Human gastrin-releasing peptide receptor gene regulation requires transcription factor binding at two distinct CRE sites

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Chinnappan D, Qu X, Xiao D, Ratnasari A, Weber HC. Human gastrin-releasing peptide receptor gene regulation requires transcription factor binding at two distinct CRE sites. Am J Physiol Gastrointest Liver Physiol 295: G153–G162, 2008. First published May 15, 2008; doi:10.1152/ajpgi.00036.2008.—Ectopic expression of the gastrin-releasing peptide (GRP) receptor (GRP-R) occurs frequently in human malignancies of the gastrointestinal tract. Owing to paracrine and autocrine interaction with its specific high-affinity ligand GRP, tumor cell proliferation, migration, and invasion might ensue. Here we provide the first insights regarding molecular mechanisms of GRP-R regulation in gastrointestinal cancer cells. We identified by EMSA and chromatin immunoprecipitation assays two cAMP response element (CRE) binding sites that recruited transcription factor CRE binding protein (CREB) to the human GRP-R promoter. Transfection studies with a wild-type human GRP-R promoter reporter and corresponding CRE mutants showed that both CRE sites are critical for basal transcriptional activation in gastrointestinal cancer cells. Forced expression of cAMP-dependent effectors CREB and PKA resulted in robust upregulation of human GRP-R transcriptional activity, and this overexpression strictly required intact wild-type CRE sites. Direct cAMP stimulation with forskolin resulted in enhanced human GRP-R promoter activity only in HuTu-80 cells, but not in Caco-2 cells, coinciding with forskolin-induced CREB phosphorylation occurring only in HuTu-80 but not Caco-2 cells. In summary, CREB is a critical regulator of human GRP-R expression in gastrointestinal cancer and might be activated through different upstream intracellular pathways.

THE REGULATORY GASTRIN-RELEASING PEPTIDE (GRP), the mammalian homologue of the amphibian peptide bombesin, is widely distributed in the central nervous system and gastrointestinal tract, where important physiological functions include regulation of satiety, thermoregulation, smooth muscle contraction, and the release of other peptide hormones (15, 27, 42). Recent studies in mice elucidated that expression of the GRP receptor (GRP-R) in the central nervous system is associated with learned fear (34) and its expression in the spinal cord mediates the itch sensation (40). GRP exerts its effects through activation of specific, high-affinity G protein-coupled receptor (GRP-R) (1, 15, 20, 27, 38, 39). Robust human GRP-R (hGRP-R) expression was detectable in the normal human pancreas and stomach, but normal human colon, lung, and prostate tissues were negative for hGRP-R expression (45). Substantial evidence has accumulated that both GRP and its receptor are aberrantly expressed in a variety of human malignancies including those of gastrointestinal origin, where they may initiate intracellular signals of cell proliferation, differentiation, and migration through autocrine and paracrine mechanisms (13, 17, 20, 23, 27). But molecular mechanisms of ectopic hGRP-R expression and its regulation in tumorigenesis still remain elusive. Previously, robust basal promoter activity was determined in various gastrointestinal and prostate cancer cells which required genomic sequence between 97 and 247 bp upstream of the transcription start including a putative consensus CRE site at 112 bp upstream of the RNA start (26). This begged the question whether the CRE sites might recruit CREB transcription factors and whether CREB is involved in transactivation of the hGRP-R gene.

The cyclic AMP pathway is a widely used signaling process that mediates the responses of cells to hormones, growth factors, and neurotransmitters to the downstream effector. CREB has been recently proposed to possess a direct role in carcinogenesis (35) and represents a very important transcription factor in the regulation of numerous genes mainly through activation of protein kinase A (16, 31). By comparison, in gastrointestinal cancer cells, studies regarding the cAMP signaling-dependent pathways as well as its downstream effectors and target genes have been limited (3, 4, 8, 9, 18, 22, 25, 28, 33, 37, 43). Therefore, the aim of this study was to establish whether transcription factor CREB might interact with consensus CRE sites within the hGRP-R gene and determine its role in receptor regulation. We identified two functional CRE sites within 1 kb of the immediate hGRP-R promoter region. Our results demonstrated that both wild-type (wt) CRE sites bind CREB transcription factor and that cooperative recruitment was necessary for basal and CREB/PKA-induced hGRP-R transcriptional activation. Our data suggest a novel molecular mechanism of CREB transcription factor complex formation at two distinct CRE sites of the hGRP-R promoter that may regulate basal and cAMP effector-dependent hGRP-R transcription in gastrointestinal cancer cells.

