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Decreased Expression of the Amplified *mdr*1 Gene in Revertants of Multidrug-Resistant Human Myelogenous Leukemia K562 Occurs without Loss of Amplified DNA

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Amplification and increased expression of the *mdr*1 gene associated with multidrug resistance in human tumors were found in multidrug-resistant sublines of human myelogenous leukemia K562 selected with vincristine (K562/VCR) or adriamycin (K562/ADM). In two revertant cell lines of K562/ADM, amplification of the *mdr*1 gene was maintained at the same level as in K562/ADM, but expression of the 4.5-kilobase *mdr*1 mRNA was greatly decreased, indicating that amplified genes may be inactivated at the level of transcription without a corresponding loss of amplified DNA.

Pleiotropic drug resistance in mammalian cells, also known as multidrug resistance, results from decreased intracellular accumulation of different cytotoxic drugs. The changes in drug transport in multidrug-resistant cells correlate with increased expression of a glycoprotein (P-glycoprotein) with a molecular mass of 170 to 180 kilodaltons on the cell surface (1, 13, 14, 17). The functional role for P-glycoprotein in multidrug resistance is suggested by the findings that P-glycoprotein is capable of direct drug binding (3, 23). We have established two multidrug-resistant human myelogenous leukemia K562 cell lines selected with vincristine (K562/VCR) (28, 29a) or adriamycin (K562/ADM) (29). These cell lines also express a membrane glycoprotein of similar molecular mass. Monoclonal antibodies against this glycoprotein were shown to modulate drug transport and inhibit the growth of resistant cells (12).

Two independent lines of investigation have provided genetic evidence that P-glycoprotein is responsible for multidrug resistance. cDNA clones corresponding to P-glycoprotein have been isolated from Chinese hamster cells and used to show that P-glycoprotein genes are amplified and overexpressed in a number of multidrug-resistant cell lines (4, 19, 24, 31). In another series of experiments, the technique of in-gel DNA renaturation (20) was used to clone a gene, designated *mdr*, which was commonly amplified in different multidrug-resistant Chinese hamster cell lines (9, 21). A human homolog of the *mdr* gene, designated *mdr*1, is amplified and overexpressed in several multidrug-resistant human cell lines (22, 25). Transfer and expression of the human *mdr*1 or hamster *mdr* gene correlate with multidrug resistance in transfectants of mouse NIH 3T3 cells, obtained after DNA-mediated (26) or chromosome-mediated (10) gene transfer. Expression of the cloned full-length mouse *mdr* cDNA is sufficient to induce multidrug resistance in drug-sensitive cells (11). Recently, human *mdr*1 and P-glycoprotein clones were shown to cross-hybridize, indicating that *mdr* gene codes for P-glycoproteins (30). Sequence analysis of the human (2), hamster (7), and mouse (8) cDNA clones has shown that P-glycoproteins contain potential channel-forming transmembrane domains and share homology with energy-coupling components of bacterial active transport systems, suggesting that they function as energy-dependent efflux pumps in multidrug-resistant cells.

In the present study, we examined the amplification and mRNA expression of the *mdr*1 gene in human K562 leukemia cell lines with various levels of drug resistance. The multidrug-resistant lines K562/VCR and K562/ADM were established by in vitro selection of K562 cells with increasing concentrations of vincristine (up to 30 nM) and adriamycin (up to 450 nM), respectively, as described previously (29, 29a). The revertant cell lines used in this study were independently obtained spontaneous revertants of K562/ADM. Briefly, K562/ADM cells were cultured in the absence of adriamycin for about 6 months and then subcloned by the limiting dilution technique. The two most sensitive clones were chosen for further analysis. All of the cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 μg of kanamycin per ml at 37°C in humidified air containing 5% CO_2. Sensitivities to adriamycin and vincristine in these cell lines were evaluated by inhibition of cell growth after incubation at 37°C for 72 h. The concentrations of the drugs producing 50% growth inhibition are shown in Table 1. All of the resistant and revertant cell lines showed cross-resistance to both drugs.

Isolation and characterization of plasmid pMDR1 corresponding to the *mdr*1 gene was described elsewhere (22). High-molecular-weight genomic DNA was gently extracted from K562 cell sublines (15) and digested with 10 U of *Eco*RI per μg of DNA at 37°C for 16 h under the conditions recommended by the supplier. After ethanol precipitation, DNA samples of 10 μg per well were run on a 1% agarose gel in 40 mM Tris hydrochloride (pH 8.3)–2 mM EDTA–20 mM sodium acetate buffer, stained with 5 μg of ethidium bromide per ml, photographed, and transferred from the gel to nitrocellulose filters by the method of Southern (27). The filters were dried in vacuo and baked at 80°C for 2 h.

