DISCRETE MUTATIONS INTRODUCED IN THE PREDICTED NUCLEOTIDE-BINDING SITES OF THE mdr1 GENE ABOLISH ITS CAPACITY TO CONFER MULTIDRUG RESISTANCE

by

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Mutational analysis of the nucleotide-binding sites of mdr1

ABSTRACT

Multidrug resistance conferred by the mouse mdr1 gene is associated with an ATP-dependent drug efflux mechanism. Two putative nucleotide-binding sites (NBS) are present within the predicted amino acid sequence of the MDR1 protein. To assess the functional importance of these NBS, amino acid substitutions were introduced in the consensus NBS, GXGKST. Mutants bearing the sequence GXAKST or GXGRST at either NBS and a double mutant harboring the sequence GXGRST at both NBS were generated. Our results demonstrate that the integrity of the two NBS is essential for biological activity of mdr1. Failure to reduce intracellular accumulation of [^{3}H] vinblastine parallels this loss of activity. However, the mutant proteins retain the ability to bind the ATP analog 8-azido ATP. This suggests that an essential step subsequent to ATP-binding is impaired in these mutants. Our results also suggest that both NBS function in a cooperative fashion, as mutations in a single NBS completely abrogate the biological activity of mdr1.

RESUME

Le phénomène de résistance multiple aux drogues, qui peut être conféré par le gène mdr1 de souris, est associé à un mécanisme de relargage des drogues impliquant l'ATP. Deux séquences consensus de liaison aux nucléotides (NBS) sont présentes à l'intérieur de la séquence en acides aminés déduite pour la protéine MDR1. Le rôle fonctionnel de ces NBS a été étudié en introduisant une série de mutations ponctuelles à l'intérieur du NBS consensus, GXGKST. Quatre mutants porteurs des séquences GXAKST ou GXGRST dans un seul NBS ainsi qu'un double mutant portant la séquence GXGRST dans les deux NBS furent créés. Nos résultats démontrent que l'intégrité de ces deux NBS est essentielle à l'activité biologique de mdr1. L'incapacité de réduire l'accumulation intracellulaire de [^{3}H] vinblastine accompagne la perte d'activité biologique de ces mutants. Par contre, les protéines mutantes ont conservé la propriété de se lier à un dérivé de l'ATP, le 8-azido ATP. Ceci indique qu'une étape subséquente à la liaison avec l'ATP, essentielle au fonctionnement normal de la protéine, est affectée chez les mutants. D'autre part, nos résultats suggèrent que les deux NBS interagissent de façon coopérative, puisque la mutation d'un seul NBS peut éliminer complètement l'activité biologique de mdr1.

PREFACE

The work described in this thesis has been submitted for publication to Molecular and Cellular Biology. The work is entirely my own. Randy Kaufman provided the expression vector p91023b, Erwin Schurr prepared the polyclonal antiserum ES4 and Victor Ling provided the monoclonal antibody C219.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

(i) Demonstration that highly conserved amino acid substitutions introduced at the putative nucleotide-binding sites of the mouse *mdr1* gene abrogate its ability to confer multidrug resistance but do not affect the ability of the mutant proteins to bind ATP

(ii) Demonstration that both homologous halves of *mdr1* do not function as independent monomers but rather operate in a cooperative fashion to mediate drug efflux

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LIST OF ABREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolylphosphate p-toluidine salt
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
Ci	Curie
CO_2	carbone dioxyde
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
dTTP	deoxythimidine triphosphate
E. coli	Escherichia coli
EDTA	ethylene diamine tetraacetate
G418	Geneticin
$G418^r$	G418 resistant
Hyd	hydrophobic amino acid
kb	kilobase

kDa	kilodalton
mRNA	messenger RNA
NABV	N-(p-azidobenzoyl)-N'- -aminoethylvindesine
NASV	N-(p-azidosalicyl)-N'- -aminoethylvindesine
NBS	nucleotide-binding site
NBT	nitroblue tetrazolium chloride
Neo^r	neomycin resistant
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
UV	ultraviolet
VBL	vinblastine
xg	times gravity

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CHAPTER I

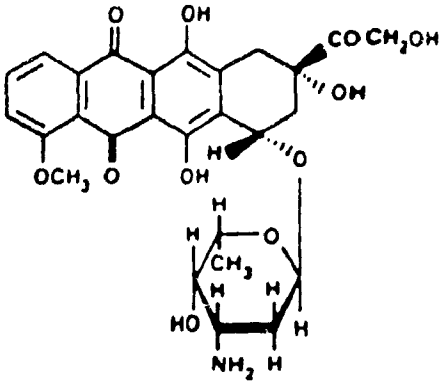
INTRODUCTION

I.1 The Multidrug Resistance Phenotype

The treatment of human cancers with cytotoxic agents of increased specificity for the rapidly dividing tumor cells was initiated four decades ago by the administration of aminopterin to leukemic children (1). However, the emergence and outgrowth of multiple drug resistant populations of tumor cells represents a major obstacle to the successful treatment of cancer by chemotherapy. Multidrug resistance is characterized by the simultaneous acquisition of resistance to a broad range of lipophilic compounds unrelated both in structure and intracellular targets. Many of these agents are natural products, such as the Vinca alkaloids (vinblastine, vindesine, vincristine), anthracyclines (Adriamycin, daunomycin), colchicine, actinomycin D, epipodophyllotoxins (etoposide, teniposide) and some protein synthesis inhibitors (2-6) (Figure 1). No obvious common chemical feature exists between these compounds other than their hydrophobicity due to the presence of several aromatic rings and their tendency to carry a positive charge at neutral pH (7,8). These drugs affect cellular metabolism by targeting different intracellular components. For example, anthracyclines bind to DNA (9), whereas Vinca alkaloids and colchicine induce mitotic arrest by binding to microtubules and tubulin, respectively (10,11).

FIGURE 1

Structural features of typical compounds forming the multidrug resistance spectrum



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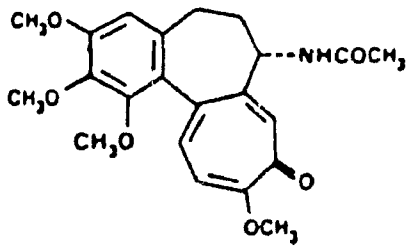

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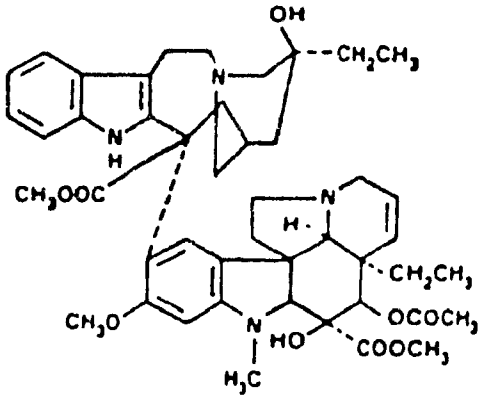
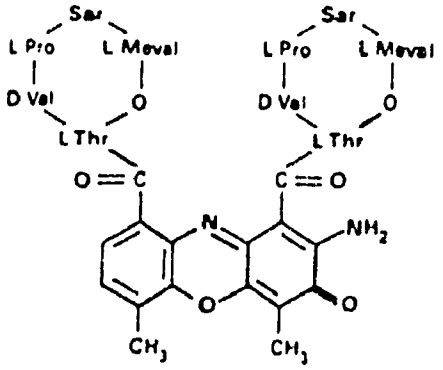


VINBLASTINE



ACTINOMYCIN D

I.2 Characteristics of Multidrug Resistant Cell Lines

Multidrug resistance has been studied extensively in highly drug resistant cell lines developped *in vitro* by stepwise selection in increasing concentration of a single cytotoxic agent (12-16). Although these cell lines were originally exposed to a single drug, they display cross-resistance to the wide range of products that form the multidrug resistance spectrum. Occasional cross-resistance to alkylating agents (melphalan, nitrogen mustard and mitomycin C) is observed in these cells (5). In addition, increased sensitivity (collateral sensitivity) to membrane-active agents such as non-ionic detergents, local anaesthetics, steroid hormones, calcium channel blockers and calmodulin inhibitors is associated with the appearance of multidrug resistance (14,17-21). The degree of cross-resistance of a specific cell line to a particular drug cannot be predicted *a priori*. Usually, but not always, the cell line is more resistant to the drug used during the initial selection (4,5).

It has been observed by several investigators that drug resistant cells accumulate less drug than their sensitive counterparts (14, 22-28). In these cell lines, the extent of intracellular drug accumulation is inversely proportional to the degree of drug resistance. In addition, this decreased accumulation is observed not only for the

drug used in the original selection but also for other drugs to which the cell line is resistant to (22,26,28). When drug resistant cells are depleted of cellular ATP by preincubation with inhibitors of the oxidative phosphorylation cycle (such as potassium cyanide, 2-deoxyglucose or sodium azide) in the absence of glucose, the steady-state level of accumulated drug increases to the levels observed in drug sensitive cells (22,24,29,30). Therefore reduced drug accumulation in the resistant cells is strictly dependent on intact intracellular ATP pools.

Since drug uptake is proportional to the drug concentration over a broad range of concentrations, entry of drug molecules into the cell is believed to occur via passive diffusion across the lipid bilayer (22,29,31). When cells are preloaded with radiolabelled drug under conditions of ATP-depletion and that normal ATP levels are subsequently restored by addition of glucose to the medium, the drug is more rapidly released from the resistant cells than from the sensitive cells (24,26,32). The observations of passive inward drug diffusion, reduced drug accumulation and increased drug efflux led Dano to postulate that an energy-dependent drug efflux mechanism underlies the multidrug resistance phenotype (22). This hypothesis has been confirmed by others (24,26,32) and is now a widely accepted working model. However, it is generally agreed that in highly drug resistant cell lines, multidrug

resistance is a complex phenomenon, possibly involving multiple factors such as lower influx, higher active drug extrusion or lower affinity for intracellular binding sites (7,22,24,28).

Juliano and Ling were the first to report overexpression of a 170 kDa glycoprotein, designated P-glycoprotein, in membrane fractions of the colchicine resistant Chinese hamster ovary cell line $CH^{R}C5$, which was undetectable in its drug sensitive counterpart AuxB1 (33). The level of overexpression of P-glycoprotein increased concomitantly with the degree of resistance in cell lines of increasing drug resistance. This finding has since then been verified by many laboratories with multidrug resistant cell lines derived from distinct tissue types by different drug selection regimens (15, 34-37).

Variations in the level of other polypeptides have been reported in multidrug resistant cell lines: increased expression of a 19 kDa acidic cytosolic protein V19 (38), now identified as the calcium-binding protein sorcin (39), or decreased expression of a group of membrane glycoproteins of 72 to 75 kDa (40) or 100 kDa (27,34) have been noted. In addition, alterations in ganglioside expression, whose synthesis becomes blocked at the level of hematosides, have also been observed (41). The role of these biochemical modifications in the development of multidrug resistance, if any, has still to be proven. The

overexpression of the 170 kDa P-glycoprotein(s) constitutes therefore the most ubiquitous biochemical marker of the multidrug resistant phenotype.

In the absence of selective drug pressure, highly drug resistant cells often revert to lower levels of resistance (42). This phenotypic instability, together with extremely high levels of P-glycoprotein overexpression, suggest that gene amplification may underlie the emergence of high levels of drug resistance. Markers of gene amplification such as double minute chromosomes and homogeneously staining regions have indeed been observed in multidrug resistant cells (42-46). In addition, multidrug resistant cells often lose these markers when they revert to lower levels of drug resistance (42,46).

I.3 Molecular Genetics of Multidrug Resistance

The biological implication of gene amplification in multidrug resistant cells was analyzed by the in-gel renaturation technique (47). A total of nine commonly amplified DNA fragments spanning 55 kb were detected in the two independently derived multidrug resistant Chinese hamster cell lines LZ and C5 (48). A 1.1 kb BamHI fragment commonly amplified in LZ and C5 was purified and cloned. Southern analysis demonstrated that the degree of

amplification of this fragment correlated with the degree of resistance in different multidrug resistant cell lines. This fragment was used as a probe to isolate cosmid clones encompassing a contigous 120 kb domain commonly amplified in the multidrug resistant cell lines LZ and C5 (49). This cloned domain was shown to contain a transcriptionally active gene, designated mdr, that encoded a 5.0 kb mRNA. In a series of multidrug resistant cells, the level of expression of this mRNA correlated with the degree of resistance and with the level of gene amplification. Interestingly, a number of cross-hybridizing bands of weak intensity were detected on Southern blots of drug resistant cells probed with fragments of the cloned hamster domain, suggesting that the cloned gene (mdr) may be part of a multigene family (49).

The presence of commonly amplified sequences in independently derived human multidrug resistant KB carcinoma cell lines was also detected by in-gel renaturation (46). Amplification of two different DNA sequences homologous to the hamster mdr gene, were reported in these human drug resistant cells and were designated mdr1 and mdr2 (50). Probes specific for these two sequences, pMDR1 for mdr1 and pMDR2 for mdr2, were isolated. Northern analysis using the pMDR1 probe detected a mRNA of 4.5 kb, whose level of expression correlated with the degree of drug resistance in independently selected

human KB cell lines (51). No expression of a corresponding mRNA with the pMDR2 probe was detected. These results suggested that mdr1 was primarily responsible for the development of multidrug resistance in human tumor cells.

