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Identification by Functional Cloning from a Retroviral cDNA Library of cDNAs for Ribosomal Protein L36 and the 10-kDa Heat Shock Protein that Confer Cisplatin Resistance

Ding-Wu Shen, Xing-Jie Liang, Toshihiro Suzuki, and Michael M. Gottesman

Laboratory of Cell Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland

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ABSTRACT

Based on analyses of two-dimensional gel and cDNA microarrays, our laboratory and others have demonstrated that a number of genes show altered expression during the development of cisplatin resistance (CP-r) in human cancer cells, including genes associated with DNA damage repair, proto-oncogenes, apoptosis, stress-response, and transcription factors. To verify these results and find genes that are directly responsible for CP-r, as opposed to those reflecting a secondary response induced by cisplatin treatment or resulting from CP-r, we constructed a retroviral cDNA library in the vector pLNCX2 from KB-CP.5 (KCP.5), a cell line selected in one step after exposure to cisplatin at 0.5 μg/ml. Using a library of cDNAs (1.8×10^6 cDNA clones) and an intermittent cisplatin selection system to allow more effective functional cloning, 11 expressed cDNAs were identified in a primary pool of 93,000 transfected cell clones. Metallothionein 2A, a known CP-r gene, was among these 11 genes found in the transfectants after CP selection. Several other genes, including those encoding ribosomal proteins (e.g., RPL36) and heat shock protein (e.g., HSP10), were also found among the cisplatin-selected clones. Transfection of either the RPL36 cDNA or HSP10 cDNA conferred on KB-3-1 cells 2.5- to 3-fold resistance to cisplatin by clonogenic assays. A subsequent transfection also identified RPL36 as a CP-r gene. The finding that a ribosomal protein gene, *RPL36*, contributes to CP-r should stimulate study of the role of ribosomal proteins in multifactorial mechanisms of cisplatin resistance.

Cisplatin (*cis*-diamminedichloroplatinum II) has revolutionized chemotherapy by improving the treatment of a wide spectrum of solid tumors. However, despite the high efficacy of the compound, the ability of cancer cells to become resistant to the drug remains a significant impediment to successful chemotherapy. Intensive efforts have been made through biochemical characterization, cellular, and genetic approaches to determine the basis of resistance and define genes that are involved in acquisition of cisplatin resistance. Recent studies using two-dimensional gel analysis (Shen et al., 1995), gene knockout (Niedner et al., 2001), differential display (Francia et al., 2004), subtractive hybridization (Yasui et al., 2004), and cDNA microarrays (Roberts et al., 2005) have documented that a large number of genes are either up-regulated or down-regulated in cisplatin-resistant cells, including genes that encode transcription factors, DNA damage-repair proteins, stress-response proteins, cell-cycle checkpoints, apoptosis mediators, and transporters (Gottesman et al., 2002; Wang and Lippard, 2005).

Functional cloning and retroviral cDNA libraries have been applied to define genes responsible for drug resistance, apoptosis, and so forth (Perez-Victoria et al., 2003; Mourtada-Maarabouni et al., 2004). In identifying genes related to cisplatin resistance, the approaches that have been used have a major drawback because of continuous stepwise challenge with increased cisplatin concentrations and may reflect secondary changes in genotype and phenotype during multistep selection of cisplatin-resistant cells. To explore genes primarily involved in cisplatin resistance, we inserted double-stranded cDNA into a retroviral expression vector, pLNCX2, from cisplatin-resistant KB-CP.5 cells that were selected by a single step of cisplatin at 0.5 μg/ml. An intermittent cisplatin selection procedure was designed for functional cloning, and subsequent identification of genes related to cisplatin resistance was obtained by PCR and sequencing. By this technique, 11 genes were found in the individual clones after functional cloning, including metallothionein,

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ABBREVIATIONS: HSP10, 10-kDa heat shock protein; RPL36, ribosomal protein L36; CP-r, cisplatin resistance; PCR, polymerase chain reaction; kb, kilobase(s); RT-PCR, reverse transcription-polymerase chain reaction.

which has been reported to be associated with cisplatin resistance (Kelly et al., 1988), and a chaperone, 10-kDa heat shock protein (HSP10), which had been found previously to be inducible by a metal salt, cadmium chloride (Lee et al., 2002). A ribosomal protein, RPL36, was identified in this work, and in a second independent transfection, it was shown to confer ~2.5-fold increased CP-r on transfected stable clones in comparison with the control vector-only-transfected cells. Therefore, this functional retroviral cloning system and modified intermittent selection provides a powerful tool for further cloning of genes able to confer CP-r.

