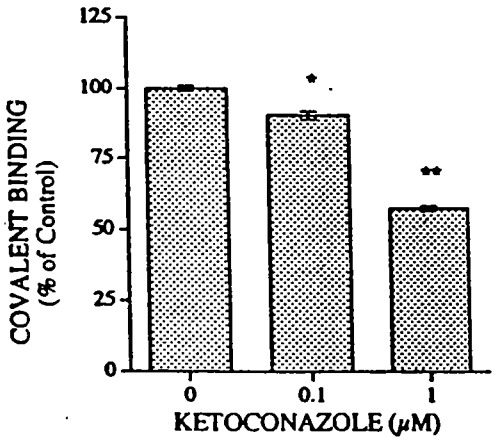


FIG. 6. *Inhibition by KZ of covalent binding of [^{14}C] 4-OH-tam to proteins from human liver microsomes.*

Human liver microsomes that contained high level of CYP3A and CYP2D6 enzymatic activity (0.25 mg protein) were incubated with [^{14}C] 4-OH-tam (5 nmol/10,000 dpm) in the presence of NADPH-regenerating system for 60 min at 37°C in a final volume of 0.5 ml. Values represent a mean of triplicate measurements. Control covalent binding (i.e., lacking KZ) = 153 pmol/mg protein. $^{*}p \leq .07$; $^{**}p \leq .0002$

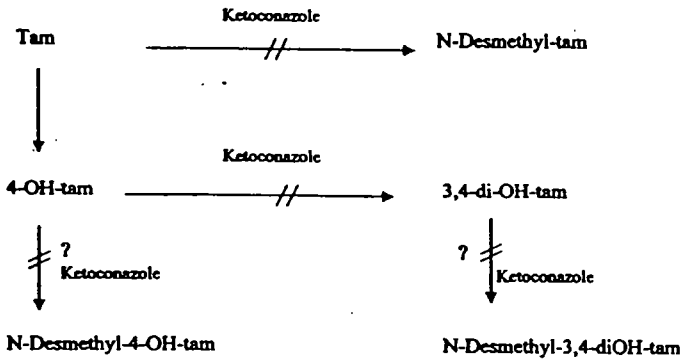
TABLE 4

Effect of KZ on catechol formation from 4-OH-tam by human liver microsomes

KZ	Methylated catechol	% of Control
μM	*nmol/mg protein*	
0	6.84 ± 0.25	100
0.1	5.18 ± 0.17 ($p \leq .0002$)	76
0.5	5.43 ± 0.42 ($p \leq .0025$)	79
1.0	9.64 ± 0.58 ($p \leq .0003$)	141
5.0	26.71 ± 1.71 ($p \leq .0001$)	391

Human liver microsomes (HAM donor # 225; 10μg), 4-OH-tam (25 μM), DTT (50 μM), COMT (120 U), SAM (200 μM/1 μCi), and NADPH-regenerating system were incubated in a final volume of 0.4 ml for 30 min at 37°C. Values represent a mean ± S.D. of triplicate measurements.

Note: Liver #225 contained high level of CYP3A (8.0 nmol 6β-OH-testosterone/mg protein/min) and low level of 2D6 (261 pmol dextromethorphan-*O*-demethylation/mg protein/min) activity.



SCHEME 1. *Sites of KZ inhibition of tam metabolism.*

An additional complication of KZ action is depicted in Scheme 1, whereby N-demethylation and *ortho* hydroxylation (both CYP3A4 activities) are inhibited. We observed that N-desmethyl tam is not as good a substrate for catechol formation as tam and therefore inhibition of N-demethylation of tam may have resulted in increased catechol formation. The possibility that the observed inhibition of catechol formation from 4-OH-tam by KZ was merely due to inhibition of COMT-mediated catechol methylation was excluded. KZ had no

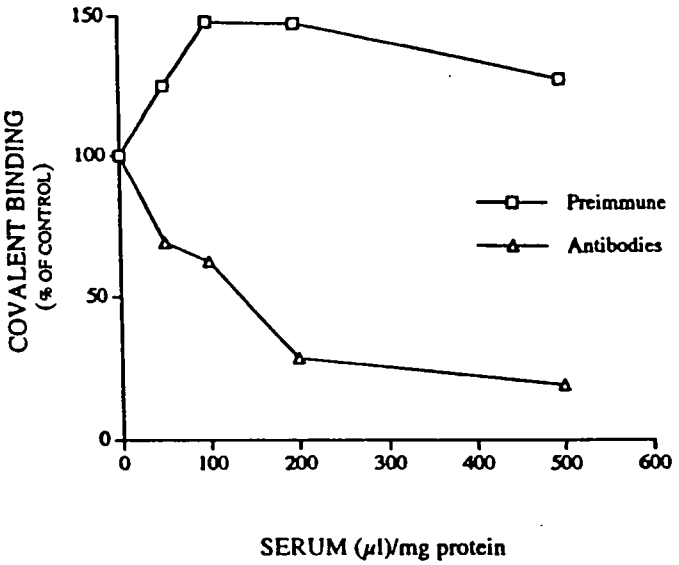


FIG. 7. *Effect of polyclonal antibodies against CYP3A4 on covalent binding of [^{14}C] 4-OH-tam to proteins from human liver microsomes.*

Human liver microsomes (50 μg protein; HAM donor #225 that contained high CYP3A and low 2D6 enzymatic activity) were incubated with [^{14}C] 4-OH-tam (5 nmol/10,000 dpm) in the presence of NADPH-regenerating system for 60 min at 37°C in a final volume of 0.4 ml. Values represent a mean of duplicate measurements.

effect on COMT-catalyzed methylation of two substrates (2-hydroxyestradiol and 3,4-di-OH-tam) in incubations with rat and human liver microsomes in the absence of NADPH (not shown).

The involvement of CYP3A4 in catechol formation (Fig. 4) and in subsequent covalent binding (Fig. 7) was examined with monospecific anti-3A4-antibodies; this resulted in inhibition of catechol formation and covalent binding by more than 80%. These findings provide further support for CYP3A4 involvement in both catechol formation and covalent binding and in the participation of tam-catechol as a proximate intermediate in covalent binding.

Discussion

Earlier findings demonstrated that *ortho* hydroxylation of several structurally diverse compounds (among these estradiol, monohydroxy-methoxychlor and *p*-nitrophenol) is catalyzed by CYP3A4 (Aoyama et al. 1990; Stresser and Kupfer, 1997; Zerilli et al., 1997). The current study demonstrates that human CYP3A enzymes catalyze the *ortho* hydroxylation of monohydroxylated TPEs, e.g., 4-OH-tam and 3-OH-tam. Additionally, CYP2D6 exhibits significant *ortho* hydroxylation activity. The evidence that CYP3A4 and 2D6 enzymes catalyze TPE catechol formation from 4-OH-tam and 3-OH-tam stems from the following: 1) there was a good correlation between testosterone 6β-hydroxylation (CYP3A4 activity) and tam catechol formation in human liver microsomes, 2) among cDNA-expressed human P-450s in supersomes, CYP3A4 and 2D6 (both without coexpressed b_5) demonstrated significant levels of tam catechol formation, however, surprisingly 3A5 had no activity, 3) low levels of KZ inhibited tam catechol formation in human liver microsomes, 4) monospecific antibodies against CYP3A4 strongly inhibited tam catechol formation, and 5) human liver microsomes containing high levels of 3A4 and low or high 2D6 activity exhibited high levels of catechol formation; by contrast, livers with low CYP2D6 and low 3A4 activity formed less tam catechol. Collectively, these findings demonstrate that 3A4 and, to a lesser extent, 2D6 are the prime catalysts of tam-catechol formation in human liver.

Previous studies indicated that CYP3A4 is involved in catalysis of tam activation and covalent binding to proteins (Mani et al., 1994) and data was obtained that suggested the participation of tam catechol in the covalent binding of tam and **4-OH-tam** (Dehal and Kupfer, 1996). In the current study, the observed inhibition of covalent binding of **4-OH-tam** by low levels of KZ and by anti-3A4 antibodies provides support for that pathway.

Of additional interest is the observation that CYP2D6 catalyzes both the 4-hydroxylation of tam (Dehal and Kupfer, 1997; Crewe et al., 1997) and the subsequent catechol formation from **4-OH-tam** (current study). It has been reported that **4-OH-tam** is formed by CYP3A4 as well (Crewe et al., 1997), however, the rate of CYP3A4-mediated catalysis was extremely low. This indicates that the contribution of CYP3A4 to 4-hydroxylation of tam is minimal. CYP2D6 is polymorphic (Skoda et al., 1988), hence, human subjects with low levels or inactive 2D6 will form only little **4-OH-tam** and consequently low levels of tam catechol (independent of whether these subjects had high CYP3A4 activity). Because **4-OH-tam** is an approximately 100-fold more potent antiestrogen than tam and could be the active antiestrogen in tam treatment (Borgna and Rochefort, 1981), the low rate of formation of **4-OH-tam** may result in diminished tamoxifen therapeutic efficacy. Because CYP3A4 is the major isoform in human liver microsomes (Shimada et al., 1994), catechol formation from **4-OH-tam** would primarily be due to CYP3A4 activity; however, it is conceivable that when 3A4 is limiting by being present at low levels (Table 3) or by being inhibited, then 2D6 would be a significant contributor to catechol formation. Of interest is the observation that cytochrome b_5 markedly stimulated the *ortho* hydroxylation of **4-OH-tam** by CYP3A4. Earlier findings demonstrated that b_5 augments the catalytic activity of 3A4 toward 6β-hydroxylation of testosterone and that the b_5 effect is due to a positive effector mechanism rather than to an electron transfer (Yamazaki et al., 1996). The question of whether the b_5 participation in *ortho* hydroxylation of phenolic compounds involves donation of the second electron or activation of 3A4 by protein-protein interaction requires elucidation.

Several studies demonstrated the formation of steroidal catechols from estradiol and estrone (Hoffman et al. 1980; Martucci and Fishman, 1993). CYP3A and 1A2 are the major P-450s catalyzing those reactions (Aoyama et al., 1990; Shou et al., 1997). The interest in catechol estrogens stems from findings of their involvement in covalent binding and possibly in hormonal carcinogenesis (Tabakovic et al., 1996; Yager and Liehr, 1996). The catechol metabolites forming semiquinones and quinones, undergoing redox cycling mediated by P-450 reductase, could lead to carcinogenicity via oxidative damage to DNA (Yager and Liehr, 1996). It has been shown that the formation of quinones by oxidation of certain semiquinones reduces O_2 to superoxide anion radical ($O_2^{\cdot}$; Nelson, 1982) and metabolism of tam by hepatocytes was found to generate $O_2^{\cdot}$; Turner et al., 1991), suggesting that tam semiquinone was formed presumable via the initial formation of tam catechol. In turn, the covalent binding of tam and **4-OH-tam** demonstrated by us (Mani and Kupfer, 1991; Dehal and Kupfer, 1996) appears to be associated with catechol formation. This conclusion was supported by the observations that inhibition of CYP3A enzymes by antibodies inhibited equally both catechol formation and covalent binding. Because TPE catechols are not highly reactive and are not expected to bind covalently to proteins, it is probable that the catechols undergo further metabolism to yield the RIs. The identification of the structures of the TPE-RIs and characterization of the hepatic acceptor proteins binding these RIs require further studies.

