Identification of a novel calcitonin-response element in the promoter of the human p21<sup>WAF1/CIP1</sup> gene

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ABSTRACT

The cyclin-dependent kinase inhibitor p21/WAF1/CIP1 is induced in many cell types in response to a variety of extracellular signals and causes cell cycle arrest in both the G1 and G2/M phases of the cell cycle. We reported previously that calcitonin (CT) receptor (CTR)-mediated growth inhibition of HEK-293 cells stably transfected with the human CTR is accompanied by a rapid and sustained induction of p21 and cell cycle arrest in G2. In the present study we have shown that CT stimulates transcription from a p21 promoter-luciferase construct. Using deletion and mutation analysis of the p21 promoter we have demonstrated that transcriptional activation of p21 by CT is p53-independent and is mediated through specific activation of Sp1-binding sites in a region of the promoter between −82 and −69, relative to the transcription start site. CTR-mediated transcriptional activation of p21 was specific for the insert-negative isoform of the human CTR. Butyrate, which was shown previously to activate the same Sp1 site, synergised with CT to increase further p21 promoter activity. These results provide the first demonstration that CTR can induce gene transcription through the constitutively expressed transcription factor Sp1, and define a mechanism of cell growth suppression that may have implications for other members of the seven-transmembrane domain G protein-coupled receptor superfamily.

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INTRODUCTION

Calcitonin (CT) is a 32 amino acid peptide hormone of thyroid origin in mammals, which interacts with a receptor (CTR) that belongs to a subclass of the large seven-transmembrane domain (7 TMD) G protein-coupled receptor (GPCR) family (Sexton et al. 1999). Multiple isoforms of CTR have been described, which result from alternative splicing of the primary mRNA transcript (Moore et al. 1995, Sexton et al. 1993). The human CTR (hCTR) is primarily expressed as two functionally different isoforms, namely an insert-negative form and a form that contains 16 additional amino acids inserted in the first intracellular loop (Gorn et al. 1995, Moore et al. 1995). Unlike the insert-negative form, the insert-positive form does not signal via phospholipase C/Ca<sup>2+</sup>-mediated pathways, although it retains a cell type-dependent capacity to signal via coupling to adenylate cyclase (Nussenzveig et al. 1994, Gorn et al. 1995, Moore et al. 1995).

The reported physiological actions of CT are diverse and include the inhibition of osteoclastic bone resorption (Chambers & Magnus 1982), promotion of embryonic implantation and development (Wang et al. 1998, Zhu et al. 1998), regulation of renal 25-hydroxylvitamin D₃-1α-hydroxylase (Shinki et al. 1999), inhibition of prolactin secretion from the anterior pituitary (Shah et al. 1996), and various effects in the central nervous system (Aboufatima et al. 1999, Sexton et al. 1999). In addition, we (Evdokiou et al. 1999) and others (Ng et al. 1983) have found that CT can suppress cellular proliferation. We recently reported that CT treatment of HEK-293 cells stably transfected with either the rat CTR or the insert-negative hCTR strongly suppressed cell growth (Evdokiou et al. 1999). In contrast, the insert-positive hCTR had no
effect on cellular proliferation. Growth inhibition was associated with an accumulation of cells in the G2 phase of the cell cycle and resulted in a rapid and sustained induction of mRNA encoding the cyclin-dependent kinase inhibitor, p21WAF1/CIP1 and concomitant inhibition of Cdc2 kinase activity. There was a corresponding increase in intracellular levels of p21WAF1/CIP1 protein. In experiments using antisense oligonucleotides, we provided evidence that p21 induction was causative of the CT-mediated growth inhibition (Evdokiou et al. 1999).

In most cases, the mechanism by which CT exerts its effects has not been elucidated. The action most intensively studied to date is the CT-induced receptor down-regulation in mouse (Rakopoulos et al. 1995, Wada et al. 1995, 1996, Ikegame et al. 1996) and human (Takahashi et al. 1995, Inoue et al. 1999) osteoclasts. In mouse osteoclasts, CTR activation caused a rapid and sustained down-regulation of the CTR mRNA, due to a cAMP-mediated destabilisation of receptor mRNA (Rakopoulos et al. 1995) with perhaps an effect on transcription also (Inoue et al. 1999). In contrast, glucocorticoid treatment of osteoclasts increased their expression of CTR, by increasing transcription from the CTR gene (Wada et al. 1997). There is currently no information on the manner by which CT affects gene transcription, and no identification of the promoter elements involved.