DNA-mediated gene transfer of multidrug resistance was initially reported by Debenham (52) and Robertson (53). However, direct proof of the transfer of donor-cell DNA sequences to the recipient cells awaited the isolation of cloned fragments of the gene amplified in resistant cells. Chromosome-mediated gene transfer was used by Gros et al. to transfer portions of the drug resistant Chinese hamster LZ genome to drug sensitive mouse LTA cells to produce drug resistant LTA cells (54). The similar transfer of genomic sequences associated with multidrug resistance were observed independently by two groups using the DNA-mediated gene transfer technique (55,56). In one instance, the increased expression of P-glycoprotein was shown to be of donor origin using a species-specific monoclonal antibody (56). These experiments strongly suggested that the transferred genes were responsible for expression of multidrug resistance.

Several approaches were used to isolate cDNA clones corresponding to the 4.5 to 5.0 kb mRNA overexpressed in multidrug resistant cells. To isolate transcriptionally active mouse mdr genes, Gros et al. screened a cDNA library prepared from a drug sensitive mouse preB cell line (57)

with cloned hamster mdr probes derived from the genomic domain amplified in LZ and C5 cells. The cDNA clones isolated fell into three classes based on their respective restriction enzyme patterns. Two transcripts were expressed at equal frequency and identified as clones DR11 and DR29 (57). A third cDNA, DR27, corresponding to a much less abundant mRNA was also obtained (A. Devault and P. Gros in preparation). The DR11 clone encoded a full-length 4.3 kb cDNA for the mouse mdr1 gene. DR29 and DR27 encoded only partial cDNA clones for the mouse mdr2 and mdr3 genes, respectively. Full-length cDNA clones for mdr2 and mdr3 were later reported by the same group (58, A. Devault and P. Gros in preparation).

The biological activity of these cDNA clones was tested in transfection experiments after insertion into appropriate mammalian expression vector systems. Upon transfection and overexpression in otherwise drug sensitive Chinese hamster or mouse cell lines, mdr1 could confer the complete multidrug resistance phenotype as tested in Adriamycin, colchicine and vinblastine (57,59,60). These results unambiguously demonstrated that overexpression of a single member of the mdr gene family was capable of conferring multidrug resistance. Since this cDNA was isolated from a drug sensitive cell line, mutations in the primary sequence of the mdr1 gene were not necessary for expression of multidrug resistance. In the same

transfection system, mouse mdr2 did not express any biological activity (58). Conversely, mouse mdr3, as mouse mdr1, could convey multidrug resistance to otherwise drug sensitive cells (A. Devault and P. Gros, in preparation). Interestingly, mdr1 and mdr3 display different patterns of cross-resistance, mdr3 conferring higher levels of resistance to actinomycin D and vinblastine than those conferred by mdr1 while the opposite is true for Adriamycin

To isolate cellular transcripts encoding for the P-glycoprotein overproduced in Chinese hamster drug resistant cells, Riordan and coworkers screened an expression library prepared from the colchicine resistant cell line, $CH^{R}B30$ (61) with the monoclonal anti-P-glycoprotein antibody C219 (36). A 630 bp cDNA clone, designated pCHP1, was isolated and shown to hydridize to a 4.7 kb mRNA overexpressed in multidrug resistant cell lines. Southern analysis also demonstrated that this pCHP1 clone, in addition to hydridizing to hamster sequences, cross-hybridized to DNA sequences amplified in human and mouse multidrug resistant cell lines, suggesting that the gene encoding for this cDNA was conserved across species (61). Subsequently, the pCHP1 clone was used by Endicott (62) and Ng (63) to isolate cDNA clones from a Chinese hamster drug sensitive cell line, $E_{29}Pro^{+}$. Three partial but distinct cDNA clones were isolated and designated pgp1, pgp2, and pgp3. The determination of the biological activity of these genes

awaits the isolation of full-length cDNA clones.

The technique of differential hybridization was used by Van der Bliek et al. to isolate genes overexpressed in the colchicine resistant Chinese hamster cell line $CH^{R}C5$ (64). Five classes of cDNAs were identified based on the length of the corresponding mRNA transcripts detected by Northern analysis. Clones belonging to class 2 hybridized to an overexpressed 4.7 kb mRNA transcript and were also found to cross-hybridize to the P-glycoprotein cDNA clone, pCHP1. These class 2 genes were the only consistently amplified genes in independently derived multidrug resistant Chinese hamster cell lines (65). A cluster of three genes, designated 2a, 2b and 2c was present within the class 2 domain. These three genes have been shown to correspond to the three hamster pgp genes isolated by Endicott and Ng (66).

Human cDNAs were isolated by two different groups. Ueda et al. isolated a set of partial cDNA clones from the colchicine resistant KB carcinoma cell line KB-C2.5 (67), using a human genomic mdr1 probe. When engineered into a mammalian expression vector, mdr1 was biologically active and could confer resistance to colchicine, vinblastine and Adriamycin to drug sensitive mouse or human cells (68). A second human mdr gene was identified in cDNA libraries prepared from human liver (69). This clone which was only detected in liver was designated mdr3 because of its

apparent homology to hamster pgp3. The full-length clone for mdr3 cannot confer multidrug resistance in transfection experiments (70).

Ng recently demonstrated the existence of a total of three mdr/pgp genes in rodents (mouse, hamster) and two mdr genes in primates (human, rhesus monkey and orangutan) (63). Sequence comparison of the cloned genes isolated from mouse, hamster and human origins indicates that the mouse mdr1, mdr2, and mdr3 genes correspond to the hamster pgp2, pgp3, and pgp1 genes, respectively. Human mdr1 and mdr3 genes are homologous to mouse mdr3 and mdr2, respectively. No counterpart to mouse mdr1 is detected in humans.

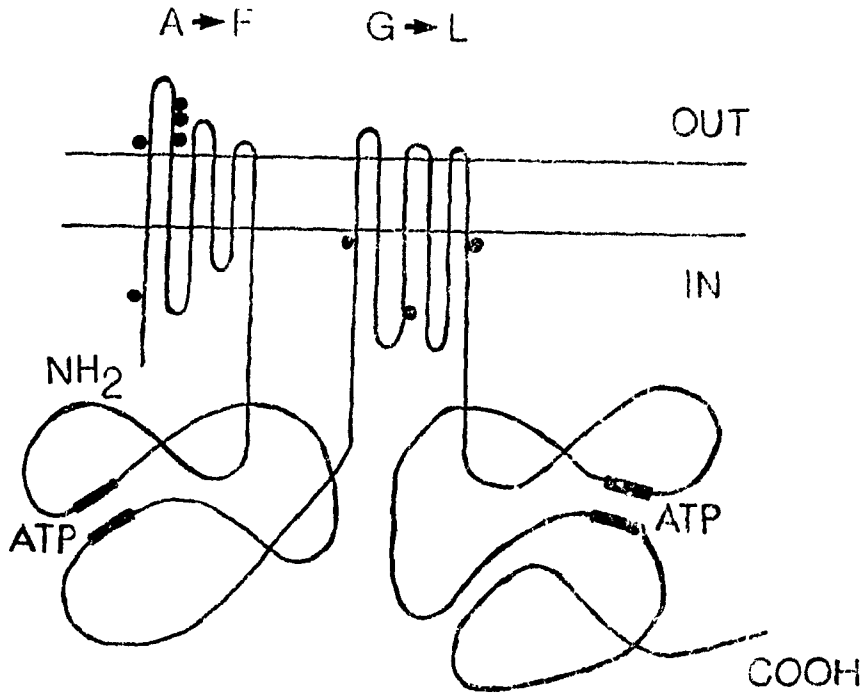
I.4 Model for MDR/P-glycoprotein

A schematic representation of the predicted structure of the mouse MDR1 protein deduced from nucleotide sequencing of the biologically active mdr1 cDNA clone is presented on Figure 2. This polypeptide contains 1276 amino acids and has a minimum molecular weight of 140 kDa (71). A cluster of putative N-linked glycosylation sites is present near the amino terminus of the MDR1 protein. A striking feature of this protein is the presence of a large internal duplication that consists of approximately 500 amino acids. Hydropathy analysis predicts the presence of three transmembrane loops within each duplicated segment. In

FIGURE 2

Schematic representation of the predicted mouse MDR1 protein

The predicted orientation of the protein in the cell membrane is presented. The putative glycosylation sites (filled dots), transmembrane domains and nucleotide-binding sites are indicated (reproduced from ref. 71).


A→F
G→L
OUT
IN
NH2
ATP
ATP
COOH

addition, a consensus sequence for nucleotide-binding (NBS) is found in each half of the protein. A gradient of sequence homology is observed between each duplicated segment of MDR1. The strongest sequence conservation is present near the carboxy terminus of each duplicated unit at the position of the NBS, where a sequence homology greater than 85% is observed. This suggests that strong selective pressure has been applied on these sequences during evolution and that very few amino acid substitutions can be tolerated at these sites to maintain activity. Therefore, these NBS likely encode domains that are functionally essential for expression of multidrug resistance. A similar model was deduced for the putative proteins encoded by the mouse mdr2 (58), mdr3 (A. Devault and P. Gros, in preparation), human mdr1 (72), mdr3 (70) and hamster pgp1 and pgp2 (62,73) cDNA clones.

The 200 amino acid region surrounding the putative NBS of MDR1 also shares striking sequence similarity with the energy-coupling subunits of several bacterial periplasmic transporters (71,74). These bacterial proteins are involved in the inward transport of various substrates across the inner membrane, such as oligopeptides (OppD, OppF) (75), histidine (HisP) (76), maltose (MalK) (77), ribose (RbsA) (78), phosphate (PstB) (79), molybdate (ChlD) (80) and vitamin B12 (BtuD) (81) [see ref. 82 for a review]. They are multicomponent systems typically composed of one

periplasmic binding protein and three membrane-bound polypeptides, two of which are hydrophobic and a third one which is more hydrophilic. This last component contains a putative NBS and corresponds to the energy-coupling subunit homologous to the mammalian MDR/P-glycoprotein.

An even stronger homology, extending well into the predicted transmembrane domains, is detected between the MDR/P-glycoprotein and the bacterial HlyB (83) and NdvA (84,85) proteins. HlyB is involved in the export of hemolysin, a 110 kDa toxin secreted by certain enterotoxic strains of E. coli (86,87), whereas NdvA is required for the export of ß-1,2-glucan, an important step in the production of nitrogen-fixing nodules in Rhizobium meliloti (84,85). This sequence similarity suggests a functional homology between bacterial transporter proteins and the mammalian MDR/P-glycoprotein, possibly in coupling energy to the transport process in agreement with the proposal that MDR/P-glycoprotein functions as an energy-dependent drug efflux pump.

A model for the evolution of the mdr/pgp gene family has been proposed by Gros et al. (58). An ancestral subunit encoding nucleotide-binding sequences would have produced the bacterial energy-coupling subunits. Addition of membrane-associated sequences to this ancestral element would have given rise to the precursor of HlyB and NdvA. A subsequent duplication event of this membrane-associated

energy-coupling subunit would have produced the mdr/pgp genes. In this respect, the recent cloning of an mdr gene from chloroquine resistant strains of the malaria parasite, Plasmodium falciparum is of interest (88). Increased chloroquine efflux has been observed in chloroquine resistant parasites (89,90). The predicted protein is duplicated and shares 54% of similar residues with the mammalian MDR/P-glycoprotein. The strongest homology is found at the putative NBS. Based on the homology with the bacterial permeases, Foote et al. proposed that the closely related mdr genes form a superfamily of transmembrane pumps (88).

The involvement of MDR/P-glycoprotein in transport is also suggested by its cellular localization in normal tissues. Expression of MDR/P-glycoprotein is detected at the apical surface of many detoxifying organs, such as liver, kidney and colon (91), at the luminal surface of the secretory epithelial cells of the pregnant uterus (92), and on endothelial cells at blood-brain barrier sites (93). These proteins might therefore be important for regulating the transport of specific molecules at different anatomical sites.

I.5 Molecular Mechanism of Action

The consensus sequence for nucleotide-binding found in

MDR/P-glycoprotein was first described by Walker (94). This sequence was derived by comparing the amino acid sequence of the α and ß subunits of bovine and E. coli F_1-ATPase to a number of enzymes that use ATP in catalysis, such as adenylate kinase, phosphofructokinase and myosin. This consensus sequence contains two elements, the A and B motifs, that are believed to come in close spacial proximity to form the nucleotide-binding pocket (95). The A motif features the sequence G-$(X)_4$-G-K-(T)-$(X)_6$-I/V. The glycine residues in the sequence are thought to form a flexible loop between an alpha helix and a beta strand (95). Upon ATP-binding, this glycine-rich loop can undergo a conformational change which is believed to influence access of the nucleotide to the substrate binding cleft (96-98). The conserved lysine residue following this loop is most likely involved in binding one of the phosphate groups of ATP (99). NMR and x-ray crystallography studies suggest that the alpha phosphate of ATP interacts with this lysine (95,96). The B motif features the sequence R/K-$(X)_3$-G-$(X)_3$-L-$(Hyd)_4$-D. It forms a hydrophobic beta-sheet structure at the back of the nucleotide-binding pocket (94,95) that would interact with the adenine-ribose moiety of the ATP molecule. The hydrophobic residues might also help to exclude water from the reaction center to minimize hydrolysis (96). The aspartate residue flanking this sequence is thought to be involved in binding the magnesium

ion of MgATP (94).

