Identification of a functional glucocorticoid response element in the promoter of the cyclin-dependent kinase inhibitor p57^{Kip2}

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Abstract

Glucocorticoids are known regulators of the cell cycle, normally exerting an anti-proliferative effect. We have previously shown that glucocorticoids stimulate expression of p57^{Kip2}, a member of the Cip/Kip family of cyclin-dependent kinase inhibitors which, in some cell types, may account for the anti-proliferative responses seen after glucocorticoid treatment. The induction of p57^{Kip2} involves primary transcriptional effects where no *de novo* protein synthesis is necessary, suggesting a direct interaction of the glucocorticoid receptor with the p57^{Kip2} gene. In this study we have identified a functional glucocorticoid response element (GRE), located 5 kilo bases (kb) upstream of the transcription start site in the human p57^{Kip2} promoter. This GRE was functional also when isolated, suggesting a direct transcriptional effect of the glucocorticoid receptor. Furthermore, mutation of this GRE abolished glucocorticoid induction of the reporter gene, whereas mutation of a nearby Sp1 site did not. Using electrophoretic mobility shift assays, we have shown that the -5 kb p57^{Kip2} promoter GRE was able to compete with a well-known GRE for glucocorticoid receptor binding. Sequence comparisons with the mouse genome showed that this GRE is highly conserved, further strengthening the biological importance of this site. All these data emphasize the involvement of this GRE in the glucocorticoid-mediated induction of p57^{Kip2} expression.

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Introduction

Glucocorticoids are steroid hormones, produced in the adrenal cortex, that play important roles in metabolism, immune responses, cell proliferation and differentiation. They exert their function by binding to the intracellular glucocorticoid receptor (GR), which is a ligand-activated transcription factor. When bound to its ligand, GR homodimerizes and translocates to the nucleus where it interacts with specific DNA sequences, known as glucocorticoid response elements (GREs), in target genes. Thereby, transcription from these genes is increased (Beato *et al.* 1995). The GREs are normally palindromic, with two hexameric half-

sites separated by three nucleotides, the perfect palindromic GRE reading 5'-AGAACAnnnTGTTCT-3'. However, a consensus GRE derived from comparison of functional GREs in target genes reads 5'-GGTACAnnnTGTTCT-3' (Zilliacus *et al.* 1995). In addition, more recent results have demonstrated that the GR can stimulate the expression of target genes without directly contacting a GRE (Teurich & Angel 1995, Stocklin *et al.* 1996, Rüdiger *et al.* 2002, Subramaniam *et al.* 2003). This also seems to be the case for other nuclear receptors, e.g. the progesterone receptor, which has been shown to transduce an effect through Sp1 in the p21^{Cip1} gene (Owen *et al.* 1998).

Glucocorticoids (GC) inhibit proliferation in many tissues and cells, including those of lymphoid, fibroblastic, epithelial, and bone origin. This anti-proliferative effect is normally represented by arresting the cells in the G₁-phase of the cell cycle (Sanchez *et al.* 1993, Frost *et al.* 1994, Rhee *et al.* 1995, Corroyer *et al.* 1997, Rogatsky *et al.* 1997). Progression through G₁- and entry into S-phase is positively regulated by the activation of cyclin-dependent kinase 2 (CDK2), which is complexed with cyclin A or cyclin E (Sherr 1993). However, proteins that directly interact with the CDK-cyclin complexes, the so-called CDK inhibitors, can inhibit the activity of CDK2. The family of CDK inhibitors that inhibits CDK2 activity, the Cip/Kip family, consists of three members: p21^{Cip1}, p27^{Kip1} and p57^{Kip2} (Sherr & Roberts 1999). We have previously identified the cyclin-dependent kinase inhibitor p57^{Kip2} as a glucocorticoid-induced protein that inhibits cell proliferation in HeLa cells by accumulating the cells in the G₁-phase (Samuelsson *et al.* 1999). This increase in p57^{Kip2} protein expression correlates with an induced expression of the p57^{Kip2} mRNA which, at least in part, implicates transcriptional regulation. Furthermore, the response is direct as no *de novo* protein synthesis is required, suggesting a direct interaction of the GR with regulatory elements in the p57^{Kip2} gene.

