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Overexpression of a Transporter Gene in a Multidrug-Resistant Human Lung Cancer Cell Line

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The doxorubicin-selected lung cancer cell line H69AR is resistant to many chemotherapeutic agents. However, like most tumor samples from individuals with this disease, it does not overexpress P-glycoprotein, a transmembrane transport protein that is dependent on adenosine triphosphate (ATP) and is associated with multidrug resistance. Complementary DNA (cDNA) clones corresponding to messenger RNAs (mRNAs) overexpressed in H69AR cells were isolated. One cDNA hybridized to an mRNA of 7.8 to 8.2 kilobases that was 100- to 200-fold more expressed in H69AR cells relative to drug-sensitive parental H69 cells. Overexpression was associated with amplification of the cognate gene located on chromosome 16 at band p13.1. Reversion to drug sensitivity was associated with loss of gene amplification and a marked decrease in mRNA expression. The mRNA encodes a member of the ATP-binding cassette transmembrane transporter superfamily.

Small cell lung cancer (SCLC) accounts for 20 to 25% of all lung cancers. It differs from other forms of lung cancer, known collectively as non–small cell lung cancers (NSCLC), because it is initially much more responsive to chemotherapy. Up to 90% of SCLC tumors respond to chemotherapy, but patients almost always relapse with multidrug-resistant disease. The initial response rate of NSCLC tumors is much lower and, for the most part, these tumors display inherent drug resistance. The molecular basis of drug resistance in both SCLC and NSCLC is poorly understood.

Overexpression of the transmembrane transport protein P-glycoprotein has been detected in many multidrug-resistant tumor cell lines and in a variety of tumors from cancer patients with both acquired and inherent drug resistance (*1*). This protein is encoded by the human *MDR1* gene, and in vitro studies have shown that it confers resistance to a range of natural product xenobiotics that are used as chemotherapeutic drugs (*2*). However, despite the widespread occurrence of drug resistance in human lung tumors, overexpression of P-glycoprotein is infrequent, which indicates the existence of alternative resistance mechanisms (*3*, *4*).

NCI-H69 (H69) is an SCLC cell line that has been used in experimental studies of lung cancer (*5*). We have described a multidrug-resistant variant of this cell line, designated H69AR, which was obtained by stepwise selection in doxorubicin. H69AR displays a drug resistance pattern typical of that associated with increased amounts of P-glycoprotein. However, as with most clinical specimens of SCLC, H69AR does not overexpress this protein or its cognate mRNA (*6*–*8*). Consistent with this observation, major differences in net drug accumulation or efflux do not appear to be part of the resistance phenotype of the H69AR cell line (*8*). Another feature that distinguishes H69AR from cell lines that overexpress P-glycoprotein is the inability of cyclosporin A and several other chemosensitizing agents to reverse doxorubicin resistance in these cells (*7*, *9*).

As part of a search for proteins responsible for the multidrug resistance displayed by H69AR cells, a randomly primed cDNA library was constructed from H69AR mRNA and screened by differential hybridization with total cDNA prepared from H69 and H69AR mRNA (*10*). One of the