Materials and Methods

Cell Lines and Cell Culture. The human epidermoid carcinoma cell lines KB-3-1 and an early-stage cisplatin-resistant cell line, KB-CP.5, which was isolated at 0.5 μg/ml cisplatin from KB-3-1 (Liang et al., 2003), were studied in this work. The RetroPack PT67 cell line was purchased from Clontech (Mountain View, CA). All cell lines were grown as monolayer cultures at 37°C in 5% CO_2 using Dulbecco's modified Eagle medium with 4.5 g/l glucose (Invitrogen, Carlsbad, CA), supplemented with L-glutamine, penicillin, streptomycin, and 10% fetal bovine serum (BioWhittaker, Walkersville, MD). Cisplatin (Sigma, St. Louis, MO) was added to the medium for KB-CP.5 (0.5 μg/ml).

Construction of a Retroviral cDNA Library. Double-stranded cDNA was synthesized from a single-step selection of the cisplatin-resistant cell line KB-CP.5, which was maintained in medium containing cisplatin 0.5 μg/ml using a BD SMART cDNA library construction kit (Clontech) as described by the manufacturer. The synthesized double-stranded cDNA was digested with the restriction enzyme Sfi and then column-purified by a CHROMA SPIN-400. The lower cutoff cDNA size was 500 bp. Ligation of the Sfi-digested double-stranded cDNA to a retroviral expression vector pLNCX2 (Clontech) with Sfi-cut was performed according to the manufacturer's instructions, and the library was named KB-CP.5/pLNCX2. The retroviral cDNA library was transformed into DH5α competent cells (Invitrogen) and resulted in 1.8×10^6 clones (library complexity).

Retroviral Transduction and Programmed Cisplatin Selection. Retroviral supernatants were collected from KB-CP.5/pLNCX2-transfected retroviral packaging PT67 cells and then used to infect the recipient KB-3-1 cells. After selection with G418, a pool of 96,000 clones containing the KB-CP.5 cDNA was established. A programmed selection with cisplatin is shown in detail in Fig. 1B.

Isolation of Genomic DNA, PCR Amplification, and Sequencing. Genomic DNA was extracted by Miller's method (Miller et al., 1988) from each clone from a total of 112 clones that were generated by programmed cisplatin selection. PCR amplification was performed in a total volume of 20 μl per reaction using BD Advantage 2 PCR kits (BD Biosciences, Palo Alto, CA). The retroviral vector pLNCX2 sequencing oligonucleotides were used as PCR primers for the identification of inserts: 5′-AGCTCGTTTAGTGAACCGTCAGATC-3′ (forward), and 5′-ACCTACAGGTGGGGTCTTTCATTCCC-3′ (reverse). These two primers were also used for sequencing, which was performed at the Core Facility of the Center for Cancer Research, National Cancer Institute (Bethesda, MD).

Preparation of RNA, and RT-PCR. For the determination of expression levels of genes of interest, RNAs were isolated from cells using an RNeasy kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. RT-PCR was performed using a GeneAmp kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Specific primers for tested genes are listed below: ribosomal protein RPL36, 5′-AAATCCATTGCCCGTGTTC-3′ (forward), and 5′-TCTTGGTCTTCAGGTTCTCC-3′ (reverse); heat shock protein HSP10, 5′-AAGTTTCTTCCACTCTTTGACC-3′ (forward) and 5′-TGAATCTCTCCACCCTTTCC-3′ (reverse); γ-catenin, GAGAGTGTGCTGAAGATTCTG (forward), and TGATTCGTCCTTGTCACC (reverse), which were used for verification of the quality and the size of genes in the library by PCR.