The aim of the present study was to identify putative GREs in the human p57^{Kip2} promoter responsible for this transcriptional induction. For this purpose we used the human p57^{Kip2} promoter coupled to a luciferase reporter gene to perform transient transfections. The results demonstrated that the induction of p57^{Kip2} by glucocorticoids is mediated by a GRE, located 5 kilo bases (kb) upstream of the transcription start site, an effect that is GR dependent. Moreover, glucocorticoid responsiveness was lost by mutating this GRE. Electrophoretic mobility shift assays further confirmed binding of GR to this GRE.

Materials and methods

Promoter constructs

A 40 kb cosmid containing the human p57^{Kip2} promoter was a kind gift from Dr Laura Hink Reid (University of North Carolina, USA) (Reid *et al.* 1996). The p57^{Kip2} promoter fragments were

cloned into the polylinker of a luciferase-based reporter vector, pGL3-Basic vector (Promega), by restriction enzyme digestion and ligation. The different promoter constructs include different lengths of the human p57^{Kip2} promoter (numbers refer to location corresponding to transcriptional start site=+1): Hinc 6·3: -6339 to +14; Kpn 4·2: -4236 to +14; Sac 4·0: -3987 to +14; Sca 3·1: -3107 to +14; Kpn 2·2: -2201 to +14; Hinc 1·6: -1552 to +14; Bgl 1·0: -1025 to +14; Min: -110 to +14; K-S/min: -5461 to -5015 + -110 to +14; WT/min: -5082 to -5042 + -110 to +14.

In the WT/min plasmid, the sequence -5082 to -5042 was synthesized and ligated into the Min plasmid. The TAT-GRE plasmid was obtained by ligation of a pair of synthetic oligonucleotides containing a GRE from the tyrosine amino transferase (TAT) promoter (Jantzen *et al.* 1987) into the Min plasmid. The sequence of the oligonucleotides used was (with the GRE underlined; dotted underlined represents not sequence specific)

TAT-GRE forward oligo: 5'-GTACCATTAC
TAGAACATCCTGTACAGTCGACA-3'

TAT-GRE reverse oligo: 5'-CGCGTGTGCGAC
TGTACAGGATGTTCTAGTAATG-3'

Plasmid mutations

Plasmids including mutations of the putative GRE or Sp1 binding site were obtained by site-directed mutagenesis (Borns *et al.* 2000). The same mutations were obtained in both the short 40 bp insert (WT/min plasmid) and in the long 446 bp insert (K-S/min plasmid). Hence, four different mutant versions of the plasmids were obtained. Mutated GRE in WT/min vector (GREmut/WT); mutated Sp1 site in WT/min (Sp1mut/WT); mutated GRE in K-S/min (GREmut/KSm); Sp1 site mutation in K-S/min (Sp1mut/KSm). In addition, two point mutations were made in the WT/min to mimic the mouse sequence of the -5 kb GRE (mouseGRE).

For the site-directed mutations, 50 µl reaction containing 5–25 ng WT/min or K-S/min plasmid, 2·5 U *PfuTurbo* DNA polymerase (Stratagene), 5 µl 10 × reaction buffer (Stratagene), 0·2 mM of each dNTP (A, C, G, T), and 125 ng of each (forward and reverse) mutation primer was used. The mutation primers were ordered from and synthesized by

Thermo Hybaid, Interactiva Division (Thermo Hybaid GmbH, Ulm, Germany).

The sequence of the primers used was: GRE mutation forward primer: 5'-GCAGCTGCCCCAAGACAAGCTTACCCTGGGGGGCGGGGC-3', GRE mutation reverse primer: 5'-GCCCCGCCCCCAGGGTAAGCTTGTCTTGGGGCA GCTGC-3'.

Sp1 mutation forward primer: 5'-GAACAGCC TGTCCTGGGAAGCTTGAACGAGCCTTGAG CTCTTACG-3', Sp1 mutation reverse primer: 5'-CGTAAGAGCTCAAGGCTCGTCAAGCTT CCCAGGACAGGCTGTTC-3'. Mouse GRE forward primer: 5'-GCCCCAAGAACAGCCCGT CCT[Ⓢ]GGGGGCGGGGCGAGCC-3', mouse GRE reverse primer: 5'-GGCTCGCCCCGCC CCC[Ⓢ]AGGACGGGCTGTTCTTGGGGC-3'.