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BIOCHEMISTRY OF MULTIDRUG RESISTANCE MEDIATED BY THE MULTIDRUG TRANSPORTER[1]

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CONTENTS

THE CLINICAL PROBLEM AND PHENOTYPE OF MULTIDRUG RESISTANCE . . . 386
ISOLATION OF THE *MDR1* GENE . . . 389
Cloning and Sequence Analysis of mdr Genes . . . 389
The Superfamily of MDR-Related Transporters . . . 393
Vectors Using the MDR1 cDNA as a Selectable Marker . . . 395
REGULATION OF EXPRESSION OF THE *MDR1* GENE IN NORMAL TISSUES AND TUMORS . . . 397
Tissue-Specific Expression, Developmental Regulation, and Other Influences on Expression of the MDR1 Gene . . . 398
Expression of the MDR1 Gene in Human Cancers . . . 400
Analysis of mdr Promoters . . . 401
STRUCTURE-FUNCTION STUDIES . . . 403
Mutational Analysis of P-Glycoprotein . . . 403
Drug Binding and Photoaffinity Labeling . . . 407
Posttranslational Modifications . . . 408
IN VITRO TRANSPORT AND ATPase ACTIVITIES . . . 409
Transport Activity in Vesicles . . . 410
Purification and Reconstitution into Lipid Vesicles of Drug-Dependent ATPase Activity Associated with P-Glycoprotein . . . 410
MECHANISM OF ACTION . . . 412
Other Phenomena and Epiphenomena Related to Multidrug Resistance . . . 412
Models for the Mechanism of Action of the Multidrug Transporter . . . 415
CLINICAL RELEVANCE OF BIOCHEMICAL STUDIES ON THE MULTIDRUG TRANSPORTER . . . 418

THE CLINICAL PROBLEM AND PHENOTYPE OF MULTIDRUG RESISTANCE

This year there will be approximately 1,000,000 new cases of cancer in the United States. Close to half of these are still localized at the original site when the patient comes to the physician and will be cured by ablative treatment such as surgery or radiotherapy. The remaining cancers include systemic cancers, such as leukemia and lymphoma, and unifocal tumors that have spread by metastasis. The only hope for cure of these cancers resides in systemic treatments such as chemotherapy and immunotherapies. Chemotherapy has proven to be effective in several disseminated cancers, and has led to the cure of many childhood and adult cancers such as leukemias, lymphomas, sarcomas, choriocarcinoma, and testicular cancers. Chemotherapy can improve long-term survival in cancers such as breast cancer, where there is no evidence of metastasis at the time of presentation, but where there is a statistical likelihood of recurrent cancer from undiagnosed, microscopic metastatic disease. Unfortunately, these examples of long-term survival after chemotherapy represent the minority of cases, perhaps 5–10% with current treatments. Most metastatic cancers are either resistant to chemotherapy (intrinsic resistance), or respond to chemotherapy but later recur as cancers that have acquired chemotherapy resistance.

Broad-spectrum resistance to chemotherapy in human cancer has been called multidrug resistance. A search for the cause or causes of multidrug resistance has occupied the attention of cancer researchers for more than four decades. The primary approach to this problem has been to isolate lines of cultured cells selected for resistance to various anticancer drugs. In studies using mouse and Chinese hamster cells (1–4), as well as human cells (5–9), recently reviewed by Beck & Danks (10) and Sugimoto & Tsuruo (11), the isolation of tissue culture cells with a broad pattern of cross-resistance to many natural product anticancer drugs was frequently reported. This resistance is due to decreased accumulation of drugs in cells because of an energy-dependent drug transport protein.

The usual pattern of cross-resistance includes a large variety of cytotoxic agents that do not have a common structure or a common cytotoxic intracellular target. Table 1 lists classes of agents in clinical or laboratory use to which multidrug-resistant cells are resistant, or that are thought to interact with the transporter responsible for resistance. This list includes dozens, and perhaps hundreds or more, of hydrophobic natural products (i.e. derived from plants, or micro-organisms), semi-synthetic analogs of such products, and synthetic organic compounds. Although no chemistry is shared by these diverse compounds, they are amphipathic compounds that are preferentially soluble in lipid.

Table 1 Classes of agents that interact with P-glycoprotein

Anticancer drugs	Agents that reverse drug resistance
Vinca alkaloids, e.g. vinblastine	Calcium channel blockers, e.g. verapamil, nifedipi dihydroperidines, azidopine
Anthracyclines, e.g. doxorubicin	Anti-arrhythmics, e.g. quinidine, amiodarone
Epipodophyllotoxins, e.g. etoposide	Antihypertensives, e.g. reserpine
Antibiotics, e.g. actinomycin D	Antibiotics, e.g. hydrophobic cephalosporins
Others, e.g. mitomycin C, taxol, topotecan, mithramycin	Antihistamines, e.g. terfenadine
Other cytotoxic agents	Immunosuppressants, e.g. cyclosporine A, FK506, rapamycin
Antimicrotubule drugs, e.g. colchicine, podophyllotoxin	Steroid hormones, e.g. progesterone
Protein synthesis inhibitors, e.g. puromycin, emetine	Modified steroids, e.g. tirilazad, tamoxifen
DNA intercalators, e.g. ethidium bromide	Lipophilic cations, e.g. tetraphenylphosphonium
Toxic peptides, e.g. valinomycin, gramicidin D, N-acetyl-leucyl-leucyl-norleucinal (ALLN)	Diterpenes, e.g. forskolin
	Detergents, e.g. Tween-80
	Antidepressants, e.g. tioperidone
	Antipsychotics, e.g. phenothiazines
	Many other hydrophobic, amphipathic drugs and the analogs

The list in Table 1 also includes agents that are not cytotoxic by themselves, but reverse the multidrug resistance phenotype by competing for the transport system responsible for resistance. These "reversing agents" or "chemosensitizers" share solubility properties with agents to which multidrug-resistant cells are resistant. As we speculate below, these physical features may be an important component needed for the recognition of these compounds.

Initial physiologic and pharmacologic studies of multidrug-resistant mutant cell lines revealed that the mechanism of resistance is reduced accumulation of drugs within the cell (1). This reduced accumulation was initially attributed to either increased drug efflux (3) or decreased cell permeability (4). A recent detailed review of the extensive literature on this subject suggests that for most cell lines, and for most drugs analyzed, both increased efflux and decreased influx can be demonstrated (3, 12–16). This unexpected result is shown schematically in Figure 1, and discussed with respect to mechanism in a later section. One important feature of this resistance, demonstrated by these isotopic labelling studies and by immunofluorescence studies on single cells (17), is the requirement for a source of cellular energy.

The first analyses of the biochemistry of multidrug-resistant cell lines suggested that one or more major protein alterations affected plasma membrane proteins (5, 9, 18, 19). One of these alterations, increased expression of a cell surface phospho-glycoprotein, termed P-glycoprotein by Ling and his colleagues (19, 20), has been shown to be encoded by the *mdr* gene in

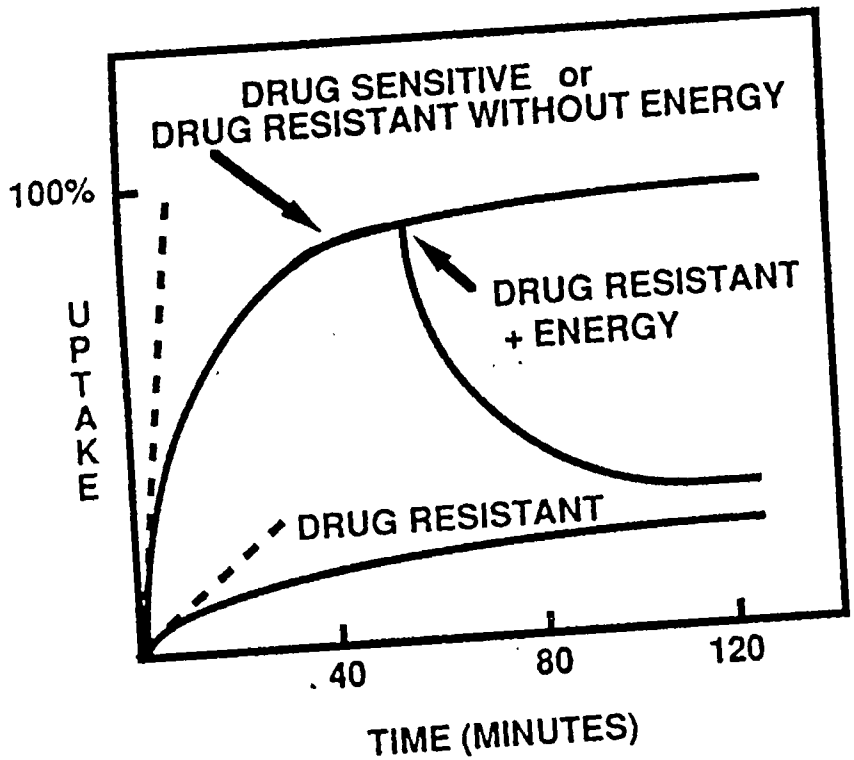

Figure 1 Expression of the multidrug transporter decreases drug influx and increases energy-dependent drug efflux: kinetic analysis. This figure is a cartoon presenting idealized curves from analyses of several different multidrug-resistant cell lines described in the literature (3, 13–15, 79). The dotted lines represent the initial rates of drug influx in drug-sensitive and -resistant cells.

both rodents and humans (21–23). Using genetic approaches of gene transfer, the *mdr* gene has been proven to be necessary and sufficient for multidrug resistance in both cultured cells and animals, since it encodes the multidrug transporter, which is responsible for both the increased drug efflux and decreased influx seen in multidrug-resistant cells (see below). More recent studies, using purified, reconstituted P-glycoprotein, provide biochemical evidence in accord with the hypothesis that P-glycoprotein is the transporter itself.

As more sophisticated studies of mutant cells exhibiting multidrug resistance have been performed, it has become clear that there may be several mechanisms of multidrug resistance with variations in the phenotype of such resistance depending on the mechanism. Two relatively well-studied examples

of cross-resistance in cultured cells include resistance to alkylating agents due to alterations in glutathione metabolism, especially glutathione S-transferase (24), and alterations in topoisomerase II, which is a target for many natural product anticancer drugs (25). Other non-P-glycoprotein-mediated mechanisms also appear to affect intracellular accumulation of drugs (26–29), but the molecular bases and physiological significance of these mechanisms are not known.

This review updates current knowledge of multidrug resistance mediated by the multidrug transporter, or P-glycoprotein, encoded in the human by the *MDR1* gene (also known as the *PGY1* gene). Because of the obvious clinical relevance of this subject, several thousand publications have covered multidrug resistance in the past few years. This review focuses on issues of biochemistry, molecular biology, and mechanism. For additional information the reader is referred to a recent multiauthor volume dedicated entirely to P-glycoprotein-mediated drug resistance (30), and recent general reviews on this subject from our laboratory and from several other laboratories (31–47).

ISOLATION OF THE *MDR1* GENE

Three independent approaches were taken in six different laboratories to isolate *mdr* cDNAs from mouse, Chinese hamster, and human cells. All of these approaches took advantage of the fact that *mdr* genes are overexpressed at very high levels in multidrug-resistant cells, which have been selected in multiple steps to high levels of resistance. In many of these highly selected cell lines, the *mdr* genes are amplified, and this phenomenon has been used to facilitate cloning of these genes.

Cloning and Sequence Analysis of mdr *Genes*

Cytogenetic analyses of highly selected multidrug-resistant cells indicated the presence of homogeneously staining regions (HSRs) and minute and double-minute chromosomes (2, 16, 48), both of which are characteristic of amplified genes. Based on this observation, a novel technique, known as in-gel renaturation (49), which allowed direct cloning of amplified segments of genomic DNA from gels, was used to isolate amplified genomic sequences in multidrug-resistant hamster cells (50, 51). An amplified genomic fragment that detected mRNAs in drug-resistant cells was then used to identify and isolate human genomic fragments from multidrug-resistant KB cells, which contained amplified genes (52, 53), and was used as a probe to detect cross-hybridizing cDNAs in the mouse (54). Gudkov and collaborators also took advantage of the amplification of drug-resistance genes in multidrug-re-

sistant Djungarian hamster cells to isolate genomic segments of the Djungarian hamster multidrug-resistance gene (55).

Analysis of the genomic fragments and mRNAs encoded by these *mdr* genes soon made it clear that there was more than one *mdr* gene in mouse, human, hamster, and rat. The human probes detected two *MDR* genes, called *MDR*1 and *MDR*2, but only the *MDR*1 probes consistently detect mRNAs of approximately 4.5 kb in drug-resistant cell lines (53, 56). As is discussed below, in humans the *MDR*1 gene is responsible for multidrug resistance; the function of the closely related *MDR*2 gene is not known. The mouse has three *mdr* genes (57–60). The mouse genes corresponding to the human *MDR*1 gene have been called *mdr*1a and *mdr*1b (61) since they both encode functional multidrug transporters, but as originally isolated they were designated *mdr*3 and *mdr*1, respectively. The *mdr*2 gene is closely related to the *mdr*1 genes in both mouse and human systems, but has no known function (62). The *mdr*2 genes are expressed in liver and some other tissues, including leukemias (58, 63–65).

