Mutagenesis of the putative nucleotide-binding domains of the multidrug resistance associated protein (MRP). Analysis of the effect of these mutations on MRP mediated drug resistance and transport

Melvin S. Center, Qichao Zhu & Hongwei Sun

Division of Biology, Kansas State University, Manhattan, KS 66506, U.S.A.

Received 25 May 1998; accepted 25 May 1998

Key words: drug accumulation, drug efflux, drug resistance, *in vitro* drug transport, mutagenesis, multidrug resistance protein, nucleotide-binding domains

Abstract

Studies were conducted to examine the functional role of the nucleotide-binding domains of MRP in drug resistance and drug transport in isolated membrane vesicles. *In vivo* studies were conducted by preparing stable transfectants of HeLa cells with wild-type MRP cDNA or MRP cDNAs which had been mutated at certain nucleotide binding domains (NBD). Stable transfectants producing equivalent amounts of the MRP encoded protein P190 were used in this study. The results demonstrated that deletions in the C-motif of NBD1 or the A-motif of NBD2 have a pronounced effect in reducing resistance levels to chemotherapeutic agents. Certain single-site mutations in lysines in these same motifs also reduce IC₅₀ values. It has also been observed that mutation of the MRP NBDs results in an increase in drug accumulation and a reduction in drug efflux. Additional studies have been carried out in which recombinant baculovirus containing either wild-type MRP or MRP containing mutated NBDs was prepared and used to infect SF21 insect cells. Using this system we have analyzed the effects of these mutations on *in vitro* transport of leukotriene C₄ (LTC₄) 17 β -estradiol 17 (β -D-glucuronide)(E₂17 β G) and daunomycin in membrane vesicles prepared from baculovirus infected cells. The results demonstrate that deletions and site-specific mutations in MRP NBDs greatly reduce the ATP dependent transport of all three substrates. The results of these studies conducted both *in vivo* and *in vitro* demonstrate that the NBDs of MRP function in a cooperative manner and are critical for the transport activity of the MRP encoded protein P190. These studies also identify specific lysines in NBD1 and NBD2 which are important for optimal MRP activity.

Introduction

Experimental cell lines isolated for multidrug resistance frequently overexpress either of the genes MDR1 (Endicott and Ling, 1989) or the Multidrug Resistant Protein (MRP) gene (Cole *et al.*, 1992). Transfection experiments indicate that both MDR1 (Gros *et al.*, 1986) and MRP (Grant *et al.*, 1994; Zaman *et al.*, 1994; Kruh *et al.*, 1994) can confer drug resistance to sensitive cells. The product of MDR1 is a phosphoglycoprotein which migrates in SDS gels as a 170–180 kd protein (Endicott and Ling, 1989). P-glycoprotein which has been extensively studied contains 2 nucleotide-binding domains (NBD) defined by the Walker A and Walker B se-

quences (Walker *et al.*, 1982). Previous studies have demonstrated that both NBDs of P-glycoprotein are needed for functional activity (Azzaria, *et al.*, 1989). P-glycoprotein contains ATPase activity (Hamada and Tourao 1988; Ambudkar *et al.*, 1992) but only one NBD seems to be necessary for functional activity of this enzyme (Shimabuka *et al.*, 1992). The MRP encoded protein P190 (Krishnamachary and Center, 1993) is also a phosphoglycoprotein (Krishnamachary *et al.*, 1994; Ma *et al.*, 1993; Almquist *et al.*, 1995) which is located predominantly in the plasma membranes and endoplasmic reticulum of resistant cells (Krishnamachary *et al.*, 1994; Barrand *et al.*, 1995). The protein is highly phosphorylated with phosphate groups contained in at least 9 tryptic peptides (Ma

