Induction of Cell Growth Regulatory Genes by p53

Stephen L. Madden,1 Elizabeth A. Galella, Deborah Riley, Arthur H. Bertelsen, and Gary A. Beaudry

Department of Molecular and Cellular Biology, PharmaGenics, Inc., Allendale, New Jersey 07401

ABSTRACT

Transcriptionally regulated growth-response genes play a pivotal role in the determination of the fate of a cell. p53 is known to transcriptionally regulate genes important in regulating cell growth potential. Using differential reverse transcription-PCR analysis of rat embryo fibroblast cells containing a temperature-sensitive p53 allele, we were able to isolate several transcripts up-regulated specifically in cells harboring functional p53 protein. Two of these genes, SM20 and microsomal epoxide hydrolase (mEH), are previously described genes. Two previously uncharacterized cDNAs, cell growth regulatory (CGR) genes CGRIJ and CGRJ9, were isolated. The predicted amino acid sequence of these novel proteins contain known motifs; EF-hand domains (CGR11) and a ring-finger domain (CGR19), suggestive of function. CGRIJ and CGRJ9 appear to be primary response genes expressed to moderate levels in functional p53 cells. Both CGRIJ and CGRJ9 are able to inhibit the growth of several cell lines.

INTRODUCTION

Significant advances have been made recently toward the identification of numerous potential growth effectors within cells. Although previous analyses have evaluated the phenotypic effects of cellular environment, growth factors and stress, it is only recently that the underlying molecular events responsible for perturbing cell growth are beginning to be defined. In a large number of cases, the cause of a particular cellular growth response can be attributed to transcriptional attenuation (1, 2). Indeed, the recent gene discoveries directly relevant to cell cycle regulation have been based, to a significant degree, on differential gene expression analyses (2—4).

The p53 gene remains the most frequently mutated gene in human cancers, undoubtedly reflecting an important regulatory function for this gene in controlling cell growth (reviewed in Ref. 5). p53 function can be attenuated by interaction with viral or cellular proteins (6—9) and cytoplasmic sequestration (10, 11), ultimately leading to alterations in cell growth potential. Although p53 is thought to exert growth-regulatory functions in response to DNA damage and by directly inhibiting DNA replication (12, 13) or inducing apoptosis (9), a large body of data indicates that the primary function of p53 is to transcriptionally regulate downstream effector genes. p53 contains a potent transcriptional activation domain and is able to bind DNA in a sequence-specific manner (14—16), allowing for both transcriptional activation and repression of target genes (17, 18). p53-mediated transcriptional repression has also been proposed to occur via p53 protein interaction with general transcription factors (19).

Identification of the p53 transcriptional responsive genes p21WAF1/CIP1 (2, 20), MDM2 (1, 8), GADD45 (21, 22), HIC (23), cyclin G (24, 25), and BAX (26—29), the products of which have been suggested to have direct effects on cell growth, emphasizes the pivotal role of p53 in modulating expression levels of growth-response genes. To date, two of the best characterized direct effectors of cell growth that are transcriptionally regulated by p53 include p21WAF1/CIP1 (2, 20) and MDM2 (1, 8). p21WAF1/CIP1 functions as a cyclin-dependent kinase inhibitor by directly interacting with cyclin-dependent kinase proteins during progression of the cell cycle (20). Overexpression of p21WAF1/CIP1 via transfection suppresses cell growth in a wide array of cancer cell lines. In contrast, MDM2 functions by negatively regulating p53 activity, and overexpression of MDM2 leads to uncontrolled cell growth and tumorigenesis (8). However, p53-induced transcriptional responses appear ill-conserved, suggesting that cell type-, genetic-, and species-dependent factors may contribute to p53 responsiveness; for example, both p21WAF1/CIP1 and MDM2 are transcriptionally activated in a p53-dependent manner in rodent cells harboring a temperature-sensitive p53 allele, but only MDM2 is induced in an analogous human system (30). In addition, activation of p21WAF1/CIP1 has been shown to be both p53-dependent and -independent, suggesting alternate pathways for regulation of its expression (31, 32). Furthermore, it appears that the levels of apoptotic regulatory gene products, such as BCL2 or E1B, are influential in determining if a cell will undergo p53-dependent growth arrest or apoptosis (9, 33). These results suggest that a complex regulatory cascade, using numerous effector gene products, determines cell growth potential. The complexities of cell growth regulation underscores the need to further define the relevant molecular determinants.