Affected transcription factor/receptor binding elements are underlined, mutations are shown in bold, and eliminated bases are shown as strikethrough superscript.

The 50 µl reaction was overlaid with 30 µl mineral oil (Sigma) before it was put in a Perkin-Elmer Gene Amp 9600 thermocycler for PCR. An initial 30 s 94 °C heat pulse was followed by 16 cycles of 95 °C for 30 s, 55 °C for 1 min and 68 °C for 10/11 min (10 min for WT/min plasmid and 11 min for K-S/min plasmid respectively), after which samples were chilled to 4 °C. The PCR products were cleaved with 10 U DpnI enzyme (Sigma) in 37 °C for 1 h. The DpnI enzyme cleaves only methylated DNA, hence only the parental template strand was cleaved, whereas the mutated synthesized DNA could be used for transformation. DH5α competent bacteria (Life Technologies, Paisley, UK) were transformed with the mutated plasmids according to the manufacturer's instructions. Plasmids were prepared using the Qiagen Plasmid Maxi kit (Qiagen) according to the manufacturer's instructions. Plasmids were sequenced (Cybergene, Huddinge, Sweden) to assure correct mutations were obtained.

Cell culture

A-549 cells are of human epithelial lung carcinoma origin (Lieber *et al.* 1976) and 293 cells are epithelial cells of human embryonic kidney origin (Graham *et al.* 1977). The 293 cells used in this study are a Flp-In cell line (Invitrogen). A-549 cells and 293 cells were grown at 37 °C in 5% CO₂ in

a medium containing 45% F12 (HAM) (Life Technologies), 45% DMEM (Life Technologies), 9% fetal calf serum (Integro b.v., Zaandam, Holland), penicillin/streptomycin 10 IU/ml and 100 µg/ml respectively (Life Technologies), and 2 mM L-glutamine (Life Technologies). The Flp-In-293 cells were grown in the presence of 100 µg Zeocin/ml (Invitrogen); stably transfected Flp-In-293 cells were grown in the presence of 100 µg Hygromycin/ml (Invitrogen). Cells were split 1:5 – 1:10, 2–3 times per week.

Stable transfections

The Flp-In-293 cells were stably transfected with pcDNA5/FRT expression vector containing the rat glucocorticoid receptor gene (Godowski *et al.* 1988) according to instructions from the manufacturer. Clones expressing GR were selected and maintained in Hygromycin-containing medium.

Transient transfections

Lipofectin reagent (Gibco BRL/Invitrogen) was used, according to the manufacturer's instructions, to transfect A-549 and 293 cells with the different plasmids. As an internal control, pRL-SV40 vector (Promega), containing the SV40 early enhancer/promoter region, providing a strong constitutive expression of Renilla luciferase, was used.

The day before the experiment a total of 60 000 A-549 cells/well were seeded out in 6-well culture dishes. An equimolar amount of the plasmid tested (corresponding to 1 µg Min plasmid), together with 25 ng pRL-SV40 vector (as an internal control), were used for each 9.5-cm² well. Following transfection, the cells were treated with 1 µM (or 100 nM for Fig. 3B) dexamethasone (Sigma) (or 0.01% ethanol as control) for 22–24 h before harvesting.

The day before the experiment, 293 cells were seeded out at 40 000 cells/well in 24-well culture dishes. Two hundred nanograms Min, WT/min or GREmut/WT plasmid, together with 5 ng pRL-SV40 vector were used for each 2-cm² well. Following transfection, the cells were left to recover for 18–20 h, and subsequently treated with 100 nM dexamethasone (Sigma) (or 0.001% ethanol as control) and/or with 1 µM RU-486 (Mifepristone, Sigma) (or 0.01% ethanol as control) for 24 h

before harvesting. All treatments were carried out in triplicate.

Dual-Luciferase assay

The Dual-Luciferase Reporter assay system (Promega) was used according to the manufacturer's instructions. All samples were measured consecutively for firefly luciferase and Renilla luciferase expression within the same plate. The individual firefly luciferase values (promoter activity) were then divided by the Renilla values (internal control) thus correcting for differences in transfection efficiency. The averages of these values (in triplicate) were used to calculate the dexamethasone inducibility (fold induction) of reporter expression.

