequenced by the Maxam and Gilbert method (C+T) (**lane 1**), or incubated with no protein (**lane 2**), 1.25 μg CREB-1 bZIP (**lane 3**) and five footprint units of c-jun (**lane 4**). All incubations were performed at room temperature for 45 min, digested with four units of *DNase I*, and DNA fragment were extracted with phenol:chloroform:isoamyl alcohol and precipitated with ethanol, and electrophoresed in 10% polyacrylamide gels with 7 M urea in 0.5 × TBE running buffer. The gels were dried and autoradiographed at −80°C.

nucleotides, namely, the first two 5′-nucleotides in mutant 5 (C→A at nt 1) and mutant 6 (T→A at nt 2), as well as, mutation of A→T in nt 3 (mutant 1), decreased CREB-1 bZIP binding only partially.

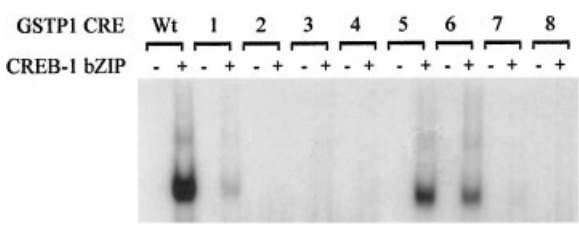

Fig. 4. Mutations in the core GSTP1 CRE sequence abolish CREB-1 bZIP binding. DNA gel mobility shift and supershift assays were performed using [γ-^{32}P]-ATP-endlabeled GSTP1 and somatostatin CREs and mutant CREs, as described in ''Materials and Methods''. Two nanograms of ^{32}P-labeled wild-type and mutant GSTP1 CRE were incubated without (−) and with (+) 0.25 μg CREB-1 bZIP. All mixtures were electrophoresed in an 8% non-denaturing polyacrylamide gel with 0.33 × TBE. The gel was dried on Whatman filter paper and exposed to X-ray films.

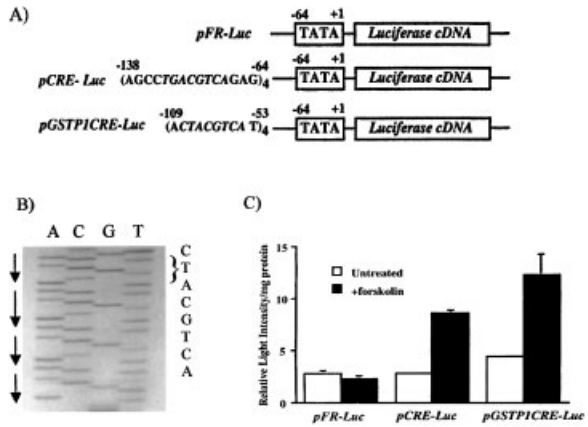

Fig. 5. GSTP1 CRE is required for the cAMP-induced luciferase gene activation. (**A**) The structure of the 5′-promoter region of the vector constructs, *pFR-Luc*, *pCRE-Luc*, and *pGSTP1CRE-Luc* is summarized. Each of the reporter plasmids contains a TATAA box in its basal promoter. Four copies of CREs are present in *pCRE-Luc* and *pGSTP1CRE-Luc*. The CRE sequences are in italics. Nucleotide positions are numbered relative to the translation start site of the luciferase cDNA. (**B**) Nucleotide sequence of the GSTP1 CREs in *pGSTP1CRE-Luc* confirmed by Maxam-Gilbert DNA sequencing method. (**C**) GSTP1 CREs transcriptionally activates a TATAA box-containing basal promoter. *Firefly* luciferase expression vectors containing the somatostatin CREs (*pCRE-Luc*) or the GSTP1 CREs (*pGSTP1CRE-Luc*) were transfected into Calu-6 cells as described in ''Materials and Methods.'' Twenty-four hours after transfection, cells were treated with vehicle (0.025% DMSO) or 25 μM forskolin for another 24 h. Parental *firefly* luciferase expression plasmid (*pFR-Luc*) and *Renilla* luciferase expression plasmid (*pRL-CMV*) were used as negative controls and for monitoring transfection efficiency, respectively. Luciferase activity is expressed as relative light intensity normalized for transfection efficiencies and protein content. The results represent the average of three independent transfections.

