Identification of Genes Highly Expressed in G2-Arrested Chinese Hamster Ovary Cells by Differential Display Analysis

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Abnormal cell cycle regulation is believed to be an important step in tumorigenesis. In mammalian cells, DNA damage commonly leads to cell cycle arrest in G2; however, little is known about the detailed biochemical mechanisms underlying the DNA damage-induced G2 arrest. In order to identify genes differentially expressed in association with G2 arrest, differential display analysis was performed between exponentially growing Chinese hamster ovary (CHO) cells and G2-arrested CHO cells induced by etoposide, SN-38, or X-radiation. We identified five

cDNA clones whose expression was up-regulated in G2-arrested CHO cells. Sequence analysis revealed that three clones were homologous to known genes: isogene I of translation initiation factor eIF-4A, ribosomal protein L13, and translation repressor NAT1. The remaining two clones showed no homology to known genes. These results indicate that DNA damage can alter the expression of multiple genes, including translational regulators. *J. Clin. Lab. Anal.* 14: 314–319, 2000. © 2000 Wiley-Liss, Inc.

Key words: cell cycle; G2/M; differential display; eIF-4A; ribosomal protein L13; NAT1

Mammalian cells are exposed to a wide variety of genotoxic stresses from both the environment and endogenous processes. Genotoxic stress, such as ionizing radiation, can cause chromosomal aberrations and alterations in DNA ploidy (1). When DNA damage is induced by unprogrammed extrinsic events, activating cell cycle checkpoints arrest the cell cycle in G1 and G2 to allow repair of the damaged template before replication (2).

Human checkpoint genes, such as ATM (ataxia telangiectasia mutated), p53, and p21, are often mutated in tumor cells. These genes are mainly involved in the G1 checkpoint. The G1 arrest after DNA damage results from the activation of p53, which induces transcription of the cyclin-dependent kinase inhibitor p21 (3–5). Among mammalian checkpoints, the G1 checkpoint is well known. However, the molecular basis for the DNA damage-induced G2 arrest is unclear. The G2/M checkpoint monitors completion of the S phase and integrity of the genome and then acts to prevent the onset of mitosis when damage is detected. The timing of the G2/M transition is dependent on the inhibitory phosphorylation of protein kinase Cdc2 (6). In eukaryotes, Chk1 phosphorylates Cdc25C on Ser216, leading to the binding of 14-3-3 protein, and inactivates Cdc25C ability to dephosphorylate Cdc2, thus preventing activation of the Cdc2–cyclin B complex and mitotic entry (7,8). In colorectal cancer cells and many other epithelial cell types, cells treated with radiation or DNA-damaging agents mainly arrest in G2 (9). For this reason, the

mechanism by which altered gene expression occurs in response to G2 arrest is an important topic for investigation.

Additionally, checkpoint dysfunctions lead to the generation of chromosomal aberrations, such as abnormal ploidy (aneuploidy) and chromosomal rearrangements, which are commonly observed in cancer cells. These kinds of genomic changes are thought to result from dysregulation of the G2/M phase of the cell cycle (10–12).

In the present study, we tried to evaluate changes in gene expression in G2-arrested CHO cells by differential display, which is an effective means of isolating genes that are differentially expressed in different states of the cell (13). As a result, we identified five genes highly expressed in G2-arrested cells. Partial sequencing of these cDNA clones revealed that two of them were novel genes whereas the other three clones corresponded to parts of known genes.

MATERIALS AND METHODS

Cell Culture and Treatment

CHO cell lines were maintained as monolayer cultures at 37°C in RPMI 1640 containing 10% FCS in a 5% CO₂ atmo-

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sphere. For drug and radiation treatments to trigger G2 arrest, 10^6 CHO cells were plated in 10-cm tissue culture dishes 48 hr before treatment. Cells were incubated with 25 μ M etoposide (Nihon Kayaku, Tokyo, Japan) or 1 μ M SN-38 (active form of CPT-11; Daiichi Pharmaceutical, Tokyo, Japan) for 1 hr at 37°C. Following drug treatments, cells were rinsed twice with drug-free medium and were reincubated. Cells were also exposed to 8 Gy of X-radiation at a dose rate of 3.2 Gy/min using 15 MV X-rays generated by a linear accelerator.

Cell Cycle Analysis

At indicated times after drug or radiation treatment, cells were harvested by trypsinization and pelleted by centrifugation. Pelleted cells were then washed with ice-cold PBS, treated with RNase A (500 units/ml), and finally stained with propidium iodide (50 μ g/ml). Samples were analyzed using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA). Experiments were repeated at least twice, and 2×10^4 events were analyzed for each sample. The percentage of cells in each phase of the cell cycle was determined using the SFIT model program (Becton-Dickinson).

