Isolation of a Novel Gene, CABC1, Encoding a Mitochondrial Protein That Is Highly Homologous to Yeast Activity of bc1 Complex

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Abstract

To search for p53 target genes throughout the human genome, we applied a cDNA microarray system using adenovirus-mediated transfer of p53 into p53-deficient U373MG (glioblastoma) cells. In this manner, we detected dozens of genes that appeared to be regulated by wild-type p53. We describe here characterization of one such gene, termed CABC1 [chaperone-activity of bc1 complex in Schizosaccharomyces pombe (ABC1)-like], which encodes a 647-amino acid peptide with significant sequence similarity to activity of bc1 complex (ABC1) in Arabidopsis thaliana and S. pombe. The CABC1 product was located in mitochondria, and colony-formation assays with cancer cell lines indicated its ability to suppress cell growth. Inhibition of CABC1 expression by transfection with antisense oligonucleotide significantly reduced the apoptotic response induced by wild-type p53. These results suggest that CABC1 may play an important role in mediating p53-inducible apoptosis through the mitochondrial pathway.

Introduction

The p53 tumor suppressor protein protects cells from malignant transformation, and the development of nearly half of human cancers appears to be associated with functional loss of p53 (1). Several cellular stresses, including DNA damage, are known to increase expression of p53 and modify p53 protein by phosphorylation or acetylation (2, 3). This protein inhibits cell growth by activating transcription of multiple genes that are involved in cell cycle arrest and/or apoptosis. For example, p21\textsuperscript{Waf1}, one of the most important p53 targets, induces cell cycle arrest (4), and another target, p53R2, plays a critical role in supplying nucleotides for repair of damaged DNA (5). Among the many physiological functions regulated by p53, the ability to induce apoptosis is thought to be central to its tumor suppressor activity (6), which is mediated through transcriptional activation of multiple genes (7). In recent years, several important genes involved in this apoptotic pathway have been isolated; these include genes that encode for mitochondrial proteins that are likely to contribute to the release of cytochrome c from mitochondria, e.g., Bax (8), Noxa (9), p53AIP1 (10), and PUMA (11, 12). A variety of key events in apoptosis occur in mitochondria, including release of caspase activators (such as cytochrome c), changes in electron transport, loss of mitochondrial transmembrane potential, and alteration of cellular oxidation-reduction status (13, 14).

To fully clarify the physiological role of p53, to apply such information to a better understanding of human carcinogenesis, and to develop novel therapeutic agents for cancer therapy, the complete set of genes regulated by p53 must be identified. Toward that end we previously applied two different strategies: One involved a yeast enhancer-trap system that allowed direct cloning of p53-binding sequences from human genomic DNA (10, 15–17); the other was a differential-display method using a colon cancer cell line in which expression of wild-type p53 can be regulated under the control of the lactose operon (5, 18, 19). As a third approach, we recently established a cDNA microarray system to identify additional genes that might be up-regulated by p53.

Here we report the discovery of a novel gene, designated CABC1 because its product is similar in amino acid sequence to ABC1 of lower eukaryotes. In yeast, ABC1 acts as a chaperone-like protein essential for proper conformation and functioning of the bc1 complex and neighboring complexes in the respiratory chain. We show evidence to suggest an important role of CABC1 in the p53-inducible mitochondrial apoptotic pathway.

Materials and Methods

Cell Lines. Human cancer cell lines U373MG (glioblastoma), MCF7 (mammary carcinoma), T98G (glioblastoma), Cos7 (monkey kidney), H1299 (non-small cell lung carcinoma), and LS174T (colorectal adenocarcinoma) were purchased from the American Type Culture Collection. All cells were cultured under conditions recommended by their respective depositors.

Microarray. Poly(A)\textsuperscript{+} RNAs were isolated from U373MG cells infected with adenovirus vectors designed to express either LacZ (AdlacZ) or wild-type p53 (Adp53), and were used as templates for synthesis of Cy3- or Cy5-labeled cDNA probes. The probes were hybridized to cDNA microarrays containing ~23,000 genes (20), which were then washed and scanned with an Array Scanner according to the supplier’s protocol (Amersham Pharmacia Biotech). Signal intensities of Cy3 and Cy5 from individual spots were quantified and analyzed by substituting backgrounds, using Array Vision software (Imaging Research Inc.).