The well-characterized murine temperature-sensitive p53 mutation (VAL135) has been used to further characterize potential transcriptional responses of wild-type p53 protein. Growth of rat embryo fibroblast cells (REF-112) transformed with activated RAS and p53-VAL135 is temperature sensitive (10, 34). Growth of REF-112 cells at 38°C maintains the p53 protein in a nonfunctional conformation complexed with HSP70 in the cytoplasm (10, 35). When the cells are shifted to 32°C, the p53 protein adopts a functional, wild type-like conformation and is able to elicit both apoptotic- and growth-specific effects, which are dependent on the presence of functional p53. Two of these genes are previously undescribed and encode proteins containing known functional motifs. We demonstrate here that these genes have growth-suppressive potential in several cell lines examined.

MATERIALS AND METHODS

Cell Culture. Rat embryo fibroblast cells REF-112 (p53-VAL135) and REF-132 (p53-PHE132; kindly provided by B. Vogelstein (Johns Hopkins University, Baltimore, MD) and M. Oren (Weizmann Institute, Rehovot, Israel)), were grown in DMEM containing 10% fetal bovine serum in 5% CO2.
at either 38°C or 32°C. Cells were split and seeded 48 h before any temperature shifts. Temperature shifts were performed by simple transfer of subconfluent flasks to preequilibrated incubators without media changes. For transfections, 4 × 10^5 cells (T98G and SW480 cells) or 0.8 × 10^5 cells (SKOV3-IP1) were seeded in six-well dishes. Transfections were performed using lipofectin (Life Technologies, Inc.) as described by the manufacturer. Briefly, 40 μl of reduced serum medium (OptiMEM; Life Technologies, Inc.) was added to 2 μg of DNA (pCEP4 derivatives; Invitrogen). A 50-μl mixture containing 10 μl of lipofectin and 40 μl of Optimem was added to the DNA mixture. After a 15-min room temperature incubation, 1 ml of Optimem was added, and the mixture was overlaid onto Optimem-washed cells. Cells were allowed to incubate for 5 h in a 37°C/5% CO₂ incubator, following which the transfection mixture was replaced with normal growth medium. After 44 h, cells were split and selection medium containing hygromycin (0.25 mg/ml). After 12–14 days, colonies were stained with 2% methyl blue in 50% ethanol and counted. Only colonies containing >50 cells were scored.

**Plasmid Constructs.** pCEP4 derivatives were constructed as follows: rCGR19 and rCGR11 were cloned as KpnI/HindIII fragments, respectively, from the original, full-length 5' RACE clone in pCR and rCGR19 were cloned as KpnI/XhoI and NotI/BamHI fragments, respectively, from the original, full-length 5' RACE clone contained in pCRII. Colonies containing >50 cells were scored.

**RNA Isolation.** Total RNA was isolated by direct lysis in RNazol B (Tel-Test, Inc.) as described by the manufacturer. Poly(A)^+ RNA was isolated from total RNA preparations using a MessageMaker kit (Life Technologies, Inc.) as described by the manufacturer.

**Oligonucleotides.** Twelve anchored oligo(dT) primers ([dT<sub>12</sub>(A,C,G)- (A,C,G,T) combinations] and 25 random 10-mers (3) were used for RT-PCR reactions. For cyclin G RT-PCR reactions, dT<sub>12</sub>G and 5'-TCTTCACTGTC-3' primer pairs were used to amplify a ~300-bp fragment.