The ATP-binding ability of MDR/P-glycoprotein was investigated in cross-linking assays with the photoactivatable analog of ATP, 8-azido ATP (100,101). In the presence of UV light the azido moeity forms a very reactive, short-lived nitrene group that can covalently bind to nearby amino acid residues. A 170 kDa photoaffinity labelled protein was immunoprecipitated from membrane preparations of the human multidrug resistant cell line KB-V1 selected in vinblastine (100) and in a multidrug resistant Chinese hamster cell line transfected with the mouse mdr1 cDNA (101). Tryptic mapping of the photolabelled mouse MDR1 protein revealed that the cross-linking was specific and limited to two discrete fragments of the protein (101). This result suggests that the two putative NBS of mouse MDR1 may be capable of binding ATP.

The 170-180 kDa P-glycoprotein overexpressed in the human drug resistant cell line K562/ADM selected in Adriamycin was recently purified by immunoaffinity chromatography using the monoclonal antibody MRK16 (102,103). This purified protein displayed ATPase activity, providing strong evidence that ATP hydrolysis by P-glycoprotein might be coupled to active drug efflux. Because of the strong sequence conservation of the putative NBS during evolution, these consensus sites are likely to play an important role in this mechanism.

To determine if P-glycoprotein is capable of direct binding to drug molecules, two radioactive, photoactive derivatives of vinblastine, NABV and NASV, were synthesized (104). These drug analogs were used to photoaffinity label membranes of highly resistant Chinese hamster lung cell lines selected in vincristine (DC-3F/VCRd-5L) or actinomycin D (DC-3F/ADX) (104). A photolabelled 150-180 kDa doublet was detected in the resistant cell lines but not in the parental drug sensitive cell line DC-3F and could be immunoprecipitated with a specific polyclonal antibody. Addition of a 200 fold excess of vinblastine could inhibit virtually all photoaffinity labelling. The vinblastine analogs NABV and NASV could also label a 150-170 kDa protein in membranes of drug resistant human KB cells selected in colchicine (KB-C4) or vinblastine (KB-V1) (105). Photoaffinity labelling was inhibited by excess vinblastine or vincristine but not by similar excess of colchicine. These experiments suggest that P-glycoprotein is capable of interacting directly with drug molecules, in agreement with the drug efflux proposal. The results also indicate that P-glycoprotein might possess two distinct drug-binding sites.

The transport of [^{3}H] vinblastine into membrane vesicles of drug resistant KB-V1 cells was investigated (106). Vinblastine transport was ATP-dependent and occurred against a concentration gradient. The nonhydrolyzable ATP

analog, 5'-[ß,γ-imido]triphosphate acted as a competitive inhibitor during the transport process. The binding of [^{3}H] vincristine to membranes of the human drug resistant K562/ADM cell line was also ATP-dependent (107) and was abolished in the presence of a nonhydrolyzable analog of ATP. The dissociation constant of vincristine binding was 40 times lower in the absence of ATP than in the presence of 3 mM ATP. These results suggested that ATP hydrolysis was coupled to decreased drug uptake in these cells and led Naito to suggest that hydrolysis of ATP by P-glycoprotein exposes high affinity drug-binding sites on the protein (107).

Finally, the ability of MDR/P-glycoprotein to bind agents that reverse the multidrug resistance phenotype, such as the calcium channel blockers azidopine and verapamil, was also tested. These agents are small, hydrophobic, positively charged molecules similar in size to the drugs belonging to the multidrug resistance spectrum. Many of these compounds such as verapamil, reserpine and quinidine can also effectively inhibit P-glycoprotein photoaffinity labelling by the vinblastine analogs, NABV and NASV (108). Three independent studies demonstrated [^{3}H] azidopine binding to membrane vesicles of multidrug resistant hamster (109) and mouse cells (110) and of a stable mouse mdr1 transfectant (101). In all cases binding was competed by a number of nonradioactive calcium

channel blockers including nimodipine, nicardine, nifedipine and verapamil (101,109,110). Almost complete inhibition of labelling could be achieved with excess vinblastine while a much weaker inhibition was observed with actinomycin D, Adriamycin and colchicine. The binding of [^{3}H] verapamil and of two photoactivatable radioactive analogs of verapamil was also reported in multidrug resistant human KB-V1 and Chinese hamster DC-3F/VCRd-5L cells (111,112). Together these studies indicate that the antagonist effects of these calcium channel blockers are likely due to the direct interaction of these agents with MDR/P-glycoprotein. Therefore this protein can recognize and bind a number of different substrates, in agreement with the hypothesis that MDR/P-glycoprotein is an efflux pump of broad specificity.

Putative drug-binding sites on MDR/P-glycoprotein have not yet been identified. However, Choi recently reported that substitution of a glycine for a valine residue at amino acid position 185 of the human MDR1 protein was responsible for preferential resistance to vinblastine over colchicine (113). According to the predicted protein model (72), residue 185 is located within the first transmembrane domain of MDR1. Since the drugs belonging to the multidrug resistance phenotype are hydrophobic, it is possible that they exhibit affinity for hydrophobic segments of the MDR1 protein.

The exact molecular mechanism of action of the MDR/P-glycoprotein remains elusive. Based on the experimental evidence presented here, it seems reasonable to postulate that the MDR/P-glycoprotein interacts directly with drug molecules and mediates drug extrusion by coupling the energy generated by ATP hydrolysis to the transport process. The recent report that a stable transfected cell clone of the biologically active mouse mdr1 gene displays ATP-dependent drug efflux properties (114) similar to those initially described in multidrug resistant cells derived in vitro (22,24,32), reinforces the notion that MDR/P-glycoprotein functions as an energy-dependent drug efflux pump. Therefore, mutational analysis of specific amino acid residues of MDR/P-glycoprotein might allow us to gain further insight into the intricate function of this protein.

CHAPTER II

MATERIALS AND METHODS

II.1 MATERIALS

Oligonucleotides were synthesized by H. Theoret (McGill University, Montreal, Quebec). E. coli strains, JM105 and RZ1032 were provided by J. Pelletier (McGill University, Montreal, Quebec). The Chinese hamster ovary cell line LR73 was a gift of C. P. Stanners (McGill University, Montreal, Quebec). The expression vector p91023b was a gift of R. Kaufman (Genetics Institute, Cambridge, MA). The antibody C219 was provided by V. Ling (Ontario Cancer Institute, Toronto, Ontario) and the antibody ES4 was prepared by E. Schurr (McGill University, Montreal, Quebec).

Restriction enzymes, agarose, nucleotides, Ficoll 400 and Protein A-Sepharose were purshased from Pharmacia (Baie d'Urfe, Quebec). Klenow fragment of DNA polymerase I of E. coli (6 units/μl) and En^{3}Hance were obtained from New England Nuclear (Lachine, Quebec) and T4 DNA ligase (400 units/μl) from New England Biolabs (Beverly, MA). All tissue culture ware and tissue culture media were from Gibco (Burlington, Ontario). DTT, EDTA, BSA, Tris, salmon sperm DNA, trasylol, leupeptin and pepstatin were obtained from Boehringer Mannheim (Indianapolis, IN). Colchicine, guanidine hydrochloride, polyvinyl pyrollidone and sodium deoxycholate were purshased from Sigma (St-Louis, MO). Phenol was from Aldrich (Milwaukee, WI), Chloroform and

SDS from Anachemia (Lachine, Quebec) and formamide from Fluka (Ronkonkoma, NY). Acrylamide and the Biorad Protein assay were obtained from Biorad (Mississaugua, Ontario). The anti-rabbit second antibody and the substrates NBT and BCIP were purshased from Biocan (Mississaugua, Ontario). Silicone oil was obtained from Armstrong Packaging (Richmond Hill, Ontario). Adriamycin was obtained from Adria (Mississaugua, Ontario). Sucrose (enzyme grade) was from Bethesda Research Laboratories (Gaithersburg, MD) and sodium citrate from BDH (Toronto, Ontario). [α-^{32}P] dATP (3000 Ci/mmol), [^{35}S] Methionine (800 Ci/mmol) and [^{3}H] vinblastine sulfate (23 Ci/mmol) were obtained from Amhersham (Arlington Heights, IL). [α-^{32}P] 8-azido ATP (7 Ci/mmol) was purshased from ICN Biomedicals (Montreal, Quebec). Diethylpyrocarbonate and X-AR autoradiographic films were from Eastman-Kodak (Rochester, NY) and intensifying screens from Dupont (Mississaugua, Ontario). All reagent grade chemicals and solvents were purshased from Fisher Scientific (Fair Lawn, NJ).

II.2 METHODS

II.2.1 General Cloning Procedures

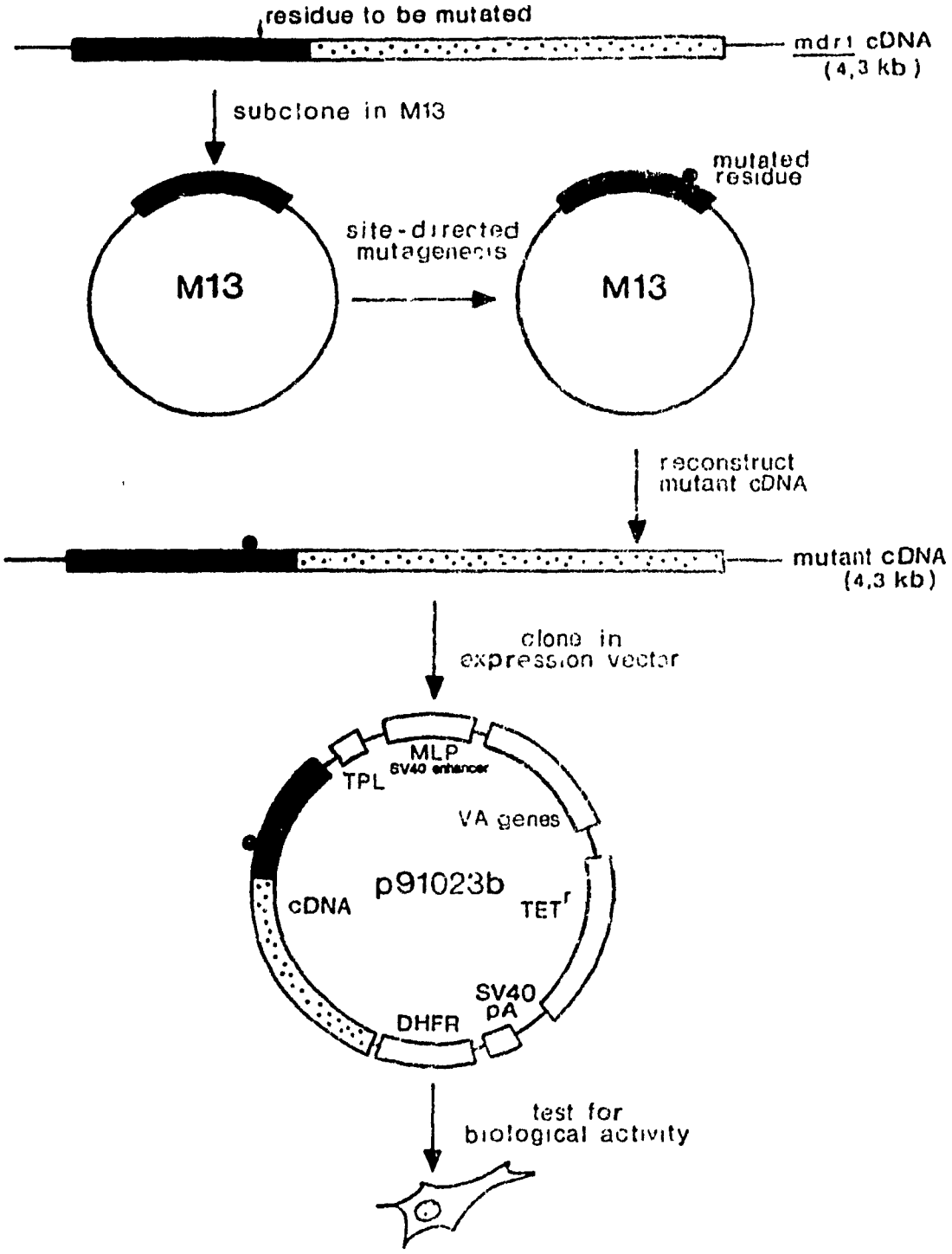
Restriction enzyme digestion of DNA, agarose gel electrophoresis, DNA ligation and large scale purification of plasmid DNA were performed according to standard protocols (115). DNA fragment extraction from agarose gels was carried out according to Langridge et al. (116).