MATERIALS AND METHODS

Reporter gene construct and site-directed mutagenesis. As described in detail previously (26), the 1,397-bp hGRP-R promoter fragment was generated by PCR using P1 DNA as template and subsequently cloned into the pGL3-basic luciferase reporter vector (Promega, Madison, WI) and referred to as −1120hGRP. Mutations of four core nucleotides in the putative cyclic AMP response element (CRE) sites CRE1 and CRE2 (ACGT → GTAC) in the reporter plasmid −1120hGRPR were introduced by using the QuickChange kit (Stratagene; La Jolla, CA). Synthetic oligonucleotides used in mutagenesis and EMSA are listed in Fig. 1. The authenticity of mutations was verified by restriction enzyme analysis and automated DNA sequencing of the recombinant plasmid DNA. Large-scale
Differences between relative luciferase activity levels were considered significant if the Student’s t-test resulted in P values <0.05.

**EMSA and supershift assay.** Nuclear extracts were prepared from confluent HuTu-80 and CaCo-2 cells as described by Inan et al. (11). Oligonucleotides were synthesized (Invitrogen; Carlsbad, CA) corresponding to the wt hGRP-R genomic CRE1 and CRE2 sites and their respective mutations at −112 and −1108 bp, respectively, upstream of the transcription start site as shown in Fig. 1. The CRE consensus oligonucleotide was synthesized according to the sequence provided by Promega. Synthetic oligonucleotides were annealed and double-stranded DNA probes were then 5'-end labeled with T4 polynucleotide kinase (Promega) and [γ-32P]ATP (PerkinElmer, Boston, MA). Binding reactions were performed by mixing 5 μg of nuclear extract with 20,000 cpm of 5'-end-labeled DNA probes in 10 μl of EMSA buffer [10 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 5% glycerol and 0.05 mg/ml poly(dI-dC)-(dI-dC)]. For competition experiments, a 100-fold molar excess or as indicated of competitor oligonucleotide was also added to the mixture. After incubation at room temperature for 20 min, the mixture was separated in a 6% nondenaturing polyacrylamide gel in 0.5 × Tris borate-EDTA electrophoresis buffer at 4°C followed by gel drying and autoradiography. For supershift assays, 2 μg of antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were incubated with nuclear extract for 20 min at room temperature before addition of radiolabeled DNA probes. Experiments were repeated at least twice.

**Western blot.** Caco-2 and HuTu-80 cells were lysed in sample buffer [62.5 mM Tris-HCl (pH 6.8 at 25°C) 2% w/v SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% wt/vol bromophenol blue]. Equal amounts of protein from the different treatments were loaded in 10% SDS-polyacrylamide gel as soon as it resolved, transferred on to nitrocellulose, then incubated with primary antibody at 4°C overnight and the secondary antibody for 1 h. The antigen-antibody complex was detected with ECL reagent (Amersham Pharmacia Biotech, Arlington Heights, IL). Experiments were performed at least twice.

**ChIP assay.** Caco-2 and HuTu-80 cells were cultured in DMEM, and chromatin immunoprecipitation (ChIP) assay was performed according to the manufacturer’s recommendation (Millipore, Temecula, CA). Briefly, cells were treated with 1% formaldehyde at 37°C for 10 min, washed with cold 1× PBS twice, and centrifuged at 4°C for 4 min at 2,000 rpm. Pellets were resuspended in 250 μl of lysis buffer. After incubation on ice for 10 min, lysates were sonicated to shear DNA to a length between 300 bp to 1 kb while the sample was kept on ice. After sonication, the samples were microcentrifuged at 14,000 rpm for 10 min at 4°C and the sonicated cell suspension was diluted with ChIP dilution buffer. Pellets of precleared chromatin samples were divided into four tubes and incubated at 4°C rotator overnight with 1 μg of anti-CREB antibody or 1 μg rabbit IgG (Santa Cruz Biotechnology). Immunoprecipitated samples and input samples were then digested and incubated with proteinase K at 45°C overnight to reverse the cross-linking. DNA fragments were recovered by phenol/chloroform extraction and ethanol precipitation. Primers encompassing the regions of the putative CREB binding sites CRE1 were sense: 5'-tagtggagcaaggattgg-3', antisense: 5'-ctcaaatcctatc-3', and, for CRE2, sense: 5'-ctctacctcctccct-3', antisense: 5'-ggctcagcttccctccg-3' to yield amplicons of 200- and 237-bp size, respectively. PCR was run for 35 cycles and amplicons were analyzed in 2% agarose gel electrophoresis. Independent experiments were repeated at least twice.