et al., 1993). All phosphate groups are present at serine residues (Ma *et al.*, 1993). It has also been observed that certain protein kinase inhibitors such as chelerythrine are capable of reducing P190 phosphorylation and reversing drug resistance (Ma *et al.*, 1993). Further studies are needed however to clearly determine if phosphorylation plays some role in the function of P190. The MRP encoded protein contains 2 putative NBDs as determined by sequence analysis (Cole *et al.*, 1992). Further evidence for the presence of NBDs in P190 has been previously obtained by labeling the protein with the photoactive agent 8-azido (α 32P) ATP (McGrath *et al.*, 1989). Although P-glycoprotein and P190 have only 15% sequence homology both function in an energy requiring drug efflux system which reduces the intracellular accumulation of drug (Endicott and Ling, 1989; Loe *et al.*, 1996; Versantvoort *et al.*, 1992). Both proteins can mediate resistance against a broad spectrum of structurally unrelated drugs and P-glycoprotein appears capable of binding and transporting drug (Endicott and Ling, 1989), whereas studies conducted thus far have been unable to demonstrate that P190 is capable of binding free drug (McGrath *et al.*, 1989). P190 is however capable of binding to and transporting the glutathione-S-conjugate LTC₄ in isolated membrane vesicles (Jedlitschky *et al.*, 1994; Leier *et al.*, 1994; Muller *et al.*, 1994). The protein is also capable of transporting *in vitro* oxidized glutathione (Leier *et al.*, 1996) cholestatic glucuronidated steroids (Loe *et al.*, 1996) and chemotherapeutic agents such as vincristine (Loe *et al.*, 1996) and daunomycin (Saptarshi *et al.*, 1996). Vincristine transport *in vitro* seems to be greatly enhanced by the presence of glutathione (Loe *et al.*, 1996), whereas daunomycin transport can occur in the absence of glutathione (Saptarshi *et al.*, 1996). A major question concerning MRP function is whether *in vivo* drug is transported in the form of an anionic conjugate.

Two reviews have recently appeared which describe in detail the biology of the MRP system (Loe *et al.*, 1996; Lautier *et al.*, 1996).

***In Vivo* Analysis of the Involvement of MRP NBDs in Multidrug Resistance**

Although extensive studies have been carried out to characterize MRP mediated multidrug resistance (Lautier *et al.*, 1996; Loe *et al.*, 1996) there have been no previous reports concerned with analyzing

functional domains of the MRP encoded protein P190 through the use of site directed mutagenesis. To initiate studies of this type we have mutagenized MRP cDNA at putative nucleotide-binding domains (NBDs) and have used mutated and wild-type DNA in expression vectors to produce stable transfectants in HeLa cells. The effect of these mutations on levels of drug resistance, drug accumulation and drug efflux were examined in detail. Mutagenesis of NBDs resulted in deletion mutants in the NBD1, C-motif (dN₁) and the NBD2, A-motif (dN₂) (Figure 1). A double deletion mutant dN₁dN₂ was also constructed (Figure 1). In addition single site mutations were introduced into lysine of NBD1, C-motif converting this amino acid to methionine (sN₁), and, also lysine in the NBD2 A-motif (sN₂) (Figure 1). A double mutant containing both single-site mutations was also prepared (sN₁sN₂). The presence of the various mutations in the MRP cDNA was verified by sequence analysis (Sanger *et al.*, 1977). Membranes were prepared from HeLa cells transfected with the wild-type and mutated MRP cDNAs and the levels of P190 were determined by Western blot analysis. The results of this study clearly demonstrated that about equal levels of wild-type and mutated P190 were contained in membranes of transfected cells. The electrophoretic mobility of the mutated proteins in SDS gels demonstrated the presence of full length proteins with a molecular mass of about 190 kd. The stable transfectants were thereafter analyzed for resistance levels against adriamycin, vincristine and VP-16. It was observed in agreement with other studies (Grant *et al.*, 1994; Zaman *et al.*, 1994; Kruh *et al.*, 1994) that wild-type MRP could confer drug resistance to drug sensitive HeLa cells (Table I). For reasons that are not known wild-type transfectants showed a higher level of resistance to vincristine as compared to adriamycin and VP-16. Detailed studies were thereafter carried out to determine the effect of the various NBD mutations on MRP mediated cell resistance. It was observed that all NBD mutants had a pronounced effect in reducing resistance to all 3 chemotherapeutic agents tested (Table I). Reduction in resistance was found to be somewhat greater with the deletion mutants as compared to the single-site mutants. It is of interest to note that deletion of either the NBD1 C-motif or the NBD2 A-motif results in a major reduction in resistance thus indicating that both NBDs are needed for optimal MRP resistance and that one NBD cannot compensate for another. At the present time it is not known however if the 2 MRP NBDs have similar or different functions