The pMDR1 probe was labeled to a specific activity of 2×10^8 cpm/μg by nick translation (18) or 10^9 cpm/μg by the oligolabeling method (5, 6) using [α-^{32}P]dCTP (3,000 Ci/mmol). The baked filters were incubated for 5 to 8 h at 65°C in 10× Denhardt solution (1× Denhardt solution is 0.2 g of Ficoll 400, 0.2 g of polyvinylpyrrolidone, and 0.2 g of bovine serum albumin per liter) with 0.5% sodium dodecyl sulfate and then hybridized for 16 h at 65°C in 3 ml of hybridization

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TABLE 1. Drug resistance and *mdr*1 gene amplification and expression in K562 sublines

Cell line	Mean ± SD IC_{50} (nM) of[a]:		Fold *mdr*1 gene amplification[b]	Fold *mdr*1 mRNA expression[b]
	ADM	VCR		
K562	13 ± 3	1.7 ± 0.4	1	1
K562/VCR	130 ± 10 (10)	120 ± 32 (71)	8	25
K562/ADM	1,750 ± 200 (135)	1,030 ± 300 (606)	30	100
R1-3	37 ± 12 (3)	6.6 ± 0.6 (4)	45	5
R1-5	28 ± 9 (2)	2.9 ± 0.2 (2)	25	3

[a] The data are based on three determinations. Shown in parentheses are degrees of resistance (fold) relative to that of the parental cell line. IC_{50}, Drug concentration that caused 50% growth inhibition.

[b] Levels of *mdr*1 gene amplification and mRNA expression were determined by densitometry of the autoradiograms with an RFT scanning densitometer and calculated from the ratio of pMDR1/β-actin signals for each slot. Amplification was calculated four times by using two independent autoradiograms for each cell line. The ranges were within 20% of the means. These mean ratios were compared with the ratio for K562, which was assigned a value of 1.

mixture containing 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.2), 5× Denhardt solution, 0.25% sodium dodecyl sulfate, 100 μg of sonicated and denatured *Escherichia coli* DNA per ml, 40 μg of poly(A) per ml, and 10^7 cpm of ^{32}P-labeled probe. Filters were washed three times for 30 min each time at 65°C in 2× SSC and twice in 0.5× SSC and then autoradiographed at −70°C on Kodak XAR-5 film with an intensifying screen. Under these hybridization conditions, the pMDR1 probe does not cross-hybridize with the related *mdr*2 gene (22).

Total cellular RNA and poly(A)$^+$ RNA were prepared by the method of Maniatis et al. (15) with minor modifications. RNA samples of 5 μg per well were run on a 1% agarose gel in morpholine propane sulfonic acid (MOPS) buffer (40 mM MOPS, 10 mM sodium acetate, 1 mM EDTA, pH 7.0) containing 13.4% formaldehyde and transferred directly to a nitrocellulose filter. The filter was dried, baked, and hybridized with ^{32}P-labeled pMDR1 probe as described above. After hybridization, the nitrocellulose filter was washed twice at 65°C in 0.1× SSC–0.1% sodium dodecyl sulfate.

To quantitate the degrees of amplification and mRNA expression of the *mdr*1 gene in K562 sublines relative to those of the parental line, serial dilutions of DNAs or RNAs from various cell lines were prepared, and hybridization was carried out as described above. Levels of gene amplification or mRNA expression were determined by densitometry of the autoradiograms. To normalize the amounts of loaded DNA or RNA, the slot blots were rehybridized to a cDNA clone for the β-actin gene, which was kindly provided by K. Kakunaga (16). We have previously reported that the amount of actin protein of K562/ADM remained unchanged (29). The values were calculated from the pMDR1/β-actin signal ratio for each cell line and expressed as values relative to that of parental K562 cells.

Two revertant clones, designated R1-3 and R1-5, were established. The degrees of resistance of the revertants are presented in Table 1. The parent line (K562/ADM) was not cloned before selection for the revertants and is likely to be heterogeneous. K562/ADM cells had been maintained in medium containing 600 nM adriamycin for more than a year. The revertant clones, however, could not proliferate even at 60 nM adriamycin. These clones, therefore, could not have existed in the original K562/ADM population. The *mdr*1 gene was amplified in multidrug-resistant K562/VCR and K562/ADM cells (Fig. 1; Table 1). The degrees of gene amplification were estimated densitometrically from slot blots (data not shown) to be approximately 8-fold for K562/VCR and 30-fold for K562/ADM. Spontaneous revertant clones R1-3 and R1-5, derived from K562/ADM, maintained the amplified *mdr*1 sequences at levels (25- to 45-fold) similar to that in K562/ADM, although the degrees of resistance of these revertants to adriamycin were only 3-fold and 2-fold, respectively, compared with the 130-fold degree of resistance of K562/ADM.

