CRE-Transcription Factor Decoy Oligonucleotide Inhibition of MCF-7 Breast Cancer Cells: Cross-Talk with p53 Signaling Pathway

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ABSTRACT: The CRE, 5′-TGACGTCA-3′, has been described as the consensus sequence for the *cis*-element that directs cAMP-regulated gene expression. Many transcription factors bind to this element and regulate the expression of a wide variety of cellular and viral genes. We have shown that CRE-transcription factor decoy oligonucleotide restrains the growth of cancer cells in vitro and in vivo [Park, Y. G., Nesterova, M., Agrawal, S., and Cho-Chung, Y. S. (1999) *J. Biol. Chem. 274*, 1573−1580]. The growth inhibition was accompanied by changes in cell morphology and apoptosis. To elucidate the molecular mechanism-(s) of the growth inhibition by the CRE-decoy oligonucleotide, we investigated the p53 signaling pathway. Herein, we report that CRE-decoy oligonucleotide treatment results in an increase in the p53 protein level in MCF-7 human breast cancer cells that express wild-type p53. The p21WAF1/Cip1 protein levels were also increased in the CRE-decoy oligonucleotide treated cells accompanying a reduction in Cdk2- and cyclin E-dependent kinase activity and pRb phosphorylation. Pulse−chase experiments reveal that the p53 upregulation was due to increased stability of the protein. The decoy oligonucleotide treatment also enhanced the p53 promotor-directed transcription in vivo along with the increase in p53-CBP (CREB-binding protein) complex formation. Thus, the stabilization and activation of p53 may have contributed to the growth inhibition induced by CRE-transcription factor decoy oligonucleotide in MCF-7 breast cancer cells. This decoy oligonucleotide approach offers great promise as a tool for defining cellular regulatory processes and treating cancer and other diseases.

Recent studies have shown that synthetic double-stranded phosphorothioate oligonucleotides with high affinity for a target transcription factor can be introduced into target cells as decoy *cis*-elements to bind the factor and alter gene transcription *(1, 2)*. Unlike the mRNA antisense approach, the transcription factor-decoy oligonucleotides provide a means for directly targeting transcription factors rather than mRNA. Furthermore, the decoy oligonucleotides provide novel methods for globally controlling the expression of genes that are regulated through an enhancer, unlike the antisense method, which only targets mRNA for one specific gene product.

The CRE,[1] 5′-TGACGTCA-3′, has been described as the consensus sequence for the *cis*-element that directs cAMP-induced gene transcription *(3)*. The CRE-transcription factor complex is a pleiotropic activator that participates in the induction of a wide variety of cellular and viral genes. We have recently shown that a 24mer single-stranded oligonucleotide of CRE-palindrome (trioctamer of TGACGTCA) oligonucleotide, that self-hybridizes to form a duplex/hairpin, can penetrate into cells, specifically interferes with CRE-directed transcription in vivo, and induces growth arrest in cancer cells in vitro and in vivo *(4)*. Moreover, the CRE-oligonucleotide effectively competed with the native CRE-enhancer for binding transcription factors and inhibited the basal CRE-gene transcription in a wide variety of cell types *(4)*. A recent study showed, by the use of oligonucleotide containing the CRE-consensus site derived from the c-fos promoter, that the sensitivity toward DNA damaging drugs is regulated by cAMP-induced gene activation and the p53-dependent pathway *(5)*. Furthermore, the cross-talk between cAMP and p53-generated signals has been shown in the induction of steroidogenesis (differentiation) and apoptosis in granulosa cells *(6, 7)*. An inverse relationship between the expression of bcl-2 and p53 has been shown in several breast cancer cell lines *(8)*. The tumor suppressor protein p53 is a transcriptional regulator whose ability to inhibit cell growth is dependent upon its transactivation function *(9−11)*. In addition, the cellular levels of the p53 protein are important. It has been shown that low or moderate levels of p53 lead to cell cycle arrest, whereas high levels trigger apoptosis *(12)*.