**RT-PCR Reactions.** Reverse transcription reactions were performed using 200 ng of total RNA in 5 mM MgCl₂/10 mM Tris, pH 8.3, 10 mM KCl, 20 μM deoxynucleotide triphosphates, 20 units RNase inhibitor (Perkin-Elmer), 50 μM T<sub>12</sub>NN, and 50 units Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer) by heating samples (without reverse transcriptase) to 65°C for 5 min, and then placing the reaction at 37°C for 5 min. Reverse transcription reactions were allowed to proceed for 55 min at 37°C. Reverse transcription reactions were inactivated by incubating for 5 min at 95°C. PCR reactions were performed with 2 μl of the reverse transcriptase reaction in 1.5 mM MgCl₂, 10 mM Tris (pH 8.3), 10 mM KCl, 4 μM deoxynucleotide triphosphates, 2.5 units Taq polymerase (Perkin-Elmer), 10 μM of [α-<sup>35</sup>S]dATP (12.5 μCi/μl; DuPont NEN), 50 μM T<sub>12</sub>NN, and 0.2 μM random 10-mer. PCR reactions were performed in a thermocycler for 40 cycles: 94°C for 3 s, 40°C for 2 min, and 72°C for 30 s. Reactions were terminated by the addition of 38% formamide, 8 mM EDTA, 0.02% bromophenol blue, and xylene cyanol. Samples were heated for 2 min at 70°C and run on a 6% denaturing polyacrylamide gel. PCR products were purified after drying the gel onto Whatmann 3MM paper and exposure to film. Excised bands were resuspended in 120 μl of H₂O for 10 min at room temperature, followed by 15 min of boiling. Debris was pelleted by centrifugation, and 10 μl of 3 M sodium acetate, 5 μl of 10 mg/ml glycerol, and 400 μl of ethanol were added to 100 μl of the eluted DNA. DNA was allowed to precipitate overnight at ~20°C. The DNA was pelleted, amplified using the same primers used for the original RT-PCR, gel purified, and cloned into pCRII (Invitrogen). RT-PCR amplification of specific clones was achieved using the following primer pairs: CGR11, dT<sub>12</sub>AT/5'-TACACAGGAGG-3'; SM20, dT<sub>12</sub>GG/5'-GATCATAGCGGAG-3'; CGR19, dT<sub>12</sub>GCG-5'-GATCATAGGCC-3'; and mEH, dT<sub>12</sub>CAG/5'-GATCATAGTGC-3'.

**5' RACE.** RACE was performed with the Marathon kit (Clontech) using REF-112 RNA harvested from 32°C-maintained cells as described by the manufacturer. Oligonucleotides specific for the 5' ends of rat CGR11 and CGR19 were used in the procedure.

**Northern Analysis.** Northern analysis was performed using 10–20 μg of total RNA electrophoresed on 1.2% formaldehyde gels. Blotting and probing was essentially as described (38). Probes were gel-purified cDNA fragments α-<sup>32</sup>P-labeled by random priming (Boehringer Mannheim Biochemicals). Rat tissue blots were obtained from Clonetech and probed, stripped, and reprobed as recommended by the manufacturer. Rat p21<sup>WAF1/CIP1</sup> cDNA was kindly provided by B. Vogelstein.

**DNA Sequencing.** Sequencing of all cDNA clones was performed by manual, Sanger dideoxy sequencing (United States Biochemical Corp.) using primers that allowed for sequence determination of both strands along the entire length of the cDNAs. Multiple clones were sequenced for both rat and human CGR11 and CGR19 cDNAs.

**Database Searching.** All homology searches (FASTA), alignments (BESTFIT), and structural features (MOTIFS) were performed using The Genetics Computer Group (Madison, WI) software programs. Final searches were performed using GenBank version 94.0.

**RESULTS**

**Characterization of REF-112 RNA.** To initially characterize total RNA prepared from REF-112 cells grown at both 38°C and 32°C, we evaluated the expression of genes known to be regulated by p53. Northern analysis of p21<sup>WAF1/CIP1</sup> showed a rapid induction of transcription, with relatively high levels of p21<sup>WAF1/CIP1</sup> RNA observed by 8 h after shifting from 38°C (Fig. 1a). Furthermore, we have observed a corresponding induction of apoptosis within 8–10 h after shifting to 32°C (data not shown). Based upon these observations, we chose an 8-h induction time for the differential transcript analyses presented here. In addition, rat cyclin G Northern analysis showed high levels of transcript at the growth-inhibitory temperature of 32°C but little or no such transcript in cells growing exponentially at 38°C.
(Fig. 1c). Both cyclin G and p21WAF1/CIP1 appeared to be induced to similar levels (data not shown; see below).

RT-PCR reactions were performed on total RNA isolated from both growth conditions with primers specific for the 3' end of rat cyclin G to characterize the RNA preparation prior to identifying novel p53-regulated genes. As anticipated, a differentially expressed band of 300 bp was detected in the 32°C-induced RNA but was absent in the RNA from uninduced cells (Fig. 1b). Excision of the cyclin G RT-PCR band and subsequent cloning and Northern analysis (Fig. 1c) confirmed the identity of this band as rat cyclin G (data not shown).