The promoter of the p21 gene contains multiple p53-response elements and induction of p21 has been shown to occur under p53-dependent conditions (Li et al. 1994, Wu & Schonthal 1997). However, a number of diverse agents have been described which activate transcription of p21 by p53-independent mechanisms. These include phorbol esters, okadaic acid, transforming growth factor β (TGF-β), extracellular calcium, butyrate, histone deacetylase inhibitor, trichostatin A and nerve growth factor (NGF) (Datto et al. 1995, Biggs et al. 1996, Nakano et al. 1997, Prowse et al. 1997, Sowa et al. 1997, Yan & Ziff 1997). These agents act through multiple signal transduction pathways, which modulate transcription of p21 by facilitating binding of different transcription factors to specific elements located within the p21 promoter. The proximal region between −122 and the start of transcription of the human p21 promoter contains multiple binding sites for members of the Sp family of transcription factors and plays an important role in the p53-independent regulation of p21 transcription (Gartel & Tyner 1999). Sp1 is a member of a multigene family of GC box-binding transcription factors that includes Sp1, Sp2, Sp3 and Sp4, which share extensive structural and sequence homology (reviewed in Courey & Tjian 1992). Three members of this family recognise the same DNA-response motif, while Sp2 recognises a distinct site. Whereas Sp1 is exclusively an activating transcription factor, Sp3 was shown to act as either a transcriptional activator or repressor in a manner dependent on the particular promoter and cell type (Kennett et al. 1997). The Sp1 transcription factor is found in glycosylated and phosphorylated forms, but little is known about how these modifications affect function. Because Sp1 is constitutively expressed and Sp1-binding sites have been described in many promoters, it was long thought that its activity is necessary only for basal transcription (Courey & Tjian 1992). However, the increasing number of studies demonstrating that Sp1-dependent transcription is regulated by activation of different signalling pathways, in response to a variety of signals, suggests that it may regulate expression by facilitating the interaction of other differentially expressed transcription factors.

To determine the mechanism by which CT activates p21 expression and thus exerts its antiproliferative effect, we have performed a detailed deletion and mutational analysis of the p21 promoter. Our results show that transcriptional activation of p21 by CT is p53-independent and is mediated by a region of the promoter between −82 and −69, relative to the transcription start site. This sequence contains two binding sites for the transcription factor Sp1, which are required for transcriptional activation of the p21 gene. We further showed that the CTR-mediated transcriptional activation of p21 is receptor-isoform specific and that the presence of a 16 amino acid insertion in the first intracellular loop of hCTR abolishes promoter activity in response to CT.

MATERIALS AND METHODS

Cell culture

Cells were maintained in Dulbecco’s Modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin, as previously reported (Chen et al. 1998). Stably transfected HEK-293 cells were maintained in 200 µg/ml geneticin (G418) (Life Technologies, Inc., Melbourne, Vic, Australia), which was removed prior to commencement of experiments. Cells were grown at 37°C in a humidified atmosphere with 5% CO2.

Cell lines

The cell lines designated D11 and HR12, which express the rat C1a isoform of the rat CTR and the
insert-negative isoform of hCTR respectively, have been described previously (Chen et al. 1998, Evdokiou et al. 1999). The hCTR insert-positive cDNA (a gift from Zymogenetics Inc., Seattle, WA, USA) was cloned into the mammalian expression vector pRc-CMV (Invitrogen, Carlsbad, CA, USA). Using a modified calcium phosphate transfection method (Wigler et al. 1979), cells were transfected with 10 µg plasmid DNA, together with 20 µg herring sperm DNA (Sigma Chemical Co., St Louis, MO, USA), in 25 cm² culture flasks, at a cell density of approximately 50%. Twenty-four hours after transfection, G418 selection was commenced with the addition of 400 µg/ml G418 and maintained for 2 weeks. Neomycin-resistant colonies were picked manually, propagated in the continual presence of G418 (200 µg/ml), and screened for binding of 125I-sCT. A number of clones were obtained with different levels of receptor expression. The cell line designated Hi12, which contains the insert-positive isoform of hCTR, expressing 2-29 × 10⁶ receptors/cell, and that bound 125I-sCT with a Kₐ of ~3-3 nM, was used for this study.

