Expression Complementary DNA Library Transfer Establishes mrp as a Multidrug Resistance Gene

Gary D. Kruh, 2 Andrew Chan, Kim Myers, Kathleen Gaughan, Toru Miki, and Stuart A. Aaronson

Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111 [G. D. K., K. G.]; Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, Maryland 20892 [A. C., K. M., T. M.]; and the Derald H. Ruttenberg Cancer Center, New York, New York 10029 [S. A. A.]

Abstract

The emergence of drug-resistant cancer cells is a major obstacle to cancer treatment. Resistant cells often display a multidrug-resistant phenotype that reduces the promise of combination chemotherapy, the classic approach to the prevention of drug resistance. mdr1, a member of the ABC cassette superfamily of transporters which encodes an energy-dependent drug efflux pump, is the only gene known to confer the multidrug-resistant phenotype. Other multidrug resistance mechanisms must exist, since cell lines which have this phenotype in the absence of mdr1 overexpression have been described. We report here the application of a novel approach involving expression complementary DNA library transfer to the identification of drug-resistant genes. Using this approach we establish that mrp, a member of the ABC cassette superfamily of transporters, is capable of conferring a multidrug-resistant phenotype. This approach should be useful in the identification of other novel resistance genes.

Introduction

The emergence of resistant cancer cells after repeated courses of chemotherapy is a major obstacle to cancer treatment and is exacerbated by the development of multidrug resistance. Multidrug resistance, which involves a wide array of lipophilic drugs including anthracyclines, Vinca alkaloids, epipodophyllotoxins, and dactinomycin, has reduced the promise of combination chemotherapy, the classic approach to the prevention of drug resistance (1). mdr1 is the only gene known to confer the full multidrug-resistant phenotype. P-glycoprotein, the Mr 170,000 protein product of the mdr1 gene, is an energy-dependent efflux pump which functions to decrease the intracellular concentration of lipophilic drugs (2, 3). Although P-glycoprotein plays a major role in multidrug resistance, other mechanisms must exist, since cell lines which have this phenotype in the absence of P-glycoprotein overexpression have been described (4, 5).

Defining the complete molecular framework that underlies the multidrug-resistant phenotype is critical to improvements in cancer chemotherapy. In the present report we demonstrate the application of a novel approach involving expression cDNA library transfer to the identification of drug resistance genes. Using this approach we transferred the resistance gene from a non-mdrl multidrug-resistant cell line to drug-sensitive NIH/3T3 cells. The transferred cDNA was identified as mrp, a member of the ABC cassette superfamily of transporters (6). The NIH/3T3 transfecants were shown to be resistant to several structurally unrelated drugs, thus establishing that mrp is capable of conferring a multidrug-resistant phenotype.

Materials and Methods

Expression cDNA Library Preparation and Transfection of NIH/3T3 Cells. Total cellular RNA and polyadenylated RNA was prepared from HL60R cells as described (7). A phagemid library was prepared from the HL60R polyadenylated RNA as described (8), using the improved ApCEV27 vector (9). NIH/3T3 cells were seeded at 1.5 X 10^5 cells/dish in Dulbecco’s modified Eagle’s medium 5% calf serum and transfected with 5 µg of phagemid DNA and 40 µg of calf thymus carrier DNA, using the CaCl2 precipitation method (10). Beginning the next day, cells were fed twice weekly with medium containing 40 nM Adriamycin.

Analysis of Drug Sensitivities. Drug sensitivities were determined by the tetrazolium salt assay (11). Cells (4000–8000/well) were seeded in 150 µl of Dulbecco’s modified Eagle’s medium 10% calf serum in 96-well plates. After overnight growth drug was added in various concentrations. After 48 h of incubation in the presence of drug, 40 µl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were added per well. Cells were lysed after a 2-h incubation at 37°C by the addition of 100 µl of extraction buffer [20% (w/v) sodium dodecyl sulfate-50% N,N-dimethylformamide (pH 4.7)]/well. After overnight incubation at 37°C, the absorbance at 570 nm was measured (Bio-Rad Microplate Multiscan). Experiments were performed in triplicate.

Northern and Southern Blot Analysis. Genomic DNA was prepared as described (12), and 20-µg samples of restriction endonuclease-digested DNA were fractionated by agarose gel electrophoresis, followed by transfer to nitrocellulose membranes. Blots were hybridized with 32P-labeled mrp probe at 42°C in 50% formamide -5 x SSC (12) -2.5 x Denhardt’s solution -7 mM Tris-HCl (pH 7.5) -0.1 mg/ml denatured calf thymus DNA and washed at 60°C in 0.1 x SSC. An mrp probe comprising nucleotides 3427-4254 of the mrp cDNA (6) was prepared by polymerase chain reaction using the HL60R cDNA library DNA as template and labeled using a nick translation kit (Amersham).