II.2.2 Site-directed Mutagenesis

The general mutagenesis strategy is outlined in Figure 3. Briefly, two fragments of the 4.3 kb mouse mdr1 cDNA containing the amino terminal (1.7 kb SmaI fragment) or the carboxy terminal NBS (1.4 kb KpnI fragment) were independently subcloned in phage M13mp18 or mp19. The following oligonucleotides were used for site-directed mutagenesis: 5'CCGACACGTTTTTCG 3' (G to A, mut55), 5'GACACCTTCTTCGTGTTG 3' (K to R, mut85), 5'GGCTGTGCGAAGAGC 3' (G to A, mut53), 5'GCTGTGGAAGGAGCACAG 3' (K to R, mut83), 5'GCAAAGAAATAAGGCAACTG 3' (K to R, mut20) and 5'GCTTTTTCCACAGCC 3' (A to G, rev15). Site-directed mutagenesis was performed essentially by the two primer method described by Zoller and Smith (117). Briefly, single-stranded phage DNA was prepared from recombinant M13

FIGURE 3

General strategy for the construction of mutant mdr1 cDNA clones


residue to be mutated
mdr1 cDNA
(4,3 kb)
subclone in M13
mutated
residue
site-directed
mutagenesis
M13
M13
reconstruct
mutant cDNA
mutant cDNA
(4,3 kb)
clone in
expression vector
MLP
SV40 enhancer
TPL
VA genes
p91023b
cDNA
TET^r
SV40
pA
DHFR
test for
biological activity

constructs by infecting the dut-, ung- strain of E. coli, RZ1032. This allows production of phage in which several thymidine are replaced by uracil residues and increases the efficiency of mutagenesis by about 10 fold compared to current methods (118). Single stranded phage was isolated by polyethylene glycol 8000/NaCl precipitation according to standard protocols (119). Twenty pmol of 5' phosphorylated mutagenic oligonucleotide and 10 pmol of M13 sequencing primer (5'TCCCAGTCACGACGT 3') were mixed with one pmol of single-stranded template DNA in annealing buffer (20 mM Tris, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT pH 7.5) in a final volume of 10 μl. The mixture was then heated for 5 minutes at 90 C and allowed to anneal for 30 minutes at 20 C. Extension/ligation followed by adding 10 μl of solution C (20 mM Tris, 10 mM $MgCl_2$, 10 mM DTT, 1 mM each dNTP (dATP, dCTP, dGTP, dTTP), 1 mM ATP, 400 units T4 DNA ligase and 20 units of DNA polymerase I) to the annealed DNA and incubating the reaction for one hour at 30 C. A second 10 μl aliquot of solution C was added and the mixture was further incubated for 3 hours at 30 C. Competent JM105 prepared according to Hanahan (120) were transformed with 10 μl of mutagenic mixture. Mutant phages were identified by differential plaque hybridization to the appropriate radiolabelled mutagenic oligonucleotide. Presence of the desired mutation was verified by Maxam-Gilbert (121) or Sanger dideoxy sequencing (122). The revertant clone rev15

was generated by mutagenizing the mut55 mutant cDNA to the wild-type mdr1 sequence. Full-length mutant mdr1 cDNAs were reconstructed by inserting the mutagenized fragments into the full-length wild-type mdr1 cDNA. The double mutant mut88 was produced by replacing the KpnI subclone of mut83 into the full-length mut85. The biological activity of mutated cDNAs was tested after cloning into the unique EcoRI site of the mammalian expression vector p91023b. This vector system uses the major late promoter of Adenovirus and SV40 enhancer to direct high levels of expression of cloned cDNAs, as well as the Adenovirus VA genes and tripartite leader to increase translation of exogenous messages (123) and has been used previously to express mdr1 (57).

II.2.3 Cell Culture and Transfection

All cell lines were cultured at 37 C in 5% CO_2 in alpha minimal essential medium supplemented with 10% fetal calf serum, 2 mM glutamine, 50 units/ml penicillin and 50 μg/ml steptomycin. Mutated or control wild-type (sense and antisense orientation) mdr1 cDNAs cloned into p91023b were introduced by transfection into drug sensitive Chinese hamster ovary LR73 cells (124) as calcium phosphate coprecipitates according to standard protocols (125). Briefly, LR73 cells (10^6 cells per 100 mm dish) were

exposed for 20 minutes at 20 C to a DNA-calcium phosphate coprecipitate containing 10 μg of circular plasmid DNA in 0.50 ml Hepes buffered saline (140 mM NaCl, 5 mM KCl, 5 mM glucose, 25 mM Hepes, 0.70 mM sodium phosphate pH 7.05). After a 4 hour incubation at 37 C in complete medium, the cells were shocked for 3.5 minutes at 37 C in 15% glycerol/Hepes buffered saline (v/v). Expression of the multidrug resistance phenotype was tested by subculturing the transfected cells in medium containing either Adriamycin (80 ng/ml) or colchicine (200 ng/ml) for a period of three weeks. The mutant and wild-type mdr1 cDNA clones were also introduced in LR73 cells by cotransfection with the dominant selectable marker Neo (transposon Tn5) gene contained in the mammalian expression vector pSV2-Neo (126). A 1:10 molar ratio of pSV2-Neo to test plasmid DNA was used for transfection and Neo^r colonies were selected and maintained in medium containing the antibiotic G418 at 0.50 mg/ml. Mass populations and individual $G418^r$ clones were harvested with trypsin 14 days after transfection and aliquots were kept frozen at -90 C in complete medium containing 10% DMSO. Individual clones expressing mutant MDR1 proteins were conserved as several frozen aliquots and a fresh tube was thawed out for every subsequent experiment. The cell lines #8 and 1A used as positive controls in our assays are multidrug resistant cell clones obtained by transfection of LR73 cells with mdr1 and have

been previously described (57).

II.2.4 Drug Survival Measurements

The drug survival characteristics of mass populations of G418^r cells cotransfected with the mutant or wild-type mdr1 cDNA clones were determined by plating 20 000 cells in 24-well titer dishes in medium containing increasing concentrations of either Adriamycin (0, 10, 20, 50, 100, 200 ng/ml) or colchicine (0, 20, 40, 100, 200, 400 ng/ml). Two weeks later the cells were fixed in 4% formaldehyde and stained with 1% methylene blue. For drug survival assays on individual G418^r clones expressing mutant or wild-type MDR1 proteins, 500 cells were plated in triplicate on 60 mm dishes in medium containing increasing concentration of either Adriamycin (0, 5, 10, 20, 30, 50, 100, 200, 500 ng/ml) or colchicine (0, 10, 20, 40, 60, 100, 250, 500, 1000 ng/ml). One week later the cells were fixed, stained and colonies containing more than 50 cells were scored. The results are expressed as the percentage of cells surviving at a given drug concentration compared to control dishes containing drug free medium.

II.2.5 Northern Analysis

Total cellular RNA was extracted from individual G418^r

clones as described elsewhere (127). Briefly, cells grown to 70% confluency in one T-75 cm^2 flask were lysed on ice in a mix containing 5.7 M guanidine hydrochloride and 100 mM potassium acetate pH 5.0 (prepared in water containing 0.1% diethylpyrocarbonate), sonicated and precipitated with ethanol. Differential precipitation of RNA over DNA was performed by resuspending the pellet in a solution of 5.4 M guanidine hydrochloride, 95 mM potassium acetate and 25 mM EDTA pH 7.0 followed by a second ethanol precipitation. The RNA pellet was then phenol/chloroform extracted. RNA yields were determined by measuring the optical density at 260 nm. For Northern hybridization, 10 μg of total cellular RNA was serially diluted (1:1) and applied to a Hybond-N membrane using a BRL manifold apparatus. Prehybridization was performed for 6 hours at 42 C in 50% formamide, 5 X SSPE (900 mM NaCl, 50 mM sodium phosphate, 5 mM Na_2EDTA pH 7.7), 5 X Denhardt's (0.1% BSA, 0.1% polyvinyl pyrollidone, 0.1% Ficoll 400) and 20 μg/ml denatured salmon sperm DNA. A specific mdr probe was prepared by labelling an internal BglII fragment of mdr1 to high specific activity by the oligo-labelling technique described by Feinberg and Vogelstein (128). Hybridization was carried out at 42 C for 2 days with 10^6 cpm/ml of the mdr-specific probe in the same solution as for the prehybridization. The blots were washed in 2 X SSC (300 mM NaCl, 30 mM sodium citrate), 0.1% SDS for 30 minutes at 20 C, then in 2 X SSC, 0.1% SDS for

30 minutes at 55 C and finally in 0.1 X SSC and 0.1% SDS for 30 minutes at 55 C and exposed overnight with an intensifying screen at -80 C on Kodak X-AR autoradiographic film.

II.2.6 Membrane Preparations

Plasma membrane extracts were prepared as follows. Cells clones were grown to confluency in three 150 mm culture dishes, washed twice with TNE (10 mM Tris, 100 mM NaCl, 10 mM EDTA pH 7.5) and homogenized on ice in hypotonic buffer (10 mM Tris pH 7.0, 1 mM $MgCl_2$) containing protease inhibitors (Aprotinin 2 μg/ml, Leupeptin 5 μg/ml, Pepstatin 0.4 μg/ml) with a Dounce homogenizer. Membrane vesicles were separated from intact cells and nuclei by centrifugation at 2,500 xg for 5 minutes at 4 C. The supernatant was spun at 50,000 xg for 30 minutes at 4 C and the membrane pellet was washed in 1 mM Tris pH 8.0. This crude membrane preparation was further purified by resuspending it in 45% sucrose and loading it onto a discontinuous density gradient of 60%, 45%, 35% and 30% sucrose followed by centrifugation at 150,000 xg for 3 hours at 4 C. Membrane vesicles present at the 30%, 35% and 45% interfaces were collected, washed in 1 mM Tris pH 8.0 and stored frozen at -90 C in TNE containing 40% glycerol.

II.2.7 Detection of Mutants Proteins

Mutant or wild-type MDR1 proteins were identified in cotransfected cell clones by immunoprecipitation of whole cell lysates labelled in vivo with [^{35}S] Methionine. Briefly, one million cells plated on a 60 mm culture dish were incubated overnight in the presence of 40 μCi/ml of [^{35}S] Methionine in alpha minimal essential medium lacking methionine, 10% dialysed fetal calf serum and 2 mM glutamine. Labelled cells were harvested with a rubber policeman in PBS, lysed 30 minutes at 4 C in 0.50 ml of immunoprecipitation buffer (20 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% sodium deoxycholate) and immunoprecipitated overnight at 4 C with 20 μl of the polyclonal antiserum ES4. Immune complexes were purified with 50 μl of Protein A-Sepharose beads, resolved on a 7.5% polyacrylamide gel according to Laemmli (129) and detected by fluorography (130). The samples were not heated prior to loading on the gel. For immunoprecipitation of 8-azido ATP photolabelled MDR1 proteins, this same procedure was used except that membrane preparations were immunoprecipitated with the monoclonal antibody C219 (10 μg/ml) in a buffer containing 10 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA and 1% Triton. The samples were autoradiographed with an intensifying screen at -80 C. Western analysis was performed essentially as described by Towbin et al. (131), using a 1/50 dilution

of the polyclonal antibody ES4. Immune complexes were detected by incubation with an anti-rabbit second antibody coupled to alkaline phosphatase and developed in alkaline phosphatase buffer (100 mM Tris pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) with the substrates NBT and BCIP.

II.2.8 Drug Accumulation

The capacity of the drug sensitive LR73 cells, the multidrug resistant positive control 1A clone and the $G418^r$ cotransfected cell clones expressing the different MDR1 proteins to accumulate [^{3}H] vinblastine was determined by a method that we have previously described (132). Briefly, 50 million cells grown to 75% confluency were harvested with trypsin, allowed to stand 2 hours at 20 C in complete culture medium and incubated in PBS containing 1 mM glucose, 1 mM glutamine and 20 nM [^{3}H] vinblastine sulfate. At specific time points cell-associated drug molecules were separated from free drug molecules by layering an aliquot of the mixture (10^6 cells) onto a silicone oil:mineral oil cushion (4:1, v/v) and centrifugation for 10 s at 12,000 xg. The aqueous phase and oil were removed and the cell pellet was digested overnight at 20 C in 1 N NaOH. The cell-associated radioactivity was counted by liquid scintillation. The protein content of the pelleted cells was determined according to Bradford (133). The results are

expressed as the percentage of [^{3}H] vinblastine accumulated in the different cell lines (calculated in pmol VBL/mg protein) compared to the maximum amount of [3H] vinblastine accumulated in the drug sensitive cell line LR73.

II.2.9 Photoaffinity Labelling

Photoaffinity labelling of mutant and wild-type MDR1 proteins with [α-32P] 8-azido ATP was carried out essentially as described elsewhere (134). Briefly, sixty micrograms of purified membranes (1 mg/ml) were resuspended in reaction buffer (50 mM Tris pH 7.5, 50 mM KCl, 2 mM magnesium acetate, 0.1 mM DTT, 6% glycerol) in the presence of 10 μCi of [α-32P] 8-azido ATP and UV irradiated on ice for 10 minutes with a 15-watt General Electric G15T8 germicidal lamp at a distance of 5 cm. Photoaffinity labelled MDR1 proteins were immunoprecipitated with the monoclonal antibody C219 and analyzed by SDS-PAGE. Competition of 8-azido ATP photoaffinity labelling was performed under the same conditions except that a 10 or 100 molar excess of unlabelled ATP or ADP was present in the reaction mixture.