RNA extraction and Northern blot analysis

For Northern blot analysis, cells were seeded at a density of 1 × 10⁵ cells/25 cm² flask, allowed to attach for 2 days and incubated for 24 h in the presence or absence of 10 nM sCT or 10 nM (8–32) sCT. Total RNA was isolated at the indicated times using TRIZOL Reagent (Life Technologies), according to the manufacturer’s instructions. Total RNA (10 µg) was electrophoresed in formaldehyde/1% agarose gels, transferred to Hybond N+ nylon membranes (Amersham, Castle Hill, NSW, Australia), and immobilised by UV cross-linking. Membranes were prehybridised for 3 h at 42°C in 1 M NaCl, 1% SDS, 10% dextran sulphate, 50% formamide, 100 µg/ml heat-denatured herring sperm DNA and hybridised with p21[WT]–CIP/CIP' cDNA (a kind gift from Dr Helen Richardson, The University of Adelaide, South Australia, Australia) that was radiolabelled with [α-32P]dCTP by random priming, using the Giga prime kit (Bресatec, South Australia, Australia). To allow quantitation of mRNA signals, the same filters were stripped and then reprobed with a 450 bp 32P-labelled PCR-generated DNA fragment of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Northern blots were analysed using the PhosphorImager SF (Molecular Dynamics Inc., Sunnyvale, CA, USA).

Western blotting

Cells were lysed in lysis buffer containing 10 mM Tris–HCl, pH 7-6, 150 mM NaCl, 1% Triton X-100, 0-1% SDS and stored at −70°C until ready to use. Cell extracts were mixed with an equal volume of sample buffer containing 12 mM Tris–HCl pH 6-8, 6% SDS, 10% β-mercaptoethanol, 20% glycerol, and 0-03% bromophenol blue. Protein samples were boiled for 5 min and electrophoresed under reducing conditions in 14% polyacrylamide gels. Separated proteins were electrophoretically transferred to PVDF transfer membrane (Dupont NEN, Boston, MA, USA) and blocked in PBS containing 5% blocking reagent (Amersham) for 1 h at room temperature. Immunodetection was performed overnight at 4°C in PBS/blocking reagent containing 0-1% Tween 20, using a mouse monoclonal antibody to p21 (C24420) (Transduction Laboratories, Lexington, KY, USA). Filters were rinsed several times with PBS containing 0-1% Tween 20 and incubated with 1:5000 dilution of anti-mouse or anti-rabbit alkaline phosphatase conjugate (Amersham) for 1 h. Bound proteins were detected and quantitated using the Vistra ECF substrate reagent kit (Amersham) on a Fluorimag (Molecular Dynamics Inc.).

Reporter constructs

The human wild type p21 promoter-luciferase fusion plasmid, WWP-Luc, was a kind gift from Dr B Vogelstein (Johns Hopkins Medical School, Baltimore, MD, USA). A series of p21 promoter deletion constructs (pWWP, pWP124, pWP101, pWPdel-Sma I) and mutant constructs (pWP101-mt Sp1–3, pWP101-mt Sp1–4, pWP101-mt Sp1–5–6 and pWP101-mt TATA) have been described previously (Nakano et al. 1997). Briefly, the 2.4 kb pair genomic fragment containing the transcription start site was subcloned into the luciferase reporter vector, PGL3-Basic (Promega Corporation, Madison, WI, USA), to generate pWWP. The construct termed pWWP-124 consists of sequences corresponding to the region of the promoter between −124 and the start of transcription and contains all six Sp1 consensus-binding sites. pWWP-101 consists of a minimal promoter region between −101 relative to the transcriptional start site and contains four Sp1-binding sites termed Sp1–3, Sp1–4 and Sp1–5–6. The construct termed pWP-del-Sma I was generated by digesting pWWP with SmaI and by religating. The wild type luciferase-reporter plasmid Sp1-Luc, consisting of three consensus Sp1-binding sites was a kind gift from Dr Peggy J Farnham (Slansky et al. 1993), whereas the mutant type Sp1-Luc was constructed by Dr Sowa (Kyoto Prefectural University of Medicine, Kyoto, Japan) and was previously described (Sowa et al. 1997). A vacant vector pGL3-Basic was purchased from Promega and was used.
as a control reporter plasmid. The pRcCMV-β-galactosidase reporter construct was used to standardise for transfection efficiency.