As noted above, *mdr* cDNAs were also cloned using more traditional methods based on overexpression of *mdr* mRNAs and their product, P-glycoprotein. Both Melera's group and Borst's group used differential cDNA libraries to isolate cDNAs corresponding to mRNAs overexpressed in highly multidrug-resistant Chinese hamster cells (66–68). Ling's group used a monoclonal antibody to P-glycoprotein that they had isolated (69) to clone a fragment of a cDNA that encoded a hamster P-glycoprotein whose gene was amplified in multidrug-resistant cells (70, 71). The three rat *mdr* genes have more recently been cloned (72).

Genetic approaches were used to prove that the cloned *mdr* genes were indeed responsible for multidrug resistance. Initially, gene transfer experiments were performed using high-molecular-weight genomic DNA to show linkage between *mdr* sequences and drug resistance (73–75). When full-length, functional cDNAs for *mdr* genes were isolated, these were shown to confer the full phenotype of multidrug resistance on drug-sensitive cells either after DNA-mediated transfer (54, 76) or after retroviral gene transfer (77, 78).

Because of a general interest in the biology of gene amplification, and because of a desire to determine whether other genes that are co-amplified with *mdr* genes have any effect on the drug resistance phenotype, a number of studies on the *mdr* amplicon have been carried out. Borst's group has explored the arrangement of genes in the hamster *mdr* (*pgp*) gene cluster by isolating cDNA probes for the genes, which are co-amplified with *mdr* using pulse-field gel electrophoresis (66, 67, 79). By comparing overlapping patterns of gene amplification and expression, which they observed in several different cell lines, they have concluded that only the functional *mdr* genes

are associated with drug resistance. Maps of the mouse (80) and human (81) *mdr* loci have been obtained by pulsed-field gel electrophoresis. The human *MDR* genes are adjacent to each other on the long arm of chromosome 7 (82, 83) near 7q21.1 (84; reviewed in 85) on a 600-kb *Nru*I fragment, and the entire *MDR*1 coding region is contained on a 120-kb *Xho*I fragment (81). The human *MDR*1 gene has been cloned on a series of overlapping cosmids and has been shown to contain 28 exons with a total span of greater than 100 kb (86). The mouse gene has a similar structure, but is somewhat smaller (87) and is located on mouse chromosome 5 (88). The hamster *mdr (pgp)* genes map to chromosome 1q26 (89, 90).

As noted above, amplified *mdr* genes may be present either on homogeneously staining regions or as autonomously replicating extrachromosomal DNA segments. Recent analysis of highly multidrug-resistant human KB cell lines has demonstrated the presence of extrachromosomal DNA segments ranging in size from 600–750 kb (91) to several megabases (92, 93). In the case of several KB cell lines selected for increasing colchicine resistance, increased copy number of the *MDR*1 gene has occurred by formation of large extrachromosomal DNA circles following sequential dimerization of a smaller episome (92). The possible presence of linear extrachromosomal elements as intermediates in the formation of HSRs has been suggested by pulsed-field gel electrophoresis of *mdr*-containing DNA from highly multidrug-resistant mouse J774.2 cells (94).

The availability of full-length *mdr* cDNAs was followed quickly by their sequencing and the analysis of these sequences. The initial publication of both mouse *mdr*1a (23) and human *MDR*1 (22) cDNA sequences led to the current model of P-glycoprotein (35). The human cDNA encodes 1280 amino acids. Sequence analysis indicates the presence of 12 transmembrane domains in two homologous halves each containing six transmembrane regions and a large intracytoplasmic loop encoding an ATP site (Figure 2). Although the two halves have 43% sequence identity in the human, the lack of homologous placing of introns (86) suggests that the two halves of the molecule might have either evolved independently or have undergone major intron movement after a duplication event (87).

Although the overall model of 12 transmembrane regions has been generally well-supported by antibody localization data (95), an alternative model has recently appeared based on coupled in vitro translation and translocation of P-glycoprotein into dog pancreas microsome membranes (96). This study suggests, based on the presence of a glycosylated segment in the carboxy-terminal half of P-glycoprotein, that under some conditions in mouse P-glycoprotein, the eighth and ninth transmembrane domains, and the amino acids between transmembrane domains 8 and 9, might be extracellular rather than cytoplasmic as suggested in the model shown in Figure 2. These results are

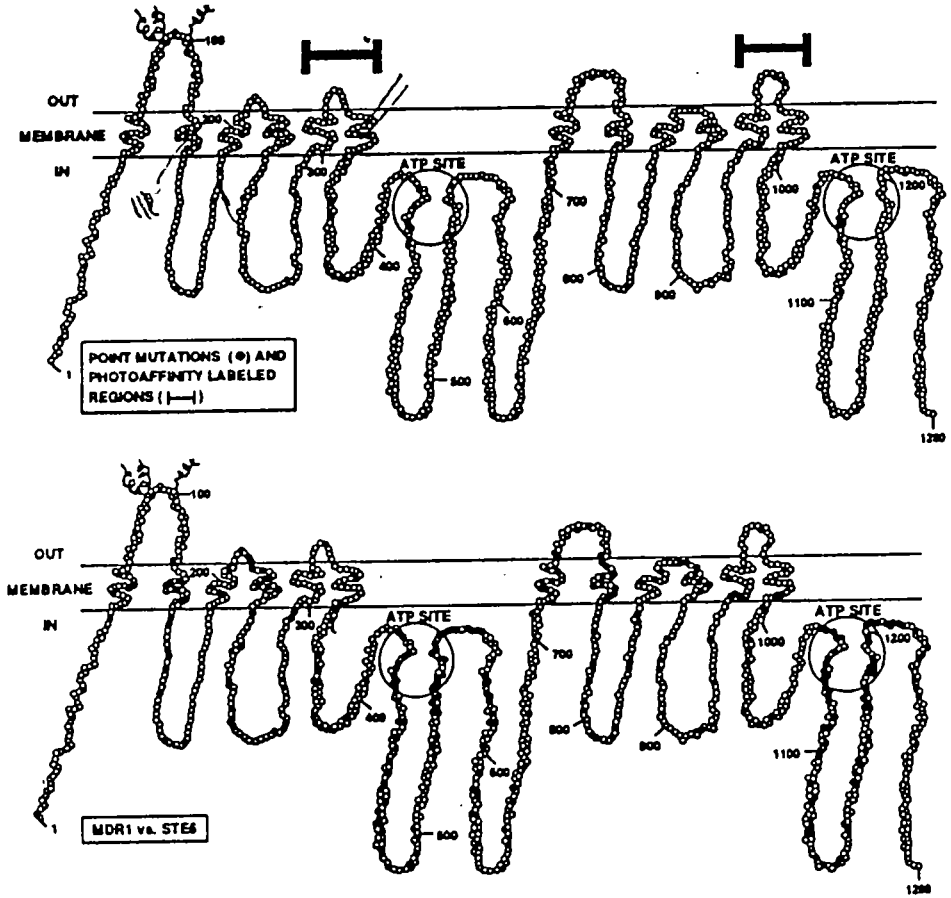

Figure 2 Model of human P-glycoprotein derived from sequence analysis (22). *Upper panel:* Representation of photoaffinity-labeled sites and point mutations that affect transport specificity of P-glycoprotein. The brackets above the model show the regions of major photoaffinity labelling by photoactivatable drug analogs. The filled-in balls are amino acid residues in which mutations have been shown to alter drug transport specificity. Some of these positions are approximate, since some of the original mutations were described in nonhuman P-glycoproteins, which have a different total number of amino acids. *Lower panel:* Comparison of the *MDR*1 sequence with that of yeast *STE6*. The filled-in balls represent amino acids that are identical in *MDR*1 and *STE6* to show extent of identity and regions of greatest identity. Alignments were made by eye; therefore, in some cases the exact position of the sequence identity may differ from other proposed alignments.

supported by in vivo expression of truncated P-glycoprotein chimeras in *Xenopus* oocytes, as well as by in vitro expression of intact human P-glycoprotein in a cell-free translation-translocation system (97). However, study of proteolytic fragments of photoaffinity-labelled P-glycoprotein indicates that there does not appear to be a glycosylated region in the carboxy-terminal half of mature human P-glycoprotein, arguing against this model of 10 transmembrane regions for a form of P-glycoprotein capable of binding drugs (98, 99). However, it is possible that this region is indeed extracellular, but not glycosylated, or that P-glycoprotein with this variant topology does not survive to the cell surface because of intracellular degradation. The precise topology of P-glycoprotein on the mammalian cell surface needs further study.

Since the original publication of the human *MDR*1 and mouse *mdr*3 (*mdr*1a) sequences, full-length sequences have appeared for mouse *mdr*1b (also called *mdr*2) (59), mouse *mdr*2 (57), human *MDR*2 (100), hamster *pgp*1 (similar to *mdr*1a) (101), and the rat *mdr*1b gene (102). Comparison of these sequences shows a considerable amount of sequence identity and homology among *mdr* family members (101). For example, the human *MDR*1 and *MDR*2 coding sequences are 76% identical despite different abilities to transport drugs, and the mouse *mdr*1a and human *MDR*1 sequences, with similar function, are 88% identical, despite 50 million years or more of evolution. Among the *mdr* genes that function as multidrug transporters, the regions of greatest homology are the ATP-binding/utilization regions, and the first and second intracytoplasmic loops in each half of the molecule. The least conserved regions are the first extracytoplasmic loop, where even glycosylation sites are not preserved, the intracellular linker region connecting both halves of the molecule, and the amino and carboxy termini.

The Superfamily of MDR-Related Transporters

All of the original publications describing the sequence of *mdr* cDNAs pointed out two regions of significant sequence identity with many other energy-dependent transport proteins that corresponded to ATP-binding sites in these molecules (22, 23, 71). These sequences include the familiar A and B binding folds of the Walker motifs for nucleotide binding (103), as well as substantial sequence identity and homology between and around these two folds, suggesting that ATPase activity specific to a superfamily of transporters may be encoded in this region. This family has become known as the ATP-binding cassette (ABC) family (104–106) or the family of Traffic ATPases (107). As has been appreciated over the past several years, there are at least 40 members of this ABC superfamily of proteins in bacteria, including nutrient, peptide, polysaccharide, toxin, and drug transporters (104–106), with virtually all of them being transporters except for proteins involved in UV repair and protein translation. There are many examples of these ABC proteins in eukaryotic

cells, as well, including a pigment transporter in *Drosophila melanogaster* (108), a pump that appears to mediate chloroquine resistance in *Plasmodium falciparum, pfmdr* (109, 110), a transporter for the a peptide mating factor of yeast called *STE6* (111, 112), CFTR, the product of the cystic fibrosis gene (113, 114), a peroxisomal membrane pump (115) which when mutant results in a fatal cerebro-hepato-renal dysfunction known as the Zellweger syndrome (116), and two linked genes associated with transport of peptides into the endoplasmic reticulum for class I antigen presentation recently named Tap-1 and Tap-2 (for transporter associated with antigen processing) (117). The STE6 gene product from yeast and the *P. falciparum* gene product involved in chloroquine resistance have significant homology with the *mdr* genes outside of the ATP sites, suggesting that these transporters may recognize similar substrates as well. The mouse *mdr*3 (*mdr*1b) has recently been shown to complement the mating defect in yeast that results from a mutation in *ste*6 (118), and a similar result has been obtained for human *MDR*1 (K. Kuchler, J. Thorner, M. M. Gottesman, unpublished data). A comparison of the STE6 protein sequence with that of human MDR1 (Figure 2, *lower panel*) shows strong sequence identity in the ATP sites, and significant sequence identity in several of the transmembrane and other cytoplasmic regions, pointing to possible regions of P-glycoprotein that might be essential for transport of peptides such as the yeast a factor (111).