Total cellular RNA was extracted by a one-step guanidinium isothiocyanate-phenol-chloroform extraction procedure, as described (13). RNA samples (20 µg) were separated by electrophoresis through a 1% agarose gel containing 1% formaldehyde and transferred to Magna NT membrane filters (Micron Separations, Westborough, MA). Membranes were hybridized with 32P-labeled mrp probe at 45°C in 50% formamide -3 x SSC -0.05 M sodium phosphate (pH 6.5) -10% dextran sulfate -0.1 mg/ml herring sperm DNA and washed at 65°C with 0.1 x SSC -25 mM EDTA -5% sodium dodecyl sulfate -0.05 M Tris-HCl (pH 7.5).

Results

Expression cDNA Library Transfer of Adriamycin Resistance to Drug-sensitive NIH/3T3 Cells. Transfection of cDNA libraries prepared with the ApCEV27 expression phagemid has been applied to the molecular cloning of oncogenes and growth factor receptors, the expressed cDNAs of which were identified based on the induction of the transformed phenotype (9, 14-16). To apply this approach to the isolation of cDNAs the expression of which confers the selectable phenotype of drug resistance, we prepared a ApCEV27 library from HL60R, an Adriamycin-resistant cell line that displays a multidrug-resistant phenotype in the absence of mdr1 overexpression (17), and attempted to transfer Adriamycin resistance to NIH/3T3, a drug-sensitive cell line that can be transfected at high efficiency. Following transfection of NIH/3T3 cells with HL60R phagemid library DNA, cells were grown in the presence of an Adriamycin concentration (40...
Fig. 1. Adriamycin sensitivity of clone 57 compared to control NIH/3T3 cells. Drug sensitivity was analyzed by the tetrazolium salt assay as described in "Materials and Methods." Experiments were performed in triplicate. Points, means; bars, SD.

Fig. 2. Southern analysis of PstI-digested genomic DNA. Genomic DNA was digested with PstI and fractionated by agarose gel electrophoresis. A nitrocellulose filter containing the transferred DNA was hybridized with a 32P-labeled mrp probe. 1°, primary Adriamycin-resistant NIH/3T3 colony; 2°, secondary Adriamycin-resistant colonies arising from transfection of clone 57 DNA; NL Human, DNA prepared from human placenta; NIH3T3, untransfected NIH/3T3 cells; HL 60R, Adriamycin-resistant cell line; HL 60, nonresistant HL60 cells; kb, kilobase.

Fig. 3. Southern analysis of Sall-digested genomic DNA. Genomic DNA was digested with Sall and fractionated by agarose gel electrophoresis. A nitrocellulose filter containing the transferred DNA was hybridized with a 32P-labeled mrp probe. 1°, primary Adriamycin-resistant NIH/3T3 colony; 2°, secondary Adriamycin-resistant colonies arising from transfection of clone 57 DNA; NL Human, DNA prepared from human placenta; NIH3T3, untransfected NIH/3T3 cells; HL 60R, Adriamycin-resistant cell line; HL 60, nonresistant HL60 cells; kb, kilobase.

Fig. 4. Northern analysis of mrp expression. Arrows, positions of the HL 60R mrp transcript (left ordinate) and the phagemid mrp transcripts in the Adriamycin-resistant NIH/3T3 transfectants (right ordinate). Labels and probe are as described in the legend to Fig. 2.

Molecular Analysis of Adriamycin-resistant NIH/3T3 Transfectants. In an attempt to verify the presence of a phagemid responsible for Adriamycin resistance, genomic DNA was prepared from clone 57 and used in a second cycle of transfection. We observed >20 Adriamycin-resistant colonies/dish of NIH/3T3 cells transfected with 50 µg of genomic DNA, confirming the presence of a dominant acting resistance gene. Three second cycle transfectants, designated 2, 3, and 8, were isolated, and each was found to exhibit G418 resistance, indicating phagemid had been transferred as well.

In the course of these studies the nucleotide sequence of mrp, a gene that encodes a predicted protein with structural features of the ABC superfamily of transporters, was reported (6). Although mrp was isolated by subtractive hybridization as a gene expressed at high levels in an Adriamycin-resistant cell line, neither the biological activity of mrp nor its mechanism of action have been reported. Using polymerase chain reaction oligonucleotide primers based on the mrp nucleotide sequence, we prepared a probe containing sequences within a predicted 1.7-kilobase PstI fragment of the mrp cDNA. When filters containing genomic DNA digested with PstI were hybridized
with this probe, intense 1.7-kilobase bands were readily detected in clone 57 as well as second cycle clones 2, 3, and 8, but not in control NIH/3T3 cells (Fig. 2). Moreover, intense mrp genomic fragments were detected in HL60R, but not the parental HL60 cells, indicating that mrp was amplified in HL60R as well. Comparison of the intensities of the 1.7-kilobase bands to the faint single copy genomic mrp fragments revealed that mrp was amplified ~20-40-fold in HL60R as well as the drug-resistant NIH/3T3 transfectants. This result strongly suggested that mrp was responsible for conferring the Adriamycin-resistant phenotype to NIH/3T3 cells and that its amplification contributed to the drug-resistant phenotype of HL60R.