**Statistical analysis.** Results are expressed as means ± SE. Student’s t-test was utilized for statistical analysis. A P value of 0.05 or less was considered statistically significant.

**RESULTS**

Characterization of basal hGRP-R promoter activity in gastrointestinal cancer cells. We have previously demonstrated that genomic sequence between 97 and 247 bp upstream...
of the hGRP-R transcription start was critical for its promoter activity in gastrointestinal cancer cells (26, 45). A schematic hGRP-R gene structure and all oligonucleotides used in this study are depicted in Fig. 1. To determine the role of the CRE sites in the human GRP-R gene, we first tested the basal hGRP-R promoter activity in transient transfection experiments in both gastrointestinal cancer cell lines. As reported previously (26), we confirmed robust promoter activity using the wt −1120hGRPR promoter reporter (Fig. 2) resulting in ∼10-fold enhancement compared with control (Fig. 2). When corresponding mutant plasmid reporters of either the CRE1 and CRE2 sites alone or their combined mutation in −1120hGRPR (−1108/−112mut) were tested, we demonstrated significant reduction of hGRP-R promoter activity compared with the wt promoter reporter (Fig. 2) in all instances that were tested. In Caco-2 cells, inactivation of either one CRE site alone resulted in a significant 70% reduction and mutation of both in the −1108/−112mut promoter reporter yielded an activity less than promoterless control levels (0.4 ± 0.1; P < 0.05). In HuTu-80 cells, we observed virtually a complete loss of transcriptional activity when CRE2 alone was inactivated or in combination with CRE1, whereas mutation of CRE1 alone only resulted in a 50% (10.8- to 5.3-fold) reduction compared with wt genomic sequence (Fig. 2). These data suggest strongly that both CRE sites are significantly involved in the regulation of basal transcriptional hGRP-R activity in gastrointestinal cancer cells, whereby inactivation of both sites results in loss of promoter activity in Caco-2 and HuTu-80 cells, and inactivation of the distal CRE2 site alone also abolished basal hGRP-R transcriptional activity in HuTu-80 cells.

Overexpression of cAMP pathway-dependent effectors and hGRP-R promoter activity. Owing to the critical role of both CRE sites in transcriptional activation as shown above, we first tested the responsiveness of the −1120hGRPR reporter to stimulation of cAMP-dependent signals with forskolin (25 μM). Promoter activity of −1120hGRPR in duodenal cancer HuTu-80 cells significantly increased from 7.8- to 15.4-fold (P < 0.05) compared with control (Fig. 3) whereas stimulation of all CRE mutant constructs did not result in increased transcriptional activity (data not shown). Furthermore, in colon cancer cells Caco-2 there was no significant enhancement of hGRP-R promoter activity with forskolin (data not shown). We then tested the ability of forced expression of cAMP-dependent effector molecules PKA α subunit and CREB to regulate hGRP-R expression as measured by transient transfection of the −1120hGRPR promoter reporter and its corresponding CRE mutants. Because in HuTu-80 cells −1120hGRPR showed responsiveness to forskolin stimulation, we chose to use the more upstream cAMP effector PKA α subunit in HuTu-80 cells and CREB in Caco-2 cells. Forced expression of the PKA α subunit and CREB resulted in a significant (P < 0.05) increase of promoter activity in HuTu-80 (3.5-fold) and Caco-2 cells (3.7-fold), respectively (Fig. 4). In similar experiments using the −1120hGRPR CRE mutant constructs, we observed a significantly reduced PKA-dependent hGRP-R enhancement in HuTu-80 cells compared with the wt −1120hGRPR reporter. CRE1 and CRE2 mutants alone reduced the PKA-dependent increase significantly by 23 and 40%, respectively, in HuTu-80 cells, but changes observed in Caco-2 cells were not significant (Fig. 4). The CRE double-mutant −1108/−112mut yielded an even further reduced PKA-induced enhancement in HuTu-80 cells to 40% of wt −1120hGRPR levels. This was also significantly lower than the reduction detected with single CRE site mutants (Fig. 4A). In Caco-2 cells, CREB-induced hGRP-R promoter activity enhancement was significantly reduced to unstimulated levels in the CRE double mutant. The 43% reduction that was observed with the CRE1 single mutant demonstrated a strong trend but did not quite reach statistical significance.
HuTu-80 cells) and CREB (cotransfected along with expression plasmids of the plasmid and corresponding CRE mutant constructs as described in Fig. 1 were activity and is dependent on intact CRE sites. The wt HuTu-80 and Caco-2 cells, respectively, enhanced hGRP-R transcriptional Fig. 4. Forced expression of PKA and CRE binding protein (CREB) in HuTu-80 and Caco-2 cells, respectively, enhanced hGRP-R transcriptional activity. Double mutant/CREB (* P < 0.001 Student's t-test) levels. These data suggest that the observed transcriptional upregulation of hGRP-R by cAMP-dependent effector molecules as measured by promoter reporter assays was mediated via transcription factor CREB.