The *mdr*1 gene encodes a 4.5-kilobase mRNA, the steady-state levels of which are proportional to the degree of multidrug resistance in several human cell lines (25). Poly(A)$^+$ RNA was prepared from the parental and drug-resistant sublines of K562. Five micrograms of each poly(A)$^+$ RNA was electrophoresed on a 1% agarose gel containing formaldehyde, and Northern (RNA) blot hybridization was carried out. Under conditions of high stringency, pMDR1 hybridized to a single mRNA of 4.5 kilobases, which was highly expressed in K562/ADM cells (Fig. 1). K562/VCR also expressed a relatively high amount of the 4.5-kilobase mRNA, but two revertant lines expressed only low amounts of the 4.5-kilobase mRNA. This mRNA was barely detectable by Northern hybridization in the parent, K562 (Fig. 1). The degrees of *mdr*1 mRNA expression were

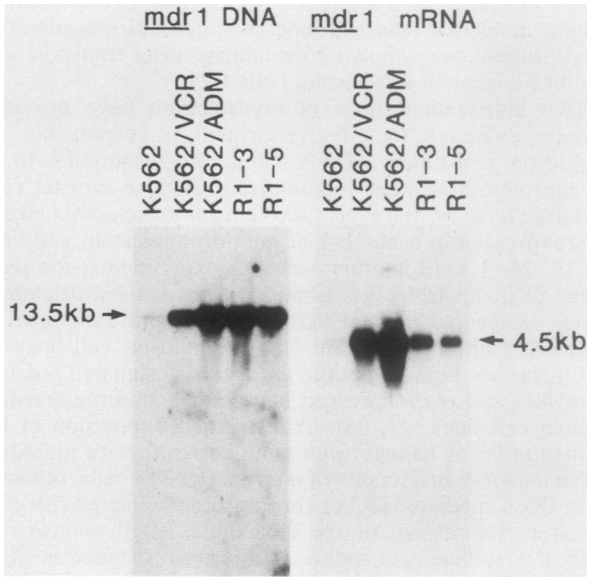

FIG. 1. (*mdr*1 DNA) Southern blot hybridization of DNA from K562 cell lines. DNA (10 μg) was digested with *Eco*RI, electrophoresed in an agarose gel, and transferred to a nitrocellulose filter. The DNA was hybridized with the pMDR1 probe labeled by nick translation. *Hin*dIII-digested λ phage DNA was used as a size standard. (*mdr*1 mRNA) Northern blot hybridization of RNA from K562 cell lines. Poly(A)$^+$ RNA (5 μg) was denatured, electrophoresed in an agarose gel, and transferred to a nitrocellulose filter. The RNA was hybridized with the pMDR1 probe labeled with ^{32}P by oligolabeling.

estimated by densitometry of slot blots (data not shown) to be 25-fold for K562/VCR and 100-fold for K562/ADM, which are proportional to the degrees of resistance. In the revertant cell lines R1-3 and R1-5, the mRNA was expressed at a low level, although these revertants contain a highly amplified *mdr*1 gene (Fig. 1; Table 1). The degrees of *mdr*1 mRNA expression of R1-3 and R1-5, compared with that of the parental line, were only fivefold and threefold. In contrast, the degrees of amplification of the *mdr*1 gene were 45- and 25-fold, respectively. These results indicate that the steady-state level of *mdr*1 mRNA in the revertants is low relative to the gene copy number.

In this study we found strong amplification and overexpression of the *mdr*1 gene in multidrug-resistant sublines of human myelogenous leukemia K562. These results are in agreement with previous findings which implicate expression of the *mdr*1 gene as the mechanism for multidrug resistance in cell lines of different origins.

Surprisingly, amplification of the *mdr*1 gene was also detected in revertants derived from K562/ADM. However, expression of the *mdr*1 gene was greatly decreased in the revertants. This result is in contrast with the findings on the revertants of multidrug-resistant human KB cells (22, 25) and Chinese hamster V79 cells (9, 21), in which a decrease in *mdr*1 mRNA expression was accompanied by either complete or partial loss of the amplified gene copies. To the best of our knowledge there are no findings in any other system in which decreased expression of amplified genes would occur in revertant cells without a corresponding loss of amplified DNA. We have analyzed only two revertant clones which were the most sensitive to adriamycin. It is feasible that other revertants of K562/ADM may utilize a different mechanism for decreasing *mdr*1 gene expression. The reasons for transcriptional or posttranscriptional inactivation of multiple *mdr*1 gene copies in R1-3 and R1-5 cells are presently under investigation.

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