The objective of this study was to investigate the mechanism(s) by which CRE-decoy oligonucleotide affects the growth in MCF-7 breast cancer cells. We report that the decoy oligonucleotide inhibits the growth of MCF-7 cells, at least in part, through the activation of the p53 signaling pathway.

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[1] Abbreviations: CRE, cyclic AMP response element; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate; CREB, CRE-binding protein; CBP, CREB-binding protein; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; CAT, chloramphenicol acetyltransferase.

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MATERIALS AND METHODS

Oligonucleotides. CRE-decoy (CRE) and control oligonucleotides (CREC) used in the present studies are phosphorothioate oligonucleotides. Their sequences are as follows: CRE, 24mer CRE-palindrome: 5′-TGACGTCA TGACGTCA TGACGTCA-3′; CREC, 24mer nonsense-sequence palindrome control oligonucleotide: 5′-CTAGCTAG CTAGCTAG CTAGCTAG-3′. The nonsense-decoy control oligonucleotide exhibited lack of binding to other factors *(4)*. The synthesis of the oligonucleotides was carried out as previously described *(4)*.

Treatment of Cells in Culture with CRE-Oligonucleotides. MCF-7 cells [(1−1.5) × 10^6 cells] plated in a 10 cm dish were grown in improved minimal essential medium (Biofluids) supplemented with 10% heat-inactivated fetal bovine serum and gentamycin (25 μg/μL) at 37 °C. To increase the delivery of oligonucleotide into the cell, the cationic lipid *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate (DOTAP) (Boehringer Mannheim) was used in the oligonucleotide treatment. The CRE-decoy and control oligonucleotides were added (1 day after seeding) to the dish at concentrations of 50−150 nM in the presence of DOTAP (DNA:DOTAP ratio = 1:5). At 12−18 h of incubation, the medium was removed, and fresh medium without oligonucleotide and DOTAP was added. Cells were harvested at the indicated times, and used for the experiments.

RT-PCR. Total cellular RNAs were prepared from MCF-7 cells untreated or treated with CRE-decoy or control oligonucleotide using Trizol reagent (Gibco). RNAs (2 μg) were subjected to reverse transcription using oligo(dT) primers by the method of SuperScript preamplification system (Gibco).

Aliquots of first-strand cDNA were amplified directly with the primers specific for p53 and G3PDH (Clontech). The PCR reactions were performed using the step-cycle program set to denature at 94 °C for 30 s, anneal at 50 °C for 30 s, and extend at 72 °C for 1 min for 35 cycles, and then the PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide.

Immunoblotting and Coimmunoprecipitation. MCF-7 cells were washed in cold phosphate-buffered saline (PBS) and lysed in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40, 0.5 mM sodium orthovanadate, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/mL each of leupeptin and aprotinin). The lysates were centrifuged at 15000*g* for 15 min, and the supernatant was collected and assayed for protein concentration using a Bradford assay (Bio-Rad). Total protein (40 μg) was run on 6% SDS−PAGE (for CBP, pRb), 10% SDS−PAGE (for p53, TFIIB), and 12% SDS−PAGE (for p21, p27). The separated proteins were then transferred to nitrocellulose membranes. Blots were then blocked overnight and probed with polyclonal or monoclonal antibodies specific to human p53 (Santa Cruz DO-1 for western, Oncogene pAb421 for immunoprecipitation), p21 (Transduction Laboratories), p27 (Santa Cruz F-8), TFIIB (Transduction Laboratories), pRb (Pharmingen), and CBP (Santa Cruz A-22). Blots were then washed and incubated with horseradish peroxidase-conjugated secondary antibodies and visualized using the Amersham ECL system. For coimmunoprecipitation, 1−1.5 mg of proteins was incubated with first antibody overnight at 4 °C and then cross-linked to protein A−Sepharose (Sigma) for 2 h at 4 °C. The beads were washed 4 times with wash buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.5% NP-40, 1 mM EDTA), and the proteins were eluted in SDS sample buffer. The separation and blotting of proteins and detection of the bands followed the same methods as that described in the immunoblotting method.