Recently, using cross-hybridization to isolate other closely related genes, *mdr*-like genes have been found in *Drosophila melanogaster* (119), *Arabidopsis thaliana* (120), marine sponges (121), *Caenorhabditis elegans* (33), *Leishmania donovanii* (122), and *Leishmania tarantolae* (123). It is likely that many other plants and micro-organisms will have such genes [reviewed in Schinkel & Borst (33)]. Although the precise function of these genes is not yet known, in *Drosophila* overexpression of one *mdr*-like gene results in colchicine-resistance of larvae (119), and in *Leishmania*, *mdr*-like genes are amplified on episomes that carry other resistance genes. *Mdr* genes in *Leishmania* and *Drosophila* appear to confer resistance with a similar pattern to the mammalian *mdr* genes.

The general structure of these transporters includes a set of six transmembrane domains, which are generally not homologous other than sharing amino acids with hydrophobic properties, followed by an ABC. In the bacterial systems, the subunit with the transmembrane domains and the ABC proteins may be separate or fused. In eukaryotic systems, a set of six transmembrane domains may be fused to a single ABC protein, as for the peroxisomal membrane protein, endoplasmic reticulum peptide transporters, *Drosophila* pigment transporter, and the antigen peptide transporters, or two of these may be fused together to give 12 transmembrane domains and two ATP sites, such

as the CFTR, MDR, and STE6 proteins. Although this family has not yet been fully described, it appears that the ABC transporters localized to intracellular membranes contain only one set of six transmembrane domains and one ATP site, whereas the transporters localized to the plasma membrane have 12 transmembrane domains and two ATP sites. Several secondary transporters, such as the glucose carrier, anion exchanger, and Na/H exchanger, which do not have ATP sites, when localized to the plasma membrane also contain 12 transmembrane domains, but homologous intracellular organelle transporters contain six transmembrane domains and function as dimers (124, 125). Based on this analogy, it is tempting to suggest that the minimal functional unit of the ABC transporters may also require 12 transmembrane domains and two ATP sites.

Although we may presume that the minimal functional unit consists of 12 transmembrane domains and 2 ATP sites for all of these transporters, it is quite possible that for P-glycoprotein and many of the other transporters the functional transporter is a multisubunit protein resulting from association of the minimal subunit into dimers (24 transmembrane domains) or even tetramers (48 transmembrane domains). This idea is supported by data suggesting that the size of the P-glycoprotein complex in mammalian membranes, as estimated from freeze-fracture and radiation inactivation studies, is larger than predicted from a monomeric structure (41, 126–128), and dimers can be isolated after chemical cross-linking (129).

Vectors Using the MDR1 *cDNA as a Selectable Marker*

The availability of full-length cDNAs for functional *mdr* genes has made it possible to develop vector systems that exploit the flexibility of this dominant selectable marker (54, 76). The human *MDR*1 gene has been expressed in eukaryotic cells under control of Moloney and Harvey retroviral promoters, the actin promoter, the cytomegalovirus promoter, the SV40 promoter, and the metallotheinein promoter, each of which gives multidrug-resistant clones at a frequency of approximately 10^{-4} to 10^{-3} under usual calcium phosphate transfection conditions (76, 130–134) (P. Marino, S. Currier, I. Pastan, M. M. Gottesman, unpublished data). To date, in our hands the Harvey retroviral promoter system appears to give the highest expression of the *MDR*1 gene both after transfection and after retroviral infection (78). The *MDR*1 gene has also been expressed in insect Sf9 cells, using a recombinant baculovirus (135), allowing high-level expression of P-glycoprotein for biochemical studies (see below) and in *Xenopus* oocytes (136). Three groups have reported expression of mammalian P-glycoproteins in yeast (118, 137, 138), where it appears able to substitute to some extent for the normal function of the STE6 a factor

transporter (118). Expression as a beta-galactosidase fusion protein in *Escherichia coli* has also been described (139).

*MDR*1-based expression vectors have been shown to be useful for cotransfection and high-level expression of proteins encoded by cDNAs carried on other vectors (131), and for co-amplification of cDNAs that are cloned into the *MDR*1 vector itself under control of a second promoter (132). A detailed analysis of the co-amplification process in this latter case suggests that when clones are selected that are highly multidrug resistant, due to a high level of expression of the *MDR*1 gene, the unselected second gene cloned into the *MDR*1 vector and the *MDR*1 gene are both present at high copy number in the transfectants. This co-amplification process appears to represent enrichment of cell lines carrying vectors present at high copy number in the original transfectant population, rather than amplification during the relatively short selection protocol (132).

Because of the ease with which *MDR*1-containing vectors can be introduced into apparently all drug-sensitive cells, and the wide range of drugs that can be used to select for expression of the *MDR* phenotype, the *MDR*1 gene has been suggested as a possible selectable marker for human gene therapy (140). Through use of both DNA-mediated gene transfection and retroviral transfection, the *MDR*1 gene has been introduced into a large variety of cultured human and rodent cells, as well as muscle cells (141) and bone marrow both in vitro (142) and in vivo (143, 144). The in vivo expression of the *MDR*1 gene in mouse bone marrow cells after retroviral infection confers a selective advantage on these cells after treatment of the mice with the *MDR* drug taxol (143, 144).

Although the expression of the *MDR*1 cDNA confers selective advantage on cells exposed to drug selection, a second, unselected gene, which is cloned on the same vector under control of a different promoter, may or may not be expressed depending on host cell factors that determine the activity of the second promoter. To circumvent this fickleness of eukaryotic promoters, we have designed *MDR*1 vectors in which the complete P-glycoprotein cDNA is translationally fused to the desired unselected gene at its 3′ end, thereby encoding chimeric P-glycoproteins. The result is a chimeric protein in which the amino-terminal part of the molecule is a functional P-glycoprotein, and the carboxy terminus carries the second protein. Through use of this approach, the human *MDR*1 transporter was fused to adenosine deaminase, and both activities were present in the chimeric protein after transfection (145) or retroviral infection (146). The use of P-glycoprotein chimeric proteins for gene therapy promises to make possible the in vivo selection of genes in humans whose introduction into recipient cells would otherwise result in no selective advantage to the target cell.

REGULATION OF EXPRESSION OF THE *MDR*1 GENE IN NORMAL TISSUES AND TUMORS

The identification of the gene and protein responsible for multidrug resistance made it possible to determine in what tissues P-glycoprotein was normally expressed, and whether it was commonly expressed in multidrug-resistant human cancers. Gene expression could be quantitated by measurement of *MDR*1 RNA, or by semi-quantitative detection of protein (147). *MDR*1 RNA could be detected by Northern blot or slot blot analysis (148), by RNAse protection (149), by in situ hybridization and autoradiography (150–152), or by a variety of quantitative polymerase chain reaction (PCR)–based assays (153–158). Despite the possible presence of the closely related *MDR*2 transcript in some human tissues, with appropriate care these assays can be made quite specific and relatively quantitative (151).

Expression of P-glycoprotein has also been measured using antibodies. Because samples of tissue and blood are readily available for pathological examination, analysis of P-glycoprotein levels in cancers, and particularly in individual cancers cells, should be useful in designing new therapies and may have prognostic value as well. A variety of polyclonal (159–161) and monoclonal antibodies, including monoclonal antibodies that recognize internal determinants, such as C219 (69), C494 (69), and JSB-1 (162), and antibodies that recognize external epitopes such as MRK-16 and MRK-17 (163), 265/F4 (164), HYB-612 (165), MAb 57 (166), 17F9 (167), 4E3.16 (168), and UIC-2 (169), have been developed.

Of these monoclonal antibodies, C219 has been extensively characterized. When it binds to its epitope near the ATP-binding sites of P-glycoprotein (170), ATP binding is inhibited (171). This antibody is very useful for immunoprecipitations and Western blotting analysis for semi-quantitative determination of P-glycoprotein levels in tissues (172, 173), and for immunohistochemical detection of P-glycoprotein (174, 175), but it is not entirely specific since it recognizes a determinant that is present in some other proteins, such as the *MDR*2 product and certain forms of myosin (176). Monoclonal antibody MRK-16 is quite specific for human P-glycoprotein (163, 176, 177), and is an excellent antibody for FACS analysis or magnetic sorting of P-glycoprotein-bearing cells (178, 179), but it cannot be used for quantitative immunoprecipitations or Western blots. UIC2 (169) shares many of these properties with MRK-16, but in addition it is a potent inhibitor of the multidrug transport function of P-glycoprotein. Although MRK-16 reverses drug resistance in vitro only slightly (163), it appears to be a more effective reversor of drug resistance in vivo, perhaps via indirect effects on cell membranes mediated by the immune system (180–182). MRK-16 has

also been linked to *Pseudomonas* exotoxin to form an immunotoxin that specifically kills P-glycoprotein-expressing cells (182–184).

Some authors have used a quick, functional assay for P-glycoprotein based on extrusion of fluorescent dyes, such as rhodamine 123 (185), which accumulates to a lesser extent in multidrug-resistant cells. If a reduction in dye accumulation can be reversed with agents such as verapamil, known to inhibit the multidrug transporter, then preliminary, but not definitive, evidence for expression of P-glycoprotein is obtained.

Tissue-Specific Expression, Developmental Regulation, and Other Influences on Expression of the MDR1 *Gene*

The first studies on expression of the *MDR1* gene in normal tissues measured *MDR1* RNA levels (148), and this was quickly followed by an immunohistochemical analysis with antibody MRK-16 (177). A very high level of expression was found in human adrenal cortical cells, the brush border of renal proximal tubule epithelium, the lumenal surface of biliary hepatocytes, small and large intestinal mucosal cells, and pancreatic ductules. Subsequently, P-glycoprotein was found in capillary endothelial cells of the brain and testis (176, 186), in placenta (187), in secretory glands of the pregnant endometrium (188, 189), in peripheral lymphocytes (189a), and in CD34 positive bone marrow stem cells (190). The major species-specific differences between rodents and humans relate to the fact that rodents have two *mdr*1 genes (*mdr*1a and b). Many tissues in hamsters and mice that correspond to human tissues expressing the *MDR1* gene express either *mdr*1a, *mdr*1b, or both (58). One plausible hypothesis is that different functions subsumed by the *MDR1* gene in the human are specialized to either the *mdr*1a or the *mdr*1b gene in rodents. This hypothesis is supported by evidence that these genes have somewhat different transport specificities in the mouse (59), and their promoter elements differ, indicating they are probably differently regulated (see below).

Based on the known cellular localization of P-glycoprotein in human and rodent tissues, and the known activities of P-glycoprotein, plausible speculation has been made about the "normal" function of the multidrug transporter. High-level expression in the adrenal gland suggests a role in steroid secretion or the protection of the membranes of steroid-secreting cells. The observation that progesterone is an inhibitor of P-glycoprotein (191), and that several steroids may be substrates for transport in epithelia expressing P-glycoprotein (K. Ueda, personal communication), support this idea. In addition, in mouse adrenal Y-1 cells, inhibitors of P-glycoprotein block secretion of steroids (192), and ablation of one copy of the *mdr*1b gene (the major *mdr* gene

expressed in mouse adrenal) in adrenal Y-1 cells affects steroid secretion (S. Altuvia, I. Pastan, M. M. Gottesman, unpublished data).

Expression of P-glycoprotein in transporting epithelia in kidney, liver, pancreas, and intestine, and capillary endothelia in brain and testis either promotes transepithelial and transendothelial transport of toxic xenobiotics or prevents their absorption. In addition, certain endogenous metabolites that are substrates for the multidrug transporter would be handled in a similar manner. This idea is supported by studies in which the *MDR1* cDNA has been expressed in monolayers of kidney epithelia grown on filters. In these epithelia, basal to apical transport of various drugs and other agents can be demonstrated (78, 193, 194). However, the recent demonstration that P-glycoprotein is associated with a volume-regulated chloride channel activity (195, 196) raises the interesting possibility that this function is distinct from the ability of P-glycoprotein to act as a multidrug transporter, and that the expression of P-glycoprotein in certain tissues may reflect its function as a chloride channel, instead of, or in addition to, its activity as a drug transporter. The observation that the multidrug transporter and cystic fibrosis genes have complementary patterns of expression in many epithelial tissues supports the idea that P-glycoprotein may be an alternate chloride channel (197). For example, in the intestine, the CFTR gene is expressed in crypt cells, and the *MDR1* gene is expressed in more differentiated epithelial cells of the villi, suggesting that the intestine is a tissue in which volume regulation may be an important activity of P-glycoprotein (197). In the brain and testis, P-glycoprotein may serve a blood-tissue barrier function, keeping toxic metabolites and xenobiotics out of these tissues. Further information about the normal function of P-glycoprotein will come from studies in which the *mdr* genes are ablated in transgenic animals. A recent preliminary report (198) indicates that *mdr* genes have been mutagenized in embryonic stem cells by insertional mutagenesis, and the production of mice lacking functional P-glycoproteins seems imminent.