Presence of the GSTP1 CRE is Required for Induction of Luciferase Reporter Gene Expression by cAMP

Figure 5 summarizes the results of these analyses of the functionality of the GSTP1 CRE examined by its ability to activate a TATAA box-containing luciferase reporter gene following activation of the cAMP pathway. The structure of the three luciferase expression plasmid vectors, *pFR-Luc*, *pGSTP1CRE-Luc*, and *pCRE-Luc*, are shown in Figure 5A. The nucleotide sequence and direction of the GSTP1 CREs in *pGSTP1CRE-Luc* was confirmed by the Sanger's DNA sequencing method [Sambrook et al., 1989] and the results are shown in Figure 5B.

Figure 5C shows that 24 h following treatment of the transfected cells with 25 μM forskolin, there was a significant threefold

increase in luciferase activity in the cells transfected with *pCRE-Luc*, and 2.8-fold in cells transfected with *pGSTP1CRE-Luc*. No significant luciferase induction was observed following forskolin treatment of cells transfected with the parent vector, *pFR-Luc*.

The GSTP1 Gene is Transcriptionally Activated in Cells following Activation of the cAMP Signaling Pathway

The results of the studies on the effect of activation of the cAMP/CREB-1 pathway on the transcriptional activity of the GSTP1 gene in Calu-6 cells are summarized in Table III and in Figure 6. Table III summarizes the result of examination of cAMP levels in Calu-6 cells treated with 25 μM forskolin for 0, 30, and 60 min and shows that forskolin effectively and significantly increased the intracellular cAMP levels in Calu-6 cells. Thirty minutes following treatment, cAMP levels increased by 12-fold above baseline levels, declining slightly to, approximately ninefold, after 60 mins.

The results of the effect of the activation of the cAMP pathway on GSTP1 gene transcription are summarized in the Northern blots in Figure 6A. Thirty minutes after exposure of Calu-6 cells to 25 μM forskolin, there was a rapid sevenfold increase in the level of GSTP1 gene transcripts relative to controls. This increase in GSTP1 transcripts continued time-dependently to a maximum of 12-fold at 2 h. Figure 6B shows that the forskolin-activated GSTP1 transcription in Calu-6 cells was suppressed dose-dependently by a 2-h pre-treatment of the cells with 0.1, 1.0, and 10 μM of the adenylate cyclase inhibitor, DDA.

TABLE III. Induction of Intracellular cAMP Following Treatment of Calu-6 Cells With Forskolin

Forskolin treatment (min)	cAMP concentration (pmol/mg protein)	Fold-increase
0	145.7 ± 1.2	—
30	1,714.3 ± 5.6	12.0
60	1,285.7±4.5	8.8

Intracellular cAMP levels were determined by enzyme immunoassay in extracts of exponentially growing Calu-6 cells treated with 25 μM forskolin for 0, 30, and 60 min. A standard curve was generated using serially diluted cAMP standards and used to calculate cAMP concentrations in cell lysates. The cAMP concentrations are expressed as pmol/mg protein after normalization with total protein content of the cell lysates, as determined by the Bradford assay.