CHAPTER III

RFSULTS

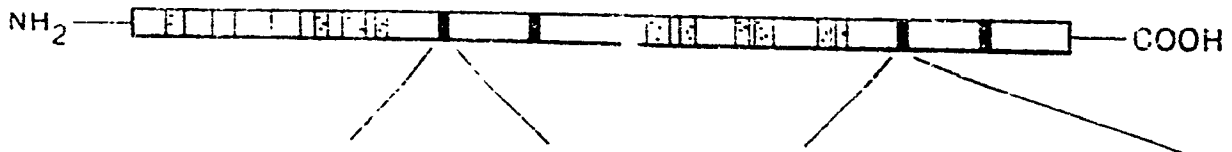
III.1 Construction of Mutant mdr1 cDNA clones

Site-directed mutagenesis was used to introduce discrete mutations within the A motif of the two predicted NBS of the biologically active mouse mdr1 cDNA. Subfragments overlapping these sites were first subcloned in M13 phage vector, mutagenized and reintroduced in the full-length wild-type cDNA. The amino acid substitutions engineered are depicted in Figure 4. The wild-type mdr1 cDNA contains two consensus GXGKST sequences described by Walker as the A motif of a nucleotide-binding fold (94). Within these sites, we targeted the two adjacent glycine and lysine residues (Figure 4) which are precisely conserved in a number of eukaryotic and prokaryotic proteins known to bind ATP (135, 136). Conservative glycine to alanine and lysine to arginine substitutions were introduced at these sites with the intent of interfering as little as possible with the overall three dimensional protein conformation. Mut55 and mut53 have a single glycine to alanine substitution at positions 431 and 1073 respectively, while mut85 and mut83 contain a single lysine to arginine substitution at positions 432 and 1074, respectively. The double mutant mut88 has both lysines 432 and 1074 replaced by arginine residues. Two additional mutants were included. First, to create a mutant mapping outside the core consensus sequence, lysine 1100, located

FIGURE 4

Effect of discrete mutations introduced at the NBS of the MDR1 protein on expression of drug resistance

A schematic representation of the predicted structural domains of the MDR1 protein is presented. The A and B motifs of the NBS consensus sequence are depicted as black boxes and dotted areas represent putative transmembrane domains. The positions of the amino acid residues targeted by site-directed mutagenesis are identified in the wild-type sequence and mutated residues are underlined in the mutants. All mutants were generated by using the wild-type sequence as a template except for the revertant rev15 where mut55 was used as a template. The capacity of the mutant cDNAs to confer (+) or not (-) multidrug resistance upon direct transfection into drug sensitive cells is indicated.



			multidrug resistance phenotype
wild-type	G C G K S T 431 432	G C G K S T.....K E I K 1073 107 1100	+
mut 55	G C A K S T	G C G K S T.....K E I K	-
mut 85	G C G R S T	G C G K S T.....K E I K	-
mut 53	G C G K S T	G C A K S T.....K E I K	-
mut 83	G C G K S T	G C G R S T.....K E I K	-
mut 88	G C G R S T	G C G R S T.....K E I K	-
mut 20	G C G K S T	G C G K S T.....K E I R	+
rev 15	G C G K S T	G C G K S T.....K E I K	+

26 residues downstream the conserved lysine of the A motif, was replaced by an arginine residue in mut20. Secondly, the overall mutagenesis procedure was tested by reverting the mutant alanine at position 431 in mut55 back to a glycine residue to recreate the wild-type sequence in rev15.

III.2 Effect of Mutations on the Biological Activity of mdr1

The wild-type and mutated mdr1 cDNAs were introduced in the expression vector p91023b and their ability to confer drug resistance was tested by transfecting the drug sensitive Chinese hamster cell line LR73, followed by drug selection in Adriamycin or colchicine. In this assay, the wild-type mdr1 cDNA and the revertant rev15 were able to confer multidrug resistance (Figure 4) whereas none of the 5 mdr1 mutant cDNAs harboring mutations within the A motif of either NBS (mut53, 55, 83, 85 and 88) displayed this ability. In contrast, the lysine to arginine substitution at position 1100 in mut20 did not alter expression of drug resistance by this clone (Figure 4).

The direct transfection and drug selection method used to test the activity of mdr1 mutants may not have allowed the detection of limited residual activity which might have been noticed under less stringent drug selection conditions. Thus, individual mutant mdr1 cDNAs (mut53, 55,

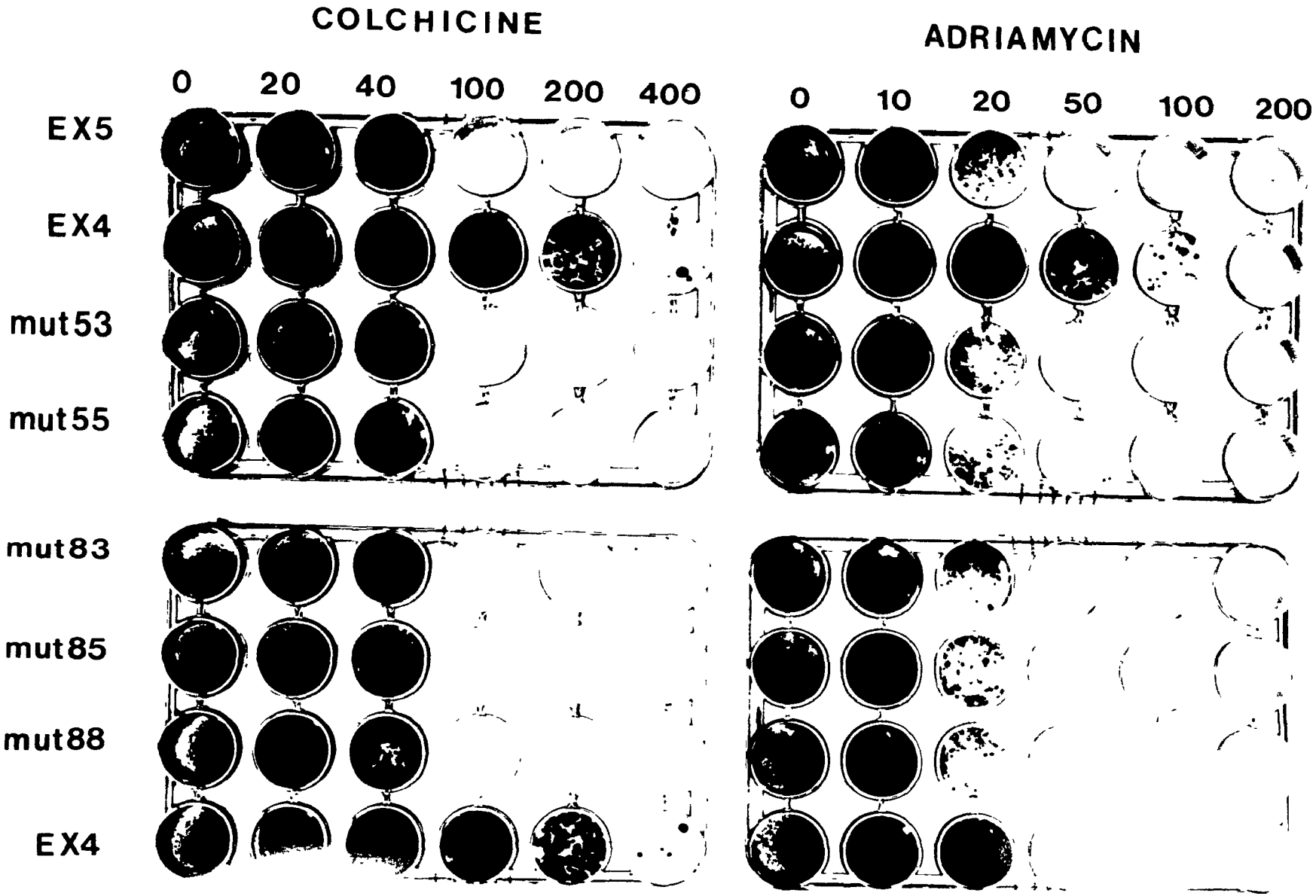
83, 85 and 88) were introduced in drug sensitive LR73 cells by cotransfection with the plasmid pSV2-Neo and mass populations of G418^r colonies were selected.

The drug survival characteristics of these mass populations were tested in medium containing increasing concentrations of the drugs Adriamycin or colchicine (Figure 5). G418^r mass populations cotransfected with the 5 inactive mdr1 mutants showed levels of Adriamycin and colchicine resistance indistinguishable from that of the control cell population cotransfected with the EX5 construct (wild-type mdr1 in antisense orientation). Only the mass population cotransfected with the control EX4 construct (wild-type mdr1 in sense orientation) was capable of forming colonies in medium containing higher drug concentrations (Adriamycin 100 ng/ml and colchicine 400 ng/ml). These observations confirm the results obtained by direct transfection and drug selection (Figure 4) and suggest that the glycine and lysine residues of the A motif of either NBS are essential for expression of multidrug resistance by the mdr1 gene. Even highly conserved amino acid substitutions cannot be tolerated at these positions. In addition, our results suggest that two intact NBS are required for the biological activity of mdr1 since mutations introduced in either of the two sites completely abolish their ability to confer drug resistance.

FIGURE 5

Drug survival characteristics of mass populations of G418^r cells cotransfected with mutant mdr1 cDNA clones

Twenty thousand cells from mass populations of G418^r cells cotransfected with control (EX4 and EX5) or mutant (mut53, 55, 83, 85 and 88) mdr1 cDNA clones were plated in each well and allowed to grow for two weeks in drug containing medium. Concentrations of Adriamycin and colchicine are expressed in ng/ml. The cotransfected controls EX5 and EX4 carry the wild-type mdr1 cDNA cloned into the mammalian expression vector p91023b in the antisense and sense orientation, respectively. The mutants tested in this assay are presented in Figure 4.


COLCHICINE
ADRIAMYCIN
0
20
40
100
200
400
0
10
20
50
100
200
EX5
EX4
mut53
mut55
mut83
mut85
mut88
EX4

III.3 Expression of Mutant MDR1 Proteins

Structural and functional characteristics of the biologically inactive mutant mdr1 cDNAs were further investigated. Individual G418^r clones cotransfected with mutant or wild-type mdr1 cDNA clones were isolated and tested for the presence of the corresponding mutant proteins. Candidate clones were initially analyzed by Southern blotting for the presence of the cotransfected mdr1 construct. The majority of clones tested in this assay contained the exogenous plasmid DNA, although the number of copies varied among the different clones (data not shown). Expression of mutant and wild-type mdr1 mRNA in cotransfectants was then analyzed by Northern blotting of total cellular RNA followed by hybridization with an mdr-specific probe (Figure 6). Very high expression of the transfected mdr1 gene is detected in the stable mdr1 transfectant clone #8 compared to the low level of endogenous hamster mdr gene in the parental drug sensitive cell line LR73. Variable mdr1 mRNA levels are expressed in individual cotransfectants typically represented by the EX4 and 88 series, transfected with the wild-type mdr1 and the double mutant mut88 cDNAs, respectively. A few number of clones, such as EX4N-7, EX4N-12, 88-2, 88-8, 88-11, 88-12 express high levels of specific mRNA.

Clones showing high levels of specific mdr1 mRNA, such

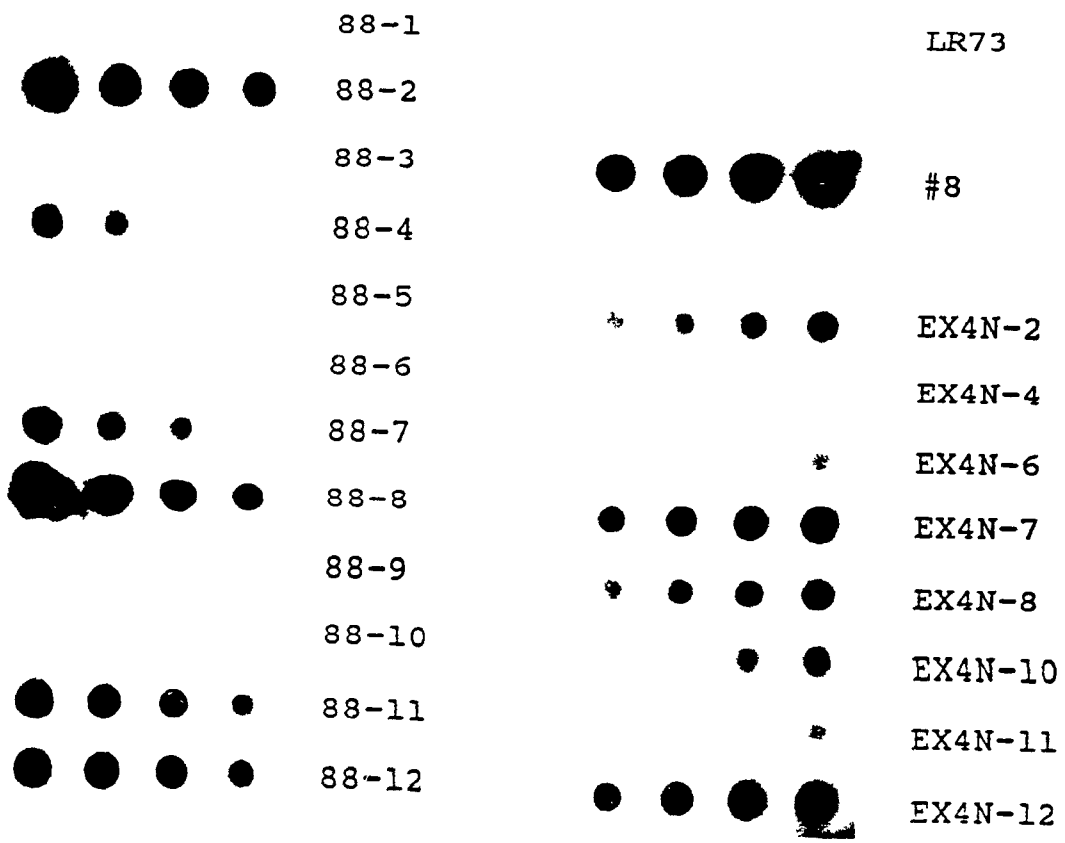
FIGURE 6

Northern analysis of mdr1 mRNA in individual G418^r cell clones

Ten micrograms of total cellular RNA were denatured, serially diluted (1:1) and applied to a Hybond-N membrane using a BRL manifold apparatus. The blot was probed with 10^6 cpm/ml of a ^{32}P labelled 1.4 kb internal BglII fragment of the wild-type mdr1 cDNA.

Upper panel: LR73 is the parental drug sensitive cell line. #8 is a stable transfectant of the wild-type mdr1 cDNA continuously maintained in 250 ng/ml of Adriamycin. EX4N-2, 4, 6, 7, 8, 10, 11 and 12 are individual G418^r clones cotransfected with the wild-type mdr1 cDNA in the sense orientation. Dilution factor is 1, 2, 4 and 8 starting from the top of the panel.