**Transient and stable transfections of reporter constructs**

Cells were seeded at a density of $1 \times 10^5$ cells/25 cm$^2$ flask, and were allowed to attach for 2 days. Cells were transfected by a calcium phosphate coprecipitation technique using 10 µg of each of the reporter plasmid DNA together with 20 µg sheared herring sperm DNA. Twenty-four hours after transfection, cells were incubated for a further 24 h with various treatments, as indicated in the figure legends, and cell lysates were collected for luciferase assay. For stable transfections, 10 µg of each reporter plasmid DNA construct were cotransfected with 20 µg herring sperm DNA. Twenty-four hours after transfection, G418 was added to the medium at a final concentration of 400 µg/ml. The medium was changed every 3 days and about 3 weeks later G418-resistant colonies were selected. For luciferase assay mass cultures of G418-resistant colonies were expanded and cells were seeded at a density of $2 \times 10^5$ cells/flask. Cells were allowed to attach for 2 days before treatment with sCT or butyrate, and cell lysates were collected for measurement of luciferase activity, as indicated.

**Luciferase and β-galactosidase assays**

The luciferase and β-galactosidase activities of the cell lysates were measured 24 h after the various treatments, as indicated in the figure legends. Luciferase and β-galactosidase were analysed using the same cell extracts according to the manufacturer’s instructions (Promega). Luciferase activities were normalised for the amount of protein in cell lysates or β-galactosidase activity as indicated.

**RESULTS**

**CT induces p21 mRNA and protein in CTR-expressing cells**

We demonstrated previously that CT treatment of HEK-293 cells transfected with either the C1a isoform of the rat CTR or the insert-negative isoform of hCTR results in growth suppression with arrest of cells in the G2 phase of the cell cycle. Evidence from p21 antisense oligonucleotide experiments suggested that p21$^{WAF1/CIP1}$ is causally involved in the CT-mediated growth suppression (Evdokiou et al. 1999). We therefore chose to examine further the expression of p21 in HEK-293 cells expressing the rat C1a CTR (D11) and the insert-negative isoform of hCTR (HR12). As shown in Fig. 1A, p21 mRNA levels were strongly induced by sCT after 24 h of exposure to the ligand in HEK-293 cells expressing either rat CTR or hCTR. In contrast, there was no effect of sCT on p21 mRNA levels in untransfected HEK-293 cells. The induction of p21 mRNA was dependent on intracellular signalling mediated by CTR receptor activation, since treatment with 10 nM of the (8–32) sCT analogue, which binds to rat CTR and hCTR but does not elicit intracellular signalling (Houssami et al. 1995), had no effect on the levels of p21 mRNA (Fig. 1B). This observation is also in agreement with the failure of (8–32)sCT to affect growth of CTR-expressing HEK cells (Evdokiou et al. 1999). Similar to the mRNA, and as indicated by Western blot analysis of cell extracts isolated from sCT treated cells, the levels of p21 protein were elevated concomitantly (Fig. 1C).