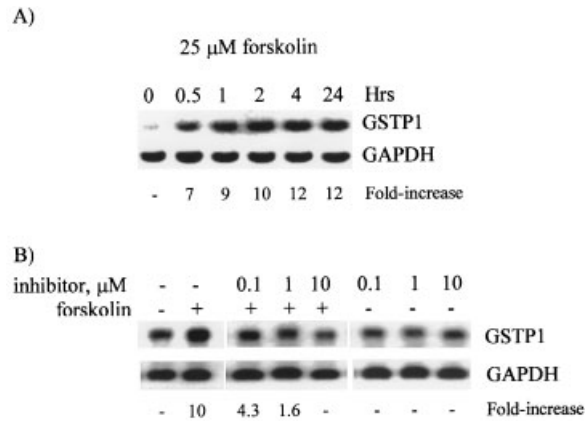

Fig. 6. GSTP1 gene expression is activated in cells with activated cAMP/CREB-1 signaling pathway. (**A**) Northern blot analyses were performed to determine whether transcription of the GSTP1 gene is affected following activation of the cAMP/CREB-1 signaling pathway. Calu-6 cells were treated with 25 μM forskolin for 0 h (**lane 1**), 0.5 h (**lane 2**), 1 h (**lane 3**), 2 h (**lane 4**), 4 h (**lane 5**), and 24 h (**lane 6**). (**B**) To suppress the activation of the cAMP/CREB-1 signaling pathway, Calu-6 cells were pre-treated with 0 μM (lanes 1 and 2), 0.1 μM (lanes 3 and 6), 1 μM (lanes 4 and 7) and 10 μM (lanes 5 and 8) DDA for 2 h prior to forskolin (lanes 2, 3–5) for 2 h. Total RNA was extracted and subjected to Northern analysiss, as described in ''Materials and Methods.'' Fold-increase of GSTP1 transcripts was determined following normalization against control GAPDH transcript levels and the levels of GSTP1 transcripts in the untreated control.

Intracellular GSTP1 and Phospho-CREB-1 (Ser133) Protein Levels are Elevated Following Activation of cAMP Signaling

Figure 7 shows the results of the analysis of CREB-1 phosphorylation following forskolin treatments of Calu-6 cells and indicates a strong correlation of CREB-1 phosphorylation with increased GSTP1 protein levels. The Western blots show a rapid, fivefold increase, in GSTP1 protein levels 30 min after forskolin treatment, with a peak at 2 h post-treatment. Simultaneously, levels of phosphorylated CREB-1 (Ser133), normalized against total CREB-1, increased by a significant sevenfold at 30 min following forskolin treatment. The extent of CREB-1 phosphorylation continued to increase, peaking at 10-fold relative to untreated controls at 4 h following forskolin treatment.

DISCUSSION

The results presented in this study demonstrate that the human GSTP1 gene is transcriptionally activated by the cAMP signaling pathway and that interaction between activated CREB-1 and the GSTP1 CRE is essential

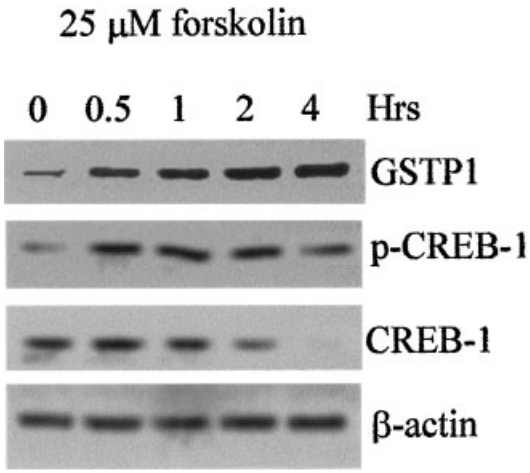

Fig. 7. Both GSTP1 and phospho-CREB-1 proteins are elevated in cells with activated cAMP/CREB-1 signaling pathway. Calu-6 cells were treated with 25 μM forskolin for 0 h (**lane 1**), 0.5 h (**lane 2**), 1 h (**lane 3**), 2 h (**lane 4**), and 4 h (**lane 5**), harvested and subjected to protein extraction and Western blot analyses. The levels of GSTP1, total CREB-1 protein, phospho-CREB-1 and β-actin proteins in the lysates were determined as described in ''Materials and Methods.''

for this transcriptional activation. The cAMP/CREB-1 signaling pathway may thus be an important mechanism for the regulation of GSTP1 gene expression in tumor cells, and may contribute to the increased and heterogeneous GSTP1 expression observed in most tumors.