Electrophoretic mobility shift assay

For the electrophoretic mobility shift assay (EMSA), the GRE from the tyrosine aminotransferase (TAT) gene (Heck *et al.* 1994) was end-labeled by T4 polynucleotide kinase using [γ -³²P] (3000 Ci/mmol, Amersham Pharmacia Biotech) (GREtat probe). DNA binding reactions of 20 μ l were carried out in a buffer containing 20 mM Tris-HCl pH 8.0, 10% (w/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol, 2 μ g poly (dI-dC) (Amersham Pharmacia Biotech), 75 mM KCl, 3% bovine serum albumin, 0.05–0.2 ng radiolabeled GRE and 10 μ g extract from baculovirus-expressed full length human GR (kindly provided by Dr Stefan Nilsson, Karo-Bio AB, Sweden) (Dahlman-Wright *et al.* 1993). Binding reactions were performed at room temperature for 30 min. When indicated, a 200-molar excess of unlabeled GREtat or the p57^{Kip2}-GRE (or mutated versions of this) was included in the binding reaction. Free and bound DNA were separated on 4% polyacrylamide gel, which was run at a constant voltage of 200 V in 22 mM Tris-borate, 0.5 mM EDTA.

The probes used for EMSA were double stranded DNA (Thermo Hybaid GmbH) with the upper strand sequence (GRE underlined): GREtat: 5'-ATTACTAGAACATCCTGTACAGTCGAC-3'; human p57^{Kip2}GRE (GRE-Sp1): 5'-GCAGC TGCCCCAAGAACAGCCTGTCCTGGGGGG CGGGGCGAGCCTTGAGCTCGGGGAAC-3'; mutated p57^{Kip2} GRE (GREmut-Sp1): 5'GCAGC

TGCCCCAAGAACAAGCTTACCCTGGGGGG CGGGGC-3'; p57^{Kip2}GRE with mutated Sp1 (GRE-Sp1mut): 5'-GCCCCAAGAACAGCCTGT CCTGGGAAGCTTGACGAGCCTTG-3'; Sp1: 5'-ATTCGATCGGGGCGGGGCGAGC-3'; AP-1: 5'-CTAGGTGTCTGACTCATGCTT-3'; mouse-p57^{Kip2} GRE: 5'-GCCCCAAGAACAGCCCGTC CTGGGGGCGGGGCGAGCC-3'.

Western blot analysis

A-549 cells were seeded at subconfluent levels in 55-cm² plates the day before treatment. Cells were treated with 100 nM dexamethasone for 2, 4, 6, 8 or 24 h or with normal growth medium, supplemented with 0.001% ethanol, as a vehicle control.

Whole cell extracts for Western blot analysis were prepared by lysing the cells in ice-cold Nonidet P-40 (NP-40) buffer (0.5% NP-40, 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM EDTA) for 10 min, after which cell debris was removed by centrifugation at 14 000 $\times g$ for 10 min at 4 °C. An equal volume of 2 $\times$ SDS gel-loading buffer was then added to the supernatant and the samples were boiled for 2 min. Protein concentration in cell extracts was quantified spectrophotometrically prior to addition of the loading buffer with the Bio-Rad protein assay kit, according to the instructions of the manufacturer (Bio-Rad, Hercules, CA, USA). Protein (20 μ g) from each whole cell extract was electrophoretically separated on a 9% SDS-polyacrylamide gel and electroblotted onto a Hybond C-extra membrane (Amersham Life Sciences, UK). For protein detection, the immunoblots were probed with a rabbit IgG polyclonal anti-p57^{Kip2} antibody (C-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) (1:100). As secondary antibody, a horseradish-peroxidase-conjugated anti-rabbit antibody (Amersham Life Sciences, UK) (1:3000) was used. The membranes were then subjected to enhanced chemiluminescence (Amersham Life Sciences, UK) and autoradiography, according to the instructions of the manufacturer. To check for equal loading and transfer, the membranes were stained with Ponceau red (Sigma) prior to addition of antibody, as well as reprobed with a mouse IgG monoclonal anti- β -tubulin antibody (N357, Amersham Life Sciences, UK) (1:3000). As

secondary antibody, a horseradish-peroxidase-conjugated anti-mouse antibody (Amersham Life Sciences, UK) (1:3000) was used.