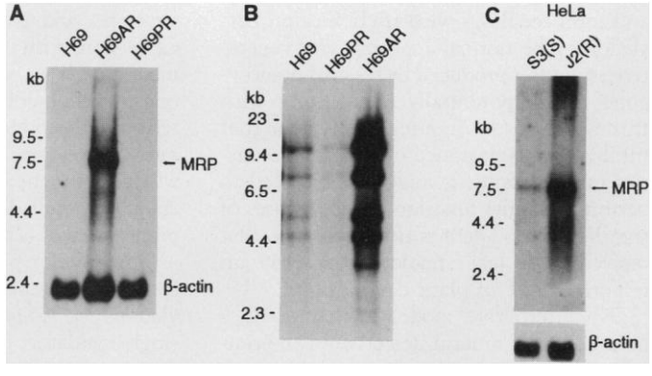

Fig. 1. (**A**) Northern blot of poly(A)$^+$ RNA from H69, H69AR, and H69PR cells hybridized with a 1.8-kb Eco RI cDNA fragment of MRP. The analysis of 1 μg of poly(A)$^+$ RNA from each cell line was carried out with standard procedures (*27*). The autoradiograph shown is a 5-hour exposure at −70°C with intensifying screens. The size of the overexpressed mRNA in H69AR cells, indicated by the arrow, was estimated to be 7.8 to 8.2 kb. Prolonged exposure of the film revealed small amounts of this mRNA in both H69 and H69PR cells. Molecular size standards are shown at the left. (**B**) Southern blot analysis of Eco RI–digested genomic DNA from H69PR, H69, and H69AR cells. DNA (10 μg) was digested with Eco RI, analyzed by electrophoresis through a 0.7% agarose gel, and blotted onto a nitrocellulose membrane. The DNA was hybridized with a 1.8-kb Eco RI cDNA fragment of MRP that was labeled by random priming with [α-^{32}P]dCTP. The autoradiograph shown is a 6-hour exposure at −70°C. On the basis of the examination of several restriction digests and normalization of the amounts of DNA loaded, no differences in the copy number of the gene in H69 and H69PR cells were detected. Molecular size standards are shown at left. (**C**) Northern blot analysis of HeLa cell poly(A)$^+$ RNA with MRP cDNA. S3 is a drug-sensitive (S) HeLa cell line, and J2c is a drug-resistant (R) one (*28*). Poly(A)$^+$ RNA (2 μg) from each cell line was analyzed by electrophoresis, blotted, and probed with MRP cDNA as described in (A). The MRP and β-actin autoradiographs shown are 18-hour and 1-hour exposures, respectively, at −70°C.

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clones isolated gave a particularly strong differential signal when analyzed on Northern (RNA) blots (Fig. 1A). This 2.8-kb cDNA clone hybridized with a single-size class of mRNA of approximately 7.8 to 8.2 kb, the concentration of which was increased 100- to 200-fold in H69AR cells relative to H69 cells. The amount of this mRNA in H69PR, a drug-sensitive partial revertant of H69AR (*11*), was approximately one-twentieth of that found in H69AR, which further substantiates the correlation of overexpression of this particular mRNA with the multidrug resistance phenotype. Southern (DNA) blot analyses of H69 and H69AR DNA indicated that the major mechanism underlying overexpression was gene amplification. Consistent with this suggestion, amplification was lost in H69PR cells (Fig. 1B). The mRNA was also overexpressed 12- to 15-fold in a doxorubicin-selected HeLa cell line that is multidrug-resistant and that does not overexpress P-glycoprotein (Fig. 1C), and the cognate gene was amplified to a comparable degree (*12*). This finding provided further evidence of the association of elevated amounts of this mRNA with multidrug resistance.

We sequenced the initial 2.8-kb cDNA clone in order to isolate overlapping clones by rescreening the H69AR cDNA library with synthetic oligonucleotides. A single open reading frame of 1522 amino acids was defined, encoding a protein we have designated as multidrug resistance–associated protein (MRP). The translated GenBank/European Molecular Biology Laboratory and SwissProt databases were searched for simi-

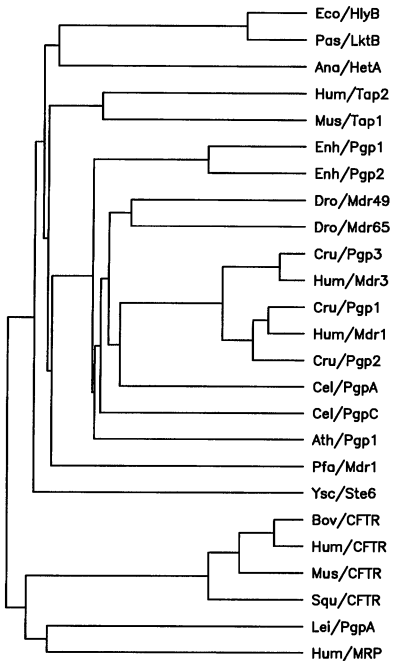

Fig. 2. Cluster analysis of the relative similarity of MRP to selected members of the ABC transporter superfamily. Shown are representative members of the superfamily that contain hydrophobic transmembrane regions, followed by nucleotide-binding folds. We created a multiple sequence alignment with the PILEUP program from the Genetics Computer Group package (version 7.1) (Madison, Wisconsin) (*29*) using a modified version of the progressive alignment method of Feng and Doolittle (*30*) with a gap weight of 3.0 and a gap length weight of 0.1. To ensure that this dendrogram was representative of the actual sequence similarity, we repeated the alignment using the following modifications: (i) subsets of the sequences in order to minimize weighting effects of similar sequences, (ii) NH_2- or COOH-terminal regions of sequences to reduce effects arising from the presence of two homologous NBFs, and (iii) variations in the gap creation and extension penalties and the position of arbitrary gaps to test the thoroughness of the alignment.