Lower panel: 88-1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 are individual G418^r clones cotransfected with the double mutant mut88. Dilution factor is 1, 2, 4 and 8 starting from the bottom of the panel.


88-1
88-2
88-3
88-4
88-5
88-6
88-7
88-8
88-9
88-10
88-11
88-12
LR73
#8
EX4N-2
EX4N-4
EX4N-6
EX4N-7
EX4N-8
EX4N-10
EX4N-11
EX4N-12

as EX4N-7, EX4N-12, 88-2 and 88-8 were further analyzed for the presence of the specific protein by metabolic labelling with [^{35}S] methionine and immunoprecipitation with the specific anti-MDR polyclonal antibody ES4 (Figure 7). A protein of molecular size of approximately 170 kDa is clearly visible in the stable mdr1 transfectant 1A clone but is undetectable in the parental drug sensitive LR73 cells. A protein of similar size is present in whole cell extracts of the cotransfected clones 88-2, 88-8, EX4N-7 and EX4N-12 but not in the EX5 control clone, cotransfected with the wild-type mdr1 cDNA in the antisense orientation. The clone EX4N-7, although expressing the same level of specific mdr1 mRNA as clone EX4N-12 (Figure 6) appears to produce much higher amounts of the MDR1 protein (Figure 7).

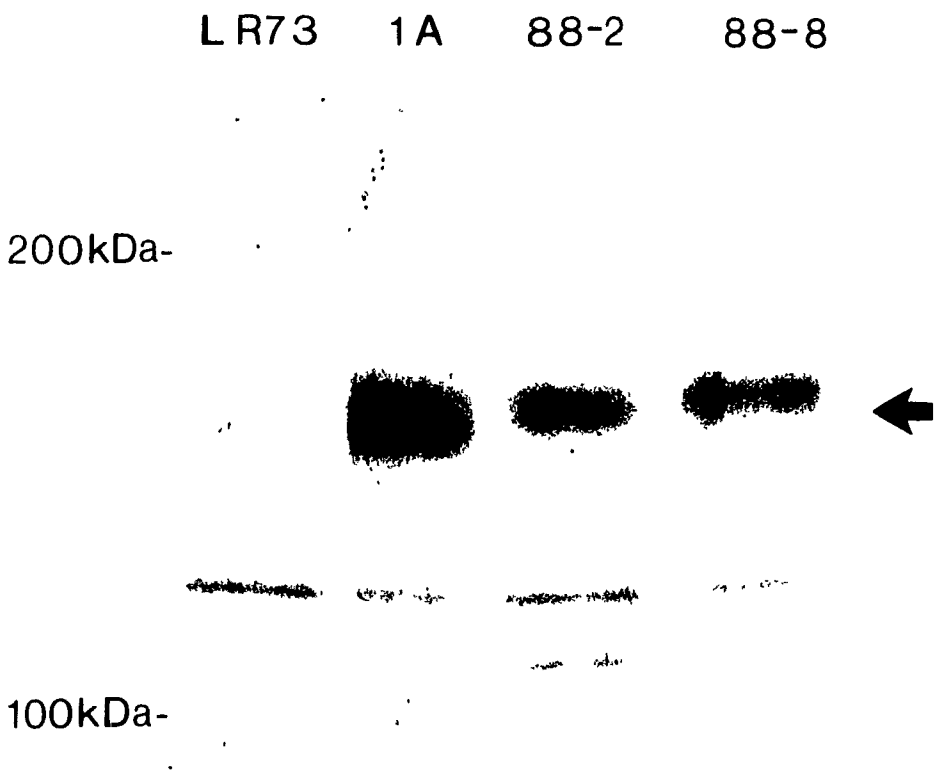
Cell membrane fractions were prepared from clones producing a clearly identifiable MDR1 protein and analyzed by immunoblotting with the specific polyclonal antibody ES4 (Figure 8). The MDR1 protein was undetectable in the parental drug sensitive cell line LR73 and very abundant in the positive control 1A clone. All mutant MDR1 proteins were readily detectable showing the expected size of approximatively 170 kDa. Wild-type and mutant MDR1 proteins were all found enriched in membrane fractions, suggesting that the mutations introduced in MDR1 did not impair its targeting to the cellular membrane compartment. Small variations in the electrophoretic mobility of mutant MDR1

FIGURE 7

Immunoprecipitation of mutant and wild-type MDR1 proteins in whole cell extracts of individual G418^r cell clones

Subconfluent cells were labelled overnight *in vivo* with [^{35}S] Methionine. Labelled cells were harvested, lysed in 1% deoxycholate and whole cell lysates were immunoprecipitated overnight in 1% deoxycholate with the polyclonal antiserum ES4. Immune complexes were purified with Protein A-Sepharose and analyzed by SDS-PAGE. The immunoreactive proteins of apparent molecular size of approximately 170 kDa are indicated by an arrow. The clones tested in this assay are presented in Figure 6 except for EX5-1 and EX5-5 which are two G418^r control clones cotransfected with the wild-type *mdr1* cDNA in the antisense orientation. LR73 is the parental drug sensitive cell line and 1A is a stable *mdr1* transfectant continuously maintained in Adriamycin at 250 ng/ml.

A


LR73
1A
88-2
88-8
200kDa-
100kDa-

B

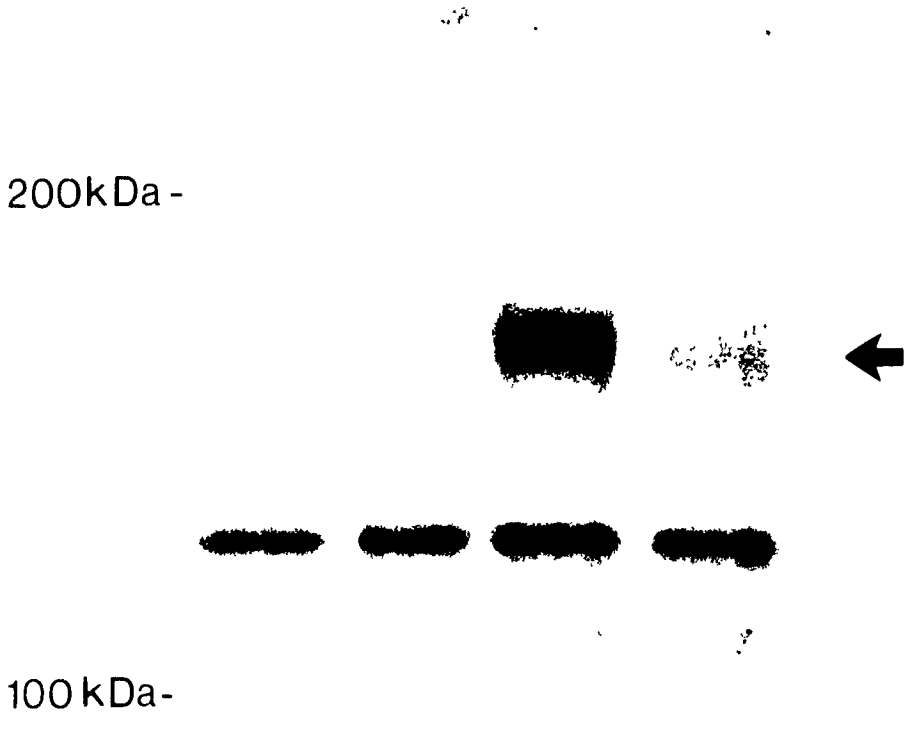
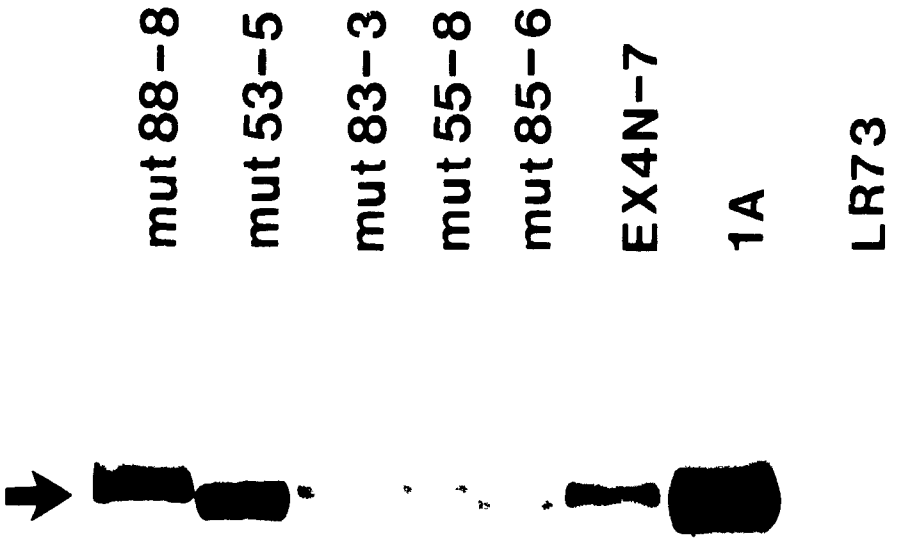

EX5-1
EX5-5
EX4N-7
EX4N-12
200kDa-
100kDa-

FIGURE 8

Western blot analysis of individual G418^r cotransfected cell clones expressing mutant MDR1 proteins

Fifty micrograms of membrane enriched fractions from individual cell clones isolated on a discontinuous sucrose gradient were separated by SDS-PAGE on a 7.5% polyacrylamide gel and transferred to a nitrocellulose membrane. The blot was incubated with a 1/50 dilution of the polyclonal antibody ES4 and immune complexes were revealed using a second anti-rabbit antibody coupled to alkaline phosphatase. The immunoreactive proteins of apparent molecular mass of approximately 170 kDa are indicated by an arrow. LR73 is the parental drug sensitive cell line. 1A is a stable mdr1 transfectant continuously maintained in Adriamycin at 250 ng/ml. EX4N-7, mut53-5, mut55-8, mut83-3, mut85-6 and mut88-8 are individual cotransfected clones maintained in G418.


mut 88-8
mut 53-5
mut 83-3
mut 55-8
mut 85-6
EX4N-7
1A
LR73

proteins were detected in some experiments (Figure 8). The reasons for these apparent differences are unknown but most likely involve experimental variations as they could not be systematically reproduced. The amount of each MDR1 protein produced by cotransfected cell clones varied among the different mutants. However, the control EX4N-7 clone produces similar amounts of the wild-type protein as the lowest MDR1 mutant producing clones (55-8, 83-3 and 85-6). This suggests that phenotypic changes detected between wild-type and mutant mdr1 expressing clones are most likely due to expression of a defective protein rather than to insufficient amount of a mutant but biologically inactive protein produced in these clones.

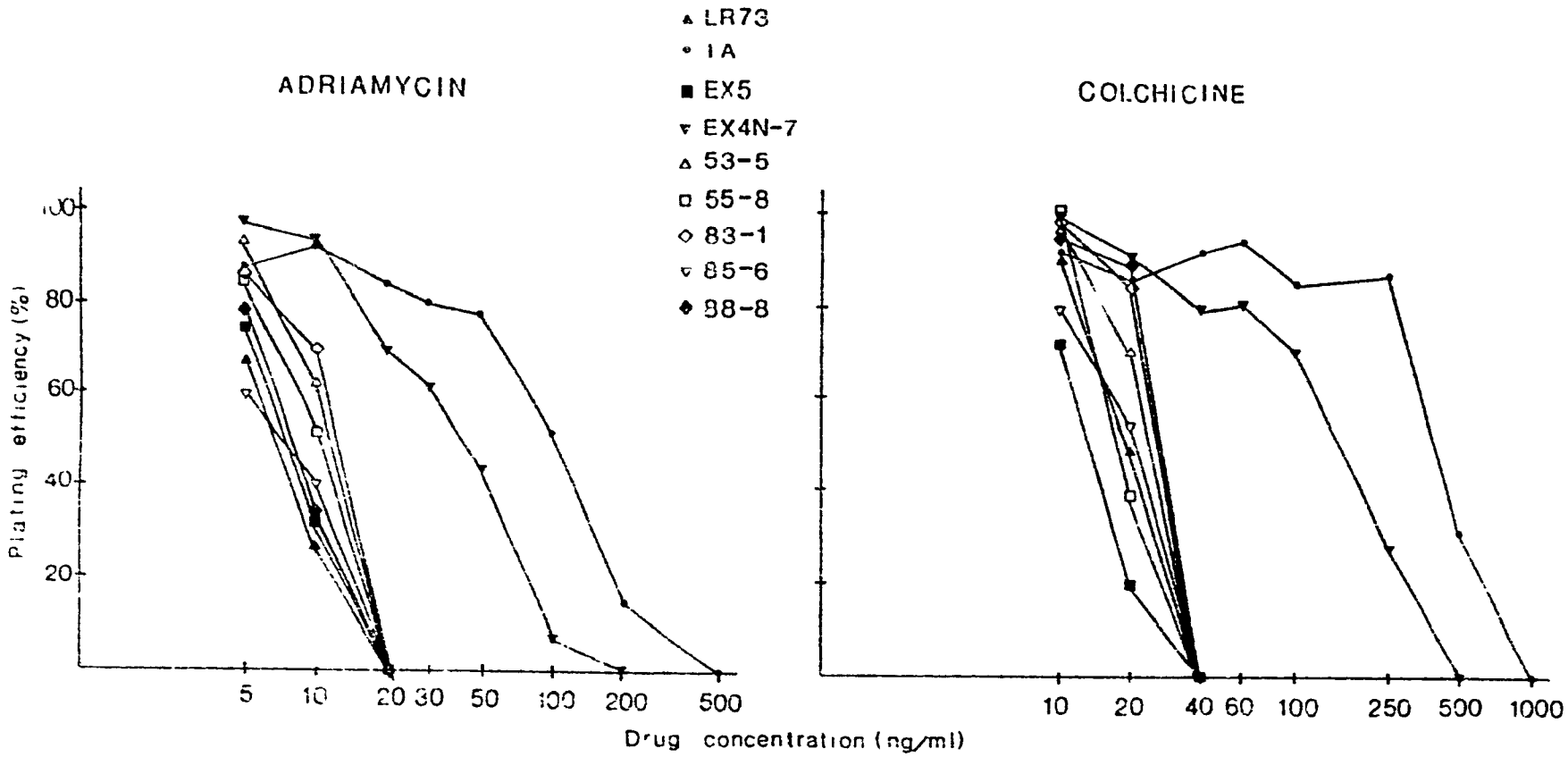
III.4 Drug Survival Characteristics of Cell Clones Expressing Mutant MDR1 Proteins