Differential Display of mRNA

Total RNA was extracted from cells using the acid guanidinium thiocyanate-phenol-chloroform extraction method as previously described (14). Possible DNA contamination was removed by treating the obtained RNA with DNase I (GenHunter, Brookline, MA) for 30 min at 37°C. First-strand cDNA synthesis was initiated from 2.5 μ g of total RNA with 50 pmol of either GT15MG, GT15MA, GT15MT, or GT15MC (where M is 3-fold degenerated for G, A, and C) as 3' composite anchor primers in a total reaction volume of 19 μ l. The solution was heated to 65°C for 5 min, cooled to 37°C for 10 min, and then 1 μ l of SuperScript II reverse transcriptase (BRL, Gaithersburg, MD) was added. After incubation at 42°C for 1 hr, the mixture was heated to 95°C for 5 min, and 30 μ l of water was added. A 2- μ l aliquot of the sample was added to 18 μ l of PCR labeling mixture. PCR was performed in the presence of [α -³⁵S] dATP (NEN Research Products, Boston, MA) using the corresponding GT₁₅MN (where N may be one of A, G, and C) and one of the arbitrary 10- to 22-mer primers. The cycling parameters were as follows: 1 cycle (94°C for 3 min, 40°C for 5 min, and 72°C for 5 min) and 35 cycles (94°C for 30 sec, 50°C for 1 min, and 72°C 45 sec). PCR-amplified fragments were separated on 6% denaturing polyacrylamide gels. The gels were dried and exposed to X-ray film. The radioactive bands present in G2-arrested cells but absent in control cells were excised from the dried gels and reamplified by PCR using the corresponding set of the primers for 30 cycles. Reamplified cDNA fragments were purified with phenol-chloroform extraction and ethanol precipitation, labeled by random priming with a DNA labeling kit (Takara, Ohtsu, Japan) and [α -³²P] dCTP (Amersham,

Arlington Heights, IL), and then used as probes for Northern blot analyses.

Northern Blot Analysis

A 10- μ g amount of total RNA was electrophoretically separated on a 1% agarose gel containing 2.2 M formaldehyde and blotted on a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) by capillary transfer in 20 $\times$ SSC. RNA was visualized with ethidium bromide to ensure that it was intact and loaded in similar amounts and to confirm proper transfer. Hybridization was performed with radiolabeled probes in 50% formamide, 5 $\times$ Denhardt's solution, 3 $\times$ SSC, 0.1% SDS, and 100 μ g/ml salmon sperm DNA at 42°C overnight. After hybridization, the membranes were washed twice for 15 min each in 1 $\times$ SSC/0.1% SDS at room temperature followed by two 10-min washes in 0.25 $\times$ SSC/0.1% SDS at 55°C. The membranes were then exposed and analyzed using a BAS2000 Bioimaging Analyzer (Fuji, Kanagawa, Japan).

DNA Sequencing and cDNA Library Screening

Reamplified cDNA fragments were subcloned into the pGem-T easy vector (Promega, Madison, WI) according to the manufacturer's instructions. Sequencing was performed using a DNA sequencer (Model 373S; Applied Biosystems, Foster City, CA) with a DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems). The subcloned cDNA fragment from differential display was used as a probe to screen a customized CHO-K1 cDNA library in a lambda ZAP II vector (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Several isolated full-length cDNA clones were sequenced in both the sense and the antisense directions.

Semiquantitative RT-PCR Analysis

The cDNAs for gene-specific RT-PCR were synthesized as described above except for the use of a random hexamer. Forward and reverse primers were designed on the basis of sequences of the subcloned cDNA fragments. The RT-PCR exponential phase was determined on 22–32 cycles to allow semiquantitative comparisons among cDNAs developed from identical reactions. The PCR conditions for each primer set were denaturation for 2 min at 94°C followed by 26 cycles of 30 sec at 94°C, 30 sec at an optimal annealing temperature (55–58°C), and 30 sec or 1 min at 72°C. The amplified products were separated on a 2% agarose gel, stained with ethidium bromide, and photographed under UV light.

RESULTS

Cell Cycle Analysis After Drug and Radiation Treatment

After treatment with etoposide (25 μ M for 1 hr), SN-38 (1 μ M for 1 hr), or 8 Gy of X-radiation, cells gradually accumu-

lated in the G2/M phase of the cell cycle (Fig. 1). The number of cells arrested in G2 was maximum at 18 hr after treatment with etoposide (81% of cells in G2/M phase), at 12 hr after treatment with SN-38 (64%), and at 12 hr after X-radiation (61%). Cells at these time points were used for further study.