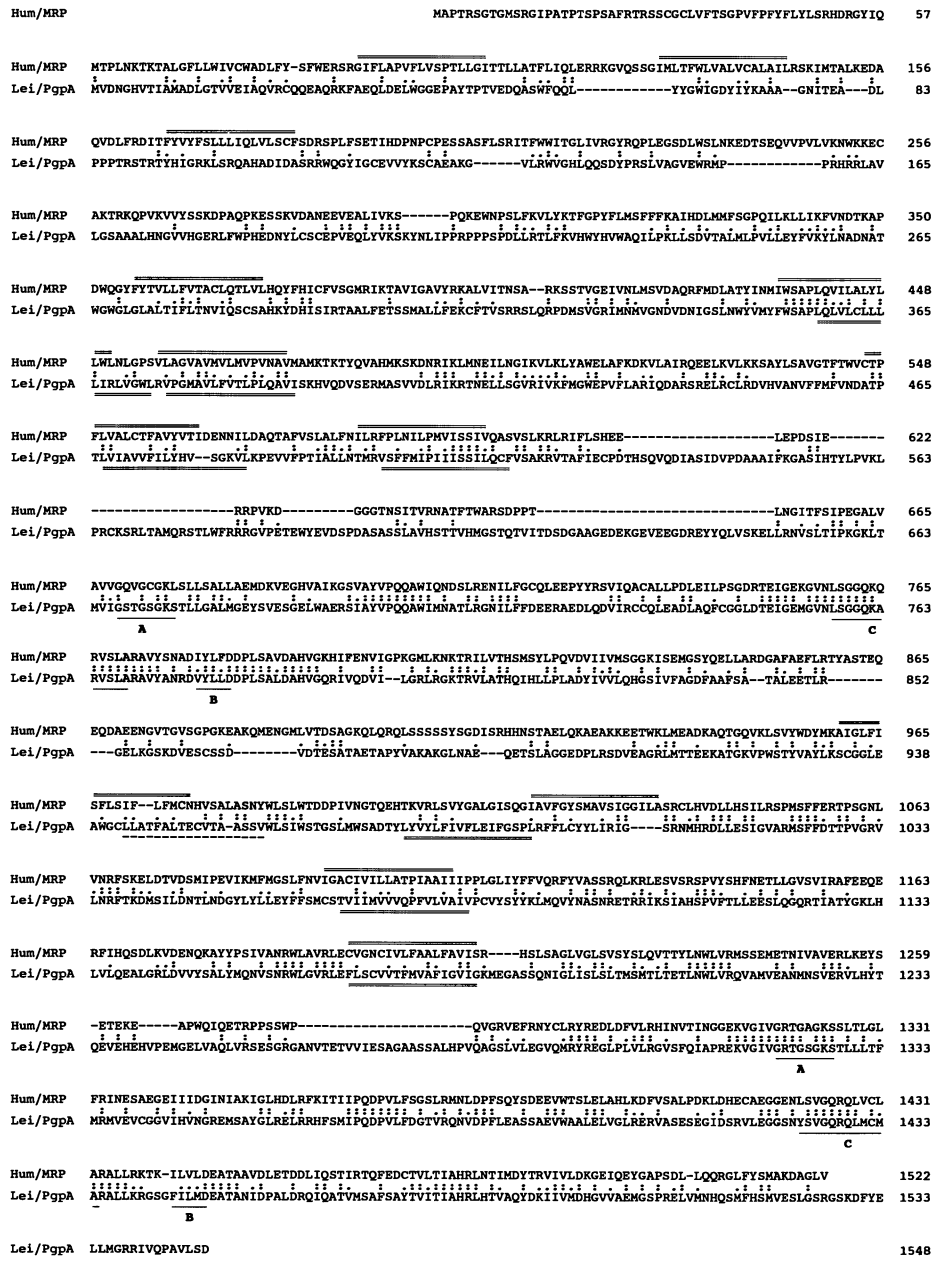

Fig. 3. Deduced amino acid sequence (*26*) of MRP aligned with the amino acid sequence of *ltpgpA* (Lei/PgpA). The alignment was generated with PILEUP as described in Fig. 2. The MRP sequence shown begins at the putative NH_2-terminal methionine and was compiled from four overlapping λgt11 cDNA clones (*31*). Alignment of the two proteins begins at a methionine residue 57 amino acids from the NH_2-terminus of MRP that aligns with the NH_2-terminal methionine of Lei/PgpA. Identical and conserved amino acids are identified by double and single dots, respectively. The Walker A and B motifs and the active transport family signature that are characteristic of nucleotide-binding folds of ABC transporters (*25*) are indicated by single lines and denoted A, B, and C, respectively, in bold. The predicted transmembrane regions of each protein are indicated by double lines (*32*). The region in Lei/PgpA underscored by a dashed line has a mean hydrophobicity value approaching that of a transmembrane region.