The drug survival characteristics of individual clones expressing the mutant MDR1 proteins were analyzed for the drugs Adriamycin and colchicine (Figure 9). The plating efficiency observed for the 5 inactive mdr1 mutants (53-5, 55-8, 83-1, 85-6 and 88-8) was very similar to that of both parental drug sensitive LR73 cells and the negative control clone, EX5. The D10 which represents the drug concentration necessary to kill 90% of the cells plated, was between 16 and 19 ng/ml for Adriamycin and between 30 and 45 ng/ml for

FIGURE 9

Drug survival characteristics of cell clones expressing individual mutant MDR1 proteins

Five hundred cells from each cell clone were seeded in triplicate in culture medium containing increasing drug concentrations and allowed to grow for one week. Colonies containing more than 50 cells were scored. Results obtained with the drugs Adriamycin and colchicine are presented on the left and right panel, respectively. The plating efficiency represents the percentage of cells surviving at a given drug concentration compared to control cells seeded in drug-free medium. The standard error for any given point does not exceed 10%. The cell lines are those presented in Figure 8 except for EX5 which is a $G418^r$ clone cotransfected with the wild-type mdr1 cDNA inserted in the antisense orientation in the mammalian expression vector p91023b.


ADRIAMYCIN
COLCHICINE
LR73
1A
EX5
EX4N-7
53-5
55-8
83-1
85-6
88-8
Plating efficiency (%)
100
80
60
40
20
5
10
20
30
50
100
200
500
10
20
40
60
100
250
500
1000
Drug concentration (ng/ml)

colchicine for the NBS mutants and the negative control cells. By contrast, the positive control clone EX4N-7 cotransfected with the wild-type cDNA showed a D10 value of 90 ng/ml in Adriamycin and 400 ng/ml in colchicine, for an overall level of resistance of 5 to 10 fold above that of the drug sensitive cells. The stable mdr1 transfectant 1A clone that produces very large amounts of the wild-type MDR1 protein (Figure 8) showed a D10 value of 270 ng/ml in Adriamycin and over 800 ng/ml in colchicine. These results indicate that although the mutant proteins are present in elevated amounts in the membrane fractions of individual cotransfected cell clones, their ability to confer multidrug resistance is abrogated.

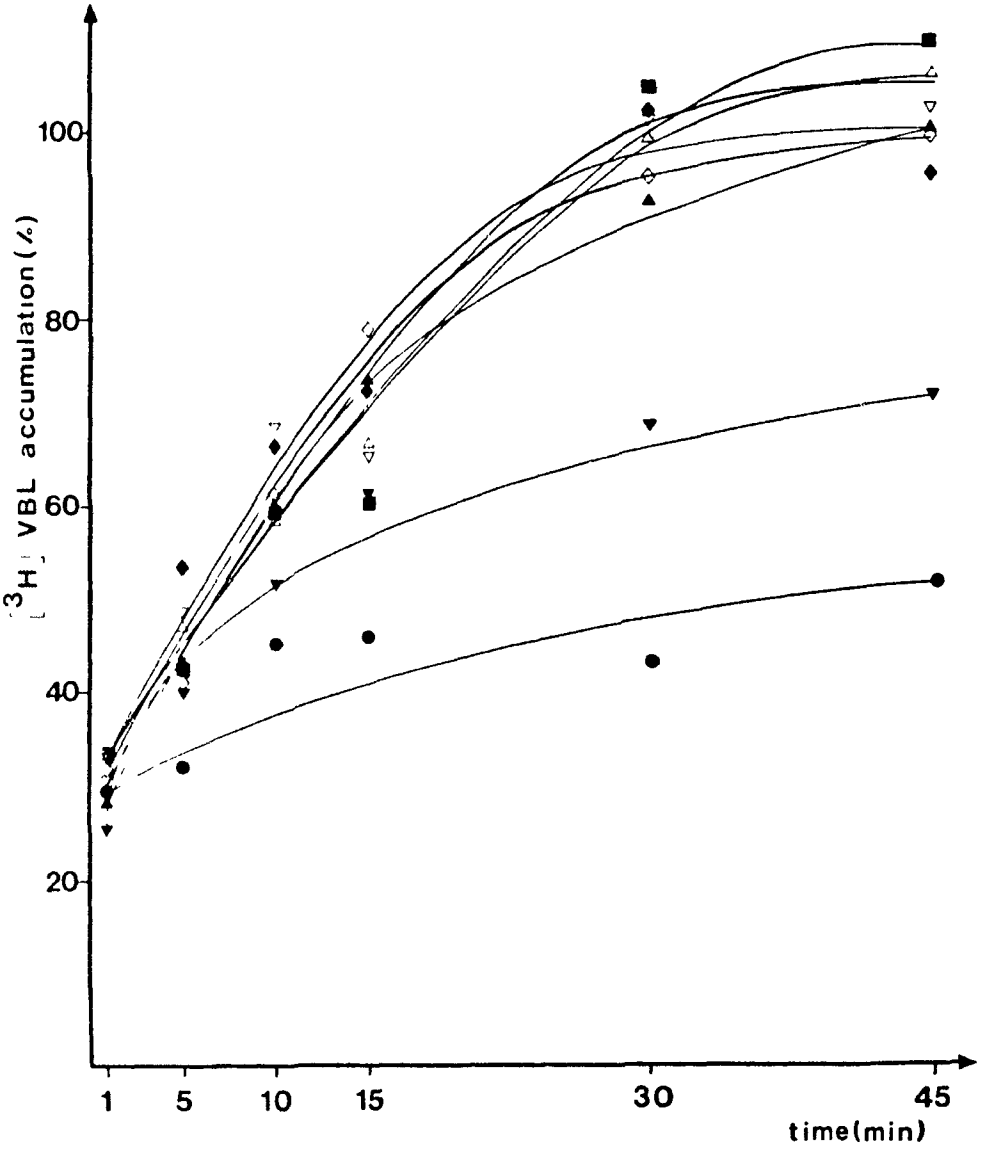
III.5 Drug Accumulation Characteristics of Cell Clones Expressing Mutant MDR1 Proteins

We have previously shown that LR73 cells transfected with the wild-type mdr1 cDNA can, over a 60 minute incubation period, maintain intracellular drug levels 2 to 3 fold lower than drug sensitive control cells (114). To determine whether the failure of the mdr1 mutants to confer drug resistance was linked to impaired capacity of these mutants to maintain decreased intracellular levels of drug, the kinetics of [^{3}H] VBL accumulation were measured in cotransfectants (Figure 10). At 1 minute, the amount of

FIGURE 10

[^{3}H] VBL accumulation in cell clones expressing individual mutant MDR1 proteins

Exponentially growing cells were harvested and incubated with [^{3}H] VBL at a final concentration of 20 nM. Increase in cell-associated radioactivity was monitored over time by centrifugation of the cell mixture through an oil cushion. The results are expressed as the percentage of [^{3}H] VBL accumulation (pmol VBL/mg protein) deduced from maximum drug accumulation in the parental drug sensitive LR73 cell line at 45 minutes. Each point was calculated from triplicate samples and the standard error for any of these points was within 10%. The cell lines used were LR73 (▲), 1A (●), EX4N-7 (▼), mut 53-5 (△), mut 55-8 (■), mut 83-1 (◇), mut 85-6 (▽) and mut 88-8 (◆).


100
80
60
40
20
[3H] VBL accumulation (%)
1
5
10
15
30
45
time(min)

accumulated [^{3}H] VBL was very similar in positive and negative controls and in cell clones expressing mutant MDR1 proteins. However, over the following 45 minute incubation period the kinetics of accumulation were very different in these cells. [^{3}H] VBL accumulation in the mutant cell clones (53-5, 55-8, 83-1, 85-6 and 88-8) was rapid and essentially identical to that observed in the parental line LR73. [^{3}H] VBL accumulation was slower in the transfected positive control cells (EX4N-7) and the maximum amount of [^{3}H] accumulated was 30% to 40% lower than that of all 5 mdr1 mutant and control drug sensitive cells. In the stable mdr1 transfectant 1A clone, [^{3}H] VBL accumulation was 40% to 50% lower than the drug sensitive controls or cell clones expressing mutant MDR1 proteins. Hence, the loss of biological activity of the mutant mdr1 cDNA clones is paralleled by an inability to lower intracellular drug levels in cell clones expressing the corresponding mutant MDR1 proteins.

III.5 ATP-Binding Characteristics of Mutant MDR1 Proteins

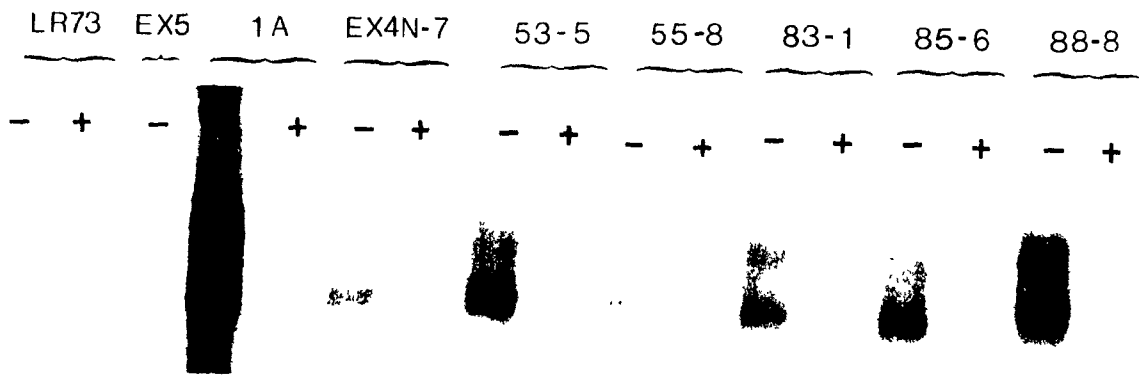
We determined if the impaired drug transport phenotype of the mutant MDR1 proteins was linked to a decreased capacity of these proteins to bind ATP. We have previously shown that the wild-type MDR1 protein present in membrane fractions of multidrug resistant 1A cells can be labelled

by the ATP photoaffinity analog, 8-azido ATP (101). We used this assay to analyze the ATP-binding properties of the mutant MDR1 proteins (Figures 11 and 12). Membrane fractions obtained from control cells and cotransfectant clones expressing mutant and wild-type MDR1 proteins were incubated with [α-^{32}P] 8-azido ATP, cross-linked with UV light and specific MDR1 proteins were immunoprecipitated with the monoclonal antibody C219 and analyzed by SDS-PAGE. A photolabelled protein of approximately 170 kDa was readily detectable in large amounts in the multidrug resistant cell clone 1A but not in the parental LR73 cells nor in the EX5 control clone (Figure 11). In membrane fractions of the multidrug resistant cotransfected clone EX4N-7 and of cell clones expressing the 5 inactive mutant MDR1 proteins (53-5, 55-8, 83-1, 85-6 and 88-8) a photolabelled polypeptide of electrophoretic mobility similar to that of wild-type MDR1 was observed. The labelling intensities were correlated by the amount of specific protein produced in each of the mutant clones as analyzed by parallel Western blotting of the membrane preparations (data not shown). A 100 molar excess of unlabelled ATP in the cross-linking reaction greatly reduced the amount of radioactivity incorporated in the wild-type or mutants 170 kDa species. These results indicate that even though the mutant MDR1 proteins can no longer confer drug resistance, they retain the property of

FIGURE 11

[α-^{32}P] 8-azido ATP binding characteristics of mutant MDR1 proteins

Sixty micrograms of membrane enriched fractions from individual cell clones isolated as described in Figure 8 were incubated with 10 μCi of [α-^{32}P] 8-azido ATP at a final concentration of 30 μM and irradiated with UV light. Specific MDR1 proteins were immunoprecipitated with the monoclonal anti-P-glycoprotein antibody C219, purified with Protein A-Sepharose and analyzed by SDS-PAGE. The (+) and (-) signs indicate respectively, the presence and absence of a 100 molar excess of unlabelled ATP in the cross-linking reaction. The cell lines used are as in Figure 9.