Histone H1 Kinase Assay. Cell lysates were diluted with lysis buffer (see immunoblotting method) to a final protein concentration of 1 μg/μL. The lysates (500 μL) were cleared by addition of protein A−Sepharose 4B (20 μL) (Sigma) for 30 min at 4 °C and centrifugation. The supernatants were incubated with 5 μg of anti-Cdk2 antibody or anti-cyclin E antibody (Santa Cruz) overnight, and 40 μL of protein A−Sepharose was added. After 2 h incubation, the precipitates were collected by centrifugation and washed 4 times with lysis buffer. The precipitates were further washed 3 times in kinase assay buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, and 1 mM dithiothreitol) and then incubated with 20 μL of kinase buffer containing 3 μg of histone H1 (Boehringer Mannheim), 1 μM ATP, and 5 μCi of [γ-^{32}P]-ATP for 30 min at 37 °C. The products of the reaction were separated on an SDS−12% polyacrylamide gel. The gels were then dried and exposed to X-ray film.

Pulse−Chase Experiments. MCF-7 cells untreated or treated with CRE-decoy or control oligonucleotide (150 nM, 24 h) were washed twice with cold PBS and preincubated for 30 min at 37 °C in labeling medium [(L-methionine + L-cysteine)-free Dulbecco's modified Eagle's medium (DMEM)(Biofluids) with 10% heat-inactivated fetal bovine serum and antibiotic−antimycotic]. Cells were then incubated for 2 h at 37 °C with prewarmed, methionine−cysteine-free DMEM supplemented with 5% dialyzed fetal bovine serum and 200 μCi/mL ^{35}S-labeled L-methionine + L-cysteine protein labeling mix (Trans ^{35}S-label, ICN). After labeling, plates were washed 3 times with PBS, and then incubated for the indicated chase times in growth medium containing 4 mM L-methionine + L-cysteine. Lysates were made at zero time (no chase) and each chase time point; p53 was immunoprecipitated, solubilized, run on SDS−PAGE, and analyzed by autoradiography. The p53 half-life was determined by direct quantification of the band intensities in the autoradiographs by image analysis *(13)*.

Transient Transfection and CAT Assay. MCF-7 cells (5 × 10^5 cells/60 mm dish) were transfected with 2 μg of PG13-CAT or MG15-CAT reporter constructs *(14)* and a 50 nM sample of CRE or control oligonucleotide using DOTAP. After 24 h, fresh medium was added, and the cells were harvested at 36−48 h and then assayed for CAT activity with [^{14}C]chloramphenicol using thin-layer chromatography *(15)*.

RESULTS

Upregulation of p53 Protein. We have previously shown that CRE-decoy oligonucleotide treatment results in the growth inhibition of MCF-7 human breast cancer cells and the growth inhibition accompanied cell morphology change and apoptosis *(4)*. We examined whether p53 protein plays a role in this growth inhibition. As shown in Figure 1A,B, CRE-decoy treatment resulted in a marked increase in the p53 level in both a time- and concentration-dependent