larities to MRP with the FASTA program (*13*). The search indicated, solely on the basis of its primary sequence, that MRP is a member of the adenosine triphosphate (ATP)–binding cassette (ABC) superfamily of transport systems (*14*). Members of this superfamily are involved in the energy-dependent transport of a variety of molecules across cell membranes in both eukaryotes and prokaryotes. Included in this superfamily are the genes for the human multidrug transporter P-glycoprotein (*MDR*1) and the cystic fibrosis transmembrane conductance regulator (*CFTR*) (*2, 15*).

The relation of MRP to various members of the ABC transporter superfamily was examined with a program that generates a dendrogram of relative protein similarities. A representative selection of a phylogenetically broad range of ABC proteins that are composed of hydrophobic transmembrane regions followed by nucleotide-binding regions, and whose sequences could be retrieved from the GenBank or SwissProt databases, were included in this analysis. With the use of a variety of alignment parameters, the analysis consistently divided this family of proteins into two major subgroups (Fig. 2). One consists of the cluster that contains MRP [human (Hum)/MRP], the leishmania P-glycoprotein–related molecule (Lei/PgpA) (*16*), and the CFTRs [Hum/CFTR, bovine (Bov/CFTR), mouse (Mus/CFTR), and dogfish (Squ/CFTR)]. The other consists of the P-glycoproteins, the major histocompatibility complex class II–linked peptide transporters (Hum/Tap2 and Mus/Tap1), the bacterial exporters (Eco/HlyB and Pas/LktB), the heterocyst differentiation protein (Ana/HetA), the malarial parasite transporter (Pfa/Mdr1), and the yeast mating factor exporter (Ysc/Ste6).

The dendrogram indicates that MRP is only distantly related to identified members of the ABC transporter superfamily. Although the analysis suggests that it is most closely related to Lei/PgpA, the similarity between MRP and Lei/PgpA resides predominantly in two regions, both containing signatures of nucleotide-binding folds (NBFs) (Fig. 3). It has been proposed that the bipartite structure of P-glycoproteins reflects duplication of an ancestral gene that occurred before the evolutionary separation of animals and plants (*17*). However, comparison of the NH_2- and COOH-terminal NBFs of MRP and Lei/PgpA revealed less similarity than typically found between the two corresponding regions of P-glycoproteins. To determine whether this was a common structural feature of MRP, Lei/PgpA, and Hum/CFTR, we aligned their NH_2- and COOH-terminal NBFs with each other and those of several P-glycoproteins. One such comparison with human P-glycoprotein (Hum/Mdr1) as an exemplar is shown (Fig. 4). The NH_2-terminal NBFs of MRP, Hum/CFTR, and Lei/PgpA share structural features that distinguish them from the NH_2-terminal NBF of Hum/Mdr1, particularly in the spacing of conserved motifs. This difference