**Transcriptional activation of p21 by CT**

To determine whether CT stimulates transcription from the p21 promoter, a construct containing the wild type full-length p21 promoter cloned in front of a luciferase reporter gene pWWP-Luc plasmid was transiently transfected into D11 and HR12 cells. Each set of transfected cells was split into equal aliquots that were treated with sCT or left untreated as controls. As shown in Fig. 2A, treatment with 10 nM sCT for 24 h resulted in a significant increase in luciferase activity (more than 5-fold in D11 cells and up to 6-fold in HR12 cells) when compared with untreated cells or untransfected parental HEK cells. In agreement with the Northern blot data (Fig. 1B) there was no increase in luciferase activity in cells treated with (8–32)sCT. These results indicate that the up-regulation of p21 by CT in HEK-293 cells is due, at least in part, to transcriptional stimulation. In addition, we have demonstrated that transcriptional activation of the p21 promoter by CTR is independent of the receptor species, since p21 promoter activation was seen equally in both the rat (D11) and human (HR12) CTR-expressing cells. To determine if p21 transcriptional activation is receptor-isoform specific, we compared the effect of CT on p21 promoter activity in cells expressing either the insert-negative isoform of the hCTR (HR12/ hCTR$^-$) or the isoform which contains a 16 amino acid insert in the first intracellular loop of the hCTR, an isoform designated (Hi12/hCTR+). As shown in Fig. 2B, CT treatment consistently activated the p21 promoter by about 5-fold in...
HR12/hCTR−, whereas Hi12/hCTR+ isoform failed to cause activation.

The dose response with respect to sCT or human CT (hCT) was determined in HR12 cells transiently transfected with the WWP-Luc reporter plasmid containing the full-length p21 promoter and luciferase activity was analysed 24 h after treatment with 10 nM sCT. Untreated cells indicated basal activity. Relative luciferase activity is shown as raw light units in cell lysates standardised to protein concentration. These results are representative of three independent experiments. Data are means ± s.d. of triplicate samples. Experiments using stably transfected cells produced similar results (not shown).

HR12/hCTR−, whereas Hi12/hCTR+ isoform failed to cause activation.

The dose response with respect to sCT or human CT (hCT) was determined in HR12 cells transiently transfected with the full-length p21 promoter construct, pWWP-Luc. The luciferase activity increased in a dose-dependent manner, up to 6-fold, by treatment with sCT when compared with untreated cells (data not shown). Similarly, treatment with hCT also resulted in a dose-dependent increase in luciferase activity with a maximum stimulation 3-fold above basal at the highest concentration used. (8–32)sCT showed no

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effect on p21 promoter activity, even at high concentrations of peptide (data not shown).

Deletion analysis of the p21 promoter
To determine the regions of the p21 promoter that respond to CT via CTR-mediated activation, we used a series of 5' deletion promoter constructs (Fig. 3). The deletion series, which spanned the 2-4 kb full-length p21 promoter, were transiently transfected into HR12 cells and luciferase activities were measured 24 h following treatment with sCT. The full-length p21 promoter construct, pWWP-Luc, was activated 8-fold by sCT when compared with untreated cells (Fig. 3). The promoter for the p21 gene contains two p53-response elements in close proximity and induction of p21 has been shown to occur under p53-dependent conditions (Li et al. 1994, Wu & Schonthal 1997). However, induction in other situations appears not to require p53 (Sheikh et al. 1994). The pWP124-Luc construct, which lacks the two p53-binding sites, was consistently activated by sCT up to 10-fold, a level slightly higher than the full-length p21 promoter (pWWP), suggesting that activation of p21 promoter by CT is independent of p53 activity. This finding is consistent with our earlier observations, which showed that CT treatment of CTR-expressing cells in fact decreases the levels of p53 mRNA (Evdokiou et al. 1999). Similarly, the pWP101-Luc construct, which contains a promoter fragment spanning 101 bp from the transcription initiation site, was also activated by CT up to 5-fold (Fig. 3). In contrast, the minimal promoter construct, pWPdel-SmaI, which contains a fragment spanning 60 bp from the transcription start site, was significantly less activated by CT (1.8-fold). Furthermore, the basal promoter activity of pWPdel-SmaI decreased to only 6-5% of pWWP, whereas those of pWP124 and pWP101 were comparable with that of the full-length promoter construct (pWWP). These results suggest that the region from 101 bp to the transcription start site harbours a putative CT-response element and defines the minimal region of the p21 promoter responsible for induction by CT.