Recruitment of CREB transcription factors at two CRE sites within the hGRP-R promoter. To characterize the DNA-protein binding pattern on both CRE1 and CRE2 sites within the hGRP-R promoter, EMSA and supershift assays were performed using synthetic oligonucleotides as listed (Fig. 1) and nuclear extracts were isolated from HuTu-80 and Caco-2 cells. Specific protein binding ability was demonstrated on the CRE1 and CRE2 sites in HuTu-80 and Caco-2 cells in a distinct pattern using wt, radioactively labeled DNA probes and corresponding competition experiments with unlabeled primers (Fig. 6, 7, and 8). Nuclear extracts from Caco-2 cells at the CRE1 (~112bp) site yielded three major protein complexes A, B, and C, of which complex A was specific because competition with increasing concentration of unlabeled primer (Fig. 6B, lanes 7–9) gradually abolished complex A. This specific DNA-protein binding complex consisted of two distinct bands A and B (Fig. 7A, lane 2). Similarly, a CRE consensus primer also abolished complex A (Fig. 6A, lanes 14–16), whereas cold DNA-binding domain and acts as a dominant repressor by forming an inactive dimer with CREB, blocking its ability to bind to CRE. As shown in Fig. 5 and observed in previous experiments, wt −1120hGRPR was significantly induced with a similar magnitude (P < 0.05) in the presence of PKA/CREB expression plasmids in both cell lines. However, cotransfection of KCREB in Caco-2 cells significantly reduced this effect to basal −1120hGRPR (0.9-fold) promoter levels. In HuTu-80 cells, KCREB expression not only abolished the PKA-dependent enhancement of hGRP-R promoter activity but also significantly reduced the activity (P < 0.01) to 40% of the wt −1120 hGRPR level (Fig. 5).

Fig. 5. Dominant-negative CREB overexpression abolished CREB/PKA-induced hGRP-R promoter activity. Cotransfection with and without the CREB dominant-negative expression killer CREB (KCREB) of the wt−1120hGRPR plasmid was performed in HuTu-80 (hatched bars) and Caco-2 cells (open bars) as described previously. Results represent data from at least 3 independent experiments, each performed in triplicate, and are expressed as the percent of relative luciferase activity of wt −1120hGRPR whose activity was arbitrarily set at 1. Overexpression of CREB/PKA resulted in significant increase of relative luciferase activity in HuTu-80 and Caco-2 cells (**P < 0.05 Student’s t-test). Inactivated CREB reduced CREB/PKA-induced activity to wt−1120hGRPR levels (**P < 0.05 compared with −1120hGRPR+CREB) in Caco-2 cells. In HuTu-80 cells activity was reduced significantly below wt−1120hGRPR (**P < 0.01 Student’s t-test) and −1120hGRPR+PKA (###P < 0.01 Student’s t-test) levels.