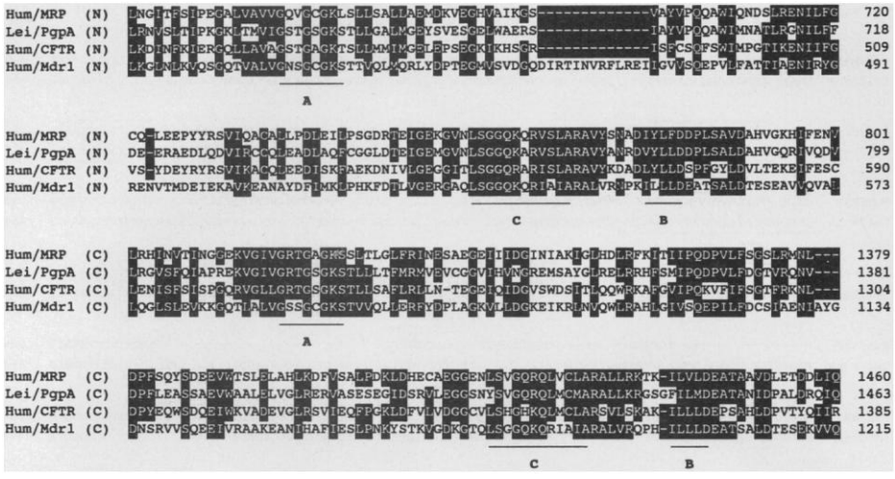

Fig. 4. Alignment of the extended nucleotide-binding regions of MRP, CFTR, and *Leishmania* and human P-glycoproteins. Shown are the NH_2-terminal (N) and the COOH-terminal (C) halves of the deduced amino acid sequence of MRP corresponding to *ltpgpA* (Lei/PgpA) (amino acids 650 to 799 and 1303 to 1463), Hum/CFTR (amino acids 441 to 590 and 1227 to 1385), and Hum/Mdr1 (amino acids 410 to 573 and 1053 to 1215). The sequences are presented as aligned by PILEUP. White type on black background indicates that three of four amino acids at that position are identical or conserved. The conserved motifs A, B, and C described in Fig. 3 are underscored by a single line. Similarity scores for the NH_2-terminal NBFs relative to MRP are as follows: Lei/PgpA, 0.93; Hum/CFTR, 0.85; and Hum/Mdr1, 0.60. Comparable COOH-terminal scores are as follows: Lei/PgpA, 0.87; Hum/CFTR, 0.84; and Hum/Mdr1, 0.73. Similarity scores for NH_2- and COOH-terminal NBFs in the same protein are as follows: MRP, 0.61; Lei/PgpA, 0.60; Hum/CFTR, 0.62; and Hum/Mdr1, 1.10.

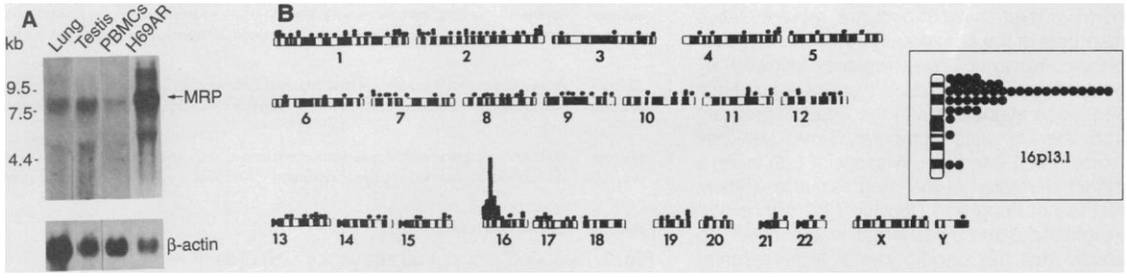

Fig. 5. (**A**) Northern blot analysis of total RNA from normal tissues with MRP cDNA. Lung and testis RNAs were obtained from Clontech Laboratories (Palo Alto, California). PBMC RNA was prepared from cells isolated from peripheral blood from healthy volunteers by centrifugation over ficoll-Isopaque (specific gravity of 1.078; Pharmacia). Total RNA from lung, testis, PBMCs (30 μg), and H69AR cells (10 μg) was analyzed as in Fig. 1A. The autoradiograph of the blot probed with a 0.9-kb Eco RI cDNA fragment of MRP is a 38-hour exposure for the normal tissue RNA and a 24-hour exposure for the H69AR RNA. The blot was stripped and reprobed with ^{32}P-labeled β-actin cDNA. The actin autoradiograph is a 24-hour exposure. Although hybridization with β-actin cDNA confirmed the integrity of RNA samples, MRP mRNA was not detectable in placenta, brain, kidney, salivary gland, uterus, liver, and spleen. (**B**) Silver grain distribution after in situ hybridization of MRP to metaphase chromosomes. In situ hybridization of a 1.8-kb Eco RI fragment of MRP cDNA was performed with the method of Harper and Saunders (*33*). The positions of 200 silver grains directly over or touching well-banded metaphase chromosomes were recorded on an International System for Human Cytogenetics Nomenclature–derived idiogram of the human karyotype. A significant clustering of grains (40) was observed in the 16p region ($P < 0.0001$), and the peak of the distribution was at 16p13.1 (inset). Approximately 160 metaphases were examined.

in spacing also contributes to the relatively low similarity between NH_2- and COOH-terminal NBFs in each of the three proteins (*18*). In addition, the COOH-terminal NBFs of MRP, Lei/PgpA, and Hum/CFTR are more similar to each other than to either the COOH- or NH_2-terminal NBFs of Hum/Mdr1 (Fig. 4). These observations, combined with the overall analysis shown in Fig. 2, suggest that MRP, Lei/PgpA, and CFTR evolved from a common ancestor that contained both NH_2- and COOH-terminal NBFs. Comparison with other ABC transporters indicates that this precursor probably differed from the ancestral gene of the P-glycoproteins or had diverged from it before the separation of animals and plants (*17*).