Although no definitive studies on expression of the *mdr* genes during fetal development have yet appeared, there are several examples in the literature of developmental regulation of expression of these genes. For example, Arceci and his colleagues have described the progesterone-dependent regulation of *mdr*1b expression in the mouse endometrium (199). In addition, *mdr* RNA levels are increased in rat hepatocytes when they are placed in tissue culture (200), and in cultured adrenal and colon cancer cell lines treated with differentiating agents such as retinoic acid, sodium butyrate, dimethyl sulfoxide, and dimethylformamide (201, 202).

A number of environmental influences have been found to increase levels of *mdr* RNA levels in cultured cells and animals, but the mechanism by which this increase is brought about has not usually been established. For example,

mdr RNA levels increase 20–50-fold in rat liver 48–72 hrs following partial hepatectomy or liver damage with cytotoxic drugs (203, 204). Because new RNA transcripts were not found to accompany the increase in *mdr* mRNA levels after hepatectomy, the elevated RNA levels were attributed to stabilization of *mdr* mRNA (205). This idea is supported by the presence in the human *MDR1* transcript of AU-rich sequences, which occur in many mRNAs with short half-lives (206). In addition, the half-life of *MDR1* RNA in cells recovering from heat shock has been shown to be short (30–60 mins) (207). Thus, *mdr* mRNA stabilization could have a dramatic effect on steady-state mRNA levels. To date there have been no direct measurements of *mdr* mRNA half-life before and after hepatectomy to confirm this hypothesis, and other studies suggest that increases in transcription, regulated by a cycloheximide-responsive trans-acting transcriptional repressor, may mediate the increase in *mdr* RNA seen in hepatocytes after exposure to cytotoxic drugs (208).

In rodent cells treated with cytotoxic agents, most of which damage DNA, *mdr* RNA levels are increased, but, once again, no increase in transcription as measured by nuclear run-off assays was detected (209). There have been single reports that the *MDR1* promoter responds directly to treatment with cytotoxic agents and serum starvation after transfection into human cells (210, 211). Heat shock, arsenite, and cadmium chloride treatment stimulate an increase (192, 212) in *MDR1* RNA levels in human renal cancer cells, but not in several other cell types or cells from other species (207). The human *MDR1* promoter has a reasonable heat-shock consensus sequence (207), and in cells transfected with *MDR1* promoter–chloramphenicol acetyl transferase (CAT) reporter constructs, this heat-shock consensus sequence is needed for the heat-shock response (213). Therefore, it seems likely that part of this heat-shock response is transcriptional, even though effects on nuclear run-off cannot be demonstrated (207). Finally, normal levels of cAMP-dependent protein kinase are essential for maintenance of basal levels of *MDR1* RNA in Chinese hamster cells and mouse adrenal Y-1 cells. Mutant CHO and Y-1 cells with alterations in this kinase have reduced *MDR1* RNA levels (192, 212).

Expression of the MDR1 *Gene in Human Cancers*

A primary goal of many cancer researchers, once the *MDR1* gene had been cloned, was to measure *MDR1* RNA levels and/or the presence of P-glycoprotein in human cancers. A preliminary survey of more than 400 different human cancers demonstrated the widespread expression of the *MDR1* gene in human cancers with both intrinsic and acquired multidrug resistance (214). This subject has been extensively reviewed elsewhere (61, 215–218). In brief, intrinsic expression of the *MDR1* gene is found in cancers derived from

kidney, liver, colon, pancreas, and adrenal (148, 214, 219–221). These are normal tissues that express the *MDR1* gene. *MDR1* RNA levels are also elevated in carcinoid tumors, and pheochromocytomas. In the case of renal cell cancer, increased expression was found in the more differentiated tumors (221), indicating that expression in these tumors is related to state of differentiation.

Some other untreated cancers often show high levels of *MDR1* RNA, and frequently the tissue of origin of the cancer does not express *MDR1* RNA at easily detectable levels. Such cancers include acute and chronic leukemias of children and adults, non-Hodgkin's lymphoma, chronic myelogenous leukemia in blast crisis, nonsmall cell lung cancer with neuro-endocrine properties (but not most other lung cancers), neuroblastoma, sarcoma, and astrocytoma (214, 222–231). In the case of childhood neuroblastoma and sarcoma, correlations have been made between the expression of P-glycoprotein at presentation and poor prognosis, which included failure to respond to chemotherapy (174, 175).

The expression of the *MDR1* gene in cancer derived from tissues that do not normally express P-glycoprotein led to the suggestion that the process of malignant transformation, per se, can activate expression of the *MDR1* gene. This suggestion was recently confirmed in a study showing that the *MDR1* promoter introduced transiently into cells as part of an *MDR1* promoter–CAT construct was stimulated by *ras* and mutant *p53*, two genes commonly associated with tumor progression (232). Another study also reports increased *MDR1* gene expression at invading margins of locally aggressive and metastatic colon cancers (152).

Increased expression of the *MDR1* gene is commonly seen in tumors treated with chemotherapy that have relapsed during the course of, or after, chemotherapy. Examples include breast cancer, ovarian cancer, lymphoma, leukemia, neuroblastoma, pheochromocytoma, rhabdomyosarcoma, and multiple myeloma (172, 214, 233–238). In these cases, it is presumed that small numbers of *MDR1*-expressing cells were present when therapy was initiated, and this population survived chemotherapy and caused the relapse, but a direct effect of chemotherapy to induce *MDR1* gene expression is also possible.

Analysis of mdr *Promoters*

Because of the obvious importance of understanding the factors that regulate expression of the *mdr* genes, potential promoter regions upstream of the *mdr*-coding regions have been isolated from rodent and human sources. As noted previously, the entire human *MDR1* gene (86) and mouse *mdr1b* gene (80), including 5′ flanking regions, have been isolated. Promoter segments of varying sizes from the human *MDR1* (239), mouse *mdr1a* (240, 241),

mouse *mdr*1b (242, 243), and hamster *pgp*1 (*mdr*1a) (240) have been isolated and analyzed.

Two different human *MDR*1 promoters have been identified based on transcripts found in multidrug-resistant cultured cells, and some drug-resistant tumors (228, 239, 244). One set of transcripts, which initiate at two adjacent sites that correspond to nucleotides 136 and 140 nucleotides upstream from the translation-initiating ATG in the cDNA, are the only major transcripts found in normal human tissues such as liver, kidney, and adrenal. The second set of transcripts has been found in drug-selected cells, and initiates at an undefined upstream promoter site, which may be a cryptic promoter activated by selection, but not normally utilized.

Studies with the human *MDR*1 promoter have focussed exclusively on the downstream promoter. This promoter contains no TATA element, but does contain a GC-rich region, a CAAT box, a heat-shock consensus element, and an AP-1-like element. When the CAT reporter group is put under control of this promoter region contained in 1 kb of genomic DNA, cell-type-specific expression, regulated by Ras and p53, is found (232). This result contrasts with a report indicating that there is a tissue-specific enhancer located 10 kb upstream from this promoter region that is essential for cell-type-specific expression (245). Recent studies indicate that this putative enhancer may not actually be linked to the *MDR*1 gene (U. Germann, N. Popescu, I. Pastan, M. M. Gottesman, unpublished data).

In vitro transcription studies have indicated a region from 5 to 127 nucleotides downstream from the major transcription initiation site that is essential for proper transcription initiation (246). This region resembles downstream regions present in viral and eukaryotic promoters thought to be involved in regulation of transcription initiation (247), and may play an important role in control of expression of the *MDR*1 promoter.

The DNA sequences of mouse and hamster *mdr* promoters differ greatly from that of the human *MDR*1 promoter, probably explaining why the rodent promoters are more responsive to certain kinds of environmental stress. Both the *mdr*1a and *mdr*1b promoters contain TATA and CAAT boxes, and putative Sp-1, AP-1, and AP-2 sites (240–243). There appears to be more than one *mdr*1a promoter, accounting, together with alternative polyadenylation sites, for the multiplicity of *mdr*1a transcripts in mouse cells (241). In the mouse *mdr*1a promoter, the AP-1 site seems to mediate negative regulation, since elimination of this site increases basal promoter activity (240). In contrast, in the hamster, the AP-1 site in the TATA-less *pgp*1 (*mdr*1a) promoter positively regulates activity and interacts with Fos and Jun (248).

The *mdr*1b promoter, which drives expression of P-glycoprotein in the adrenal and in secretory glands of the endometrium, contains several potential regulatory elements including a progesterone response element (243). In transient expression assays, this promoter has been shown to be regulated by progesterone via the progesterone receptor (S. B. Horwitz, R. Piekarz, personal communication). Raymond & Gros (242) have carried out a deletion analysis of the *mdr*1b promoter, and demonstrated that sequences between −93 and +84 were required for basal promoter activity, but addition of sequences upstream to −141 had positive and negative, cell-type-specific effects on expression of a reporter gene. Similar observations have been made for the rat *mdr*1b promoter (J. Silverman, S. Thorgeirsson, personal communication).

STRUCTURE-FUNCTION STUDIES

A major unsolved problem in the field of multidrug resistance is how a single integral membrane protein, the product of a single gene in humans, can transport drugs and hydrophobic peptides with a wide array of structures. Additional questions concern how ATP is used in this process, and how the chloride channel function relates to the drug transport activity. A variety of genetic and biochemical approaches have been taken to understand these questions, and the remainder of the review is devoted to an examination of the studies that have begun to explore the relationship between structure and function of P-glycoprotein. This section focuses on mutational analysis of P-glycoprotein, photoaffinity labelling studies with substrates for P-glycoprotein, and the potential role of posttranslational modification (i.e. phosphorylation) in P-glycoprotein function.

Mutational Analysis of P-Glycoprotein

The isolation of multidrug-resistant cells and the molecular analysis of the multidrug transporter in these lines invariably led to the discovery of mutants of P-glycoprotein that affect its function [reviewed in (249)]. As already noted, selection for drug resistance usually results in overexpression of P-glycoprotein, either by amplification of a wild-type gene, alterations in gene expression that increase levels of wild-type *mdr* RNA (56), or both. In cancer samples from patients, increased expression most often results from increased RNA levels in the absence of amplification. That amplification and expression are not necessarily related has been demonstrated in cultured multidrug-resistant K562 leukemia cells carrying amplified *MDR*1 genes in which revertants carry the amplified gene, but no longer express it (250). Although very little is known about the mutations and normal regulatory pathways that affect *mdr* gene expression, both cis-acting (affecting only one gene of a two-allele pair) and trans-acting (affecting both alleles) regulatory changes have been found in cancer cells selected in vivo and in vitro (A. T. Fojo, personal communication).

Several different human *MDR*1 cDNAs have now been sequenced. There are several polymorphic variations in the coding regions in the human population, which appear to have no effect on function (130, 251). However, several other point mutations have been described that affect substrate specificity of the transporter, and these are illustrated in Figure 2 and described below. As can be seen in Figure 2 (*upper panel*), the point mutations are scattered throughout P-glycoprotein, suggesting that drug transport specificity is a complex phenomenon that either involves higher-order structure, or can be affected by multiple independent parts of the molecule.