The induction of GSTP1 gene transcription following activation of the cAMP signaling pathway occurred early, within 30 min, of adenylate cyclase activation, characteristic of other CRE-containing genes that are responsive to cAMP [Gonzalez and Montminy, 1989; Lee and Masson, 1993; Andrisani, 1999]. As has been reported for other cAMP responsive genes, the significant, sevenfold, increase in the level of GSTP1 gene transcripts correlated with increased levels of CREB-1 phosphorylation at Ser133, but without CREB-1 de novo synthesis [Gonzalez and Montminy, 1989; Lee and Masson, 1993; Andrisani, 1999]. The sustained increase in GSTP1 transcript levels, 24 h after forskolin exposure, is also similar to that reported for several CRE-containing genes, including, those of lysyl oxidase in vascular smooth muscle cells [Ravid et al., 1999], pre-proenkephalin, and pre-prodynorphin [Simpson and McGinty, 1995].

Transcription factors of the CREB-1/ATF family are substrates of PKA and mediate gene expression following intracellular elevation of cAMP [Borrelli et al., 1992; Shaywitz and Greenberg, 1999]. Physiologically, elevation of intracellular cAMP levels results from the conversion of ATP to cAMP by activated membrane-bound adenylate cyclases. Adenylate cyclases are activated by receptor coupled G-proteins, which themselves are activated following interaction of ligands (hormones and neurotransmitters) with receptors of the sevenless transmembrane receptor-family [Dixon et al., 1986; Borrelli et al., 1992]. Transcriptional activation via cAMP signaling involves the binding of cAMP to PKA, resulting in the release of regulatory PKA subunits from the catalytic subunits. The free PKA catalytic subunits then translocate into the nucleus and activate CREB-1 by phosphorylating it at Ser133. The activated CREB-1 binds to the CRE of the targeted gene and recruits the adaptor protein, CBP, through the kinase-inducible domain containing the phosphorylated Ser133. Further recruitment of RNA helicases A, RNA polymerase II and transcription factor complex leads ultimately to gene transcription [Borrelli et al., 1992; Andrisani, 1999].

Many reports have established that multiple signaling pathways are involved in both the activation of CREB-1 phosphorylation at Ser133 and the subsequent transactivational function of phospho-CREB-1 [Lee and Masson, 1993; Andrisani, 1999]. These signaling pathways include those that are activated by cellular stress, membrane depolarization/Ca^{+2} influx, and by growth factors (nerve growth factors, NGFs, and epidermal growth factors, EGFs), and inflammatory cytokines, including, interleukin 1, IL-1, and tumor necrosis factor-α, TNF-α. Following activation of these signaling pathways, multiple kinases become activated that are capable of phosphorylating CREB-1 at Ser133, such as, extracellular-signal regulating kinases/pp90, ribosomal S6 kinases 1–3, Ca^{2+}/calmodulin-dependent kinases II/IV, and p38/MAPKAP Kinase-2 [Lee and Masson, 1993; Andrisani, 1999]. Although, our data demonstrate that the human GSTP1 gene is activated by the activated cAMP signaling pathway, it is possible that other signaling pathways may also be involved in GSTP1 gene activation via the GSTP1 CRE. Since the GSTP1 gene contains a functional CRE with high affinity for CREB-1, the signaling pathways that activate CREB-1, including, those mediated by NGFs, EGFs, Ca^{2+} influx, IL-1, TNF-α, and cellular stress, may

activate GSTP1 gene transcription. Future studies are needed to examine these possibilities.