A

B

WT EIGEKGVNLSGGQK^{*}QRVSLARAVYS GGEKVGIVGRTGAGK^{*}SSLTLGLFR
dN₁ EIGEKGVNLSGGQKQ^{*}RVSLARAVYS GGEKVGIVGRTGAGKSSLTLGLFR
dN₂ EIGEKGVNLSGGQKQRVSLARAVYS GGEKVGIVGRTGAGKSSLTLGLFR
dN₁N₂ EIGEKGVNLSGGQKQRVSLARAVYS GGEKVGIVGRTGAGKSSLTLGLFR
sN₁ EIGEKGVNLSGGQM^{*}QRVSLARAVYS GGEKVGIVGRTGAGKSSLTLGLFR
sN₂ EIGEKGVNLSGGQKQRVSLARAVYS GGEKVGIVGRTGAGMSSLTLGLFR
sN₁N₂ EIGEKGVNLSGGQM^{*}QRVSLARAVYS GGEKVGIVGRTGAGMSSLTLGLFR

Figure 1. Sequences of deletion and site-specific mutants of MRP NBDs which were analyzed in this study. A. Diagram showing relative locations of the MRP NBDs. B. Amino acid sequences which were deleted or mutated in the C-motif of NBD1 or the A-motif of NBD2. The deletions (shaded sequences) are represented by *dN₁*, *dN₂* and the double deletion *dN₁N₂*. Also K (lysine) indicated by the asterisk was mutated to M (methionine) in both the C-motif and A-motifs resulting in *sN₁*, *sN₂* and the double single-site mutant *sN₁N₂*. The materials and methods used for construction of MRP-NBD mutants, cytotoxicity assays, drugs accumulation and efflux were as previously described (Zhu *et al.*, 1997).

Table 1. Effects of different drugs on the resistance levels of HeLa cells transfected with wild type and mutant MRP cDNAs

Transfectants	IC ₅₀ (μM) ^c			Fold resistance ^d		
	Adr.	Vcr.	VP-16	Adr.	Vcr.	VP-16
pcDNA3 ^a	0.08	0.004	2.10			
pcDNA3+MRP ^b	1.00	0.088	15.6	12.5	22.0	7.4
pcDNA3+dN ₁	0.10	0.007	3.00	1.3	1.8	1.4
pcDNA3+dN ₂	0.16	0.007	4.30	2.0	1.8	2.0
pcDNA3+dN ₁ N ₂	0.10	0.002	1.90	1.3	0.5	0.9
pcDNA3+sN ₁	0.27	0.013	7.30	3.4	3.3	3.5
pcDNA3+sN ₂	0.27	0.031	4.10	3.4	7.8	2.0
pcDNA3+sN ₁ N ₂	0.23	0.016	5.10	2.9	4.0	2.4

^a Cells transfected with the pcDNA3 vector which did not contain MRP cDNA.

^b Cells transfected with the pcDNA3 vector which contained wild-type MRP cDNA.

^c The concentration of drug that inhibit cell growth by 50%. The values represent the results of an experiment performed in triplicate and are representative of several independent experiments.

^d I₅₀ of HeLa cells transfected with wild-type or mutated MRP divided by C₅₀ of cell transfected with the pcDNA3 vector.

in MRP drug transport. It was also observed that either of the single site mutants in lysines of the NBD1 C-motif or NBD2 A-motif produced a major reduction in IC_{50} values with either adriamycin, vincristine or VP-16 (Table I). Thus the single lysines at position 773 (C-motif) or 1333 (A-motif) play a major role in the functional activity of MRP. Previous studies indicate that the lysine present in the Walker A motif interacts with the phosphate group of ATP (Walker *et al.*, 1982). The function of the lysine in the C-motif is not known but it may be important in maintaining the structural integrity of this linker region (Walker *et al.*, 1982).