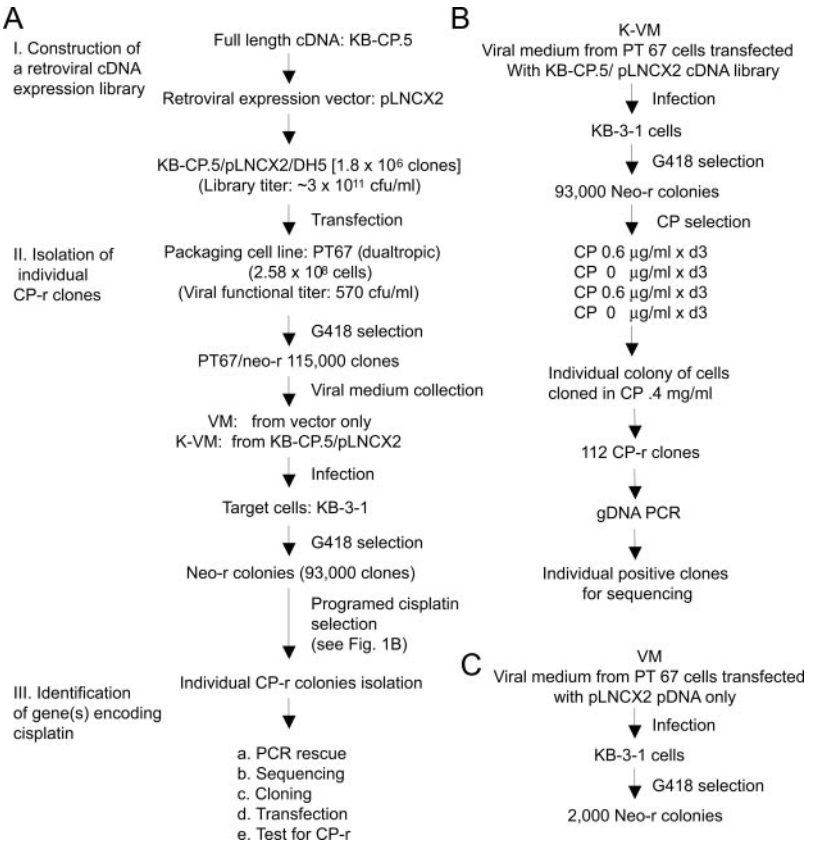

Fig. 1. Flow charts. A, strategy to functionally clone CP-r genes using a retroviral cDNA library from an early-stage cisplatin-resistant cell line, KB-CP.5 (human epidermoid carcinoma cells KB-3–1 cells selected with cisplatin 0.5 μg/ml). The flow chart shows the cloning system and is detailed in the text. B, flow diagram showing programmed/intermittent cisplatin selection for CP-r clones developed in this work, detailed in the text. C, flow chart showing isolation of 2000 neomycin-resistant clones from KB-3-1 cells, which served as a control.

Gene Transfection and Assays of Cell Resistance Levels to Cisplatin, Carboplatin, and Sodium Arsenite. Full-length cDNA for the genes encoding RPL36 and HSP10 were purchased from the American Type Culture Collection (Manassas, VA). Both genes were inserted into a mammalian expression vector, pcDNA3.1 (Invitrogen), as described by the manufacturer. Gene transfection was done with Lipofectin (Invitrogen) by following the manufacturer's instructions. Stable transfected clones were isolated after selection with G418. For testing cell sensitivities to cisplatin, carboplatin, and sodium arsenite, cells were counted after 3 days using a Coulter Counter or Cell Counting Kit (CCK8; Dojindo Labs, Gaithersburg, MD) as described in the legend to Fig. 3. A clonogenic assay was used to further confirm the ability to confer CP-r for the positive genes, such as *RPL36* and *HSP10*. Cells were seeded into 60-mm dishes (Corning Glassworks, Corning, NY). Cisplatin at a desired concentration was introduced into each dish before cell seeding. The medium was replaced every 3 days with medium containing cisplatin at the desired concentration. Colonies were stained with methylene blue at the end of 12 days of incubation and then counted. Control cells were transfected with insert-free vector only. The values are the means of triplicate determinations.