Dominant-negative CREB abolished PKA/CREB-dependent hGRP-R promoter induction. In view of the preceding results and in an effort to identify CREB a possible functional transcription factor involved in basal and PKA/CREB-induced hGRP-R regulation, we cotransfected HuTu-80 and Caco-2 cells with wt −1120hGRPR promoter construct in the presence of wt PKA and CREB expression vectors along with plasmid pCMV-KCREB (KCREB). KCREB encodes a mutant variant of the human CREB protein that contains mutations in its DNA-binding domain and acts as a dominant repressor by forming an inactive dimer with CREB, blocking its ability to bind to CRE. As shown in Fig. 5 and observed in previous experiments, wt −1120hGRPR was significantly induced with a similar magnitude (P < 0.05) in the presence of PKA/CREB expression plasmids in both cell lines. However, cotransfection of KCREB in Caco-2 cells significantly reduced this effect to basal −1120hGRPR (0.9-fold) promoter levels. In HuTu-80 cells, KCREB expression not only abolished the PKA-dependent enhancement of hGRP-R promoter activity but also significantly reduced the activity (P < 0.01) to 40% of the wt −1120 hGRPR level (Fig. 5).

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Fig. 5. Dominant-negative CREB overexpression abolished CREB/PKA-induced hGRP-R promoter activity. Cotransfection with and without the CREB dominant-negative expression killer CREB (KCREB) of the wt−1120hGRPR plasmid was performed in HuTu-80 (hatched bars) and Caco-2 cells (open bars) as described previously. Results represent data from at least 3 independent experiments, each performed in triplicate, and are expressed as the percent of relative luciferase activity of wt −1120hGRPR whose activity was arbitrarily set at 1. Overexpression of CREB/PKA resulted in significant increase of relative luciferase activity in HuTu-80 and Caco-2 cells (**P < 0.05 Student’s t-test). Inactivated CREB reduced CREB/PKA-induced activity to wt−1120hGRPR levels (**P < 0.05 compared with −1120hGRPR+CREB) in Caco-2 cells. In HuTu-80 cells activity was reduced significantly below wt−1120hGRPR (**P < 0.01 Student’s t-test) and −1120hGRPR+PKA (###P < 0.01 Student’s t-test) levels.
competition with a mutant primer did not affect it (Fig. 6A, lanes 12–13). Similar experiments with HuTu-80 nuclear extracts yielded two specific DNA-protein complexes on the CRE1 site as competition with a 100 molar excess of cold wt primer 1108 and mutant 112 primer did not affect protein binding pattern significantly (Fig. 6B). The top and bottom complex consisted of at least two distinct bands each which became apparent under different exposure conditions (Fig. 7B). The top and bottom complex of at least two distinct bands each, which became apparent under different exposure conditions (Fig. 7B).

Furthermore, in HuTu-80 cells at the CRE2 site at least four bands (A, B, C, D) in the two previously described complexes at both CRE sites (Fig. 7B; lanes 1; and 6B, lane 1) in the absence of antibodies. Antibodies against CREB-1 (24H4B and X-12; Santa Cruz) shifted band B or C to form a new band (CRE1: Fig. 7B, lanes 3 and 4; CRE2: Fig. 8B, lane 2). Anti-ATF1 antibodies resulted in a demonstrable diminution of band D at the CRE2 site (Fig. 8B, lane 3) and abolished band C at the CRE1 site (Fig. 7B, lane 5). Antibody against ATF-2 shifted band A at CRE1 (Fig. 7B, lane 2) and bands A and B were shifted at the CRE2 site (Fig. 8B, lane 4). A noticeable decrease of bands C and D was observed at the CRE2 site in the presence of anti-CRE modulator antibodies (Fig. 8B, lane 5), but not at the CRE1 site (data not shown). In Caco-2 cells at the CRE1 site three major protein complexes were detectable, of which bands A and B are greatly diminished and band C was abolished with ATF2 antibody, whereas YY1, SPI, and AP2 antibodies did not result in any substantial changes (Fig. 7A). When other antibodies were used in supershift assays in HuTu-80 and Caco-2 nuclear extracts, directed against ATF-3, ATF-4, ATF-6, SP-1, c-Jun/AP-1, and c-Fos, experiments failed to demonstrate any changes in the DNA-protein binding complex (data not shown).
shown). In summary, supershift assays demonstrated proteins of the CREB transcription factor family, including CREB, ATF-1, and ATF-2, binding to both CRE sites in the hGRP-R promoter in HuTu-80 cells and CREB and ATF-2 in Caco-2 cells. To further support these results with an independent experimental approach, we performed ChIP assay in HuTu-80 and Caco-2 cells to test binding of CREB at both CRE sites. Appropriate amplicons of 200-bp size were detectable at the CRE1 site and of 237 bp at the CRE2 site in both cell lines (Fig. 9). The results suggest strongly CREB protein binding at the −112CRE1 and −1108CRE2 sites in vivo in both HuTu-80 and Caco-2 cells.