Effect of MRP NBD Mutations on Drug Accumulation and Efflux

Previous studies have demonstrated that most experimental isolates overexpressing MRP exhibit a reduction in the accumulation of drug (Marsh *et al.*, 1986; Coley *et al.*, 1991; Versantvoort, *et al.*, 1992). In MRP transfectants reduced accumulation of drug which requires ATP is also observed (Zaman *et al.*, 1994; Cole *et al.*, 1994). It is also indicated that reduced drug accumulation is related to an energy requiring efflux system (Loe *et al.*, 1996). In the P-glycoprotein system it would be indicated that the NBDs play a role in the P-glycoprotein ATPase which releases energy for efflux of drug against a concentration gradient. At the present time the P190 protein has not been purified and there is still no evidence that this protein contains an ATPase. Thus to gain insight into the function of the MRP NBDs studies were conducted to examine drug accumulation and efflux in transfectants containing P190 mutated at nucleotide-binding domains (Figures 2 and 3). Thus in these experiments HeLa cells transfected with wild-type or mutated MRP were incubated in the presence of [3 H] daunomycin and the accumulation of drug over various time periods was determined (Figure 2). The results clearly demonstrate that wild-type transfected cells exhibit a major reduction in drug accumulation compared to cells transfected with the empty vector. Cells containing MRP mutated at NBDs exhibit a major increase in the accumulation of drug. The relative levels of drug accumulation occurring in cells containing either the dN₁ or dN₂ mutations are about the same thus demonstrating once again that deletion of either the NBD1 C-motif or NBD2 A-motif results in a cell with a drug sensitive phenotype. Thus a single intact NBD site does not seem capable of functioning to reduce drug

accumulation. Similarly the single site mutants sN₁ or sN₂ also render the NBD sites inactive resulting in a major increase in drug accumulation (Figure 2). Thus the single lysines mutated in this study seem to play a critical role in the functional activity of the MRP NBDs.

Additional studies have been carried out to examine drug efflux in HeLa cell transfectants containing either wild-type or mutated MRP cDNA. The results of this study reveal that the rate of drug efflux in wild-type transfected cells after a 90 min incubation period was about 2-fold greater than that found for the various deletion mutants (Figure 3). Efflux in cells transfected with these mutants was equivalent to that occurring in cells transfected with the empty vector thus indicating that either the dN₁ or dN₂ mutation essentially abolishes MRP mediated drug efflux. The rate of drug efflux was also reduced with either of the single-site mutants sN₁ or sN₂. The rate was somewhat further reduced in the transfectant containing the double single-site mutations sN₁N₂.

Taken together the results of these studies provide definitive evidence that drug resistance in the MRP system is related to an energy dependent efflux which reduces cellular accumulation of drug. The mechanism by which the MRP encoded protein P190 carries out the function remains to be determined.

In Vitro Analysis of MRP Mediated Substrate Transport: Effect of MRP NBD Mutations on Transport in Isolated Membrane Vesicles

Previous studies have shown that membrane vesicles prepared from cells overexpressing MRP are capable of the ATP dependent transport of a number of compounds including the leukotriene glutathione conjugate LTC₄ (Jedlitschky *et al.*, 1994; Leier *et al.*, 1994; Muller *et al.*, 1994) and also certain chemotherapeutic agents such as daunomycin and vincristine. (Saptarshi *et al.*, 1996, Loe *et al.*, 1996). In recent studies we have developed a recombinant baculovirus which contains a full length MRP cDNA (Sun *et al.*, 1996). Infection of SF 21 insect cells with this virus results in the synthesis of a deglycosylated form of the MRP protein. In SDS gels the protein has a molecular mass of about 170 kd. The P170 protein is phosphorylated and associated with cell membranes. The deglycosylated protein appears to be functional in the *in vitro* system since membrane vesicles containing P170 can transport LTC₄ in an ATP dependent reaction

Figure 2. Daunomycin accumulation in MRP transfectants. Bar graphs showing the relative levels of $[^3\text{H}]$ daunomycin accumulation in transfectants containing wild-type or mutated MRP.

Figure 3. Daunomycin efflux in MRP transfectants. Bar graphs showing the relative levels of $[^3\text{H}]$ daunomycin efflux in wild-type transfectants or transfectants containing mutated MRP.

Figure 4. Transport of MRP substrates in membrane vesicles prepared from wild-type or NBD mutated MRP cDNA contained in recombinant baculovirus. Baculovirus was also prepared with transfer vector which did not contain MRP cDNA (designated EV). Transport studies (Sun *et al.*, 1996) were carried out with membrane vesicles prepared from baculovirus infected SF21 cells. The substrates used were [³H] LTC₄ (panels A and B); [³H]E₂17βG (panels C and D) and [³H] daunomycin (panels E and F).