**Differential Analysis of REF-112 RNA.** To identify other growth-regulated genes, we performed RT-PCR reactions using 12 "anchored" oligo(dT) primers in conjunction with 25 "random" 10-mers (see "Materials and Methods"). RT-PCR reactions were performed on duplicate, independently isolated total RNA from REF-112 cells maintained at 38°C or 32°C. Primer pairs that would amplify either rat p21WAF1/CIP1 or MDM2 were omitted.

A total of 35 differentially expressed RT-PCR products were chosen for further analysis based on apparent induction of product at the induced temperature of 32°C. Although RT-PCR reactions were performed on duplicate RNA samples, all reactions containing potentially interesting transcripts were repeated in full to further avoid potential artifacts that are a common problem in differential display analyses (3, 39). Subsequent Northern analyses ruled out all but four of these RT-PCR products as being differentially expressed genes. None of these four genes showed any transcriptional induction in RNA from control cells harboring a non-temperature-sensitive p53 mutation (REF-132 cells (PHE132)) grown at 32°C, suggesting that the transcriptional induction observed was not due to the shift in temperature (data not shown). Two of the induced genes, SM20 (40, 41) and microsomal epoxide hydrolase (mEH; Refs. 42-45), have been described previously. Although correlative gene expression analyses have described these genes as being differentially expressed depending on various growth conditions, p53-dependent transcription of these genes has not been proposed previously.

Differential RT-PCR products from the two novel cDNAs, designated 11 and 19, as well as SM20 and mEH are shown in Fig. 2. Northern analysis for each of these partial cDNAs confirmed that these transcripts were differentially expressed in cells grown at 38°C or 32°C (Fig. 2). Partial cDNA fragments 11 and 19 were used as probes for expression analysis from various rat tissues (Fig. 3a). A restricted expression pattern was observed using the partial 11 cDNA probe, revealing a 1.3-kb transcript present predominantly in whole brain and kidney with limited expression in heart, lung, liver, and skeletal muscle, and no expression in spleen and testis. Using the partial clone 19 cDNA as a probe revealed a more ubiquitous expression pattern, with a 1.4-kb transcript showing the highest levels of expression in rat testis. Because neither of these genes have been described previously, we have chosen to name them cell growth regulatory genes CGRI1 and CGRJ9. Both CGRI1 and CGRJ9 also showed significant transcriptional induction at the permissive temperature in baby rat kidney cells transformed with temperature-sensitive p53 and E1A (apoptotic cells) and cells transformed with temperature-sensitive p53, E1A, and E1B (growth-arrested cells; data not shown).

Kinetics of inductions for CGR11 and CGR19 transcripts following a shift in temperature from 38°C to 32°C in REF-112 cells was performed to aid in determining whether the transcriptional induction of these genes was an early event in the cascade leading to perturbation of growth (Fig. 3b). Induction kinetics for CGR19 closely parallel those for p21WAF1/CIP1 (compare Figs. 1a and 3b). A slightly slower induction rate was observed for CGR11 (Fig. 3b), and a higher basal (uninduced) transcript level existed for CGR19 than for CGR11 (Fig. 2). Expression levels of CGR11 and CGR19 could also be indirectly assessed relative to p21WAF1/CIP1 and cyclin G in this REF-112 system. Probe hybridization to an induced REF-112 cDNA library yielded cyclin G levels 2-fold higher than p21WAF1/CIP1, with p21WAF1/CIP1 being expressed at ~0.1% of the total mRNA in the cell and cyclin G being expressed at ~0.2% (data not shown). Serial analysis of gene expression (46) with induced REF-112 cells, which permits a more quantitative assessment of message abundance, also yielded cyclin G levels at ~0.2%. Using cyclin G as the standard for quantitation, we conclude that CGR11 is expressed at 0.05%, and CGR19 is expressed at 0.01% of the total message in p53-induced REF-112 cells.