**CREB phosphorylation occurs through different pathways in HuTu-80 and Caco-2 cells.** Prompted by results of transient transfection studies that demonstrated in HuTu-80 but not in Caco-2 cells, the enhancement of hGRP-R promoter activity in response to forskolin stimulation and PKA expression, we examined CREB phosphorylation by immunoblotting experiments. As shown in Fig. 10, in Caco-2 cells the baseline level of phosphorylated CREB is highly abundant compared with levels in HuTu-80 cells and remained unchanged subsequent to forskolin (25 μM) stimulation and PKA inhibitor H89 (20 μM) treatment. In contrast, in HuTu-80 cells CREB phosphorylation was induced by forskolin and the PKA inhibitor H89 abolished forskolin-induced phosphorylation activity.

**DISCUSSION**

The human GRP-R gene is aberrantly expressed in various different human malignancies including epithelial cancers from the gastrointestinal tract, prostate, and breast, whereby agonist-dependent receptor activation may result in cell proliferation, migration, and invasion (13, 15, 17, 20, 27, 45). In this present study we provide the first molecular insights regarding the regulation of human GRP-R in gastrointestinal cancer cells. Our data demonstrated the presence of two functional CRE sites spaced 1 kb apart within the human GRP-R promoter as binding sites of coordinated CREB transcription factor recruitment. CREB transcription factor binding to both CRE sites was determined critical for basal transcriptional hGRP-R activation as well as cAMP effector-dependent regulation in Caco-2 colon carcinoma and HuTu-80 duodenal carcinoma cells.

Both wt CRE sites are requisite not only for full hGRP-R promoter activity in gastrointestinal cancer cells (Fig. 2) but also for the full responsiveness of transcriptional hGRP-R upregulation to forced expression of PKA subunit α and CREB, respectively. In the case of inactivation of either single CRE site, responsiveness was significantly diminished only in HuTu-80 but not in Caco-2 cells (Fig. 4). Forced expression of a dominant negative CREB-inactivated mutant (Fig. 5) clearly showed that hGRP-R promoter upregulation via cAMP-dependent effector molecules was completely abolished in both cell lines, confirming the CREB-dependent mechanism of hGRP-R upregulation. Direct stimulation of the promoter reporter with forskolin resulted in transcriptional enhancement in HuTu-80 cells only but not in Caco-2 cells (Fig. 3), underscoring some important differences in hGRP-R regulation in gastrointestinal cancer cell lines used in this study. In HuTu-80 cells the transcriptional responsiveness of the human GRP-R promoter to forskolin-induced cAMP accumulation required both intact CRE sites. Additional transfection studies in Caco-2 cells with the wt −1120hGRP-R promoter and the corresponding CRE sites mutants demonstrated that overexpression of PKA did not result in transcriptional enhancement (data not shown). Taken together, these findings suggest that CREB is a common hGRP-R transactivator but may be targeted via distinct upstream pathways (16, 31) that may involve PKA-dependent mechanisms as observed in HuTu-80 cells and PKA-independent ones in Caco-2 cells, whereby alternative upstream ki-
nases for the latter case were not further searched. We provided further experimental evidence of this potentially important notion with immunoblot studies demonstrating the inability of forskolin to yield CREB phosphorylation in Caco-2 cells whereas in HuTu-80 cells it expectedly resulted in PKA-dependent CREB phosphorylation (Fig. 10). We speculate that substantial differences of phosphorylated CREB levels in both cell lines might account for either the ability to activate cAMP-dependent signal pathways (low CREB-P level in HuTu-80 cells) or the inability to do so possibly secondary to substrate deficiency (high CREB-P level in Caco-2 cells) as illustrated schematically in Fig. 11.