Northern Blotting. mRNAs were isolated from MCF7 or U373MG cells after DNA damage or infection with adenovirus. A 2-µg aliquot of each mRNA was separated on a 1% agarose gel containing 1× MOPS buffer and 2% formaldehyde and transferred to a nylon membrane. Hybridization with a random-primer \textsuperscript{32}P-labeled CABC1 cDNA probe was carried out according to the instructions for the Megaprime DNA labeling system (Amersham Pharmacia Biotech).

Construction of Expression Vectors. We constructed two mammalian expression vectors in pcDNA3.1 (+), each containing the entire coding sequence of a p53-up-regulated transcript chosen from the microarray (CABC1), on either the sense or the antisense strand. A full-length expression vector (pcDNA3.1-CABC1) was constructed by cloning RT-PCR products into the pcDNA3.1 vector and verified by DNA sequencing. Expression vectors with a HA epitope tag at the NH\textsubscript{2}-terminus (pcDNA3.1/N-HA-CABC1) or the COOH-terminus (pcDNA3.1/C-HA-CABC1) were constructed as well.

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3 The abbreviations used are: CABC1, chaperone-ABC1 (activity of bc1 complex in Schizosaccharomyces pombe)-like; ABC1, activity of bc1 complex; RT-PCR, reverse transcription-PCR; HA, hemagglutinin; β2MG, β2 microglobulin; FACS, fluorescence-activated cell sorting.
Semiquantitative RT-PCR Analysis. Isolation of total RNA from cultured cells was performed using RNeasy spin column kits (Qiagen) according to the manufacturer’s instructions. cDNAs were synthesized from 10-μg aliquots of total RNAs with the SuperScript Preamplification System (Life Technologies, Inc.). The RT-PCR exponential phase was determined to allow semiquantitative comparisons among cDNAs developed from identical reactions. Each PCR regime involved a 94°C, 2-min initial denaturation step followed by 23 cycles (for CABC1), 20 cycles (for β2MG), or 25 cycles (for p53) at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, on a Gene Amp PCR system 9600 (Perkin-Elmer). β2MG served as the quantity control.

DNA-damaging Treatments. MCF7 cells were subjected to γ (50 Gy) or UV (10 J/m²) radiation or treated with Adriamycin (1 μg/ml). Cells were harvested at 0, 6, 12, 24, 48, and 72 h, or 0, 5, 6.5, 8, 9.5, 11, 24, and 48 h after treatment. We performed Northern blotting to examine expression of CABC1 in the damaged cells. Accumulation of p53 protein after DNA damage was examined by Western blotting. Expression of β-actin was used as the quantity control.

Immunocytochemistry. COS-7 cells transfected transiently with pcDNA3.1(+)-HA-CABC1 were seeded on a COL1-coated coverglass (IWAKI). Twenty-four h later, the cells were incubated in medium containing 70 mM Mito Tracker Red CMXRos (Molecular Probes) at 37°C for 30 min, fixed with PBS containing 4% paraformaldehyde, and permeabilized with PBS containing 0.1% Triton X-100 at 4°C for 2.5 min. The cells were covered with blocking solution (3% BSA and 2.5% goat serum in PBS) for 1 h at room temperature and incubated for 1 h at room temperature with a rabbit anti-HA antibody (Boehringer) diluted 1:1000 in blocking solution. This antibody was stained with a goat antirabbit secondary antibody conjugated to FITC and viewed with an ECLIPSE E800 microscope (Nikon).

Antisense Experiments. To inhibit expression of endogenous CABC1, we prepared HPLC-purified antisense oligonucleotides (antisense, 5′CCCTT-TACCTGCAACT-3′; sense, 5′AGGTGCAGGTAAGGG-3′) corresponding to sequences of the CABC1 gene. For FACS analysis, adherent and detached cells were collected 72 h after infection with Ad-p53 (100 or 200 multiplicity of infection) or 80 h after treatment with Adriamycin (0.5 or 1.0 μg/ml) and fixed with 70% ethanol in PBS at 4°C before incubation for 30 min in 1 ml of RNase (1 μg/ml) solution at 37°C. Cells were then stained in 500 μl of propidium iodide (0.1 mg/ml) and fixed with 70% ethanol in PBS at 4°C before incubation for 30 min in 1 ml of RNase (1 μg/ml) solution at 37°C. Cells were then stained in 500 μl of propidium iodide (0.1 mg/ml). A total of 5 × 10⁵ cells were then analyzed in a flow cytometer (FACScalibur; Becton Dickinson).