LR73
EX5
1A
EX4N-7
53-5
55-8
83-1
85-6
88-8
- + - + + - + - + - + - + - + - +

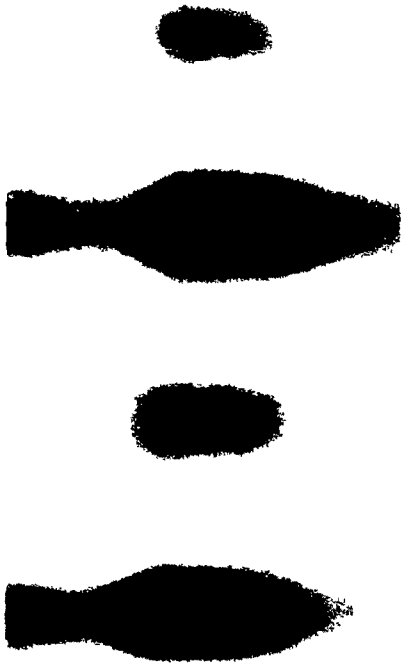
the wild-type protein to bind the ATP-analog, 8-azido ATP. For mutants carrying a point mutation at a single NBS (53-5, 55-8, 83-1, and 85-6), it is possible that the binding observed occurs at the other, unaltered, NBS. However, since the labelling intensities seem to correlate the amount of protein produced and since the double mutant 88-8 can still bind ATP, it is likely that the mutations introduced do not impair the ability to bind the ATP analog.

The characteristics of 8-azido ATP photolabelling of wild-type and mutant MDR1 proteins were further investigated. For these experiments only the 1A clone expressing the wild-type MDR1 protein and the 88-8 clone carrying lysine to arginine substitutions at both NBS were tested. Since 1A cells produce larger amounts of the wild-type protein than clone 88-8 produces of the double MDR1 mutant, the relative amounts of either MDR1 proteins were estimated in each membrane fraction by immunoblotting and adjusted to yield equal final amounts in the cross-linking assay. Results presented in Figure 12 show that the wild-type and double mutant MDR1 proteins can be photolabelled to the same intensity by 8-azido ATP. The specific labelling of the two proteins is competed to a similar extent by a 100 fold excess of unlabelled ATP or ADP. Photoaffinity labelling of both proteins was reduced to intermediate but comparable levels by incubation of the

FIGURE 12

Nucleotide specificity of [α-^{32}P] 8-azido ATP photolabelling of mutant and wild-type MDR1 proteins

[α-^{32}P] 8-azido ATP cross-linking was performed as described in Figure 11. Where indicated a 10 or 100 molar excess of unlabelled ADP or a 100 molar excess of unlabelled ATP were added to the cross-linking reactions. 1A is a stable *mdr1* transfectant that expresses the wild-type MDR1 protein. 88-8 is a G418^r cotransfected cell clone that expresses a mutant MDR1 protein carrying a lysine to arginine mutation in each NBS (Figure 4).


mut88 + 100X ADP
mut88+ 10X ADP
mut88 + 100X ATP
mut88
1A +100X ADP
1A + 10X ADP
1A + 100X ATP
1A
LR73 +100X ATP
LR73

reaction in the presence of a 10 molar excess of ADP. These results suggest that although the biologically inactive mut-88 protein carries amino acid substitutions at both NBS, it still displays the 8-azido ATP binding characteristics of the wild-type protein.

CHAPTER IV

DISCUSSION

IV.1 Mutational Analysis of Mouse mdr1

In cultured cells, overexpression of the mouse or human mdr1 gene is sufficient to confer the complete multidrug resistance phenotype to otherwise drug sensitive cells (57,68). Sequence analysis of the cloned biologically active mouse and human mdr1 cDNAs (71,72) and drug transport characteristics of multidrug resistant cell lines obtained either by stepwise drug selection (22,24,32) or direct transfection with mouse mdr1 (114), indicate that these mdr1 genes most likely encode ATP-dependent drug efflux pumps. The human mdr1 gene product overexpressed in drug selected or mouse mdr1 transfected multidrug resistant cells can bind ATP (100,101). The purified human 170-180 kDa P-glycoprotein was recently shown to have ATPase activity (102). The functional role of ATP-binding and hydrolysis in the molecular mechanism of multidrug resistance encoded by mdr1 is, however, not yet understood.

Two putative nucleotide-binding sites are present within the predicted amino acid sequence of the MDR1 protein. These consensus motifs were originally derived by Walker from sequence comparison of a large number of bacterial and eukaryotic ATP-binding proteins (94). This sequence has now been reported in a number of other proteins involved in very different cellular processes such as transport, nodulation, replication, DNA repair and

others (135,136). They are formed by the association of two highly conserved clusters of residues. The A motif, G-X-X-G-X-G-K-T-(S), is believed to form a flexible loop between a beta strand and an alpha helix. Through conformational changes induced by ATP-binding this loop would control access to the nucleotide-binding domain (96-98). The conserved lysine residue is thought to interact directly with one of the phosphate groups of the ATP molecule and thereby be essential for ATPase activity (95,96,99). The B motif, R/K-X-X-X-G-X-X-X-L-Hyd-Hyd-Hyd-Hyd-D, located 100 residues downstream from the A motif is believed to be in close proximity to the glycine-rich flexible loop (motif A). The stretch of hydrophobic residues in this motif would form the homing pocket for the adenine-ribose moeity of the ATP molecule (94,95), possibly conferring the nucleotide specificity to the binding site.

To determine the functional importance of these sequences in the proposed energy-dependent drug efflux property of mdr1, we have introduced amino acid substitutions within the core consensus sequence of the A motif in either of the two predicted NBS of the protein. The evolutionary conserved glycine and lysine residues of both NBS of MDR1 were replaced by alanine and arginine residues, respectively. The analysis by direct transfection and in mass populations of cotransfected cells of mutant mdr1 cDNAs with altered A motifs reveals that these

mutations, although extremely conservative, abolish the biological activity of the mdr1 gene. Our results also suggest that both NBS do not function in an independent fashion, as mutation of a single site, such as in mutants mut53, mut55, mut83 and mut85, causes complete loss of biological activity.

Structural and functional characterization of the inactive MDR1 proteins expressed in individual clones demonstrates that the incapacity of the mutants to convey drug resistance is paralleled by an enhanced drug accumulation compared to cell clones expressing the wild-type protein. However, the ATP-binding ability of the inactive MDR1 proteins does not seem to be altered since they retain the capacity to bind the photoactivatable ATP analog, 8-azido ATP.

Several lines of evidence indicate that the loss of biological activity in these mutants is caused by bona fide mutations at the targeted sites rather than disruption of the reading frame or additional mutations introduced elsewhere in the molecule through artefacts of the mutagenesis/cloning procedures. All mutant MDR1 proteins showed electrophoretic mobility comparable to that of the wild-type protein (approximately 170 kDa) and were recognized by MDR-specific antibodies. In addition, a revertant (rev15) of mut55 where the mutant alanine residue of mutant mut55 is replaced by the wild-type glycine

residue completely recovers its biological activity. Both of these observations suggest that there is no alteration of the reading frame in the mutant cDNAs.

Moreover, it appears that the loss of biological activity of the mdr1 mutants is due to functional inactivation of the A motif rather than to major modifications of the three dimensional structure of the proteins. Major modifications of this type could affect intracellular targeting or membrane insertion. First, a lysine to arginine replacement introduced outside the A motif at position 1100 does not alter the activity of MDR1, suggesting that lysine to arginine substitutions can be tolerated at certain positions in MDR1 without loss of activity. Second, all mutant MDR1 proteins expressed in stably cotransfected cells were predominantly associated with the membrane fractions of these cells, suggesting proper targeting of these proteins to the membrane. Third, two of two mutants tested so far, 53-5 and 88-8, retain the capacity to bind the calcium channel blocker and drug analog [^{3}H] azidopine in cross-linking experiments (data not shown), a specific property of the wild-type MDR1 protein (101,109,110). Finally, the five NBS mutants retain the ability to bind the photoactivatable ATP analog, 8-azido ATP.

The ability of the mutant proteins to bind 8-azido ATP is of particular interest since we have introduced

mutations in evolutionary conserved sequences of the predicted NBS. The reactive nitrene group of 8-azido ATP is located at position C-8 of the adenine ring in the molecule. According to Walker's model, this reactive moeity would react with residues of the hydrophobic homing pocket encoded by the B motif rather than residues of the A motif. Our results therefore suggest that the homing pocket for the adenine moiety is still intact in the mutants. In addition, competition of 8-azido ATP photolabelling by ADP and ATP was identical in the double mutant clone 88-8 and in the wild-type protein (Figures 11 et 12), also suggesting that the nucleotide specificity of the pocket (motif B) is not modified by mutations in motif A. Taken together these results suggest that the structural integrity of the mutant proteins has not been fundamentally altered. Therefore, a step subsequent to ATP-binding and essential to drug transport must be impaired in the mutants possibly ATPase activity and/or conformational change triggered in the protein by ATP-binding or hydrolysis.

Mutational analysis of the A motif in other ATP-binding proteins showing ATPase activity, such as the α and ß subunits of *E. coli* F_1-ATPase, indicate that substitutions at the conserved lysine residue usually destroys ATPase activity and, in some but not all cases, leaves the ATP-binding property intact, this possibly depending on the nature of the residue used to substitute

lysine (137-140). Of particular interest is a recent mutational analysis of the RAD3 gene product, a yeast protein implicated in the excision/repair of DNA duplexes (141). Replacement of lysine 48 (motif A) for an arginine residue in RAD3 completely abolishes ATPase activity while only partly affecting the ability to bind ATP. This mutation therefore uncouples ATP-binding and ATP hydrolysis in RAD3. It is tempting to speculate that the lysine to arginine substitutions in the A motif of both of MDR1 NBS have similar effects on the protein, possibly interfering with an essential step of the phosphotransferase reaction. The guanidinium group of arginine although bearing a net positive charge, may not be able to substitute for the primary amino group of lysine for ATP hydrolysis.

Although the biological consequences of the substitutions of the conserved glycine and lysine residues are identical, they may not involve the same molecular defect. Replacement of glycine by the similar but bulkier alanine residue might interfere with a conformational change of the flexible glycine-rich loop believed to control access to the NBS. X-ray crystallography analysis of adenylate kinase shows that residues with side chains such as alanine cannot maintain the dihedral angle of the polypeptide backbone at this position, implying that glycine is strictly required (142). In addition, the case of a mutant of the GTP-binding p21ras protein, where a

conserved glycine residue belonging to the consensus glycine-rich loop of the GTP-binding domain is replaced by a valine, is of interest. This mutant exhibits impaired GTPase activity compared to the wild-type ras protein (143). X-ray crystallography analysis of the mutant protein reveals that although the overall conformation of the protein remains unchanged, the glycine-rich loop is enlarged (144). This is suggested to lock the mutant protein in a GTP-bound conformation which would prevent GTP hydrolysis.

IV.2 Cooperative interactions between the NBS

Irrespective of the molecular mechanism responsible for the effect of the mutations introduced in MDR1, our results clearly establish that both NBS of MDR1 are functional and essential for the overall expression of multidrug resistance and therefore for the proposed drug efflux mechanism. Moreover, these two sites do not appear to function independently from each other since mutating either site completely abolishes the biological activity of mdr1.

Each of these NBS is located within the two highly homologous halves of MDR1 which themselves share considerable sequence similarity with the ATP-coupling subunit of bacterial periplasmic transport systems,

including OppD, OppF, RbsA, MalK and PstB as well as the HlyB and NdvA transport proteins (71,84). The homology observed between MDR1 and these transporters extends much further than Walker's consensus sequence, especially in the case of HlyB and NdvA. An important functional role is likely associated with this sequence, possibly in coupling energy produced by ATP hydrolysis to substrate transport. It has been shown that OppD can bind a Cibacron Blue affinity column, specific for many ATP-binding proteins (145) and also react with the ATP affinity analog, 5'-p-fluorosulphonylbenzoyladenosine (5'-FSBA). In addition, HisP has been shown to react with 8-azido ATP (146). Although ATP is required for substrate transport by the bacterial permeases (147), direct proof of ATP hydrolysis by the energy-coupling subunits of these transporters awaits the isolation of the individual components in their purified form.

In the bacterial systems, the proposed ATP-coupling subunit containing the NBS is present as a monomer except for RbsA which possesses two NBS on the same polypeptide (78). Higgins et al. have proposed that, rather than functioning as monomers, nucleotide-binding domains of periplasmic transport systems function in pairs (RbsA) or as hetero- (OppD, OppF) or homodimers (HisP, MalK, PstB) (135). Likewise, our observations indicate that the dual nature of the mammalian MDR1 protein is pivotal for drug

efflux, not merely by doubling the activity of a functional monomer, but rather suggesting cooperative interactions between the two halves of the molecule. Our results therefore provide the first experimental evidence for Higgins proposal. Duplicated NBS are also present in ArsA, the energy-coupling subunit of the bacterial arsenate transporter (148). Although the sequence homology of this protein to MDR1 is not as extensive as that of the bacterial permeases, the arsenate system is analogous to MDR1 because it contains two putative NBS and is involved in export of its substrate (149).

The nature of the cooperative interactions between the two NBS of MDR1 is unknown and can only be speculated upon at the moment. Based on sequence homology (71) and tryptic mapping analysis of the photolabelled MDR1 protein (101), it is likely that both sites bind and actively catalyze hydrolysis of ATP. They could do so in a concerted or sequencial fashion to effect efflux: concerted hydrolysis of two ATP molecules may be required to produce a conformational change responsible for efflux. Alternatively, sequencial mechanisms could involve ATP hydrolysis at one site to effect efflux and at a second site to regain the original conformation. Evaluating the ATPase activity of purified MDR1 mutants should further enhance our understanding of the molecular mechanism of action of MDR1.

CHAPTER V

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