Despite knowledge of the structure and ability of P-glycoprotein to act as a drug efflux pump, its normal physiological role has not been elucidated. Some possible clues to its function have been provided by its distribution in normal tissues. P-glycoprotein is highly expressed in secretory organs and tissues, such as the adrenal glands, kidneys, lumenal epithelium of the colon, and the murine gravid uterus. It has also been detected in the lung, although this finding is variable (*4*, *19*). On the basis of the cell types in which it is expressed, it has been postulated that P-glycoprotein may be involved in steroid transport or protection against xenobiotics. Northern blot analyses of total RNA preparations from a range of human tissues indicate that MRP displays a different tissue profile of expression. MRP mRNA is readily detectable in lung (*20*), testis, and peripheral blood mononuclear cells (PBMCs) (Fig. 5A). MRP transcripts were below the level of detection in placenta, brain, kidney, salivary gland, uterus, liver, and spleen.

The human *CFTR* and *MDR1* genes have been mapped to chromosome 7 at bands q31 and q21, respectively (*21*, *22*). The structural similarity of MRP to these proteins prompted us to examine the possibility that the MRP gene may be linked to one of these loci. However, in situ hybridization carried out on bromodeoxyuridine (BrdU)-synchronized PBMCs demonstrated that the gene coding for MRP is located on chromosome 16 at band p13.1 (Fig. 5B). At present, the size of the amplicon containing MRP is unknown. Consequently, the possibility cannot be excluded that the amplicon contains additional genes that may be responsible for or contribute to the multidrug resistance phenotype.

Although total cellular drug accumulation appears unchanged in H69AR cells (*8*), the possibility remains that sequestration of drugs in these resistant cells may be modified so that the drugs are less able to reach their intracellular sites of action (*23*). MRP may participate directly in the active transport of drugs into subcellular organelles or influence drug distribution indirectly. If MRP is involved in ion transport, its overexpression could alter cytoplasmic or intra-organelle pH. A relative decrease in intra-organelle pH would be expected to result in greater sequestration of drugs, such as the anthracyclines and *Vinca* alkaloids, which are protonated under acidic conditions (*23*, *24*). The subcellular localization of MRP and the identification of the molecules it transports should provide insight into the mechanism by which this protein is involved in multidrug resistance. The isolation and characterization of MRP provides definitive molecular evidence for the existence of a transporter that may confer or at least contribute to multidrug resistance in SCLC. The overexpression of MRP in multidrug-resistant HeLa cells and inherently resistant NSCLC cell lines (*20*) suggests that this protein may play a role in resistance in other malignancies as well.

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20. The amounts of MRP mRNA in the normal lung are considerably higher than those detected in the sensitive H69 cells. This finding raises the possibility that this SCLC cell line has either a diminished ability to express the MRP gene or that H69 is derived from a cell type in the lung that is distinct from those responsible for the relatively high amount of MRP detected in normal tissue. This observation is also compatible with the suggestion that some cell types in the lung may be inherently drug-resistant by virtue of a relatively high constitutive expression of MRP. MRP mRNA is readily detectable in Northern blots of five of five inherently resistant NSCLC cell lines.
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26. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
27. Polyadenylated [poly(A)$^+$] RNA was obtained with a Fast Track mRNA isolation kit (Invitrogen), and 1 μg was separated by electrophoresis on a formaldehyde-agarose denaturing gel. The RNA was transferred to nitrocellulose membrane and hybridized in 50% formamide, 5× SSPE (1× is 150 mM NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA at pH 7.4), 2.5× Denhardt's solution (50× is 1% bovine serum albumin, 1% polyvinylpyrrolidone, and 1% Ficoll), and sheared and denatured herring testes DNA (100 μg/ml) for 4 to 16 hours at 42°C. The blot was probed with a 1.8-kb Eco RI fragment of MRP, labeled to a specific activity of >5 × 10^8 cpm per microgram of DNA with α-^{32}P–labeled cytidylate deoxynucleotide (dCTP) (3000 Ci/mmol; Du Pont/Biotechnology Systems) by the random priming method [A. P. Feinberg and B. Vogelstein, *Anal. Biochem.* **132**, 6 (1983)]. Hybridization was carried out for 16 to 20 hours at 42°C. Blots were washed three times in 0.1% SDS and 0.1× SSC (0.15 M NaCl and 0.015 M Na citrate, pH 7.0) for 30 min each at 52°C, and then film was exposed to the blots. To estimate variation in RNA loading of the gel, we reprobed the blot with a ^{32}P-labeled β-actin cDNA (201pBv2.2)