Mutational analysis of the p21 promoter
The 101 bp fragment, which defines the minimal region of the p21 promoter for induction by CT, contains two independent and two overlapping consensus Sp1-binding sites (Nakano et al. 1997). These are termed Sp1–3, Sp1–4 and Sp1–5–6 (Fig. 4). To define precisely the region of the p21 promoter necessary for induction by CT, and to determine whether these Sp1-binding sites are involved in activation, we used a series of constructs carrying mutations in each of the of Sp1 consensus-binding sites. In addition, we included a construct containing mutations in the TATA box. The mutant constructs are shown schematically in Fig. 4 and are identical to the wild type pWP101 except for the mutations indicated in lower case letters. The
mutant constructs were transiently transfected into HR12 cells and CT-induced luciferase activity was measured 24 h after CT treatment. All mutant constructs had decreased basal activity in the absence of CT when compared with the wild type pWP101 construct. In addition only one of these Sp1 mutant constructs, pWP101-mt-Sp1–4, retained the ability to be activated by CT in a manner similar to that of the wild type pWP101. pWP101-mt-Sp1–3, pWP101-mt-Sp1–5–6 and pWP101-mt-TATA had completely lost the ability to be activated by CT. These results suggest that the sequence of the p21 promoter between –77 and –82 relative to the transcription start site, and which comprises the Sp1–3-binding site, is required for activation by CT. However, the two overlapping Sp1-binding sites (pWP101-mt-Sp1–5–6) between –60 and –51 and the TATA box may also be required for induction by CT, because mutation of these completely abolished sCT-induced luciferase activity. Furthermore, the effect of mutation in the two overlapping Sp1 consensus sites and the TATA box resulted in complete loss of activity even in the absence of CT, suggesting that transcription factor(s) binding to these sites are important in both CT-mediated transcription and basal activity of the p21 promoter.

CT regulates transcription of the p21 promoter through Sp1

The above results suggest that Sp1 transcription factors are important in the activation of the p21 promoter in response to CT. To confirm that Sp1 elements are indeed activated by CT we used a wild type reporter plasmid, pGL2-Sp1-Luc, which contains only three consensus Sp1-binding sites derived from the SV40 promoter and no TATA box. A reporter construct, pGL2-mt-Sp1-Luc that contains mutations in these Sp1 sites, and a vacant vector pGL2-Basic lacking Sp1 sites, were used as controls. When transiently transfected into HR12 cells, the wild type construct was significantly activated by CT about 6-fold, whereas neither the vacant vector nor the Sp1 mutant construct was activated by CT above background levels. In fact, the latter two constructs demonstrated near complete loss of promoter activity in the presence or absence of CT (Fig. 5). These results strongly suggest that Sp1 plays important roles in both the basal transcriptional and CT-induced activity of the p21 promoter.

CT cooperates synergistically with butyrate to activate p21 transcription

Our results thus far clearly demonstrate that Sp1–3 is critical for the CT-induced promoter activity, whereas Sp1–5 and Sp1–6 are required for basal activity. Similarly, this GC-rich region has also been shown previously to be required during p53-independent induction of p21 by a number of other agents. Recently, Nakano et al. (1997) demonstrated that the same region containing Sp1–3 is also critical for butyrate-mediated

**Figure 4.** Mutational analysis of the p21 promoter. Mutants pWP101-mt Sp1–3, pWP101-mt Sp1–4, pWP101-mt Sp1–5–6 and pWP101-mt TATA are identical to the wild type pWP101 with the exception of the sequences shown for each mutant construct. These constructs were transiently transfected into HR12 cells and sCT-induced luciferase activity was measured 24 h later. Luciferase activity was normalised to protein concentration and β-galactosidase activity. Fold induction shown on the right was calculated by comparing the luciferase activity of cells treated with 10 nM sCT and untreated controls. In each experiment triplicate transfections were performed. These results are representative of three independent experiments. Data are means ± s.d. of triplicate samples.
activation of p21 promoter in colorectal cancer cells. To determine whether butyrate also activates transcription of p21 via the same mechanism in the HR12 cells, we transiently transfected HR12 cells with each of the p21 promoter constructs and measured luciferase activity 24 h after treatment with 1 mM butyrate. Butyrate activated the p21 promoter in a similar manner to CT. In fact, when compared with the CT-induced pattern of promoter activation (Figs 3 and 4), butyrate displayed an identical pattern across all promoter constructs (data not shown). These findings suggest that CT and butyrate may work through common signalling pathways that converge onto the same Sp1–3-binding site to activate transcription of p21.