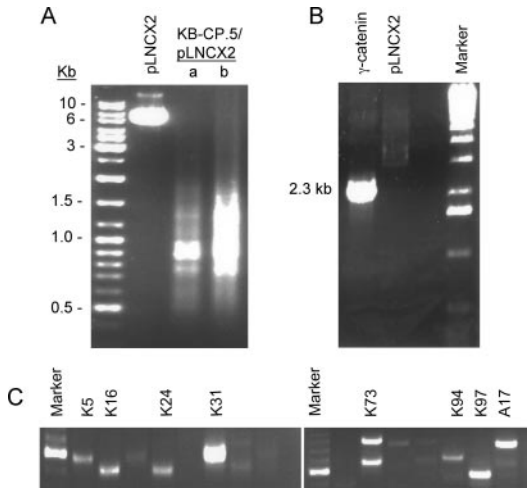

Fig. 2. Images of PCR products. A, the KB-CP.5/pLNCX2 library was primed by specific oligonucleotides of the retroviral vector pLNCX2 as described under *Materials and Methods* and then amplified by Advantage 2 PCR (CLONTECH). Two different loading amounts of KB-CP.5/pLNCX2 were applied as one third of loading (a) and full loading (b). B, γ-catenin was amplified by the specific primers described under *Materials and Methods*, showing the 2.3 kb of the full-length gene. C, images of genomic DNA PCR, showing inserts of different sizes in individual clones.

Results

Strategy for Functional Cloning. Figure 1A shows a flow chart of construction of the library, isolation of CP-r clones, and identification of genes related to CP-r. A retroviral cDNA expression library was established from an early-stage (single-step) CP-r cell line (KB-CP.5) into a retroviral expression vector, pLNX2. A total of 1.8×10^6 clones were pooled from KB-CP.5 cDNA/pLNCX2/DH5α. The titer of the unamplified library was $\sim 3 \times 10^{11}$ colony-forming units/ml. The quality and insert size of the library is shown in Fig. 2A using the primers for pLNCX2 as described under *Materials and Methods*, indicating a distribution range of the inserts from 0.5 to 6 kb and enriched between 0.7 and 1.8 kb. To determine whether there were larger intact genes in this library, γ-catenin, a known cDNA of 2.3 kb, was generated by PCR amplification and showed a full-length cDNA in the library (Fig. 2B).

The RetroPack PT67 cell line was used to package the library. This cell line expresses a dual-tropic/polytropic envelope, and the virus produced can infect a broad range of mammalian cells. The viral titer was determined by functional assay (G418 resistance) and resulted in 570 colony-forming units/ml. Transfection of the KB-CP.5 cDNA retroviral library into PT67 cells yielded 115,000 neo-r colonies after G418 selection (Fig. 1A). The viral medium (K-VM) was collected from these cells and then infected into the target KB-3-1 cells. A pool containing 93,000 neo-r clones was obtained after G418 selection (Fig. 1A). A control pool with 2000 neo-r clones was collected (Fig. 1C) using the vector only without inserts.

An intermittent cisplatin selection was developed in this work as shown in Fig. 1B. In brief, cells were intermittently selected with cisplatin at 0.6 μg/ml for 3 days and then cultured in medium without the drug for another 3 days for recovery. A second round of 3 days of cisplatin selection followed by a recovery period of 3 days was repeated. Cisplatin-resistant colonies appeared after a period of 12 days, and much larger colonies were seen in the group of KB-3-1 cells infected with the viral medium from the KB-CP.5/pLNCX2 cDNA retroviral library (K-VM), whereas the control cells, which were infected with the viral medium from the vector without inserts, showed much smaller colonies (VM). One hundred twelve individual colonies that survived in the K-VM group were cloned and propagated in 0.4 μg/ml cispla-

TABLE 1
Positive clones identified by functional cloning and sequencing

Clone	kb	Gene	mRNA	CDS
			bp	
K5	0.55	Metallothionein 2A	451	76–261
K16	0.5	RPS27 (ribosomal protein S27) (metallopanstimulin A)	344	36–290
K24	0.55	ATP synthase, H + transporting, mitochondrial F0 complex, subunit d, FITO	631	58–543
K31	0.7	RPL41 (ribosomal protein L41)	478	84–161
K73A	1.0	Nucleolar protein family A, member 2 (H/ACA small nucleolar RNPs)	879	87–548
K73B	0.7	CGI-121, a novel PRPK	660	64–591
C104	**0.65**	**RPL36 (ribosomal protein L36)**	**578**	**153–470**
A17	1.0	Hypothetical protein FLJ21174	1062	200–847
A22	0.9	RPL13A (ribosomal protein L13A)	1142	23–634
A24	**0.8**	**HSP10 (10-kDa heat shock protein)**	**556**	**115–423**
A39	0.8	Stratifin, SFN (14-3-3s)	1309	49–795
	0.7	RPL36aL (ribosomal protein L36a-like)[a]	542	96–416

bp, base pairs; CDS, coding sequence.
[a] Four clones from B8/10-1A to D.