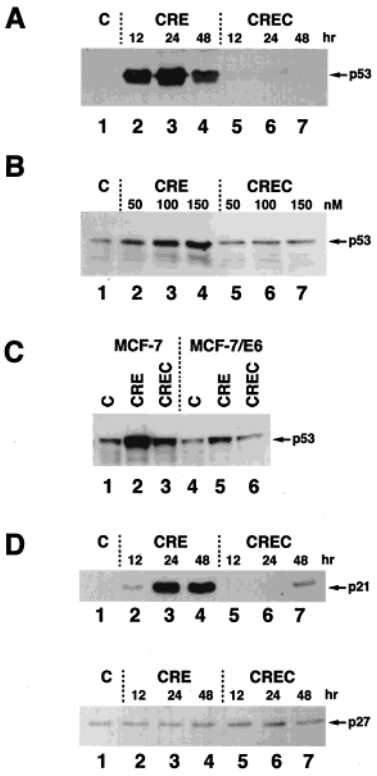

FIGURE 1: CRE-decoy oligonucleotide induces p53 and p21 WAF1/Cip1. (A) Time course of p53 upregulation. Total cellular lysates were prepared from cells treated with saline (C), CRE-decoy oligonucleotide (CRE), or control oligonucleotide (CREC) at 150 nM for 12, 24, and 48 h, and western blotting analysis was performed as described under Materials and Methods. (B) Concentration dependence of p53 upregulation. Cells were treated with CRE-decoy (CRE) or control oligo (CREC) at the indicated concentrations for 24 h, and western blotting analysis was performed as described in (A). (C) CRE-decoy-induction of p53 in wild-type MCF-7 and mutant MCF-7/E6 transfected cells. p53 levels were measured as described in (A), except that the time point is 24 h post the CRE-decoy treatment. (D) Time course for p21 and p27 protein induction. The p21 and p27 protein levels were examined by western blotting analysis. The time course and concentration of CRE-decoy are the same as in (A). The data represent one of three independent experiments that gave similar results.

manner. Quantitation of the band intensities in the autoradiograph of Figure 1B by densitometric tracings [Alpha Imager 2000, Documentation & Analysis system (Alpha Innotech Corp.)] showed that CRE-decoy increased p53 protein levels 1.5-fold at 50 nM, 5-fold at 100 nM, and 6-fold at 150 nM. Within 12 h of oligonucleotide treatment, upregulation of p53 protein was detected, by 24 h, p53 levels were maximally increased, and at 48 h, p53 levels were reduced to that of 12 h. Importantly, this time-dependent increase in p53 protein correlated with the uptake of CRE-oligonucleotide by the cell. The cellular uptake of fluorescence-labeled CRE-oligonucleotide has been shown to reach its peak level at 12 h posttreatment, and by 24 h, the intensity of fluorescence has been reduced in the cell *(4)*. Thus, the p53 protein upregulation followed the preceding cellular uptake of the oligonucleotide. By comparison, control oligonucleotide (CREC) treatment had no effect on the p53 protein level at any time points (Figure 1A) or concentrations (Figure 1B) examined.

To further examine the involvement of p53 in the CRE-decoy oligonucleotide-induced growth inhibition, we used MCF-7 cells transfected with human papilloma virus E6 gene *(16)*. In MCF-7/E6 cells, the E6 gene product stimulates degradation of p53 through the ubiquitin pathway *(17)*. Quantitation, by densitometric tracings, of the band intensities in the autoradiograph of Figure 1C showed that the p53 level in CRE-decoy-treated MCF-7/E6 cells was 40% of that in CRE-treated parental MCF-7 cells. Although E6-degradation of p53 appears to be incomplete in this MCF-7/E6 cell line, the data support the involvement of p53 in the growth-inhibitory effect of the CRE-decoy.

The upregulation of p53 by the CRE-decoy oligonucleotide treatment was not restricted to MCF-7 cells. Similar upregulation of p53 protein was observed in various cancer cells that contain the wild-type p53, including LS-174T colon carcinoma, LNCaP prostate carcinoma, and A549 lung carcinoma cells. In contrast, in cancer cells that contain the mutant p53 (such as MDA-MB-231, MDA-MB-468, T47D, and SK-BR-3 breast carcinoma cells) or null mutation in p53 (PC3M prostate carcinoma cells), the CRE-decoy had no effect on the p53 levels (data not shown.)

We next examined whether the CDK inhibitors, p21 and p27, are involved in the CRE-decoy-inhibition of cell growth. The decoy oligonucleotide markedly increased p21 levels but had no effect on p27 levels (Figure 1D). Thus, the increase in p53 by the CRE-decoy oligonucleotide resulted in increased transactivation activity of p53 and the subsequent induction of p21 protein.