Results

Isolation of a Novel p53-inducible Transcription Unit. We isolated mRNAs from U373MG cells at various time points (0, 6, 12, 24, and 48 h) after infection with adenovirus designed to express wild-type p53 (Ad-p53) or LacZ (Ad-LacZ), and hybridized this preparation to a cDNA microarray consisting of 23,000 genes. Northern blot analysis of candidate p53 target genes, i.e., cDNAs that showed patterns of induction after infection with Ad-p53 but not Ad-LacZ, confirmed a time-dependent induction of a 3.2-kb transcript (Fig. 1A) corresponding to a gene later termed CABC1 for reasons described below. Northern blot analysis using mRNAs from 16 human tissues indicated ubiquitous expression of this gene, with relatively greater abundance in heart and skeletal muscle (data not shown). Because the cDNA we obtained accounted for only part of the 3.2-kb transcript, we screened a cDNA library constructed from poly(A)+ RNA isolated from Ad-p53-infected U373MG cells and obtained cDNA sequences (GenBank accession nos. AJ278126 and BC005171) corresponding almost to the full 3.2-kb transcript. Comparison of the cDNA with genomic sequences in the public database indicated that this gene is located on chromosome 1q42.2 (21). Its 15 exons encode a protein of 647 amino acids. A homology of the predicted peptide against the public database detected significant similarity to ABC1 in Arabidopsis thaliana (47% identity) and Schizosaccharomyces pombe (46%). Furthermore, CABC1 appeared to contain a mitochondrion localization signal and an ABC1 ubiquinone biosynthesis region (22, 23), suggesting a mitochondrial location (Fig. 1B).

Induction of Endogenous CABC1 by DNA Damage. To confirm regulation of CABC1 expression by wild-type p53, we examined whether endogenous CABC1 could be induced by DNA damage from Adriamycin treatment, UV irradiation, or γ irradiation in a p53-dependent manner. All three genotoxic stresses markedly induced expression of CABC1 mRNA in MCF7 cells (Fig. 2A), which possess functional wild-type p53 (Fig. 2B), but not in p53-defective cancer cells (data not shown). The time lapse between the imposition of genotoxicity and induction of expression was slightly different according to the type of stress used, but the patterns of CABC1 induction were all very similar to those of p21Waf1 under the same genotoxic conditions.

Functional Analysis of CABC1. To determine the subcellular location(s) of CABC1, we constructed a plasmid clone designed to express CABC1 with a HA tag. We transfected the plasmid DNA into
COS7 cells and stained the HA-CABC1 protein for immunofluorescence analysis. As shown in Fig. 3, CABC1 protein appeared in cytoplasm in a dotted pattern. Counterstaining of mitochondria with red dye showed a complete overlap with the green signal of CABC1, supporting the computer-predicted mitochondrial location of CABC1 protein (Fig. 3).

We performed colony-formation assays to investigate a potential growth-suppressive function of CABC1 protein, by separately transfecting three plasmid clones [pcDNA3.1(\*H11001) vector alone and pcDNA3.1(\*H11001) derivatives designed to express sense or antisense strands of CABC1] into T98G glioblastoma cells and H1299 lung cancer cells, both of which lack wild-type p53 and express no CABC1. As shown in Fig. 4, introduction of exogenous CABC1 in these two cell lines resulted in significant suppression of growth; 2–5-fold decreases in the number of geneticin-resistant colonies were observed. These results were confirmed by three independent experiments. We then examined whether endogenous CABC1 would affect p53-dependent cell cycle arrest or apoptosis induced by Ad-p53. To suppress expression of CABC1, we designed sense and antisense oligonucleotides corresponding to a part of the CABC1 cDNA sequence. After transfection of U373MG (p53-defective) or LS174T (with wild-type p53) cells with either of the oligonucleotides for 4 h, the U373MG cells were infected with Ad-p53 and the LS174T cells were treated with Adriamycin, and both cell lines were then analyzed by FACS scan. Pretreatment of U373MG cells with the antisense, but not sense oligonucleotide, strikingly inhibited induction of CABC1 expression as well as induction of apoptosis by wild-type p53. Similarly, pretreatment of LS174T cells with the antisense oligonucleotide inhibited Adriamycin-induced apoptosis, although the expression of p53 was induced at the same levels in the cells treated with the antisense and sense oligonucleotides (Fig. 5), indicating an important role of CABC1 in the p53-dependent apoptotic pathway.