Statistics

P values for comparison of samples were obtained by using the paired *t*-test with two-tailed distribution using the Microsoft Excel program.

Results

Identification of a functional glucocorticoid response element in the human p57^{Kip2} promoter

To find a functional GRE in the p57^{Kip2} gene, the human p57^{Kip2} promoter was analyzed using fragments of the promoter, which were inserted into the pGL3-Basic luciferase reporter vector. The lengths of the promoter fragments used ranged from just over 100 bases to approximately 6.3 kb long. The resulting plasmids were transfected into A-549 cells. By Western blotting technique, A-549 cells were found to be inducible for expression of endogenous p57^{Kip2}, in a time-dependent manner, after dexamethasone exposure (Fig. 1A, upper panel), showing that these cells present a good model system for studying the underlying mechanisms for this induction. Equal loading and transfer of protein was confirmed by using a β -tubulin antibody (Fig. 1A, lower panel).

After transfection with the various p57^{Kip2} promoter constructs, A-549 cells were treated with dexamethasone, and expression of the luciferase reporter was measured using the Dual-Luciferase Reporter assay (Fig. 1B). Using the differently sized promoter constructs, a 2 kb long area of interest (–6.3 kb to –4.2 kb), which caused a threefold increase in luciferase expression following dexamethasone administration to the cells, was identified (Fig. 1B). Possible GREs, based on similarity to the consensus sequence, were searched for within this 2 kb region. A putative GRE was found around –5 kb, and a 446 bp fragment (–5461 to –5015) spanning this GRE was inserted into the pGL3-Basic vector upstream of the minimal promoter (ranging from –100 bp to +14) (K-S/min). This short promoter stretch was able to induce the luciferase expression three- to fivefold, when stimulated by the glucocorticoid agonist dexamethasone (see Figs 1B and 2A). To further pinpoint the GRE, an even shorter (40 bp)

Figure 1 (A) Western blot of p57^{Kip2} expression in A-549 cells: expression of p57^{Kip2} in A-549 cells (upper panel) following 100 nM dexamethasone treatment for 0 to 24 h (time indicated in h). As a control for equal amount of protein loading and transfer, the filter was reprobbed with an antibody against β -tubulin (lower panel). (B) Dexamethasone-induced expression of luciferase reporter gene with different p57^{Kip2} promoter constructs. The left diagram shows schematically the different promoter constructs used (numbers indicating base pairs from transcription start site). The right diagram shows the induction of luciferase reporter activity after dexamethasone treatment (1 μ M, 24 h), when the respective construct is analyzed (numbers indicating fold induction). One representative experiment, carried out in triplicate, is shown.

synthetic fragment of the p57^{Kip2} promoter (–5082 to –5042), spanning the putative –5 kb p57^{Kip2} GRE, was analyzed (WT/min). Indeed, this 40 bp sequence was sufficient to drive the glucocorticoid-induced expression of the reporter (Fig. 2A). Mutation of this putative p57^{Kip2} GRE resulted in loss of its ability to induce luciferase expression following dexamethasone treatment, in both the 446 bp fragment (GREmut/KSm) and the short 40 bp sequence (GREmut/WT) (Fig. 2A).

When studying the sequence surrounding the core GRE, a putative Sp1 binding site was found to be located immediately downstream of the GRE. To investigate possible synergistic effects between GR and Sp1, the Sp1 site was mutated, in isolation, in both the short (Sp1mut/WT) as well as in the longer 446 bp K-S/min fragment (Sp1mut/KSm). Mutations of the putative Sp1 binding site did not impair the inducibility by glucocorticoids, but rather enhanced it (Fig. 2A).