The detailed functional characterization of the CRE motif in the human GSTP1 gene performed in this study demonstrates that the GSTP1 CRE, which belongs to the class of non-palindromic, asymmetric, "imperfect" CREs, is a functional *cis*-element with high affinity and specificity for activated CREB-1 and with significant functional transcriptional activity in response to cAMP. Approximately 50% of reported CREs belong to this class of "imperfect" CREs. The GSTP1 CRE shows high similarity (only one nucleotide mismatch) to CREs in several genes, including, those encoding β2-adrenergic receptor [5′-GTACGTCA-3′; Collins et al., 1990], phosphoenolpyruvate carboxykinase [5′-TTACGTCA-3′; Short et al., 1996], tyrosine aminotransferase [5′-CTGCGTCA-3′; Boshart et al., 1990], and proenkephalin [5′-CTGCGTCA-3′; Hyman et al., 1988].

The structural requirements of CREs for binding to CREB-1 are still a subject of controversy. Benbrook and Jones [1994] found no correlations between the symmetrical nature of CRE and either their binding affinities to CREB-1 or their transactivational abilities. Furthermore, Deutsch et al. [1988] showed that the CREB-1 binding and gene transactivating abilities of CREs were strongly affected by sequences adjacent to the CREs but not by the core palindromic sequences. The results of our functional and mutational analysis of the GSTP1 CRE presented in this study support these observations. Our results of the CRE mutational analysis clearly indicate that the asymmetrical core sequence, 5′-CGTCA-3′, is critical for CREB-1 bZIP binding. These findings are also in agreement with those made by Fink et al. [1988] demonstrating that the minimal core sequence, 5′-CGTCA-3′, required for CRE functionality is shared by consensus CREs in many cAMP-responsive genes. The GSTP1 CRE containing this core sequence binds to CREB-1 bZIP despite its reported weak binding to the CREB-1/ATF family of proteins [Nicolson et al., 1993]. The high binding affinity of CREB-1 to the GSTP1 CRE may be due to the three flanking nucleotides, 5′-CTA-3′, that are proximal to the asymmetric core sequence, which may provide for a more symmetric CRE structure. This is supported by the results of the mutational analysis which showed that CREB-1 bZIP binding to the CRE was significantly reduced when the third 5′-nucleotide of the GSTP1 CRE was mutated from A → T.

Our data suggest that GSTP1 and somatostatin CREs may interact differentially with herterodimers of the CREB/ATF1 family of transcription factors. This is most evident in the results of the gel shift studies in which the somatostatin CRE competed out both shifted bands while the GSTP1 CRE out competed only the lower band. Further studies will be needed to clarify whether the GSTP1 CRE interacts with heterodimers of transcription factors of the CREB/ATF1 family and, if so, whether such interaction occurs with a similar affinity as with the CREB-1 homodimers.

Aberrant cAMP signaling is a common characteristic of neoplastic cells. Increased activities of PKAs and CREB-1 are frequently reported and associated with tumorigenesis, increased tumor growth, resistance to chemo- and radiation therapy, and metastasis [Cho-Chung et al., 1995; Xie et al., 1997; Ciardiello and Tortora, 1998; Jean et al., 1998]. The product of the GSTP1 gene has also been associated with many of these phenotypic characteristics of tumors suggesting an inter-relationship between increased activation/activity of PKAs and CREB-1 and the significant up-regulation of the GSTP1 gene associated with the malignant process. Furthermore, inhibition of cAMP signaling pathway has been shown pre-clinically and clinically to suppress tumor growth, resistance to chemo- and radiation therapy, and metastasis [Xie et al., 1997; Jean et al., 1998; Wang et al., 1999; Nesterova et al., 2000; Tortora et al., 2000]. The data we present in this study linking activation of the cAMP signaling pathway with GSTP1 transcriptional activation suggest that these two mechanisms may interact to regulate the neoplastic process and that the anti-tumor effect of strategies targeting the PKA pathways may be, in part, a result of down regulation of the GSTP1 gene expression. Furthermore, the identification of the GSTP1 gene as an early cAMP response gene describes a novel CRE/CREB-1-dependent mechanism of cAMP-mediated GSTP1 gene expression and may contribute to a better understanding of the cAMP-dependent response of normal cells to stress and growth factors.

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