Similarly, in the epithelial neuroblastoma cell line SK-N-SH, forskolin also did not activate hGRP-R promoter activity; however, transcription factor Ets1 was reported to upregulate GRP-R expression by binding to the genomic region 169 to 159 bp (24). Although ets1 has been reported expressed in gastrointestinal cancer cells and its target genes have been associated with tumor invasiveness, cell migration, and metastasis (32), screening this genomic DNA region with EMSA in HuTu-80 and Caco-2 cells did not reveal any specific protein binding (data not shown), suggesting the possibility of cell-specific differences in transcription factor recruitment to the hGRP-R promoter.

In the hGRP-R promoter transcription factors of the CREB/ATF family including CREB-1, ATF-1, and ATF-2 (Figs. 6–8) were shown to be recruited to both CRE sites in HuTu-80 cells. Although extracts of Caco-2 cells did not show binding using the −1108CRE probe in EMSA, we provided the in vivo evidence that CREB can be recruited to both CRE sites by ChIP analysis (Figs. 9 and 11). It is also consistent with the findings in transfection experiments that showed that both CRE motifs are required for the basal and regulated hGRP-R pro-

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**Fig. 8.** Transcription factors of the CREB family bind to the −1108CRE site in HuTu-80 cells. A (lanes 1–4): EMSA of nuclear extracts from HuTu-80 cells using the −1108CRE primer. Cold competition (100 × access) abolished all bands (lane 2), whereas competition with the −112CRE wt primer and −1108CREmut primer does not alter the binding pattern (lanes 3 and 4). B: the DNA-protein complex for −1108 probe showed 4 distinct bands A, B, C, and D (lane 1). Antibodies against CREB, ATF-1, and ATF-2 shifted band C; almost abolished band D, and shifted bands A and B, respectively. Using anti-CREM antibody (Santa Cruz) resulted in reduced binding of bands C and D (lane 5). Anti-ATF-3 and ATF-4 antibodies did not result in any change of the DNA-protein complex (lanes 6 and 7). Representative results of several experiments are shown.

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**Fig. 9.** Chromatin immunoprecipitation (ChIP) demonstrates in vivo binding of CREB to genomic DNA from HuTu-80 and Caco-2 cells. ChIP was performed as described in MATERIALS AND METHODS. Formaldehyde cross-linked chromatin samples from HuTu-80 and Caco-2 cells were used for immunoprecipitation (IP) with antibodies against CREB. DNA aliquots from PCR reactions performed on the extracts before immunoprecipitation (Input) were loaded on the same gel. PCR resulted in expected amplicons of 200 and 237 bp in size for the CRE1 and CRE2 sites, respectively. Representative results of several experiments are shown.

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**Fig. 10.** The hGRP-R promoter transcription factors of the CREB family including CREB-1, ATF-1, and ATF-2 (Figs. 6–8) were shown to be recruited to both CRE sites in HuTu-80 cells. Although extracts of Caco-2 cells did not show binding using the −1108CRE probe in EMSA, we provided the in vivo evidence that CREB can be recruited to both CRE sites by ChIP analysis (Figs. 9 and 11). It is also consistent with the findings in transfection experiments that showed that both CRE motifs are required for the basal and regulated hGRP-R pro-
motor activity in Caco-2 cells. Our findings represent an unusual but not unprecedented complex mechanism of gene regulation via multiple cis-regulatory CRE motifs (2, 5–7, 10, 12, 14, 19, 21, 29, 30, 36, 41). Among those previous studies (2, 5, 12, 14, 19, 30, 41), spacing of two functional CRE sites was generally reported to occur within 100–200 bp of each other within close proximity to the RNA start sites. However, in the hGRP-R gene the CRE sites are located 996 bp apart and both sites were demonstrated to be essential for basal and CREB/PKA-dependent promoter activity in a cooperative manner. Our findings in this study also suggested, not surprisingly, that transcription factor recruitment to several CRE sites within the same promoter might be different depending on the cell- or tissue-specific context and might also arise from distinct upstream signals. This complexity has been illustrated previously only for the regulation of the human insulin gene with four CRE sites. Here, transcriptional activity in HIT cells was repressed by c-Jun via all four CRE sites (10), whereas its GLP-1-dependent upregulation in INS-1 cells was mediated through only three of the four CRE sites (7).