**cDNA and Deduced Protein Sequences for Cell Growth Regulatory Genes.** Potentially full-length cDNAs for CGR11 and CGR19 were obtained from REF-112 RNA by 5' RACE (47) and by hybridization to a human fetal brain cDNA library. Deduced protein sequences for rat and human clones are shown in Fig. 4. Rat and human CGR11 cDNAs obtained are 1209 and 1113 bp in length, respectively, and have an open reading frame encoding protein products of 403 and 301 amino acids (Fig. 4a). No in-frame stop codons were observed for either cDNA upstream of the putative start site (data not shown). The use of GeneTrapper technology (Life Technologies, Inc.) did not yield cDNAs with longer 5' extensions for the human clone. Optimal 5' alignment between the rat and human CGR11 proteins truncates seven amino acids (MSRWMQ) from the first rat CGR11 ATG, disallow-

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Experiments were performed in duplicate. b @EP4 vector alone. c, human; r, rat. ND, not determined.

Table 1 Inhibition of colony formation by cell growth regulatory genes

<table>
<thead>
<tr>
<th>Clone</th>
<th>SW480 Colony number (%) of control</th>
<th>SKOV IP1 Colony number (%) of control</th>
<th>T98G Colony number (%) of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>hWAF1</td>
<td>29.0 ± 4.4</td>
<td>34.5 ± 6.8</td>
<td>20.6 ± 8.6</td>
</tr>
<tr>
<td>hp53</td>
<td>0.2 ± 0.2</td>
<td>5.1 ± 1.4</td>
<td>5.7 ± 2.3</td>
</tr>
<tr>
<td>hp53(as)</td>
<td>92.7 ± 37</td>
<td>62.4 ± 20</td>
<td>151.5 ± 29</td>
</tr>
<tr>
<td>rCGR11</td>
<td>18.2 ± 8.8</td>
<td>31.5 ± 9.3</td>
<td>25.8 ± 11</td>
</tr>
<tr>
<td>rSM20</td>
<td>4.4 ± 3.6</td>
<td>16.4 ± 8.3</td>
<td>11.4 ± 3.2</td>
</tr>
<tr>
<td>rmEH</td>
<td>73.6 ± 6.1</td>
<td>21.9 ± 7.3</td>
<td>5.2 ± 3.2</td>
</tr>
<tr>
<td>rCGR19</td>
<td>74.8 ± 7.7</td>
<td>38.3 ± 17</td>
<td>13.3 ± 7.6</td>
</tr>
<tr>
<td>hCGR11</td>
<td>ND</td>
<td>8.3 ± 0.1</td>
<td>57.6 ± 11</td>
</tr>
<tr>
<td>hCGR11ΔEF</td>
<td>ND</td>
<td>110.0 ± 5.0</td>
<td>98.8 ± 2.6</td>
</tr>
</tbody>
</table>

* Experiments were performed in duplicate.
* pCEP4 vector alone.
* h, human; r, rat. ND, not determined.

Fig. 3. Expression profiles for CGR11 and CGR19. a. Rat multiple tissue Northern blot probed with 32P-labeled cDNA fragments specific for CGR11, CGR19, and β-actin. b, induction kinetics of CGR11 and CGR19. REF-112 cells were maintained at 32°C for the indicated times (h). Total RNA was isolated, electrophoresed, blotted, and probed with 32P-labeled cDNA specific for rat CGR11 or CGR19.

Growth Suppressive Function of Cell Growth Regulatory Genes. To begin to assess the potential growth-suppressive properties of the differentially expressed genes isolated in this study, we evaluated growth inhibition via stable transfection in a colony inhibition assay. All genes examined were expressed from the cytomegalovirus promoter on episomally maintained plasmids (pCEP4), and colony formation was scored 2—3 weeks after transfection. As controls, p21WAF1/CIP1, p53, and p53 antisense constructs were also transfected. We chose three cell lines, all harboring different p53 alleles for the analysis of growth-suppressive function: the SW480 colon carcinoma cell line contains two point mutations, H1S273 and SER309; an ovarian carcinoma line, SKOV3 IP1, which is p53-null; and a glioblastoma cell line, T98G, containing a single point mutation, MET237, in p53. Rat mEH, SM20, CGR11, and CGR19 all exhibited some growth-suppressive effects, but the degree of suppression depended on the cells analyzed (summarized in Table 1). p53 consistently showed the most potent growth-inhibitory effect. p21WAF1/CIP1 exhibited between 65 and 80% inhibition, in agreement with previous results (2). Only CGR11 and SM20 showed marked growth inhibition in SW480 cells (80 and 95%, respectively), but all clones tested showed growth inhibition. The rat and human CGR11 genes are 65% identical, whereas two highly conserved putative Ca2+-binding EF-hand motifs (amino acids 82—94 and 127—139 in the human protein; overlined in Fig. 4b) share nearly 100% identity. Additionally, four clustered 17-amino acid repeats exist within the COOH-terminal portion of only the human CGR11 protein (consensus; PGPRGAEAGQAE(A/K/R/G)DA), suggesting a structure resembling four α-helical domains interrupted by distinct turns. In vitro production of rat CGR11 and CGR19 proteins yielded products of ~37 and 34 kDa, respectively (data not shown). Furthermore, the CGR11 protein is very acidic, with a net negative charge of ~29 and a pI predicted to be 4.21.