Figure 2 Expression of luciferase reporter is affected by mutations in the p57^{Kip2} GRE fragment and is dependent on GR. (A) Mutations in the p57^{Kip2} GRE cause alterations in the dexamethasone-induced luciferase reporter expression. A-549 cells were transfected with the p57^{Kip2} promoter constructs as indicated, and thereafter treated with 1 μM dexamethasone for 24 h. WT/min is the 40 bases long sequence, surrounding and including the p57^{Kip2} GRE, inserted upstream of the Min promoter. GREmut/WT is the WT/min promoter with the GRE mutated. Sp1mut/WT is the WT/min promoter with the Sp1 site mutated. K-S/min is the 446 base pair long promoter, inserted upstream of the Min promoter. GREmut/KSm is the K-S/min construct with the GRE mutated. Sp1mut/KSm is the K-S/min construct with the Sp1 site mutated. Results are shown as fold induction of dexamethasone-treated vs control. The mean of four experiments±standard deviation is shown. Student's *t*-test is used to calculate the statistical difference of fold induction compared with the Min reporter. **P*<0.05, ***P*<0.01. (B) GR-dependent reporter expression. GC-mediated induction of luciferase expression in 293 cells without GR (-GR:) or stably transfected with GR (GRwt:) After transfection with Min or WT/min plasmids cells were treated with dexamethasone (100 nM) for 24 h. Results are shown as fold induction of dexamethasone-treated vs control. The mean of three experiments±standard deviation is shown. Student's *t*-test is used to calculate the statistical difference of fold induction compared with GRwt: Min. **P*<0.05. (C) Effects of glucocorticoid agonist and antagonist on reporter expression. Luciferase expression in GR-expressing 293 cells, after transient transfection with WT/min plasmid and treatment for 24 h with dexamethasone (Dex, 100 nM) and/or RU-486 (1 μM) is shown. Results are shown as the relative expression of luciferase (firefly/Renilla (Ren.)). The mean of three experiments±standard deviation is shown. Student's *t*-test is used to calculate statistical difference. **P*<0.01 vs non-treated, ***P*<0.001 vs non-treated, #*P*<0.01 vs Dex-treated.

Figure 3 (A) EMSA showing the ability of p57^{Kip2} GRE to compete for baculovirus-expressed GR binding to the GREtat. Lane 1 (probe) is free probe. Baculovirus-expressed full-length GR (extract) is present in lanes 2–8. In lanes 3–8 the interaction of the GR with the ³²P-labeled-GREtat probe is challenged by a 200-fold excess of cold probe (GREtat, GRE-Sp1, GREmut-Sp1, GRE-Sp1mut, Sp1, and AP-1). One representative experiment out of four is shown. (B) Comparison of the inducibility of reporter gene via the p57^{Kip2} GRE and a classical GRE: expression of luciferase reporter gene under the control of a single GRE from the TAT-promoter (TAT-GRE) compared with a single GRE from the p57^{Kip2} promoter (WT/min). A-549 cells were transfected with the promoter constructs indicated, and thereafter treated with 100 nM dexamethasone for 24 h. Results are shown as fold induction of dexamethasone-treated vs control. The mean of three experiments $\pm$ standard deviation is shown. Student's *t*-test is used to calculate the statistical difference of fold induction compared with the Min reporter. ***P* < 0.01.

The analyses of luciferase expression, using the promoter fragments of the human p57^{Kip2} gene and mutations of the putative GRE, clearly showed that the GRE located –5076 to –5062 in the human p57^{Kip2} promoter was involved in the inducibility by glucocorticoids (Fig. 2A).

To verify that the effect found was dependent on the GR, 293 cells, which lack endogenous GR (–GR), were compared with stably transfected GR-expressing 293 cells (GRwt). After transfection with the Min and WT/min plasmids, the dexamethasone-induced expressions of the reporter gene clearly demonstrate that this effect is GR-dependent (Fig. 2B). In addition, the dexamethasone-induced luciferase reporter gene expression, under the control of the p57^{Kip2} GRE in these cells, was significantly attenuated by the glucocorticoid antagonist RU-486 (Fig. 2C). Although mainly working as an antagonist, RU-486 is known to have a low agonistic activity on GR (Schulz *et al.* 2002), which is seen also in this

experiment. Using the construct with a mutated p57^{Kip2} GRE (GREmut), neither dexamethasone nor RU-486 could induce the expression of the reporter gene (data not shown).

Analysis of GR interaction with the p57^{Kip2} GRE by EMSA

In order to investigate the ability of the –5 kb putative p57^{Kip2} GRE to interact with GR, its capacity to compete GR binding to a well-known GRE (GRE II) from the tyrosine amino transferase (TAT) promoter (Jantzen *et al.* 1987) was tested.