Studies regarding the role of CRE-dependent gene regulation and CREB in gastrointestinal cancers have been limited considering the vast number of CREB-regulated genes. CREB phosphorylation and subsequent induction of c-fos transcription was shown in T84 colon cancer cell proliferation in response to estradiol stimulation (8). In HT29 colon cancer cells, CREB was found increased during cell proliferation (44) and cAMP-induced c-IAP2 expression in T84 colon cancer cells through CREB phosphorylation and CRE-dependent tran-

Fig. 10. CREB is phosphorylated at Ser 133 after forskolin (FSK) stimulation in HuTu-80 but not in Caco-2 cells. CREB phosphorylation (P-CREB) occurred subsequent to FSK (25 μM) stimulation for 30 min in HuTu-80 but not in Caco-2 cells whereas baseline P-CREB levels were higher in Caco-2 cells. Pretreatment for 15 min with the PKA inhibitor H89 (20 μM) completely abolished FSK-induced CREB phosphorylation in HuTu-80 cells but did not significantly affect P-CREB levels in Caco-2 cells. Pretreatment with H89 alone did not affect P-CREB and total CREB levels in either cell line. Phosphorylated CREB at Ser 133 (P-CREB) and total CREB were detected by immunoblotting with an anti-phospho-CREB and total CREB-specific antibody, respectively (Cell Signaling, Beverly, MA) as described in MATERIALS AND METHODS. A representative immunoblot of 3 experiments is shown.

Fig. 11. Schematic illustration of CREB-dependent transcriptional hGRP-R regulation in colon carcinoma cells Caco-2 and duodenal adenocarcinoma cells HuTu-80. This illustration represents a possible model of CREB-related hGRP-R regulation. Binding of CREB at 2 CRE sites within the hGRP-R promoter (bottom; not to scale) occurs in both Caco-2 and HuTu-80 cells as demonstrated by EMSA, supershift, and ChIP assays (solid arrows) or ChIP assays only (dashed arrow). Experiments in this report demonstrated that both CRE sites in the hGRP-R promoter, located –1108 and –112 bp upstream of the RNA start site, are targets of transcription factor CREB binding. Basal transcriptional hGRP-R activity in Caco-2 and HuTu-80 cells required wt genomic DNA sequences of both CRE sites. In addition, cyclic AMP effector (CREB and PKA)-dependent enhanced hGRP-R transcription also required both intact CRE sites, whereby in Caco-2 cells (left) only exogenous CREB (not specifically designated in this diagram), but not cAMP and PKA, increased hGRP-R transcription. In contrast, in HuTu-80 cells exogenous upstream molecules cAMP and PKA resulted in transcriptional hGRP-R upregulation (right). Immunoblot experiments (Fig. 10) suggested that these findings might be due to high baseline levels of phosphorylated CREB in Caco-2 cells that did not further increase in response to cAMP accumulation (left; thin, dashed vertical arrow) whereas in HuTu-80 cells sufficient endogenous unphosphorylated CREB was present for robust PKA-inducible phosphorylation (right; thick vertical arrow). Potential upstream signals of abundant phosphorylated CREB in Caco-2 cells were not further investigated and could include a series of different kinases as reported previously (16).
scription suppressing apoptosis (18). Expression of amphiregu-
lin was found upregulated in colon cancer cells via a CRE
element in its promoter as downstream target of COX-2 de-
pendent prostaglandin E2 generation (33). Stimulation with the
regulatory peptide gastrin resulted in CREB and CRE-depen-
dent regulation of the cyclin D1 promoter in human gastric
malignant cells (22). CREB phosphorylation and CRE-depen-
dent gene expression was also demonstrated via agonist-depen-
dent GRP-R stimulation in HuTu-80 cells, derived originally from a
duodenal adenocarcinoma (25). In esophageal adenocarcinoma
segregates EG1-A, NAPDH oxidase NOX5-S promoter was in-
ducible by CREB (4).

Therefore, this present study provides valuable novel in-
sights into molecular mechanisms by which hGRP-R regula-
tion and its receptor-dependent biological effects might be
mediated in gastrointestinal cancers cells derived from the
colon and duodenum. Among those transcription factors that
were shown to bind to both CRE sites in vitro, the role of
CREB was further investigated in this study, and we propose
that basal and cAMP-effector dependent regulation of hGRP-R
gene expression is the result of cooperative CREB binding to
two distinct CRE sites and that CREB maybe activated via
different upstream signals depending on distinct host cell
factors.

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