The first functional point mutation described in P-glycoprotein, a change from Gly to Val at position 185 (illustrated in Figure 2, *upper panel*), was detected in a highly colchicine-selected multidrug-resistant cell line (KB-C1), and was shown to change the specificity of the transporter so that colchicine transport was improved, while transport of vinblastine and actinomycin D was decreased (130, 251, 252). Efforts to understand this alteration in drug specificity have led to conflicting conclusions. One study reported that reduced vinblastine transport and increased colchicine transport in the Val185 mutant is accompanied by increased photoaffinity labelling of the transporter by a vinblastine analog, and decreased labelling by a colchicine analog (253), and the authors suggested that the mutation decreases the rate at which vinblastine leaves the transporter. However, it was also shown that *Vinca* alkaloids such as vinblastine and vincristine are more effective inhibitors of photoaffinity labelling of P-glycoprotein in the Val185 mutant than in the wild-type protein, suggesting that the Val185 mutation affects the initial interaction of drugs with P-glycoprotein (99). Recent studies have shown that the Val185 mutation has complex effects on the kinetics of uptake of drugs by cells expressing this multidrug transporter (W. Stein, M. M. Gottesman, I. Pastan, unpublished data). Therefore, it seems likely that a simple explanation for the effect of this mutation will not suffice.

Melera's group has detected mutations of Gly to Ala and Ala to Pro at positions 338 and 339 in transmembrane domain 6 in P-glycoprotein in Chinese hamster cells selected for high levels of resistance to actinomycin D (254). These mutations occurred in a cell line with extremely high levels of amplification and expression of *mdr* genes, and appear to decrease resistance to several drugs, while maintaining normal resistance to actinomycin D, giving the appearance of a specific increase in actinomycin D resistance (254; P. W. Melera, personal communication). Presumably, the high-level expression of the *mdr* gene in this cell line has a deleterious effect and would not be tolerated by the cell unless P-glycoprotein is crippled by this mutation.

Two other mutations that have been engineered into P-glycoprotein also affect substrate specificity. One, first detected by Hsu et al (241) and characterized by Gros et al (255), converts a Ser to Phe at residue 941 in the mouse *mdr*1 (*mdr* 1b) gene in transmembrane domain 11 [residue 939 in the mouse *mdr*3 or *mdr*1a gene, which is described in (241)]. This mutation alters specificity to allow transport of vinblastine, but drastically decreases transport of colchicine and doxorubicin. The second mutation is a point mutation of Asn to Ser at position 183 in the first intracytoplasmic loop of P-glycoprotein (252). This was introduced into the transporter because Asn is found at this location in the *MDR*2 gene. This mutation, when combined with the Val185 mutation, has the interesting effect of increasing the vinblastine and actinomycin D resistance of cells, without decreasing their colchicine resistance, which had been increased by the Val185 mutation. Such a double mutant argues that drug transport specificity may be determined by overlapping, but not identical sites, on P-glycoprotein.

Point mutations have also been engineered into the A fold of the ATP site of P-glycoprotein; both the amino-terminal and carboxy-terminal ATP sites have been modified (see Figure 1). The conversion of a Gly to an Ala at positions 431 or 1073 or of a Lys to Arg at positions 432 or 1074 in the mouse *mdr*1 (*mdr*1b) gene results in almost complete loss of function of the transporter, despite continued binding of azido-ATP (256). However, when the equivalent Lys residues in the ATP sites are changed to Met in the human *MDR*1 cDNA, low levels of transport activity are maintained, azido-ATP binding is decreased, and the crippled transporters can be selected to high-level expression (I. B. Roninson, personal communication). Whether this small amount of remaining activity reflects movement of only small amounts of protein to the cell surface, represents low amounts of transport activity in the original mutant proteins reflecting low levels of functional ATPase activity, or whether a single functional ATP site is adequate to maintain low-level function of the transporter, is not yet known. Similar mutations in the ATP sites of the related yeast transporter, STE6, also have a "leaky" phenotype (256a). Recent reports indicate that the volume-regulated chloride channel activity of P-glycoprotein does not require a functional ATPase site, although ATP, or a nonhydrolyzable analog of ATP, must be present to detect chloride channel activity (195, 196).

Another approach to mutational analysis is the study of deletions, insertions, and substitutions in the transporter. Two naturally occurring deletions, which arose during intensive selection to high levels of drug resistance, have been described. The first occurred in the same highly actinomycin D–resistant Chinese hamster cell line that gave rise to the transmembrane 6 point mutation (254), and is a deletion of part of the first half of P-glycoprotein. A truncated mRNA that encodes this deletion, apparently arising from aberrant processing of P-glycoprotein mRNA, is present at substantial amounts in this cell line (101). This truncated P-glycoprotein cDNA has not been proven to encode a functional transporter. It is possible that such truncated molecules are

aberrations resulting from the complex rearrangements that accompany *mdr* gene amplification in highly resistant cell lines. Another smaller deletion of part of the first cytoplasmic segment in the amino terminus of P-glycoprotein, which is apparently functional, has recently been detected in a highly multidrug-resistant cell line (A. T. Fojo, personal communication).

Other deletions have been specifically engineered into P-glycoprotein (257). Deletions placed in either the carboxy- or amino-terminal half of P-glycoprotein have resulted in loss of transport function, indicating that both halves are necessary. Smaller deletions at the carboxy terminus of up to 23 residues allow maintenance of function. As noted previously, chimeric proteins can be formed with functional P-glycoprotein by addition at its carboxy-terminal end (145).

Small insertions near the carboxy-terminal ATP site are tolerated in P-glycoprotein (257), as are insertions of up to 18 amino acids with predicted random structure in the linker region that connects the two halves of P-glycoprotein (L. Airan, U. Germann, I. Pastan, M. M. Gottesman, unpublished data). However, insertions of the same size in the linker region with predicted alpha-helical structure are not tolerated, suggesting that there is critical spacing of the two halves that must be preserved.

Chimeras between the nondrug transporting *mdr*2-class molecules and *mdr*1-class molecules have also been reported. Gros's group (258) created a series of chimeras throughout the whole length of the mouse *mdr*1b gene product with segments of mouse *mdr*2, and found that only the ATP sites were interchangeable enough to produce a functional transporter. We have confirmed this result for the amino-terminal ATP site of human *MDR*2 exchanged into *MDR*1 (P. Wu, U. A. Germann, I. Pastan, M. M. Gottesman, unpublished data). These results indicate that the *mdr*2-class genes have at least one functional ATP site and support the speculation that these *mdr*2 proteins are functional transporters for unknown substrates. Dhir & Gros (259) have also used chimeras between the mouse *mdr*1(*mdr*1b) and *mdr*3 (*mdr*1a) genes to explore the basis of preferential resistance to colchicine and actinomycin D, respectively, by these genes. No simple chimera could be used to define a region of P-glycoprotein responsible for this different pattern of resistance.

A more detailed analysis of a human *MDR2/MDR1* chimera in which the *MDR1* region between amino acids 140 and 229 is replaced with the corresponding region from the *MDR2* gene has been instructive (252). In this 89-amino-acid region, including the first intracytoplasmic loop, the third transmembrane domain, and part of the fourth transmembrane domain, there are 17 amino acid differences between *MDR1* and *MDR2* divided equally between the putative cytoplasmic loop and transmembrane domain. Changing

only four of these amino acids (residues 165, 166, 168, and 169) from *MDR2* to *MDR1* residues in the cytoplasmic domain fully restores function of the chimeric molecule. This study emphasizes the similarity between *MDR1* and *MDR2*, and highlights the first intracytoplasmic loop as an important determinant of function of these different transporters.

Drug Binding and Photoaffinity Labeling

The binding of drugs that are transported by P-glycoprotein to P-glycoprotein itself was first demonstrated using ^{3}H-vinblastine, which bound to membranes prepared from multidrug-resistant cells (260). These studies were then extended to include the binding of agents, such as calcium channel blockers, which inhibit the multidrug transporter (261, reviewed in 262). That this binding was directly to P-glycoprotein was demonstrated with a photoaffinity analog of vinblastine (263), which binds to P-glycoprotein immunoprecipitated from multidrug-resistant Chinese hamster cells (264). Photoaffinity labeling was specific because it could be inhibited by an excess of various cytotoxic substrates for the multidrug transporter or nontoxic agents that reverse drug resistance, such as verapamil (263). This study, which showed the specificity of the labelling, pointed to a mechanism by which agents that overcome (reverse) drug resistance might work, i.e. as competitive inhibitors of drug binding and/or transport. Since these initial studies, a large number of substrates and substrate analogs of P-glycoprotein have been shown to be photoaffinity labels, including azidopine (95, 98, 265), verapamil (266), iodomycin (267), colchicine (253, 268), azidoprazosin (269), forskolin (270), and cyclosporin A (271), reviewed in (272).

Progress has been made in identifying the sites in P-glycoprotein labeled by these various photoaffinity labels. A carboxy-terminal site was found to be labeled by ^{3}H-azidopine in mouse P-glycoprotein (273, 274), whereas two azidopine-labeled sites were found in human P-glycoprotein (95, 98, 99). The presence of these sites, one or more in the amino-terminal part of P-glycoprotein, and one or more in the carboxy terminus, has since been confirmed in mouse P-glycoprotein using azidoprazosin photoaffinity labeling (269, 275). By proteolytic digestion, and cyanogen bromide cleavage, labeled fragments of P-glycoprotein have been identified using antibodies to specific parts of P-glycoprotein (99, 160). Both the amino and carboxy sites are labelled equally; one of these sites is in the region around transmembrane segments 5 and 6, and the other occupies an analogous site near transmembranes 11 and 12 (at least in mouse P-glycoprotein) (99, 269). With more complete digestions, and the use of an iodo-forskolin analog, as well as iodoazidoprazosin, the regions of labeling have been narrowed to the 5th or 6th transmembrane domain or the cytoplasmic domain immediately following

the 6th transmembrane region and a region within the 12th transmembrane domain or the cytoplasmic domain immediately following the 12th transmembrane region (275, 276).

These studies have identified only major sites of affinity labeling—not precise amino acids. Furthermore, in many of these experiments there are minor labeled fragments that might correspond to other parts of the molecule. Hence, it is not known if these sites are unique, or if the labeling is specific for certain amino acids. However, the results demonstrate unequivocally that two apparently symmetrical parts of P-glycoprotein can be labeled by several different photoactivatable drugs that interact with the transporter. Recent data showing that inhibition of azidopine labeling by vinblastine reduces labeling equivalently in both the amino- and carboxy-terminal halves of P-glycoprotein (99) suggests that these two binding sites are equivalent with respect to their ability to bind two different drugs, and supports a model of P-glycoprotein in which both halves of the transporter come together to form a single transport channel. This result is not necessarily in conflict with recent data suggesting that the precise binding sites of verapamil and vinblastine are not identical (277), since the transport channel for drugs, which is identified by the photoaffinity labels, and the initial binding sites, which define how drugs enter the transporter, may well be different.

P-glycoprotein has also been affinity labeled by 8-azido-ATP (262, 278, 279). This labeling can be competed with ATP or GTP, which is consistent with the finding (see below) that both ATP and GTP can provide energy for the transport reaction (280). The precise binding sites for the ATP-affinity labels have not yet been described.

Posttranslational Modifications

P-glycoprotein is known to be posttranslationally modified by glycosylation and phosphorylation (281–285). However, it seems unlikely that glycosylation significantly affects the function of P-glycoprotein for the following reasons: (*a*) There are major differences in glycosylation sites and apparent state of glycosylation between functional human and mouse P-glycoprotein (22, 23); (*b*) Tunicamycin-treatment, which blocks N-linked glycosylation, does not affect drug resistance of multidrug-resistant cells (286); and (*c*) It is possible to isolate multidrug-resistant cells from lectin-resistant mutants, which are glycosylation defective (287).

P-glycoprotein has been shown to be phosphorylated on several sites as detected by phosphorylated tryptic fragments (282). One of these phosphorylation sites is stimulated by treatment with the phorbol ester TPA (282), which increases drug resistance and decreases drug accumulation in some multidrug-resistant cell lines (288). Protein kinase C (PK-C) can phosphorylate P-glycoprotein in vitro (289, 290), and reduced accumulation of drugs in multidrug-resistant cells is associated with phorbol ester treatment, which increases phosphorylation of P-glycoprotein in association with translocation of PK-C to a membrane-bound form in vivo (291). SW620 human colon carcinoma cells treated with sodium butyrate show decreased phosphorylation of P-glycoprotein, which correlates with decreased efflux activity against some drugs. This decreased transport activity can be partially reversed by treatment with TPA, but involvement of protein kinase C has not been demonstrated (292). Membrane-associated protein phosphatases 1 and 2A appear to be involved in dephosphorylation of P-glycoprotein phosphorylated by PK-C (290). Recent demonstrations that MCF-7 cells transfected with a cDNA encoding PK-C alpha have increased drug resistance (293), and that rat fibroblasts transfected with PK-C beta1 are also multidrug resistant (294), point to a possible role for PK-C in regulating activity of P-glycoprotein. However, these genetic experiments must be interpreted with caution because they require the transfected cells to be isolated by selection in tissue culture, and therefore, PK-C may have an indirect effect on P-glycoprotein, such as by altering membrane composition or increasing mRNA levels (294a). In this regard, increased amounts of PK-C alpha have also been seen in the nuclei of MCF-7 multidrug-resistant cells (295). Proof of a direct role of PK-C-mediated phosphorylation in regulating the activity of P-glycoprotein remains to be demonstrated using pure P-glycoprotein functionally reconstituted into transporting vesicles.