Inhibition of Cdk2- and Cyclin E-Associated Kinase Activity and pRb Phosphorylation. Since p21 has been shown to inhibit the cyclin E- and Cdk2-associated kinase activity *(18*−*20)*, and the above data show that p21 is increased by the CRE-oligonucleotide treatment (Figure 1C), we examined whether Cdk2- and cyclin E-associated kinase activity is affected by the decoy treatment. Histone H1 kinase assay showed that the Cdk2- and cyclin E-associated kinase activity was markedly reduced in CRE-decoy oligonucleotide treated cells (Figure 2A). By comparison, the CRE control oligonucleotide exhibited no effect (Figure 2A).

In the CRE-decoy treated cells, the p21 levels were increased (Figure 1C) in the absence of changes in Cdk2 and cyclin E levels (data not shown), and thus the decrease in Cdk2- and cyclin E-associated kinase activity observed (Figure 2A) may be due to the increased binding of p21 to Cdk2−cyclin E complex. We therefore examined whether the p21 protein induced by CRE-decoy is associated with the Cdk2−cyclin E complex. Cell lysates were first immunoprecipitated with anti-Cdk2 antibody and followed by western blotting analysis. Probing with anti-p21 antibody showed that p21 protein was markedly increased at 24 h after CRE-decoy treatment (Figure 2B), indicating that the CRE-decoy not only induced p21 expression but also increased the level of p21 in association with Cdk2.

The cyclin E−Cdk2 complex formation reaches its peak level at the G1/S transition, and this complex contributes to phosphorylation of pRb *(21, 22)*. We examined the status of phosphorylation of pRb in CRE-decoy treated cells by western blotting analysis. A doublet band of unphosphorylated (pRb) and phosphorylated (ppRb) Rb proteins in equal amounts was detected in the control (saline-treated) cells (Figure 2C, lane 1). The CRE-decoy treatment resulted in a time-dependent reduction in phosphorylated Rb (ppRb, Figure 2C, lanes 2−4). Although exact quantification was not possible due to poor resolution of the doublet band, the

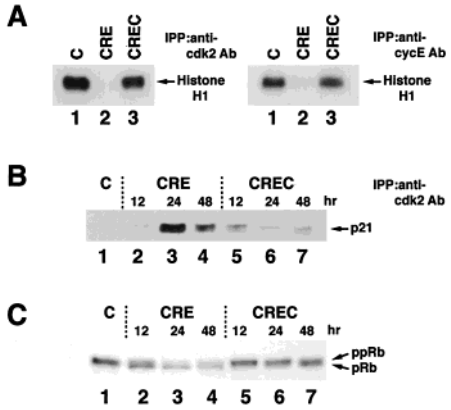

FIGURE 2: Effect of CRE-decoy treatment on Cdk2- and cyclin E-dependent kinase activity, in vivo association of p21 with Cdk2, and pRb phosphorylation. (A) Cdk2- and cyclin E-associated histone H1 kinase activity. Anti-Cdk2 antibody immunoprecipitate and anti-cyclin E antibody immunoprecipitate, obtained from an equal amount of lysates from cells treated with saline (C), CRE-decoy oligonucleotide (CRE), or control oligonucleotide (CREC) at 150 nM for 24 h, were assayed for kinase activity using histone H1 as a substrate as described under Materials and Methods. (B) In vivo association of p21 with Cdk2. Cells were treated with saline (C), CRE-decoy oligonucleotide (CRE), or control oligonucleotide (CREC) at 150 nM for 12, 24, and 48 h, and cell lysates were immunoprecipitated with anti-Cdk2 antibody followed by Western blotting analysis with anti-p21 antibody. (C) Phosphorylation of pRb. Phosphorylation of Rb protein was determined by western blotting analysis of the same lysates used in (B) using anti-pRb antibody. The data represent one of three independent experiments that gave similar results.

reduction in ppRb was most striking at 24 h post-CRE-decoy treatment (Figure 2C, lane 3). Importantly, at this 24 h time point of CRE-decoy treatment, the greatest amount of p21 protein was found to be coimmunoprecipitable with anti-cdk2 antibody (Figure 2B, lane 3). These results suggest that a reduction of cyclin E−Cdk2-associated kinase activity by p21 may be the cause for the hypophosphorylation of pRb.