The rat and human CGR19 proteins are 88% identical at the amino acid level, with consistent homology throughout the entire length of the 332-amino acid proteins (Fig. 4c). Although no in-frame stop codons are found upstream from the putative initiation codon, the rat and human protein homologues diverge immediately upstream of the start codon, further suggesting that the designated ATG is indeed the start codon for CGR19 (data not shown). The predicted protein sequence of CGR19 suggests limited homology to known proteins with the exception of a putative zinc-binding C4HC4 ring-finger domain at the NH2 terminus of the protein (Fig. 4d). No accompanying B-box domain was observed in CGR19, as has been observed in some ring-finger-containing proteins. Interestingly, we have also isolated a full-length CGR19 cDNA from REF-112 cells containing a 130-bp insert within the middle of the cDNA (data not shown). This insert contains 5' GT and 3' AG ends, consistent with exon-intron boundaries (48). This suggests that either some of the CGR19 transcript in the cells is incompletely processed, or that an alternate transcript containing a retained intron is produced. The latter CGR19 would produce an in-frame termination codon within the putative intron, resulting in a protein chimera retaining the first 140 amino acids of CGR19. RT-PCR analysis from 32°C-induced REF-112 cells reveals two bands of equal intensity, consistent with the stable generation of two CGR19 transcripts. Additional analysis of protein products encoded by these two transcripts is required to determine whether this differentially processed transcript is significant.
inhibition in SKOV3IP1 and T98G cells (60–95% inhibition), demonstrating potential conservation of CGRI1 and CGRI9 function in human cells. Although the above results are suggestive of a growth-inhibitory function for the tested cDNAs, it remains possible that overexpression of these proteins yields the inhibitory effects observed due to nonspecific toxicity. To address this possibility, we constructed an EF-hand deletion mutant within the human CGRI1 cDNA and assessed this protein for growth-inhibitory potential. Previous results with EF-hand-containing proteins suggest that a deletion of one of two EF-hand motifs results in a functional protein with respect to Ca²⁺-binding potential (49). Therefore, we deleted both of the EF-hand domains within human CGRI1 for this analysis (CGR11ΔEF, amino acids 82–139). Unlike intact CGRI1, EF-hand-deleted CGRI1 cDNA transfectants were unable to inhibit colony formation in SKOV3 IP1 or T98G cells (Table 1). Human wild-type CGRI1 cDNA suppressed cell growth to 8% (SKOV3 IP1) and 58% (T98G) relative to the tested mutant. Thus, it likely that, at least with respect to CGRI1, the growth-suppressive effects observed reflects a structure-function relationship critical to growth inhibition. A similar analysis is ongoing with respect to the ring-finger domain within CGRI9. Site-specific mutagenesis to identify the critical residues within these domains may yield additional information on the function of these domains with regard to cell growth suppression.

**DISCUSSION**

We have described the isolation of potential growth-regulatory genes from rat and human cells based on a differential transcript analysis from rat cells temperature-sensitive for p53 function. This analysis revealed the induction of four genes in cells harboring functional p53. Two of the genes isolated, SM2O (40, 41) and mEH (42–45), are previously described genes, although no correlation between p53 status and transcriptional induction of these genes had been established. SM2O was described as a platelet-derived growth factor-A-induced transcript in vascular smooth muscle cells (40); the function of SM2O has not yet been elucidated, although expression has been demonstrated in the arterial wall within smooth muscle (41). In contrast, mEH is known to be involved in the catalytic detoxification of xenobiotics, including metabolizing reactive epoxides (42–45).