P-glycoprotein is also a substrate for phosphorylation by cAMP-dependent protein kinase (283), and by a novel kinase that has not been well characterized yet (296). In the case of cAMP-dependent protein kinase, deletion of the major site of phosphorylation in mouse P-glycoprotein has no obvious effect on activity of the transporter (S. B. Horwitz, personal communication). However, basal levels of cAMP-dependent protein kinase are essential for maintaining *mdr* RNA levels in cultured CHO and Y-1 cells (192, 212).

IN VITRO TRANSPORT AND ATPase ACTIVITIES

A more complete biochemical understanding of how P-glycoprotein functions must await the complete purification and functional reconstitution of this transporter into defined lipid vesicles. Such studies are essential for an analysis of mechanism, which is an area of considerable interest and controversy. Although the complete functional reconstitution of purified P-glycoprotein has not yet been achieved, progress has been made in two areas: studies on transport function in isolated vesicles, and reconstitution of the P-glycoprotein-associated drug-stimulatable ATP hydrolysis.

Transport Activity in Vesicles

Using plasma membrane vesicles prepared from multidrug-resistant cells, it has been possible to demonstrate ^{3}H-vinblastine transport into inside-out vesicles (280, 297, 298). This transport does not occur when vesicles are prepared from drug-sensitive cells that lack P-glycoprotein, it is dependent on the constant supply of an energy source (either ATP, an ATP-regenerating system, or GTP), and it occurs against an apparent concentration gradient. Nonhydrolyzable ATP analogs such as AMPPNP do not support transport (280, 297), and vanadate strongly inhibits the transport process in a noncompetitive manner (297). The specificity of the transport was demonstrated through inhibition by unlabeled vinblastine, or many of the other cytotoxic substrates for the transporter, including vincristine, actinomycin D, daunorubicin, and colchicine (297). Agents that reverse drug resistance, such as verapamil, also inhibit transport. As indicated previously, the mechanism by which such drugs inhibit transport has been debated, and the vesicle transport system appeared to offer a possible means to test whether such inhibition was competitive, as is suggested by the fact that verapamil is itself a substrate for transport (193, 299, 300), or noncompetitive. Unfortunately, because of the errors inherent in measuring uptake of a hydrophobic compound such as vinblastine into vesicles (high background), the kinetic data are not quite good enough to conclude that other cytotoxic drugs and reversing agents are competitive inhibitors. The published data are consistent either with competitive or mixed inhibition, but do not support a model of pure noncompetitive inhibition, which would be expected if the binding sites for all of the drugs were completely different (298).

Arias and his coworkers have demonstrated transport of daunorubicin into inside-out vesicles formed from the basal membranes of rat hepatocytes (301), which are rich in P-glycoprotein (177). This transport system has the properties expected for P-glycoprotein-mediated transport, and its derivation from the basal membranes of hepatocytes in which P-glycoprotein has been shown to be present (65, 177, 301) argues that this protein is functional as a transporter in normal tissues. Similar transport ability has been demonstrated using membranes derived from the brush border of rat intestinal mucosa. Precisely what cellular metabolites or foreign substances are usually transported by P-glycoprotein in hepatocytes is yet to be determined.

Purification and Reconstitution into Lipid Vesicles of Drug-Dependent ATPase Activity Associated with P-Glycoprotein

The first purification of P-glycoprotein was reported by Riordan et al (20), who used standard biochemical techniques to prepare denatured material for antibody studies. Subsequently, Tsuruo and coworkers (302) purified P-glycoprotein by affinity chromatography over MRK-16 antibody affinity columns in the presence of the detergent CHAPS, and showed the preparation contained small amounts of ATPase activity of low specific activity that was not drug dependent. The *MDR1* cDNA has been inserted into a baculovirus expression system and used to make large amounts of P-glycoprotein in Sf9 insect cells. This preparation of P-glycoprotein has ATPase activity of high specific activity that is drug dependent (135, 279). ATPase activity has also been found, although at low levels, in immunoprecipitates of P-glycoprotein–beta galactosidase chimeras, which contain the presumptive ATP sites (139).

Several transporters have been successfully reconstituted by employing "osmolyte-mediated" reconstitution protocols, including ABC superfamily members such as the histidine and maltose permeases (303–306). Based on this approach, we have partially purified P-glycoprotein from multidrug-resistant KB-V1 cells and reconstituted it into phospholipid vesicles prepared from *E. coli* bulk phospholipids, phosphatidyl choline, phosphatidyl serine, and cholesterol (307). The ATP hydrolytic activity of the partially purified P-glycoprotein is preserved in the presence of the detergent octylglucoside. However, the cytotoxic substrate, vinblastine, and the reversing agent, verapamil, failed to stimulate ATPase activity of the soluble protein. The lack of effect of these drugs may be due to an interaction of the hydrophobic detergent with the drug-substrate binding site(s) on P-glycoprotein. Alternatively, the conformation of the soluble P-glycoprotein may not be suitable for substrate-induced activation.

When the partially purified P-glycoprotein is reconstituted into phospholipid vesicles, it is possible to show drug-stimulated ATPase activity (307). Several drugs such as vinblastine, doxorubicin, and daunorubicin, which are cytotoxic substrates for P-glycoprotein, and verapamil, a transport substrate that inhibits transport of other substrates, stimulate ATPase activity 3–4-fold without a change in the K_m for ATP (0.3 mM). In contrast, camptothecin, another hydrophobic anticancer drug which is not a substrate for P-glycoprotein (308), does not stimulate ATPase activity. The necessity to reconstitute P-glycoprotein to demonstrate drug-dependent ATPase activity is consistent with the recent observations of Davidson et al (309) that the maltose-stimulated ATP hydrolysis of the maltose permease is only seen with the reconstituted protein. On the basis of work with the partially purified P-glycoprotein (307), it appears that pure P-glycoprotein will exhibit a high level of ATPase activity (15–38 μmol/min/mg protein) comparable to the specific ATPase activity of ion-transporting ATPases. Vanadate is a potent inhibitor of both the basal and drug-stimulated ATPase activity, as was found for transport activity in vesicles (297). The activities of other ABC superfamily members, such as histidine and maltose permeases, are also inhibited by vanadate (309–311). Although

the mechanism of vanadate inhibition of ABC transporters is not clear at present, it is certainly not only a specific inhibitor of P-type ATPases (312).

MECHANISM OF ACTION

Studies on the mechanism of action of P-glycoprotein have attempted to incorporate the available data on the molecular biology, physiology, and pharmacology of this transporter to produce coherent models that generate testable hypotheses. In this section, several alternate models are briefly discussed after the presentation of some additional data relevant to mechanism.

Other Phenomena and Epiphenomena Related to Multidrug Resistance

The published literature on P-glycoprotein is replete with examples of phenomena observed in multidrug-resistant cells that are not observed in parental drug-sensitive cell lines of the same type. Most multidrug-resistant cell lines were selected over many months through many steps of exposure to drugs and mutagens. Therefore, some of the observations made concerning multidrug resistance are unrelated to action of the multidrug transporter, but may represent other types of multidrug resistance, including mutations in other drug-resistance genes, adaptive changes due to exposure to toxic substances, or genetic drift that occurs during continuous passage of cells. In this section, a variety of phenomena consistently seen in multidrug-resistant cells are reviewed, and an attempt is made to place these in the context of mechanism.

The earliest studies on high-level multidrug resistance observed a collateral increased sensitivity of multidrug-resistant cells to some steroids, tertiary amine local anesthetics, some nonionic detergents (such as Tween) (313), calcium channel blockers (such as verapamil) (299, 314), as well as sensitivity to disruption by physical shear (20). These observations are seen in virtually all highly resistant cell lines [reviewed in Cano-Gauci & Riordan (315)], but have never been satisfactorily explained. One possibility is that large amounts of P-glycoprotein in the membrane disrupt normal membrane structure, thereby sensitizing cells to membrane active agents. Another possibility is that some of these agents are increased in amount in multidrug-resistant cells, but this has generally not been the case (315). A third possibility is that the action of P-glycoprotein requires so much ATP that ATP levels become limiting for repair of drug-induced membrane damage. This idea is supported by evidence for increased turnover of ATP in the presence of verapamil (316), and by the increased sensitivity of multidrug-resistant cells to 2-deoxyglucose (317). Finally, P-glycoprotein may indirectly weaken cellular defenses against membrane-active agents by altering pH or ionic conditions, or by actively

extruding an endogenous compound needed for normal membrane integrity or cell viability. Although the phenomenon of collateral sensitivity has been suggested as an exploitable approach to clinical reversal of drug resistance, the high levels of P-glycoprotein needed to observe this phenomenon are rarely achieved clinically in multidrug-resistant cancers. A related phenomenon may be the reduced growth rate and the decreased tumorigenicity of many highly multidrug-resistant cell lines (18) (E. Stanbridge, I. Pastan, M. M. Gottesman, unpublished data).

Another phenomenon consistently observed in highly multidrug-resistant cells is an alteration of pH manifested either as increased intracellular pH, increased acid secretion, or increased intracellular pH evoked by alkalinization of medium (318–320). These data suggest that a proton may be transported out of the cell due to the action of P-glycoprotein. This could be a direct effect (i.e. P-glycoprotein is a proton pump) or an indirect effect (i.e. an endogenous substrate for P-glycoprotein is protonated, or its mechanism of action requires transport of an anion which is accompanied by a proton). We consider this phenomenon again when we speculate on mechanism.

Recently, a volume-regulated chloride channel activity has been observed in P-glycoprotein-expressing cells (195, 196). This activity is clearly related to expression of P-glycoprotein, since antisense oligonucleotides that reduce P-glycoprotein expression eliminate the chloride channel activity. Recent mutational analysis of P-glycoprotein in which ATP sites are altered indicates that chloride channel activity can be maintained despite loss of multidrug transport activity (196). Agents that inhibit the chloride channel have no effect on multidrug transport (196, 321; J. Campain, M. M. Gottesman, I. Pastan, unpublished data), but chloride channel activity is not seen in the presence of verapamil, or cytotoxic drugs such as vinblastine that are substrates for P-glycoprotein (196). One interpretation of this result is that the volume-regulated chloride channel and the multidrug transport activity are entirely separate properties of the same protein; another is that both activities cannot be expressed simultaneously, as might be seen if a regulated (rather than open) chloride channel were needed for multidrug transport.

A number of laboratories have observed that the reduced accumulation of drugs in multidrug-resistant cells does not account quantitatively for the high levels of resistance in these cells. Detailed analysis, using fluorescent drugs such as the anthracyclines, indicates that drugs may be found in intracellular vesicles in these cells (17, 322). One speculation is that these vesicles are actively accumulating drugs either because of the presence of P-glycoprotein in their membranes, or through some other mechanism. This seems unlikely because P-glycoprotein has not been demonstrated in the membranes of endocytic or other vesicles of mammalian cells (323). Furthermore, the

anthracycline found in the vesicles is not fixed to protein (17). Rather, it appears to be associated with acid endocytic vesicles because of trapping due to the weakly basic character of anthracyclines.