(Sun *et al.*, 1996). In view of this and in an effort to extend these findings we have prepared recombinant baculovirus containing wild-type MRP or MRP containing the NBD mutations described in Figure 1. Thus in these experiments insect cells were infected with recombinant virus and the effect of mutated NBDs on MRP mediated transport was examined (Figure 4). In these experiments all transport studies were conducted with membrane vesicles containing equivalent levels of MRP-P170 as determined by Western blot analysis. The MRP specific antibody 6KQ (Krishnamachary and Center 1994) was used in these studies. It was observed from these experiments that the effect of the NBD mutations on transport *in vitro* is quite similar to that found in the *in vivo* system using stable transfectants of Hela cells. Thus it was observed that transport of LTC₄, E₂17 β -D-glucuronide and daunomycin was reduced at least 75% with either deletion mutant dN₁ or dN₂ as compared to transport activity obtained with vesicles prepared from cells infected with recombinant baculovirus containing wild-type MRP (Figure 4). Similar results were obtained with the single site lysine mutants sN₁ or sN₂. Thus in the presence of either mutant *in vitro* transport with the three substrates tested was greatly reduced compared to transport obtained in the presence of wild-type MRP. The finding that single-site mutants reduce MRP mediated transport suggests that the transport alterations observed are probably not due to topological changes of the P170 protein in the membrane. The results point to the finding that lysine-773 of the NBD1 C-motif or lysine-1333 of the NBD2 A-motif play critical roles in the nucleotide binding domains of MRP.

Acknowledgement

Work conducted in our laboratory was supported by Public Health Service Grant CA-37585, National Institutes of Health.

References

- Almquist KC, Loe DW, Hipfner DR, Mackie JE, Cole SPC and Deeley RG (1995) Characterization of the Mr 190 000 multidrug resistance protein (MRP) in drug-selected and transfected human tumor cells. *Cancer Res* 55: 102–110.
- Ambudkar SV, Lelong IH, Zhang J, Cardarelli CO, Gottesman MM and Pastan I (1992) Partial purification and reconstitution of the human multidrug resistance pump: characterization of the drug-stimulatable ATP hydrolysis. *Proc Natl Acad Sci USA* 89: 8472–8476.
- Azzaria M, Schurr E and Gros P (1989) Discrete mutations introduced in the predicted nucleotide-binding sites of the *mdr1* gene abolish its ability to confer multidrug resistance. *Mol Cell Biol* 9: 5289–5297.
- Barrand MA, Robertson KJ, Neo S-Y, Rhodes T, Wright KA, Twentym PR and Scheper RJ (1995) Localization of the multidrug resistance-associated protein MRP in resistant large-cell lung tumour cells. *Biochem pharmacol* 50: 1725–1729.
- Cole SPC, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AMV and Deeley RG (1992) Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science (Washington DC)* 258: 1650–1654.
- Cole SPC, Sparks KE and Fraser K (1994) Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. *Cancer Res* 54: 5902–5910.
- Coley HM, Workman P and Twentym PR (1991) Retention of activity by selected anthracyclines in a multidrug resistant human large cell carcinoma line without P-glycoprotein hyperexpression. *Br J Cancer* 63: 351–357.
- Grant CE, Valdimarsson G, Hipfner DR, Almquist KC, Cole SPC and Deeley RG (1994) Overexpression of multidrug resistance-associated protein (MRP) increases resistance to natural product drugs. *Cancer Res* 54: 357–361.
- Gros P, Neria YB, Croop JM and Hansman DE (1986) Isolation and expression of a complementary DNA that confers multidrug resistance. *Nature* 323: 728–731.
- Hamada H and Tsuruo T (1988) Purification of the 170- to 180-kilodalton membrane glycoprotein associated with multidrug resistance – 170 to 180-kilodalton membrane glycoprotein is an ATPase. *J Biol Chem* 263: 1454–1458.
- Jedlitschky G, Leier I, Bucholz U, Center MS and Keppler D (1994) ATP dependent transport of glutathione S-conjugates by the multidrug resistance-associated protein. *Cancer Res* 54: 4833.
- Krishnamachary N and Center MS (1993) The MRP gene associated with a non-P-glycoprotein multidrug resistance encodes a 190-kDa membrane bound glycoprotein. *Cancer Res* 53: 3658–3661.
- Krishnamachary N, Ma L, Zheng L, Safa AR and Center MS (1994) Analysis of MRP gene expression and function in HL60 cells isolated for resistance to adriamycin. *Oncol Res* 6: 119–127.
- Kruh GD, Chan A, Myers K, Gaughan K, Miki T and Aaronson SA (1994) Expression complementary DNA library transfer establishes *mrp* as a multidrug resistance gene. *Cancer Res* 54: 1649–1652.
- Lautier D, Canitrot Y, Deeley RG and Cole SPC (1996) Multidrug resistance mediated by the multidrug resistance protein (MRP) gene. *Biochem Pharmacol* 52: 967–977.
- Leier I, Jedlitschky G, Bucholz U, Cole SPC, Deeley RG and Keppler D (1994) The MRP gene encodes an ATP-dependent export pump for leukotriene C₄ and structurally related conjugate. *J Biol Chem* 269: 27807–27810.
- Leier I, Jedlitschky G, Bucholz U, Center M, Cole SPC and Deeley RG (1996) ATP-dependent glutathione disulphide transport mediated by the MRP gene-encoded conjugate export pump. *Biochem J* 314: 433–437.
- Loe DW, Deeley RG and Cole SPC (1996) Biology of the multidrug resistance-associated protein, MRP. *European J Cancer* 32A: 945–957.
- Loe WL, Almquist KC, Deeley RG and Cole SPC (1996) Multidrug resistance protein (MRP) mediated transport of leukotriene C₄ and chemotherapeutic agents in membrane vesicles. *J Biol Chem* 271: 9675–9682.