tin for genomic DNA preparation and further PCR determination. We tried to select cells continuously with cisplatin at different concentrations, but this approach was unsuccessful because of either cells being killed at high concentrations of cisplatin or spontaneously mutated at low concentrations, generating a high background of low-level cisplatin-resistant clones similar to the control. Therefore, a recovery period during the first two rounds of cisplatin selection seems to be important for functional gene cloning, particularly under the conditions described above.

Identification of Genes in Association with Cisplatin Resistance. All 112 cisplatin-resistant clones were analyzed by genomic PCR using the primers for the retroviral vector pLNCX2 as described under *Materials and Methods* (i.e., only the inserts containing the vector sequences at both ends could be detected in the clones). Figure 2C shows fragment(s) of different sizes from 0.5 to 1 kb in these clones. After sequencing, 11 inserts were identified after BLAST match and are listed in Table 1. Metallothionein 2A was 1 among the 11 genes observed, and this gene had been reported to be associated with resistance to cisplatin and other heavy metals (Kelly et al., 1988; Yang et al., 1994), demonstrating that the strategy applied in this work by functional cloning and using a retroviral cDNA library has the power to identify

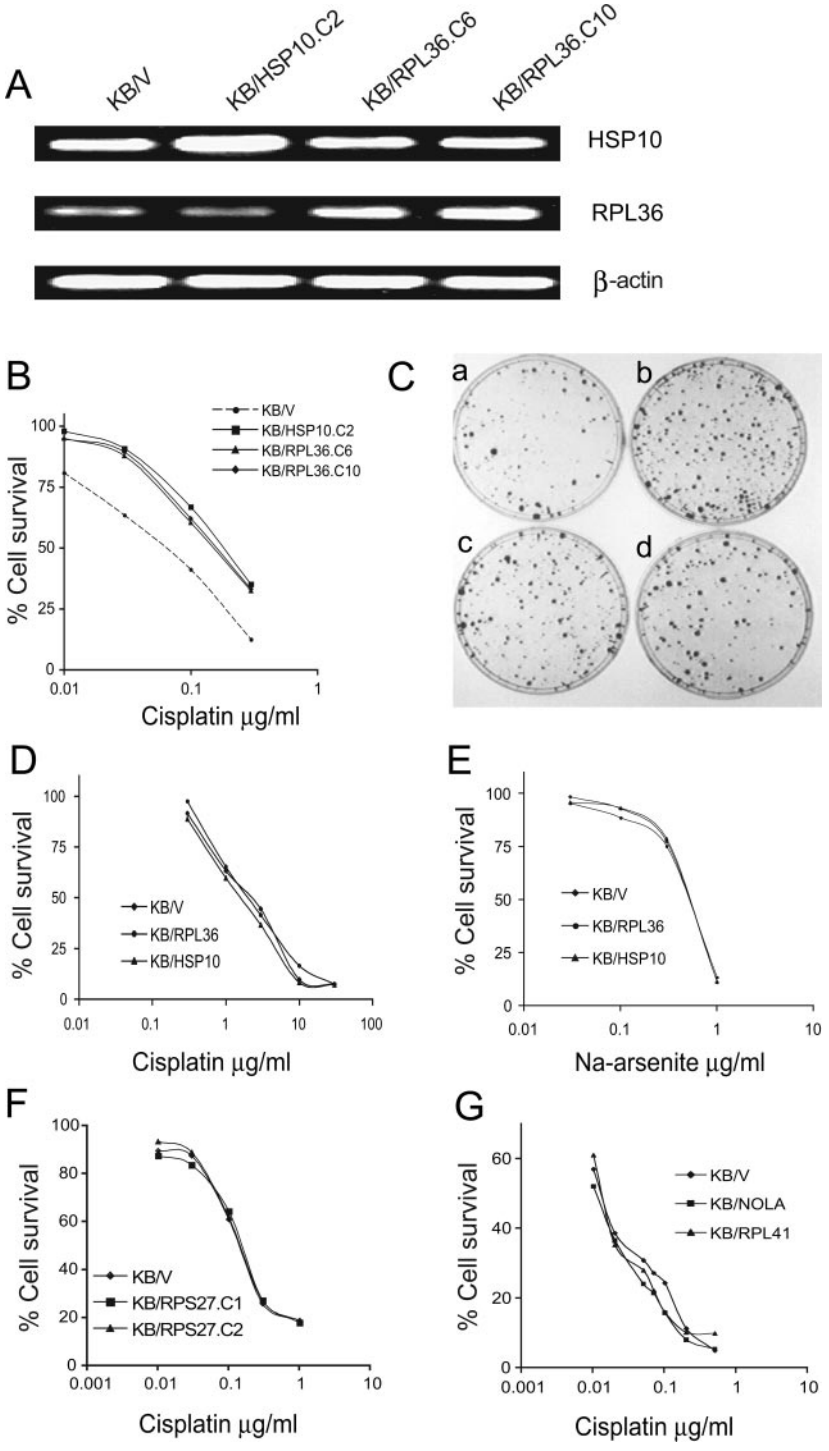

Fig. 3. RT-PCR analysis. The specific primers for RPL36 and HSP10 are described under *Materials and Methods*. A, expression levels in transfectants: KB/V, KB-3–1 cells transfected with vector pcDNA3.1 only; KB/HSP10.C2, KB-3–1 cells were transfected with HSP10, clone 2; KB/RPL36.C6 and KB/RPL36.C10 cells were transfected with the RPL36 gene, two individual clones. β-Actin served as control. B and C, colony-forming assay (CFA). B, killing curves measured 12 days after cisplatin selection, methylene blue-stained. KB/V, KB-3-1 cells transfected with vector pcDNA3; KB/HSP10.C2, KB cells transfected with HSP10/pcDNA3, clone 2; KB/RPL36.C6 and KB/RPL36.C10, KB cells transfected with RPL36/pcDNA3, 2 individual clones. C, the appearance of colonies in dishes of KB/V (a), KB/HSP10.C2 (b), KB/RPL36.C6, (c) and KB/RPL36.C10 (d) after exposure to cisplatin 0.3 μg/ml for 12 days as described in B. D, E, and G, cells were seeded at 5000 cells/well in a 96-well plate and