Incubation of ³²P-labeled TAT-GRE (GREtat) with baculovirus-expressed full length GR resulted in a complex that could be competed by a 200-fold excess of unlabeled GREtat as well as the –5 kb p57^{Kip2} GRE (GRE-Sp1) (Fig. 3A). However, the probe with the –5 kb p57^{Kip2} GRE mutated (GREmut-Sp1) could not compete out the

Table 1 Sequence of the human p57^{Kip2} GRE and mutations introduced as well as a comparison with the mouse p57^{Kip2} GRE

Human p57 ^{Kip2} -5082 to -5042	5'-GCCCCAAGAACAGCCTGTCCTGGGGGGCGGGGCGAGCCTTG-3'
GRE mutation	5'-GCCCCAAGAC A AGC T A CCTGGGGGGCGGGGCGAGCCTTG-3'
Sp1 mutation	5'-GCCCCAAGAACAGCCTGTCCTGGG A AGC T T AGAGCCTTG-3'
Mouse p57 ^{Kip2} -4028 to -3989	5'-GCCCCAAGAACAGCC CG TCCT- <u>GGGGGCGGGGCGAGCCGAG</u> -3'

The human GRE sequence has single underline and the putative Sp1 binding site has double underline. Introduced GRE- and Sp1-mutations are shown in bold with dotted underline. Differences in the mouse p57^{Kip2} GRE to human p57^{Kip2} are shown in bold italic with double underline.

labeled probe, demonstrating the requirement of an intact p57^{Kip2} GRE. Mutating the Sp1 site in the 40 bp fragment containing the p57^{Kip2} GRE did not affect GR interaction with the labeled probe, as the GRE-Sp1mut probe was still able to compete out the labeled probe. Non-specific unlabeled oligonucleotides containing a consensus site for Sp1 or AP-1 binding could not compete out the interaction between the GR and GREtat probe, demonstrating that the GR interaction with p57^{Kip2} GRE is specific.

To compare the activity of the -5 kb p57^{Kip2} GRE to the prototype GRE from the TAT gene (GRE II), both were cloned as a single moiety upstream of the p57^{Kip2} minimal promoter. Both were able significantly to stimulate reporter gene expression after dexamethasone exposure, although the p57^{Kip2} GRE was slightly less effective (Fig. 3B).

A conserved and functional GRE is present in the mouse p57^{Kip2} promoter

To find evolutionary support that the identified GRE in the human p57^{Kip2} gene is of importance in GC regulation of p57^{Kip2} expression, a sequence comparison was made with the mouse p57^{Kip2} promoter. This comparison revealed a very similar GRE present in the mouse p57^{Kip2} promoter, only differing in one nucleotide position as compared with the human p57^{Kip2} GRE (position 10 of the 15 bp GRE: T→C). Furthermore, the mouse p57^{Kip2} promoter contained a one-nucleotide deletion immediately 3' of the GRE (Table 1). In order to test whether the altered nucleotides had any functional effect on the ability of the mouse

p57^{Kip2} GRE to confer a GC response, the two differences were introduced (a T→C change in the GRE and an elimination of a G in the putative Sp1 binding site) in the context of the human p57^{Kip2} GRE. In luciferase reporter expression experiments, it was shown that the mouse GRE is functional, albeit slightly weaker than the corresponding human GRE (Fig. 4). Furthermore, the mouse p57^{Kip2} GRE was, like the human p57^{Kip2} GRE, able to compete out GR binding to the TAT-GRE probe in the EMSA (data not shown).

Discussion

The p57^{Kip2} promoter deletion studies and mutational studies suggest that the GRE located 5076 to 5062 bases upstream of the transcription start site of the human p57^{Kip2} gene is responsible for the glucocorticoid inducibility of the p57^{Kip2} gene. This GRE is functional, even as an isolated 40 bp fragment. However, we cannot exclude the possibility that there are other parts of the gene, upstream or downstream of the 6.4 kb promoter region, that may contribute to the GC regulation of p57^{Kip2} expression. Low reporter gene induction, as compared with protein, may be because of additional post-transcriptional regulation or other regulatory elements. Moreover, a putative GRE downstream of the transcription start site (+171 to +185 bp), with similarities to the consensus GRE, had no significant effect on the dexamethasone inducibility (data not shown). Previously, we have shown that the dexamethasone induction of p57^{Kip2} is transcriptionally regulated (Samuelsson *et al.* 1999), and we now conclude that this effect is