Discussion

We have described here the identification of CABC1, a novel p53-inducible gene that is likely to be involved in the p53-dependent apoptotic pathway. Transfection of Ad-p53 into U373MG cells, in which wild-type p53 is defective, induced a high level of CABC1 expression. Expression of CABC1 was also inducible by treatment
with various genotoxic agents, in a wild-type p53-dependent manner. Furthermore, overexpression of exogenous CABC1 decreased the numbers of colonies formed by two cancer cell lines that lack wild-type p53. Although those results might reflect the toxic effect of a high level of CABC1 in these cells, subsequent experiments using antisense oligonucleotide to suppress CABC1 expression yielded demonstrable inhibition of p53-induced apoptosis. The fact that CABC1 is located in mitochondria supports a role of the novel gene in apoptosis mediated by p53, because mitochondria play a key role in regulating apoptosis by the p53-dependent pathway.

Fig. 4. Colony-formation assay of two cancer cell lines, H1299 and T98G, by transfection with CABC1. Cells were transfected with constructs designed to express sense strand [CABC1/pcDNA3.1(+)] or antisense strand [CABC1/pcDNA3.1(-)] of CABC1 or with empty vector.

Fig. 5. Suppression of p53-induced apoptosis by inhibiting CABC1 expression with antisense oligonucleotides. Antisense (AS) or sense (SE) oligonucleotides (1 μg) were transfected into U373MG or LS174T cells with Lipofectin reagent (Life Technologies) for 4 h. The U373MG cells were then infected with Ad-p53, and the LS174 cells were treated with Adriamycin. A, RT-PCR analysis of CABC1 expression in the U373MG cells 48 h after infection with Ad-p53 or in the LS174 cells 48 h after treatment with Adriamycin. β2MG was used as quantity control. B, FACS analysis of the U373MG cells 72 h after infection with Ad-p53 or of the LS174 cells 80 h after treatment with Adriamycin. MOI, multiplicity of infection.
The amino acid sequence of CABC1 revealed significant similarity to ABC1, a member of an electron-transferring membrane protein complex in S. pombe, A. thaliana, and Saccharomyces cerevisiae (24–26).

The ABC1 protein is essential for the correct conformation and functioning of the bc1 complex and the neighboring complexes II and IV in the mitochondrial respiratory chain (27). The mitochondrial respiratory chain consists of complexes I, II, III (bc1 complex), and IV. Complexes I, III, and IV are proton pumps. The mitochondrial ΔΨm is generated by an electrochemical gradient through activity of a proton pump in the respiratory chain. The similarity of CABC1 to ABC1 protein suggests that it may have a physiological role in the respiratory chain, altering the electrochemical gradient by its effect on complex conformation. It may lead to changes in electron transport and in mitochondrial transmembrane potential, which are the key events of apoptosis occur in mitochondria.

Several of the genes known to be regulated by p53 are predicted to encode proteins with activities related to the redox of cells. One of these is the PIG3, an NADPH-quinone oxidoreductase that is a potent generator of reactive oxygen species (28). NADPH-quinone oxidoreductase is present in the electron-transferring membrane. Hence, we speculate that CABC1 affects the respiratory chain and is involved in apoptosis through the mitochondrial pathway by way of cooperation with other mitochondrial proteins.

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The abc1 gene in S. cerevisiae was reported to be Coq8 (29). We thank Ching Ching Ng, Chizu Tanikawa, and Motoko Unoki for helpful advice.

References

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