were treated with the desired drug for 3 days; then cell numbers were determined by Cell Counting Kit-8 (CCK8) as described by the manufacturer. The concentrations of the drugs are indicated. F, cells were seeded at 5×10^4 cells/well in a 24-well plate and treated with cisplatin for 3 days, then counted using a Coulter Counter. The values are means of triplicate determinations.

genes related to cisplatin resistance. It should be noted that there were several ribosomal protein genes in this pool, suggesting that overexpression of the ribosomal gene family might also play an important role in protecting cells from cisplatin-induced damage.

We chose a ribosomal protein, L36 (RPL36), and a chaperone gene, HSP10, in our pool to evaluate whether these two genes have roles in CP-r. Both *RPL36* and *HSP10* genes were inserted into a mammalian expression vector, pcDNA3.1, respectively, and then transfected into cisplatin-sensitive (CP-s) KB-3-1 cells separately. Individual clones were isolated from the transfectants after G418 selection. Figure 3A shows that the *RPL36* gene (middle) was overexpressed in two clones of RPL36-transfected cells, KB/RPL36.C6 and KB/RPL36.C10. Both of the control cells (KB/V), which were transfected with vector only, and another transfected clone KB/HSP10.C2 showed much lower levels of the gene. At the top of the figure, HSP10 was well-expressed in KB/HSP10.C2 cells, which were transfected with the *HSP10* gene (approximately 2-fold higher than the control KB/V), and in two other clones isolated from the *RPL36* transfection. At the bottom are shown similar expression levels of β-actin in these four clones, serving as loading controls.

The sensitivities of these clones to cisplatin were then determined by a clonogenic assay. The results are shown in Fig. 3, B and C. Increased resistance to cisplatin (approximately 2.5~3-fold) was seen in the transfectants of RPL36, clones C6 and C10, and HSP10, clone C2, compared with the control vector-transfected only (Fig. 3C). In Fig. 3C, the amount and size of colonies in the clones expressing *RPL36*, clones C6 and C10 (c and d) or HSP10, clone C2 (b), were more numerous and larger than with the control vector only (a) after exposure to cisplatin 0.3 μg/ml for 12 days. These results demonstrate that, where overexpressed, the ribosomal protein RPL36 and the heat shock protein HSP10 may play roles in cisplatin resistance.