Identification of Genes That Are Highly Expressed in Association With G2 Arrest

To identify genes up-regulated in association with G2 arrest, mRNAs expressed in control CHO cells and in G2-arrested CHO cells were subjected to screening by differential display. A total of 120 different combinations of primer sets made of four 3' composite anchor primers, GT15MG, GT15MA, GT15MT, and GT15MC, and 30 arbitrary 10- to 22-mer upstream primers were used for differential display. In most of the displays, 20–100 bands of sizes between 200 and 700 bp were identified in each lane. Representative displays are shown in Fig. 2. Fragments detected only in G2-arrested cells were reamplified by PCR with the same primers, and the resulting PCR products were used as probes for Northern blot. Differential expressions of five of these fragments were confirmed by Northern blot analysis, supporting the pattern shown on the electrophoretic differential displays (Fig. 3). Clones C5, C21, and G3 showed increased expression in all of the G2-arrested cells compared with the control cells. Clone G12 showed increased expression in only etoposide-induced G2-arrested cells compared with the control cells, and clone A2 was detected as 1.3- and 1.6-kb transcripts only in radiation-induced G2-arrested cells.

Gene Cloning

Five fragments screened by Northern blot analysis were cloned into the pGem-T easy vector and completely se-

quenced. Sequence analysis revealed that all fragments were flanked by the sequences corresponding to the particular 3' composite anchor primer and the upstream primer. Then their nucleotide sequences were analyzed by searching for homologies in databases. The results are summarized in Table 1. Clone G3 has 92.1% identity with the rat ribosomal protein L13, C21 has 90.0% identity with the mouse initiation factor eIF-4A isogene I (eIF-4AI), and G12 has 97.8% identity with the mouse translation repressor NAT1. Clones C5 and A2, however, have no significant homology to previously characterized sequences.

Isolation of Chinese Hamster Ribosomal Protein L13 cDNA

To obtain full-length cDNA of clone G3, a CHO-K1 cDNA library was screened with the cloned G3 fragment. Three independent positive clones were identified and sequenced. The longest clone, G3-1 (723-bp insert), contains a single open reading frame encoding a 211-amino acid protein with a calculated molecular weight of 24,533. A computer search of the databases indicated that G3-1 has 87.0% identity with the rat ribosomal protein L13 gene in the coding sequences. Similarly, the amino acid sequence of G3-1 is 97.6% identical to that of rat ribosomal protein L13 and 97.1% identical to that of human breast basic conserved 1 (BBC1, data not shown). We conclude that G3-1 is the Chinese hamster homologue of the ribosomal protein L13 (GenBank accession number AB014724).

eIF-4AI and Ribosomal Protein L13 mRNA Expression After X-Radiation

To determine the time course of X-radiation-induced expression of eIF-4AI and ribosomal protein L13, we isolated total RNA after 0, 6, 12, 18, 24, 30, and 36 hr of exposure of

Fig. 1. Flow cytometric analysis of cell cycle distribution following treatment with 25 μ M etoposide for 1 hr, 1 μ M SN-38 for 1 hr, and 8 Gy of X-radiation: (□) G1 phase; (◇) S phase; (●) G2/M phase. The distribution of

untreated, exponentially growing CHO cells was 50% G1, 33% S, and 17% G2/M. The experiments were repeated at least twice, and the averages are shown. Arrows indicate time points when RNA was isolated from cells.

Fig. 2. Representative band patterns on differential display showing up-regulated fragments in G2-arrested CHO cells. RNA was isolated from exponentially growing CHO cells (lane G), at 18 hr after treatment with 25 μ M etoposide for 1 hr (lane 1), at 12 hr after treatment with 1 μ M SN-38 for 1 hr (lane 2), and at 12 hr after treatment with 8 Gy of X-radiation (lane 3), subjected to differential display analysis. Three different parts of polyacry-

lamide gels are shown with one band each, corresponding to a differentially expressed gene as indicated by an arrow in a–c (C5, G12, and A2, respectively). The primer combinations used were as follows: (a) 5'-GTTGCG-ATCC-3' and GT15MC; (b) 5'-AATTCGTTTCATG-3' and GT15MG; and (c) 5'-TGCAATTCAGATAAACAAGA-3' and GT15MA. The genes corresponding to the marked bands are (b) NAT1 and (a, c) unknown.

CHO cells to 8 Gy of X-radiation and performed semi-quantitative RT-PCR experiments (Fig. 4). Ribosomal protein L13 showed a dramatic increased expression after 6 hr of X-radiation. Maximum induction continued after 16 hr post-radiation, corresponding to the cell cycle arrest in G2 phase. The induction of eIF-4AI was slightly faster than that of ri-

bosomal protein L13. The expression of the two genes remained high until at least 36 hr after radiation.