Altered Stability of p53. We examined whether p53 upregulation was due to altered stability of p53 by pulse−chase experiments. The half-life of p53 in saline- or control oligonucleotide-treated cells was approximately 45 min as measured by immunoprecipitation of ^{35}S-labeled p53 protein from cell extracts after a cold chase with unlabeled methionine. In contrast, the half-life of p53 protein in cells treated with CRE-decoy oligonucleotide was 180 min (Figure 3A). This represents a 4-fold increase in the half-life of p53 protein upon CRE-oligonucleotide treatment. Pulse−chase experiments with MDA-MB-231 cells, which contain a mutant p53, demonstrated that the half-life of p53 protein in untreated control cells was 180 min. This was 4 times greater than that in MCF-7 control cells. The CRE-decoy oligonucleotide treatment had no effect (data not shown). We next examined p53 mRNA levels in cells treated with CRE-decoy or control oligonucleotide after labeling of cells with [^{35}S]methionine under the conditions shown in Figure 3A. The p53 mRNA levels in the control and CRE-treated cells were determined by RT-PCR analysis. As shown in Figure 3B, the levels of p53 mRNA were not affected by the CRE-decoy oligonucleotide treatment. Thus, the increase in p53 protein was not due to an increase in the transcription.

Increased Association of p53 with CBP. Transcription factor, CBP (CREB-binding protein) *(23)*, which is implicated in cell proliferation and differentiation, acts as a p53 coactivator and potentiates its transcription activity *(24)*. As the above data suggest that p53 activation may be involved in the growth inhibitory effect of the CRE-decoy, we examined whether enhanced binding of p53 to CBP is involved in the growth inhibition. Lysates prepared from the control and CRE-decoy treated (24 h, 150 nM) MCF-7 cells were first immunoprecipitated with either anti-p53 antibody or anti-CBP antibody and then subjected to western blotting analysis probing with anti-CBP antibody and anti-p53 antibody, respectively. As shown in Figure 4B,D, an increased binding of p53 to CBP was observed in the CRE-decoy oligonucleotide treated cells. By comparison, the control oligonucleotide (CREC) had no effect (Figure 4B,D). In contrast, the binding of CBP to basal transcription factor, TFIIB, was not affected by the CRE-oligonucleotide treatment (Figure 4F). Importantly, the increased binding of p53 to CBP observed in the CRE-decoy treated cells was due to an increase in the amount of p53 in the cell but not CBP (Figure 4A,C); the amount of CBP did not change by the decoy treatment.