Highly selected multidrug-resistant cells occasionally have alterations in plasma membrane lipids (reviewed in 324). Although the earliest reports did not find such changes (325), subsequent studies have detected alterations in sphingomyelin, cardiolipin, phosphatidyl ethanolamine, and phosphatidyl serine (126, 326). These changes in phospholipid composition do not alter P-glycoprotein activity in a major way, since cells transfected or infected with *mdr* vectors in a single step without selection express the complete phenotype of multidrug resistance with resistance levels proportional to the amount of P-glycoprotein on the cell surface (77, 178). Nevertheless, such changes may affect membrane fluidity and thereby determine how drugs are presented to the transporter or the efficiency with which the transporter operates (see below). In support of this hypothesis is the finding that when human P-glycoprotein is put in a baculovirus expression system and used to infect Sf9 insect cells, which have a different lipid composition from mammalian cells, the pattern of drug inhibition of photoaffinity labeling by azidopine is altered dramatically (135). Recent data on P-glycoprotein expressed in yeast suggests that yeast sterols may affect its function (137, 327).

In addition to lipid changes in multidrug-resistant cells, there are many associated protein changes, virtually all of which appear to be epiphenomena associated with other resistance mechanisms, random changes in protein levels, or expression of genes co-amplified with the *mdr* amplicon. Many of these changes have been detected using two-dimensional gel analysis of several different multidrug-resistant cell lines (9). One relatively consistent change is the increased expression of sorcin, a 22-kDa calcium-binding protein, whose gene is closely linked to P-glycoprotein and frequently co-amplified with it (328, 329). No direct interaction with P-glycoprotein or effect on multidrug resistance has been demonstrated for sorcin to date. Another change is an increased expression of EGF receptors on some multidrug-resistant cell lines (330), but this expression may be related to the selective growth advantage such cells have, given the growth inhibitory effects of high levels of expression of P-glycoprotein. Decreased expression of the 72–75-kDa cell surface intestinal alkaline phosphatase in some multidrug-resistant human KB cells has been described (331), but no evidence that this phosphatase affects P-glycoprotein function has been obtained (U. A. Germann, I. Pastan, M. M. Gottesman, unpublished data). In summary, despite the occurrence of many protein alterations in multidrug-resistant cells, only the expression of P-glycoprotein appears to be necessary and sufficient for the complete phenotype of multidrug resistance. However, modulating effects of other proteins cannot be ruled out.

Models for the Mechanism of Action of the Multidrug Transporter

Speculation on the mechanism by which the multidrug transporter reduces accumulation of cytotoxic drugs in multidrug-resistant cells has evolved as the physiology, biochemistry, and pharmacology of the transporter have been elucidated. Initially, drug resistance was thought to be due to a reduction in the permeability of the plasma membrane to certain drugs (4). When the efflux pump activity of P-glycoprotein was demonstrated (3, 17), this was thought to be the sole means by which reduced drug accumulation was achieved (35, 332). More recently, it has been appreciated that the multidrug transporter can both reduce influx into the cytosol and increase efflux of drugs, and models based on removal of drugs directly from the plasma membrane have been proposed (12, 23, 333, 334).

As illustrated in Figure 3, our current model of the mechanism of action of P-glycoprotein has two major features. The most critical feature is that drugs can be detected and expelled as they enter the plasma membrane in the manner of a hydrophobic vacuum cleaner (335). This effect accounts for the decreased accumulation in the cytosol. The second feature is that transport occurs through a single barrel of the transporter.

Several lines of evidence support the conclusion that drugs are removed directly from the plasma membrane: (*a*) The kinetic data reviewed above

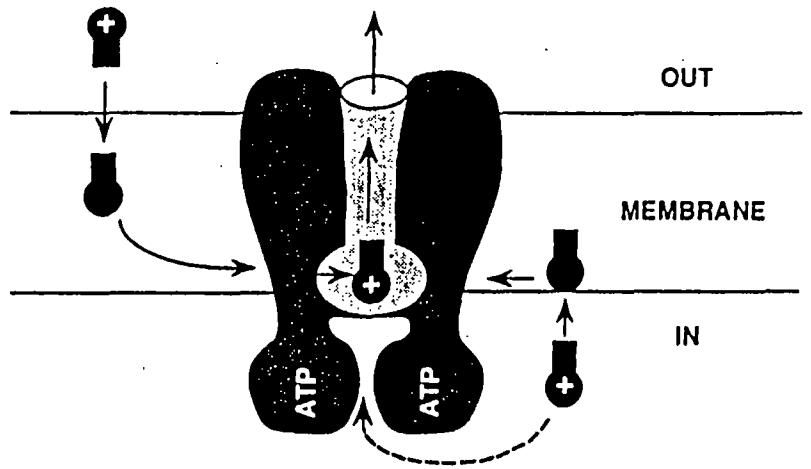

Figure 3 A possible mechanism of action for P-glycoprotein emphasizing two features: 1. Drugs may be detected in and ejected from the plasma membrane; and 2. The transporter has a three-dimensional structure involving one or more P170 subunits, which brings both halves together to form a single transport channel.

(12–15). (*b*) Virtually every study of a series of structurally related drugs that differ in their ability to be transported by the multidrug transporter demonstrates that the most important determinant of their ability to be transported is their relative hydrophobicity (e.g. 336, 337), and that drugs that are substrates for the transporter must have partition coefficients (octanol/water) of approximately 1 or greater. In addition, certain agents to which multidrug-resistant cells are resistant are hydrophobic peptides, such as gramicidin D, valinomycin (M. Horio, M. M. Gottesman, I. Pastan, unpublished data), and the calpain and cathepsin inhibitory peptide N-acetyl-leucyl-leucyl-norleucinal (ALLN) (338), all of which are thought to be highly concentrated in the plasma membrane. However, very hydrophobic agents, such as camptothecin, which are sparingly soluble in water, are not substrates, indicating that some water solubility is required for the recognition of substrates by P-glycoprotein (12, 308).

(*c*) Rhodamine 123, a highly fluorescent mitochondrial laser dye, which is an excellent P-glycoprotein substrate (185), was shown by Kessel to have different fluorescence excitation spectra in drug-resistant and drug-sensitive cells. In drug-sensitive cells, the fluorescence excitation spectrum of rhodamine 123 resembles its spectrum in octanol, suggesting a highly hydrophobic environment, while in multidrug-resistant cells the spectrum looks much more aqueous, suggesting that the drug has been removed from the plasma membrane in the resistant cells (339). This result has been confirmed using confocal fluorescence microscopy to localize rhodamine 123 in multidrug-sensitive and -resistant cells (322).

(*d*) The final evidence for the "hydrophobic vacuum cleaner" model rests on the demonstration that drugs such as doxorubicin are removed directly from the plasma membrane by the action of the transporter. By measuring the transfer of energy from doxorubicin to iodinated naphthalene azide (INA), a highly hydrophobic label of membrane constituents including transmembrane proteins, the presence of doxorubicin within the membranes of drug-sensitive cells can be easily detected. However, in drug-resistant cells, doxorubicin is only found in the plasma membrane associated with P-glycoprotein. This result indicates that the transporter has removed doxorubicin from the plasma membrane (335).

The second important feature of our current model of P-glycoprotein is that transport occurs through a single barrel of the transporter composed of one or more P170 subunits as illustrated in Figure 3. This conclusion comes from a composite of all of the data concerning photoaffinity labeling, mutational analysis, and inhibitor studies, which have already been summarized. This model in no way implies that different drugs do not enter the transporter in different ways (hence, initial regions of contact with the transporter may vary from drug to drug), but limits their passage through the transporter to a single transport channel.

How is the energy of ATP transduced to result in removal of drug from the plasma membrane of multidrug-resistant cells? Although we know that both ATP sites are needed for this activity to occur efficiently (256), and that the drugs themselves stimulate the ATPase activity (279, 307), very little is known about how the energy of ATP is harnessed in this transporter. Speculation has included the idea that ATP is constantly being hydrolyzed to produce a "moving staircase" or "waterwheel" in which any drug that happens to fall into the transport chamber will be extruded. This idea is consistent with the known basal ATPase activity of the transporter, but is not consistent with the stimulation of the ATPase in the presence of drugs (279, 307). Another idea is that the transporter is essentially a "flippase" that detects drug within the inner leaflet of the plasma membrane and "flips" it into the outer leaflet (from which it can diffuse away from the cell) or directly into the extracellular space (334). Since little is known about how phospholipid flippases function (340), this model certainly does not reduce the analysis of multidrug transport to a problem previously solved, nor have there been any data to support or refute this idea.

Another speculative model integrates the putative "proton pump" and chloride channel activity of the transporter to provide motive force for the "hydrophobic vacuum cleaner." In this model, ATP hydrolysis is linked to transport of protons into the transporter, with chloride following passively (alternatively, it could just as well be the chloride that is being transported as a result of ATP hydrolysis, with the proton, or other cation, or even the cationic drugs, following passively) (12). Once within the transporter, these ions will draw water into the transporter out of the plasma membrane. Amphipathic drugs within the membrane should follow the water, and drugs will be removed from the membrane just as a "scrubber" on a smokestack removes water-soluble materials from smoke. Although this model provides an explanation for why the physical features of the drugs (i.e. their hydrophobicity and amphipathicity) are the major determinants of whether or not they will be substrates, its kinetic plausibility has not been demonstrated, and it fails to address directly two kinds of data. First, it does not account for the existence of specific mutations in P-glycoprotein that change substrate specificity (see above). Second, it does not consider that the chloride channel activity of P-glycoprotein, which is volume regulated and not drug regulated, can be dissociated from the drug transporter activity (196). Although both of these objections can be overcome by minor modifications in the model (such as that substrate specificity is determined by access to the transporter, which is highly sensitive to P-glycoprotein folding or subunit structure within the

membrane, or that the chloride channel activity can be both "unregulated" and "regulated" during transport), these considerations raise concerns that will need to be directly addressed by experimentation. The ability to purify and reconstitute P-glycoprotein into lipid vesicles should accelerate experiments aimed at specifically testing various models.

CLINICAL RELEVANCE OF BIOCHEMICAL STUDIES ON THE MULTIDRUG TRANSPORTER

As already noted above, the presence of the multidrug transporter in multidrug-resistant human cancers has been conclusively demonstrated. One goal of current cancer research is to find ways to overcome or circumvent drug resistance due to expression of the multidrug transporter. The development of pharmacologic agents that can reverse multidrug resistance is therefore a high priority (341, 342). In addition to low-molecular-weight drugs, the use of antibodies (163, 169, 180, 181), immunotoxins (183), antisense oligonucleotides (195), and liposome-encapsulated drugs (343) have all been shown to be valid approaches to the elimination of multidrug-resistant cells. In vivo model systems with which to test these strategies have been difficult to develop (reviewed in 344). One system that has demonstrated that *MDR1* RNA levels found in human cancers can result in multidrug resistance in animals, and has been useful for the testing of various strategies for circumventing P-glycoprotein-mediated multidrug resistance, is transgenic mice in which the human *MDR1* gene is expressed in bone marrow under control of a chicken actin promoter (133, reviewed in 249, 345). The bone marrow of these animals is resistant to cytotoxic drugs that are substrates for the multidrug transporter, and hence, models such as this can be used to test agents that reverse drug resistance (346, 347). Although the original *MDR1* transgenic mice in which the *MDR1* gene was placed under control of an actin promoter have not continued to express the *MDR1* gene in the bone marrow at high levels after many generations of breeding, the construction of other *MDR1* transgenic mice using bone-marrow-specific enhancers is in progress (M. Siegsmund, I. Aksentijevich, G. Evans, G. Merlino, M. M. Gottesman, I. Pastan, unpublished data).

Another novel approach to cancer therapy that exploits the genetics and biochemistry of the multidrug transporter is the use of *MDR1* expression vectors for gene therapy. Drug-resistant bone marrow from *MDR1*-transgenic mice can be transplanted to drug-sensitive animals, resulting in a recipient with multidrug-resistant marrow (348), and similarly, bone marrow in mice has been made resistant by infection with an *MDR1* retrovirus (143, 144). These preliminary experiments suggest that the *MDR1* gene can be used as a selectable marker both for protection of bone marrow during chemotherapy, and to introduce nonselectable genes into cells (140).

We are poised to understand the mechanism of action of an important class of eukaryotic transporters, and to exploit this knowledge for the treatment of human disease. Study of the basic biochemistry, cell, and molecular biology of an obscure drug resistance phenotype in cultured cells has already reaped an important intellectual harvest, and promises to bear even greater benefits in the future.

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