To confirm that the mrp coding sequence was transferred to clone 57 by the transfected HL60R expression library, we hybridized blots containing genomic DNA digested with SalI with the mrp probe. Since SalI digestion releases cDNA inserts from ApCEV27 (9), and the 7.5-kilobase mrp cDNA does not contain SalI restriction sites (6), a 7.5-kilobase mrp genomic fragment should be released if the complete mrp cDNA were present in integrated phagemid. Fig. 3 shows that the predicted 7.5-kilobase fragment was detected in DNA prepared from the Adriamycin-resistant transfectants, but not in the NIH/3T3 or HL60 controls.

When total cellular RNA was hybridized with an mrp probe, substantial overexpression of the 7.5-kilobase mrp transcript was detected in HL60R, but not parental HL60 cells (Fig. 4). Moreover, each of the resistant NIH/3T3 transfectants, but not untransfected NIH/3T3 cells, expressed larger mrp transcripts of ~8 and ~10 kilobase, as expected for transcription from ApCEV27. Multiple transcript species are common with cDNAs expressed from ApCEV27 and result from the use of alternative transcription termination sites.

### Drug Sensitivity Analysis of NIH/3T3 Transflectant

We analyzed the drug sensitivity patterns of the primary mrp transflectant and two of the second cycle transflectants to examine the drug resistance phenotype conferred by mrp. The hallmark of the multidrug-resistant phenotype is simultaneous resistance to a spectrum of structurally unrelated natural product drugs. Three structurally unrelated cytotoxic drugs, Adriamycin, vinblastine, and VP16, which have distinct mechanisms of action were tested. As shown in Table 1, the three NIH/3T3 clones each displayed a multidrug-resistant phenotype with resistance to all three drugs. Although the relative levels of resistance to each drug varied, these results are consistent with both the resistance levels and variability observed in NIH/3T3 cells transfected with a retroviral-based mdrl expression vector (18).

### Discussion

Our present studies demonstrate the application of an expression cDNA library approach to the identification of a drug resistance gene overexpressed in the multidrug-resistant HL60R cell line. The cDNA was shown to be mrp, a member of the ABC cassette superfamily of transporters, which now includes over 50 proteins (19). Other members of this family are involved in the translocation of diverse substrates such as ions, peptides, proteins, and sugar polymers across biological membranes in prokaryotic and eukaryotic cells. Although sequence comparison indicates that mrp is as related to the cystic fibrosis 1 ion transporter as it is to the mdrl protein product P-glycoprotein (6), we established that mrp was capable of conferring a multidrug-resistant phenotype to transfected cells. In addition, we found that the calcium channel blocker verapamil can reverse the drug-resistant phenotype of mrp transflectants6 analogous to the effects of this drug on cells that overexpress P-glycoprotein (20). Verapamil acts by directly competing with lipophilic drugs for the drug-binding sites of P-glycoprotein (21). Thus the ability of verapamil to make mrp transflectants drug sensitive suggests that the mrp protein product directly transports lipophilic drugs as well. These findings are consistent with evidence that HL60R cells exhibit enhanced nuclear efflux of daunomycin (22), a property consistent with the overexpression of a transporter.

The isolation of only one Adriamycin-resistant colony, as well as the attendant mrp amplification, may be related to the relatively high drug concentration used for selection. Moreover, while cDNA amplification occurred at a level comparable to mrp gene amplification in HL60R, the mrp transcript was relatively inefficiently expressed in the transflectants. This may reflect transcript instability or some promoter abnormality specific to the transferred expression clone. Nevertheless, the potential exists for the application of expression cDNA cloning strategies to the detection and isolation of other novel drug resistance genes.

Our elucidation of the activity of mrp in conferring multidrug resistance establishes that there are at least two genes capable of conferring this phenotype and suggests that the expression of several genes likely underlies the genetic basis of in vivo multidrug resistance. This may explain in part the incomplete correlation between pretreatment mdrl expression levels and clinical outcome in tumors treated with natural product drugs (23–26), as well as the observation that the degree of multidrug resistance in untreated tumor cell lines does not invariably correlate with mdrl expression (27). The expression of mrp may be of particular relevance in the de novo resistance of tumors which express low or undetectable levels of mdrl, such as breast and non-small cell lung cancer (28). Further studies should make it possible to assess the import of mrp in de novo and acquired clinical drug resistance.

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### References


*G. D. Kruh and S. A. Aaronson, unpublished observations.*
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