Discussion

A variety of approaches have recently been applied in efforts to define genes that are related to cisplatin resistance. Our previous studies demonstrated that a pleiotropic defect occurred in the cisplatin-resistant cells, including defective influx of [^{14}C]carboplatin, and other related heavy metals or unrelated compounds, mislocalization of membrane transporters, reduced expression of folate binding protein, and increased DNA hypermethylation (Liang et al., 2003; Shen et al., 2004), indicating a multifactorial mechanism involved in CP-r.

In this work, two genes, a ribosomal protein (RPL36) and a heat shock protein (HSP10), were identified to confer cisplatin resistance by 2.5- to 3-fold using a retroviral cDNA library and functional cloning. Although the function of these two genes in CP-r is unknown, their effect may plausibly be related to increased protein synthesis and protein stabilization to protect cells from the toxic effect of cisplatin, directly or indirectly, in association with the regulation of proteins in DNA damage/repair, antiapoptotic processes, or detoxification of cisplatin. We repeated the selection using a somewhat different protocol by which cells were selected with 1 μg/ml cisplatin for 24 h, instead of intermittent exposure for a longer period of time, and then were allowed to recover with drug for a period of 8 to 10 days until cloning. By this method, we further isolated more RPL36-related genes, such as *RPL36aL*, found in 4 individual clones from a pool of 30 (Table 1, last entry). *RPL36aL* has 90% identity with the coding region of the *RPL36* gene. This result provides independent evidence that the selection of the *RPL36* gene family members is not a random event but reflects their association with CP-r. None of the transfected clones, however, were cross-resistant to carboplatin or sodium arsenite, as shown in Fig. 3, D and E, respectively. This does not necessarily indicate a different mechanism of resistance, because the original parent cell line, KB-CP.5, was more resistant to cisplatin (up to 50-fold), whereas the *RPL36* and *HSP10* transfectants showed only 2.5- to 3-fold resistance.

None of the cDNAs isolated so far from the cDNA library made from these cells confers this phenotype on the full range of resistance seen in the KB-CP.5 cells. We assume, therefore, that this phenotype either results from expression of more than one gene or that we have transfected genes whose overexpression confers CP-r by a mechanism different from that seen in KB-CP.5.

The 10-, 27-, 60-, and 70-kDa heat shock proteins and others have been linked to stress response in protein folding and unfolding in drug resistance (Mandic et al., 2002; Shan et al., 2003; Zhao et al., 2005). Ribosomal proteins have been reported recently to be associated with the regulation of apoptosis, multidrug resistance (Wendel et al., 2004), oncogenesis, and chemotherapy (Zhang and Berger, 2004). Transfection of the ribosomal protein RPL23 into gastric cancer cells was reported to induce multidrug resistance, including CP-r, and to protect cells against vinblastine-induced DNA fragmentation but had no effect on intracellular drug accumulation (Shi et al., 2004). Our finding in this work provides evidence that overexpression of *RPL36* and *HSP10* confers CP-r in KB-3-1 cells but does not confer resistance to sodium arsenite. The pattern of genes selected by cisplatin may reflect stress responses and cisplatin-specific resistance genes. Further functional cloning by selection with other agents, including carboplatin, would help to determine which genes are involved in general stress responses and which are unique to cisplatin.

Other genes were detected in the clones (Table 1), but RPS27, RPL41, and nucleolar protein family A, member 2, were tested individually and did not confer CP-r as shown in Fig. 3, F and G, respectively. Either these genes are not sufficient to confer CP-r but may be necessary in the context of other patterns of gene expression or they were simply false-positive results from the screen applied here where the background (vector alone) was not 0. The other genes listed in Table 1 could not be tested initially because full-length cDNAs were not readily available. Nevertheless, these results indicate that the functions of RPL36 and HSP10 in CP-r cells and their potential role in human cancers need to be further elucidated.

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Address correspondence to: Dr. Michael M. Gottesman, Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, 37 Convent Dr., Room 2108, Bethesda, MD 20892-4254. E-mail: mgottesman@nih.gov