Figure 4 The mouse p57^{Kip2} GRE is functionally conserved: dexamethasone-induced expression of luciferase reporter gene, under the control of the mouse p57^{Kip2} GRE (mouse GRE) as compared with the human p57^{Kip2} GRE (WT/min). Results are shown as fold induction of dexamethasone-treated vs control. The mean of three experiments $\pm$ standard deviation is shown. Student's *t*-test is used to calculate the statistical difference of fold induction compared with the Min reporter. **P* < 0.05, ***P* < 0.01.

mediated via a direct GRE interaction at the -5 kb GRE in the p57^{Kip2} promoter. Indeed, the clear induction in reporter gene expression seen when treating the cells (transfected with the Hinc 6:3, K-S/min or WT/min constructs) with the glucocorticoid agonist, dexamethasone, supports the proposal that glucocorticoid regulation is orchestrated through this GRE. However, posttranscriptional events, e.g. affecting mRNA stability or protein stability, may contribute to the overall expression of p57^{Kip2} protein. Both p21^{Cip1} and p27^{Kip1} have been shown to be upregulated on mRNA or protein levels after glucocorticoid treatment (Corroyer *et al.* 1997, Ramalingam *et al.* 1997, Rogatsky *et al.* 1997). Although transcriptional activation of p21^{Cip1} has been implicated (Ramalingam *et al.* 1997), the exact mechanisms behind GC regulation of the members of the Cip/Kip family are not fully elucidated. One exception is the GC-mediated induction of p21^{Cip1} in rat hepatoma cells, where the GR-dependent stimulation of the gene does not involve a

consensus GRE, but rather a C/EBP α -interaction (Cha *et al.* 1998, Cram *et al.* 1998). The results presented in this paper identify a functional GRE in the human p57^{Kip2} promoter, suggesting a classical GC-stimulated pathway.

The involvement of the -5 kb GRE in GC-orchestrated induction of the p57^{Kip2} promoter is supported by the observation that interaction between GR and the classical GREtat probe can be competed by an excess of cold GRE-Sp1 oligonucleotide. Furthermore, it is shown that the lack of a glucocorticoid response when the GRE is mutated is due to lack of interaction between the GR and the GRE, as the EMSA showed that the mutated p57^{Kip2} GRE oligonucleotide could not compete out the classical GREtat probe.

Although the p57^{Kip2} GRE is slightly less efficient than the TAT-GRE in driving the reporter gene expression the results clearly demonstrate that both, significantly and in the same range, can stimulate reporter gene activity after dexamethasone treatment. Moreover, it is shown that the response of the p57^{Kip2} GRE is dependent on GR and GC agonist, as 293 cells (not expressing GR) transfected with the p57^{Kip2} promoter construct show no induction of reporter expression after dexamethasone treatment, whereas 293 cells stably expressing GR, transfected with the same p57^{Kip2} promoter construct, have a response to dexamethasone similar to A-549 cells that endogenously express GR (Greenberg *et al.* 2002).

The GRE located 5 kb upstream of the transcription start site has a putative Sp1 binding site immediately downstream. Functional interaction between the GC-stimulated and the putative Sp1 pathways was investigated, since it has previously been shown that the progesterone receptor can regulate the transcription of the p21^{CIP1} gene through binding to Sp1 (Owen *et al.* 1998). Complete competition of GRE-GR interaction by excess of the oligo with a mutated Sp1 site (GRE-Sp1mut) shows that this putative Sp1 binding site has no direct effect on GR-DNA binding. However, the induction of reporter protein expression is further enhanced when this putative Sp1 binding site is mutated (see Fig. 2A), leading us to speculate that Sp1 might have a negative effect on transcription by some unknown mechanism, e.g. competition for overlapping binding sites.

The finding that the mouse GRE (homologous in sequence to the human –5 kb GRE, and located –4 kb) is functional in transient transfections, further implies that the glucocorticoid induction of human p57^{Kip2} is mediated through this GRE. This further suggests that GC regulation of p57^{Kip2} *in vivo* is of biological significance. This is presently under investigation.

In conclusion we found that the highly conserved –5 kb GRE in the p57^{Kip2} promoter is involved in the induction of p57^{Kip2} by dexamethasone and that this induction is orchestrated via a direct GR–GRE interaction.

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