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28. The multidrug-resistant J2c cell line is a derivative of the drug-sensitive S3 HeLa cell line obtained by the selection of mutagenized S3 cells in doxorubicin. The J2c cell line is about fivefold more resistant to doxorubicin relative to S3 and does not overexpress P-glycoprotein (R. Baker, personal communication).
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30. D.-F. Feng and R. F. Doolittle, *J. Mol. Evol.* **25**, 351 (1987). The final alignment is produced by a series of progressive, pairwise alignments, beginning with the two most similar sequences and continuing until all sequences have been included in the final alignment. Before alignment, the sequences are first clustered by similarity to produce a dendrogram that directs the order of the subsequent pairwise alignments. Although this dendrogram is not a phylogenetic tree, the horizontal branch lengths are inversely proportional to the degree of similarity between the sequences. Based on the FASTA search, the following protein sequences (with their GenBank/EMBL accession numbers) were selected for alignment with MRP (Hum/MRP): cystic fibrosis transmembrane conductance regulators from humans (Hum/CFTR, A30300), cow (Bov/CFTR, M76128), mouse (Mus/CFTR, M69298), and dogfish (Squ/CFTR, M83785 and M76974); P-glycoproteins from *Leishmania* (Lei/PgpA, A34207), humans (Hum/Mdr1, A25059 and Hum/Mdr3, JS0051), Chinese hamster (Cru/Pgp1, M60040; Cru/Pgp2, M60041; and Cru/Pgp3, M60042), *Drosophila* (Dro/Mdr49, M59076 and Dro/Mdr65, M59077), *Caenorhabditis elegans* (Cel/PgpA, X65054 and Cel/PgpC, X65055), *Entamoeba histolytica* (Enh/Pgp1, M88599 and Enh/Pgp2, M88598), *Arabidopsis thaliana* (Ath/Pgp1, X61370), and *Plasmodium falciparum* (Pfa/Mdr1, A32547); MHC class II–linked peptide transporters from human (Hum/Tap2, M74447) and mouse (Mus/Tap1, M55637); the mating factor exporter from yeast (Ysc/Ste6, S05789, S14174, and S05872); the bacterial toxin exporters from *Escherichia coli* (Eco/HlyB, M10133 and M12863) and *Pasteurella haemolytica* (Pas/LktB, M20730); and the heterocyst differentiation protein from *Anabaena* sp. (Ana/HetA, M31722).
31. The inserts of four λgt11 cDNA clones with a minimum of 400 nucleotides of overlapping sequence were subcloned into pGEM-3Zf(+) (Promega), and the nucleotide sequences of both strands were determined by the dideoxynucleotide chain termination method [F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977)]. We confirmed the continuity of the total sequence compiled from the four clones (5009 nucleotides) by using the reverse polymerase chain reaction to amplify overlapping segments of MRP mRNA that spanned the entire sequenced region. The compiled sequence contained a single open reading frame encoding a protein of 1522 amino acids. The putative translational initiation codon of this open reading frame was preceded by an in-frame termination codon located 141 nucleotides upstream (5′ to the coding sequence). The length of the 5′ untranslated region of the mRNA was predicted by partial sequencing of seven additional cDNA clones that were independently isolated and complementary to the 5′-proximal portion of MRP mRNA. Three clones contained untranslated leader sequences of 204 nucleotides. The other four contained leader sequences of 222 or 225 nucleotides. The location of the 5′ end of the mRNA was also determined by ribonuclease H mapping, which indicated that the untranslated leader sequence was 200 to 225 nucleotides long. The MRP cDNA sequence will appear in sequence databases under accession number LO5628.
32. P. Klein, M. Kanehisa, C. DeLisi, *Biochim. Biophys. Acta* **815**, 468 (1985).
33. M. E. Harper and G. F. Saunders, *Chromosoma* **83**, 431 (1981). Metaphase chromosomes on slides were denatured for 2 min at 70°C in 70% deionized formamide and 2× SSC and then dehydrated with ethanol. The hybridization mixture consisted of 50% deionized formamide, 10% dextran sulfate, 2× SSC (pH 6), sonicated salmon sperm DNA (20 μg/ml), and ^{3}H-labeled MRP cDNA (0.2 μg/ml). The cDNA probe was uniformly labeled to a specific activity of 8.5×10^8 cpm per microgram of DNA with [^{3}H]thymidylate deoxynucleotide and [^{3}H]adenylate deoxynucleotide (Du Pont Biotechnology Systems) and denatured in the hybridization solution at 70°C for 5 min. Probe solution (50 μl) was placed on each slide and incubated at 37°C overnight. After hybridization, the slides were washed in 50% deionized formamide and 2× SSC, followed by 2× SSC (pH 7), and then dehydrated sequentially in ethanol. The slides were coated with emulsion (NTB/2; Kodak) and developed after exposure for 5 weeks at 4°C. Chromosomes were stained with a modified fluorescence, 0.25% Wright's stain procedure [C. C. Lin, P. N. Draper, M. Braekeleer, *Cytogenet. Cell Genet.* **39**, 269 (1985)].
34. We thank R. M. Baker (Roswell Park Memorial Institute, Buffalo, NY) for providing the HeLa cell lines, S. Mirski for discussions, and M. Vasa and K. Sparks for technical contributions. Supported by grants from the Medical Research Council (MRC) of Canada (to S.P.C.C. and R.G.D.) and the National Cancer Institute of Canada (to J.H.G.). Work in A.M.V.D.'s laboratory is supported by the Canadian Networks of Centres of Excellence Program. J.E.M. and C.E.G. are recipients of MRC postdoctoral fellowships, and E.U.K. is the recipient of an MRC studentship.