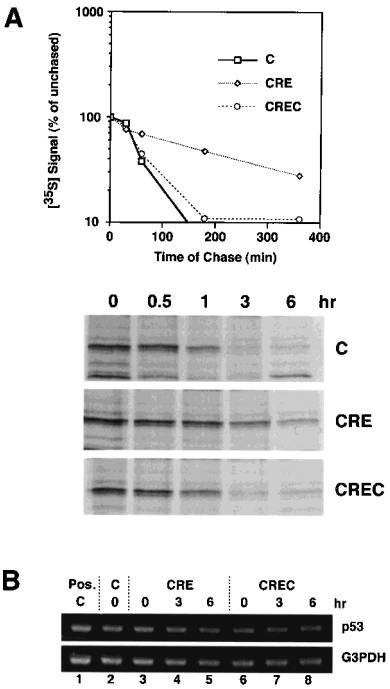

FIGURE 3: CRE-decoy causes p53 protein stabilization. (A) Stabilization of p53 protein. MCF-7 cells were treated with saline (C), CRE-decoy oligonucleotide (CRE), or control oligonucleotide (CREC) at 150 nM for 24 h, and the half-life of p53 was determined by [^{35}S]methionine pulse−chase analysis (see Materials and Methods). Lysates were made at 0, zero time (no chase), and at 0.5, 1, 3, and 6 h of chase, and p53 was immunoprecipitated, solubilized, run on SDS−PAGE, and analyzed by autoradiography. The band intensity in the autoradiographs was quantified by image analysis. Band intensities are expressed as a percentage of the control signal (pulse only, no chase). The data represent one of three independent experiments that gave similar results. (B) RT-PCR analysis of p53 mRNA levels. The RT-PCR was performed with cDNA prepared from control and CRE- and CREC-treated cells, labeled with [^{35}S]methionine followed by cold methionine chase under the conditions shown in (A), using p53 primers as described under Materials and Methods, and G3PDH mRNA as an internal control. 0, 3, and 6 h are the same time points as (A).

Increase in p53 Transactivation Activity. We examined whether CRE oligonucleotide treatment enhanced the p53

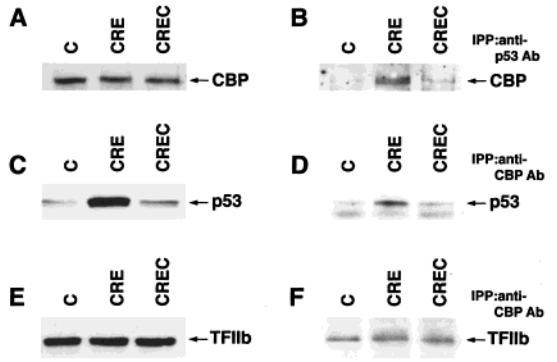

FIGURE 4: CRE-decoy induces increased interaction of p53 and CBP. MCF-7 cells were treated with saline (C), CRE-decoy oligonucleotide (CRE), or control oligonucleotide (CREC) at 150 nM for 24 h. p53 and CBP were then immunoprecipitated from total cell lysates. (A, B) Western blotting of CBP in cell lysates (A) and in anti-p53 antibody immunoprecipitates (B), respectively; (C, D) western blotting of p53 in cell lysates (C) and anti-CBP antibody immunoprecipitates (D), respectively; (E, F) western blotting of TFIIB in cell lysates (E) and in anti-CBP antibody immunoprecipitates (F), respectively. The data represent one of three independent experiments that gave similar results.

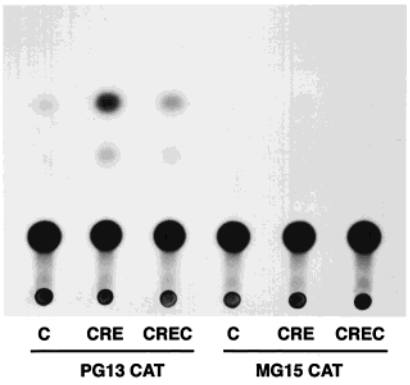

FIGURE 5: Effects of CRE-decoy on p53-mediated transactivation. MCF-7 cells were transfected with 2 μg of PG13-CAT or MG15-CAT reporter construct and a 50 nM sample of CRE-decoy (CRE), control oligonucleotide (CREC), or saline (C). The CAT activity was quantified by image analysis and normalized by β-gal activity. The data represent one of three independent experiments that gave similar results.

transactivation activity. As shown in Figure 5, transfection of MCF-7 cells with PG13-CAT *(14)*, a reporter construct containing multiple copies of the specific p53-binding sequence, plus CRE-decoy oligonucleotide (CRE, PG13-CAT) resulted in a 10-fold induction of CAT activity over that in the CRE-control oligonucleotide treated cells (CREC, PG13-CAT). In contrast, the CRE-decoy had no effect on the transcription activity of MG15-CAT, a mutant p53-CAT (Figure 5). Thus, the endogenously upregulated p53 by CRE-decoy was capable of enhancing the transactivational activity of p53.

DISCUSSION

We have shown previously that CRE-decoy oligonucleotide produces growth inhibition in cancer cells but not normal cells *(4)*. The CRE-decoy oligonucleotide markedly inhibited CRE DNA−protein complex formation, CRE-directed transcriptional activity, and endogenous cAMP-responsive gene expression *(4)*. The specific growth inhibitory effect toward cancer cells correlated with induction of phenotypic change and apoptosis.