To determine if both agents work through the same or alternative signalling pathways to induce p21 transcription, we transfected HR12 cells with the full-length p21 construct and compared luciferase activity following treatment with either CT or butyrate alone or in combination. We reasoned that if each agent alone maximally activates the same pathway, then additional activation by the combined treatment through this mechanism would be impossible. Alternatively, if each agent activates separate signalling pathways, then the combined treatment of CT and butyrate should result in a synergistic effect on p21 promoter induction. Figure 6 shows that while CT and butyrate when used individually were able to activate the p21 promoter (5-fold for CT and 11-fold for butyrate), a combination of CT and butyrate consistently resulted in a more than additive response of up to 20-fold. These findings suggest that the synergistic activity may result from the differential expression and interplay of additional transcription factors mediated by alternative signalling mechanisms that converge onto the same Sp1-binding site to increase p21 transcription.

DISCUSSION

CT is best understood in its role as a potent negative regulator of osteoclastic bone resorption (Martin et al. 1998). However, the more recent discovery of CT production (Martin et al. 1998, Zhu et al. 1998), and expression of its receptor (Kuestner et al. 1994, Martin et al. 1998), in extraskeletal sites implies that CT may have actions unrelated to calcium metabolism and protection of the skeleton. Our recent published work, which demonstrated the potent antiproliferative effects of CT on CTR-expressing cells (Evdokiou et al. 1999), as well as other published data showing that CT treatment of breast and prostate cancer cell lines influences cellular proliferation, further supports...
this notion (Ng et al. 1983, Shah et al. 1994, Ritchie et al. 1997). That CT might have a physiological role in cellular growth is consistent with a growing body of evidence for an important role for receptors of the same class as the CTR (7 TMD or GPCR) in influencing cell growth both positively and negatively, as well as cellular differentiation, tissue remodelling and repair, and oncogenesis (Dhanasekaran et al. 1995, Morisset et al. 1995, Schipani et al. 1995, Sharma et al. 1999).

At present, our knowledge of CT action in target cells is largely limited to events proximal to the plasma membrane, whereas the nuclear mechanisms involved in the CTR-mediated growth inhibition are largely unknown. It is only recently that we have begun to elucidate the intracellular signalling mechanisms by which CT influences cellular proliferation. Recently, we have shown that CT inhibits the growth of CTR-expressing cells by a mechanism involving the rapid and sustained induction of the universal cyclin-dependent kinase inhibitor p21 and arrest of cells in the G2 phase of the cell cycle, which was associated with inhibition of Cdc2 kinase activity. Evidence that p21 induction was causative of cell growth suppression was obtained from p21 antisense oligonucleotide experiments (Evdokiou et al. 1999). Therefore, it appears that this cyclin-dependent kinase inhibitor is a key target of regulation by the CTR-mediated signalling pathway that leads to growth inhibition.

To determine the mechanisms by which induction of p21 mRNA expression occurs during CT treatment in CTR-expressing cells, we performed a detailed functional analysis of the p21 promoter. Through deletion analysis we have shown that induction of p21 by CT occurs via a p53-independent mechanism since deletion of the two p53-response elements upstream of the promoter did not influence promoter activity. Furthermore, we have shown that the minimal region of the p21 promoter required for its induction maps to a stretch of 77 bp relative to the transcriptional start site. This GC-rich region harbours two independent (Sp1–3, Sp1–4) and two overlapping (Sp1–5–6) binding sites for the transcription factor Sp1, as well as a TATA box. By mutation analysis of each of these Sp1 sites, we have identified a major CT-responsive element to be defined by one of the four Sp1-binding sites (Sp1–3) present in this region. In addition, our finding that CT is capable of activating transcription from a wild type reporter plasmid, which contains only three consensus Sp1-binding sites derived from the SV40 promoter and no TATA box, provides further evidence that Sp1 or Sp1-related proteins are involved in the transcriptional activation of the p21 promoter in response to CT.