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Expression of an Inward-Rectifying Potassium Channel by the *Arabidopsis KAT1* cDNA

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Inward-rectifying potassium channels located in the plasma membrane of higher plant and animal cells contribute to cellular homeostasis and excitability. The genes encoding this specific class of K^+ channels have not been functionally identified. This report shows that a single messenger RNA transcript from the *Arabidopsis thaliana KAT1* complementary DNA confers the functional expression of a hyperpolarization-activated K^+ channel in *Xenopus* oocytes. The channels encoded by *KAT1* are highly selective for K^+ over other monovalent cations, are blocked by tetraethylammonium and barium, and have a single-channel conductance of 28 ± 7 picosiemens with 118 millimolar K^+ in the bathing solution. These functional characteristics, typical of inward-rectifying K^+ channels in eukaryotic cells, demonstrate that *KAT1* encodes an inward-rectifying K^+ channel.

Analysis and manipulation of cDNAs encoding outward-rectifying K^+ channels (*1*) has led to an understanding of how components of the primary protein structure contribute to functional characteristics such as voltage-dependent activation (*2*) and ionic conductivity (*3*). Although inward-rectifying K^+ channels regulate excitability in animal cells (*4*) and K^+ uptake in higher plant cells (*5*, *6*), little is known about the protein structure of this class of ion channels.

Two cDNAs, *AKT1* and *KAT1*, were cloned from the higher plant *Arabidopsis thaliana* (*7*, *8*) by the complementation of *Saccharomyces cerevisiae* mutants deficient in K^+ uptake (*9*). AKT1 and KAT1 share some amino acid similarity to outward-rectifying K^+ channels in a voltage-sensing domain (S4), in an ion-conducting pore-forming region (H5), and in the predicted topology of the core region of the protein (*7*, *8*). Despite structural similarity between AKT1 and KAT1 and outward-rectifying K^+ channels, these plant genes completely restored K^+ uptake to yeast mutants. From patch clamp studies on guard cells, K^+ uptake into plant cells has been ascribed to proton pump–driven K^+ influx through inward-rectifying K^+ channels (*5*, *10*–*12*). Therefore, characterization of the *KAT1* cDNA by heterologous expression in *Xenopus laevis* oocytes was initiated to determine whether the protein encoded by *KAT1* functions as a voltage-activated inward-rectifying K^+ channel.

Uninjected or water-injected control oocytes were analyzed in all experiments and showed only small currents in response to hyperpolarizing pulses (Fig. 1, A and D) that activated at membrane potentials more negative than −145 ± 14 mV [n = 20; mean ± SD]. These endogenous currents have been suggested to be carried by chloride ions (*13*). In 65 oocytes (from ten frogs) injected with mRNA synthesized from the *KAT1* cDNA (*14*), large inward currents were measured that were activated by hyperpolarization of the membrane potential to values more negative than −102 ± 13 mV (n = 14) (Fig. 1, B and D). Currents were not elicited by depolarization of the membrane potential in injected oocytes (Fig. 1, C and D). Inward current magnitude was 1.2 ± 0.5 μA (n = 23) at

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