We have shown in the present study that CRE-decoy oligonucleotide treatment of MCF-7 breast cancer cells which contain wild-type p53 resulted in upregulation of p53 protein. This upregulation of p53 by the CRE-decoy did not occur in cells that contain mutant p53 or null p53. Importantly, the CRE-decoy had a much reduced effect on p53 upregulation in MCF-7/E6 cells in which the E6 gene product stimulates degradation of p53. Thus, the CRE-decoy appears to have a specific effect on the upregulation of p53 in MCF-7 cells.

It has been shown that certain kinds of cellular stress, such as DNA damage and hypoxia, cause increased p53 protein levels through protein stabilization and induce p53-dependent biological response pathways *(25−27)*. It has also been shown that transient stabilization of p53 occurs in retinoic acid induced growth arrest of lung carcinoma cells *(13)*. Our results indicate that the CRE-decoy acts in similar ways as DNA damaging agents in terms of p53 protein upregulation and growth inhibition. The upregulated p53 induced the CDK inhibitor p21, increased p21 protein complex with Cdk2, and reduced the Cdk2- and cyclin E-associated kinase activity and pRb phosphorylation in MCF-7 cells. Therefore, p53-dependent p21 induction may be involved in the growth inhibition by CRE-decoy oligonucleotide in MCF-7 cells. The mechanism by which CRE decoy oligonucleotide brought about activation of p53 is not clear, but pulse−chase experiments reveal that an increase in stability of the p53 protein underlies the upregulation by the CRE-decoy treatment. CRE-decoy oligonucleotide specifically upregulated the wild-type p53, but not mutant p53. In fact, little or no change in the expression of mutant p53 was observed in several cell lines that contain mutant p53 after CRE-decoy treatment. These results suggest that CRE-decoy may act through modulation of protease activity, stabilizing factors, or conformation modifiers specific for wild-type p53 through depletion of transcription factor binding to the CRE-enhancer.

Recently, it has been reported that CBP is involved in the growth control pathway by the interaction with the tumor suppressor p53 *(24, 28−30)*. CBP was originally found as a CREB-binding protein, and its direct interaction with phosphorylated CREB leads to enhancement of CREB transactivational activity *(23)*. Based on the idea that the competition for a common coactivator like CBP may in part explain the cross-talk which is commonly observed between disparate signaling pathways, we speculated that CRE-decoy oligonucleotide, through reducing the CREB binding to CBP, would render CBP to be more accessible to p53, resulting in increased binding of p53 to CBP. In fact, our results demonstrated the increased binding of p53 to CBP in CRE-decoy treated cells. Thus, the growth inhibitory effect of CRE-decoy oligonucleotide may be linked to increases in p53−CBP complex formation. This interaction of p53 with CBP may increase the p53 transactivation activity, leading to induction of p21 and the downstream growth inhibitory signaling pathway.

At present, however, it cannot be excluded that decreased CRE-dependent transcription due to CRE-decoy treatment might play a role in the regulation of cell-cycle genes or in growth arrest. CREB is a member of the CREB/ATF family of transcription factors, and its transactivation activity increases after phosphorylation by cAMP-dependent protein kinase (*31*). More recently, it has become apparent that

CREB is an in vivo substrate for a variety of other kinases including calmodulin kinases II and IV (*32*) or RSK2 (*33*), implying that the CREB/ATF family of transcription factors can activate CRE-transcription in response to cAMP, Ca^{2+}, and growth factor stimulation (*34*−*37*). That the growth factor-stimulated pathways are quiescent in noncancerous cells could explain, at least in part, the tumor cell-specific inhibition of growth demonstrated by the CRE-decoy (*4*).

Although the exact mechanism of action remains to be elucidated, our present results suggest that the p53-dependent induction of p21 may be a critical event involved in the growth inhibition induced by CRE-decoy oligonucleotide in MCF-7 breast cancer cells.

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