DISCUSSION

In this study, differential display was applied to compare the expressions of mRNA in exponentially growing cells and

Fig. 3. Northern blot analysis of confirmed clones. Northern blot analysis of RNA from exponentially growing CHO cells (lane G) and G2-arrested CHO cells (lane 1, 18 hr after treatment with 25 μ M etoposide for 1 hr; lane 2, 12 hr after treatment with 1 μ M SN-38 for 1 hr; lane 3, 12 hr after treat-

ment with 8 Gy of X-radiation). A 10- μ g amount of each RNA was run in each lane, and the blotted filter was hybridized with reamplified cDNA fragments. Equal loading of total RNA was confirmed by ethidium bromide staining of 28 S rRNA.

TABLE 1. Characteristics of genes expressed at a higher level in G2 arrested cells than control in CHO cells

Clone	PCR primer (5'-3')	Fragment size (bp) ^a	mRNA size (kb) ^b	Homology ^c
C5	GTTGCGATCC GT ₁₅ MC	313	3.5	None
C21	AGAGTCTTTGCCCGAACCTG GT ₁₅ MC	280	1.9	Initiation factor eIF-4AI
G3	GGGCCAATGT GT ₁₅ MG	359	1.1	Ribosomal protein L13
G12	AATTCGTTTCATG GT ₁₅ MG	460	3.5	NAT1
A2	TGCAATTCAGATAAACAAGA GT ₁₅ MA	358	1.6, 1.3	None

^aDetermined by nucleotide sequence of the insert in the vector multicloning site.

^bApproximate mRNA sizes were calculated from Fig. 3.

^cSearched with the DDBJ/EMBL/GenBank databases using BLAST.

cells arrested in G2 due to the action of three different DNA-damaging agents, etoposide, SN-38, and X-radiation. We identified five genes whose expressions were increased in G2-arrested cells. Eukaryotic initiation factor eIF-4AI (clone C21), ribosomal protein L13 (clone G3), and clone C5 showed increased expression in all of the G2-arrested cells, whereas the expressions of NAT1 and clone A2 were induced by only etoposide and X-radiation, respectively.

The ribosomal protein L13 gene is well conserved throughout eukaryotic evolution (15), and it is also expressed in a great variety of organs (16). Its homologues have been isolated in a wide range of species, including human BBC1 and BnC24, a cold-inducible plant gene from *Brassica napus* (17). The expression of BBC1 gene is down-regulated in human breast cancer tissue as compared to benign breast lesion (18). Additionally, mutations within the BBC1 gene were detected, even at a lower incidence, in prostate cancer. Although it is suspected that they are nucleic acid binding proteins located in the nuclei, the functions of these genes are not known.

Considering the above findings together with our result showing that the homologue of this conserved gene alters its expression at cell cycle arrest, it seems that this gene might play an essential role in cell growth under conditions of stress.

eIF-4A is an RNA binding protein with RNA-dependent ATPase and RNA helicase activities (19–21). A previous study showed that the eIF-4AI expression levels in growing cells and in growth-arrested cells are almost identical, while its expression increases 3–4-fold as cells reentered the cell cycle following serum stimulation of nutrient depletion-induced G1 arrest (22). Our results showed that the expression of eIF-4AI was increased in G2-arrested cells. Because G2 arrest followed an increased percentage of S-phase cells (at 6–9 hr post-treatment, Fig. 1), it is possible that this up-regulation is a reflection of a transient increasing population of S-phase cells. On the other hand, the expression of the translation repressor NAT1 gene was induced by etoposide. Yamanaka et al. (23) suggested that NAT1 competes with eIF-4G for binding to subunits of eIF-4F and acts as a translation repressor in

Fig. 4. Effect of X-radiation on eIF-4AI and ribosomal protein L13 in CHO cells. At the indicated times after 8 Gy of X-radiation, total RNA was isolated and subjected to semiquantitative RT-PCR analysis.

cell growth regulation. We found that the expression of both the translation initiation factor and the repressor were increased after DNA damage, suggesting that mammalian cells exhibit cell cycle arrest in response to DNA damage through complex pathways.

We used three different RNA samples of the G2-arrested CHO cells for differential display in order to identify genes that are highly specific to G2 arrest. Etoposide and SN-38 induce DNA single- and double-strand breaks via interaction with topoisomerase II and I, respectively (24,25), while X-radiation induces DNA nicks and base damage in addition to DNA single- and double-strand breaks (26,27). Four of the five genes identified in this study were highly expressed in two or three different forms of G2 arrest. In addition, CHO cells are not derived from neoplastic tissue; therefore, G2 arrest after DNA damage in CHO cells is apparently an ordinary reaction. Moreover, it is highly possible that these genes are strongly associated with the DNA damage-induced G2 arrest. Thus, further investigations, including identification of full-length cDNAs of novel genes and functional analysis of these gene products, including antisense experiments, are required to elucidate the underlying mechanisms of DNA damage-induced G2 arrest. Additionally, the G2/M checkpoint plays a major role in human tumorigenesis. It would therefore be important to examine the expression and genetic alteration of these genes.

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