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5 GenBank accession numbers: U66468, U66469, U66470, U66471.
Although no known correlation exists between mEH and wild-type p53, induction of p53 activity through epoxides may aid in maintaining genomic stability in the presence of toxins.

CGR11 Northern analysis showed the highest level of induction of the four genes, followed by mEH, CGR19, and SM20. REF-112 serial analysis of gene expression has confirmed the relative abundance of CGR11 and CGR19 as being 0.05 and 0.01%, respectively, of the total mRNA in the wild-type p53-harboring cells. Both CGR11 and CGR19 amino acid sequences exhibit features described previously in proteins directly involved in regulating cell growth. CGR11 contains two putative Ca\textsuperscript{2+}-binding EF-hand domains. These domains have been described in numerous proteins including calmodulin, S100 proteins, troponin C, and CDC31 in yeast. More recently, an EF-hand-containing protein, ALG-2, was identified as being essential for FAS-induced apoptosis in T cells (49). Because calcium is a major second messenger within cells, the prevalence of calcium-binding domains within numerous cellular proteins reflects the importance of calcium-mediated regulation of cell growth.

The CGR11 protein is a very acidic protein exhibiting a net negative charge of -29 and a pi predicted to be 4.21. Interestingly, the CGR11 homologues derived from rat and human are highly conserved only in the region of the EF-hand domains, although both rat and human homologues are highly acidic. Indeed, a 17-amino acid clustered repeat region is present within the CGR11 COOH terminus but is absent from the rat homologue. It is unclear if structural features aside from the EF-hand domain are maintained within the CGR11 homologues. Both rat and human CGR11 homologues displayed growth-inhibitory potential at least as potent as p21\textsuperscript{WAF1/CIP1}. By contrast, EF-hand deletion mutants of CGR11 were unable to inhibit growth in the colony-inhibition assay, suggesting that the EF-hand domain within this protein is essential for the observed growth-inhibitory activity. CGR11 expression in rat tissues was limited to, but abundant in, rat brain and kidney, suggesting a more defined and tissue-specific role for the CGR11 protein than for the more ubiquitously expressed CGR19 transcript.

The CGR19 cDNAs isolated from rats and humans are highly conserved, exhibiting 85% amino acid identity. Structural similarities of CGR19 to other previously described genes is limited to a putative zinc-binding ring-finger domain at the very COOH terminus of the protein. In contrast to some other ring-finger-containing proteins, CGR19 lacks a ring-finger-associated B-box domain (reviewed in Ref. 50). Interestingly, a correlation between ring-finger positioning within a given protein and potential functional implications has been established. Generally, ring-finger proteins either harbor the ring-finger domain at the NH\textsubscript{2} or COOH terminus of the protein. Proteins harboring this domain at the COOH terminus have been linked to vacuolar/peroxisomal biogenesis and include the Zellweger's syndrome-associated ring-finger protein PAF1 (50). Ring-finger-containing proteins have also been implicated in regulating development, oncogenesis, and vacuolar/peroxisomal biogenesis (50). It remains unclear whether ring-finger domains are functional with respect to nuclear acid binding, protein-protein interactions, or protein-lipid interactions. Currently, we are evaluating the functional significance of the CGR19 ring-finger domain with respect to growth inhibition.

Kinetics of induction of these genes varied, although induction patterns were similar to the previously characterized p21\textsuperscript{WAF1/CIP1} transcript. This suggests that the induction of these genes is a relatively early event in the cascade leading to growth inhibition. This analysis has not definitively addressed the question of whether these genes are direct transcriptional targets of p53 or are consequences of reduced cell growth. Similarly, it remains unclear if induction of these genes is specific for apoptotic or growth-arrested cells. We are currently assessing other apoptotic and growth arrest-susceptible systems for induction of these genes. In any event, transcriptional induction of these genes may have important implications for discerning the complex regulatory cascade leading to growth perturbation.

Further work will focus on determining if clones CGR11 and CGR19 are direct targets of p53 regulation or are downstream effectors relevant in determining the growth status of a particular cell. It remains to be determined if the induction of these clones is conserved in other growth-regulatory systems, both p53 dependent and independent. Finally, work is ongoing in the hopes of defining any potential protein-protein-interacting partners for these clones in the hopes that defining these partners will aid in the elucidation of their function.

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