The same region comprising Sp1–3 was previously shown to be critical for p21 induction by a number of other agents including, TGF-β, calcium, NGF, the histone deacetylase inhibitors, trichostatin A and butyrate (Datto et al. 1995, Nakano et al. 1997, Prowse et al. 1997, Sowa et al. 1997). Taken together, these findings suggest that regulation of Sp1-related factors could be part of a common mechanism to induce expression of p21. However, since each of these agents activates distinct signal transduction pathways, it is likely that several differentially expressed transcription factors, including activators and co-activators, may converge onto the same Sp1–3-binding site to regulate p21 transcription. The interplay of such factors and the way in which they interact with the general transcriptional machinery is likely to be important in determining the degree of activation. Our observation that CT cooperates synergistically with butyrate to increase p21 promoter activity further supports this notion. We have previously shown that the insert-negative isoform of hCTR increases intracellular calcium and cAMP (Evdokiou et al. 1999). Calcium has been shown to induce expression of p21 by a mechanism involving Sp3 binding to the Sp1–3 site (Prowse et al. 1997) and butyrate acts at the same site by an Sp1 and Sp3 mechanism (Nakano et al. 1997). Although not examined in this study, these findings raise the possibility that CTR could signal via calcium and Sp3.

The mechanisms by which Sp1 or Sp1-related transcription factors mediate p21 induction are largely unknown. Regulation of Sp1-dependent transcription was shown previously to be affected by phosphorylation and glycosylation events. Phosphorylation has been shown to influence DNA binding and transactivation activities (Black et al. 1999), whereas glycosylation confers resistance to proteosome-dependent degradation by increasing the stability of the Sp1 protein (Han & Kudlow 1997). In addition, Sp1 was shown to associate directly with members of the general transcriptional machinery such as TFIID and the co-integrator CBP/p300 (Gill et al. 1994, Owen et al. 1998). Furthermore, Sp1 physically interacts and functionally cooperates with several other transcription factors including NF-kB, GATA, YY1, E2F1, SREBP-1 pRb, Smad proteins (Lee et al. 1993, Uvdadia et al. 1995, Lin et al. 1996, Hirano et al. 1998, Moustakas & Kardassis 1998) and more recently c-jun (Kardassis et al. 1999). Therefore, Sp1-regulated p21 gene transcription by multiple signalling pathways appears to be mediated by
the interaction of several differentially expressed factors.

hCTR is primarily expressed as two functionally different isoforms, comprising an insert-negative form and a form that contains 16 additional amino acids inserted in the first intracellular loop (Gorn et al. 1995, Moore et al. 1995). Unlike the insert-negative form, which we show here mediates transcriptional activation of p21 in response to CT, the insert-positive form does not. This finding is consistent with earlier observations showing that CT induces growth inhibition, specifically in cells expressing the insert-negative isoform of the hCTR, whereas cells expressing the insert-positive isoform are essentially resistant to the antiproliferative effects of CT (Evdokiou et al. 1999, Raggatt et al. 2000). This is despite the fact that both cell lines display similar binding characteristics for CT.

In summary, we conclude that CT induces p21 transcription via a p33-independent mechanism, mediated through specific activation of Sp1-binding sites in the promoter of the p21 gene. The main CT-response element is defined by the Sp1–3 site in the p21 promoter and this sequence is essential for the CT-mediated p21 transcription. We have further shown that the CTR-mediated activation of p21 promoter is receptor-isoform specific and that the presence of the 16 amino acid insert in the first intracellular loop of hCTR, which comprises the insert-positive isoform, was without effect on p21 promoter. There is currently no information on the manner by which CT affects gene transcription, and no identification of the promoter elements involved. Therefore, this is the first demonstration that CT induces gene transcription through the constitutively expressed transcription factor Sp1 and suggests that other Sp1-responsive genes might be similarly regulated by CT. These findings, together with the potent antiproliferative function of CT mediated by induction of the cyclin-dependent kinase inhibitor, p21, defines a novel signalling pathway for CT.

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