- Ma L, Krishnamachary N and Center MS (1995) Phosphorylation of the multidrug resistance associated protein gene encoded protein P190. *Biochemistry* 34: 3338–3343.
- Marquardt D, McCrone S and Center MS (1990) Mechanisms of multidrug resistance in HL60 cells: detection of resistance-associated proteins with antibodies against synthetic peptides that correspond to the deduced sequence of P-glycoprotein. *Cancer Res* 50: 1426–1430.
- Marsh W, Sicheri D and Center MS (1986) Isolation and characterization of adriamycin-resistant HL60 cells which are not defective in the initial intracellular accumulation of drug. *Cancer Res* 46: 4053–5057.
- McGrath T, Latoud C, Arnold ST, Safa AR, Felsted RL and Center MS (1989) Mechanisms of multidrug resistance in HL60 cells: analysis of resistance associated membrane proteins and levels of *mdr* gene expression. *Biochem Pharmacol* 38: 3611–3619.
- Muller M, Meijer C, Zaman GJR Borst B, Scheper RJ, Mulder NH, De Vries EGE and Jansen PLM (1994) Overexpression of the gene encoding the multidrug resistance associated protein results in increased ATP dependent glutathione S-conjugate transport (1994) *Proc Natl Acad Sci USA* 91: 13033–13037.
- Sanger F, Nicklin S. and Coulson AR (1977) Sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 74: 5466–5466.
- Saptarshi P, Belinsky MG, Shen H and Kruh GD (1996) Structure and *In vitro* substrate specificity of the murine multidrug resistance-associated protein. *Biochemistry* 35: 13647–13655.
- Shimabuka AM, Nishimot T, Ueda K and Komano T (1992) P-glycoprotein ATP hydrolysis by the N-terminal nucleotide-binding domain. *J Biol Chem* 267: 4308–4311.
- Sun H, Zhu Q and Center MS (1996) Expression and characterization of the multidrug resistance-associated protein in insect cells infected with a recombinant baculovirus. *Oncol Res* 8: 287–293.
- Versantvoort CHM, Broxterman HJ, Pinedo HM, de Vries EGE, Feller N, Kuiper CM and Lankelma J (1992) Energy dependent processes involved in reduced drug accumulation in multidrug-resistant lung cancer cell lines without P-glycoprotein expression. *Cancer Res* 52: 17–23.
- Walker JE, Saraste M, Runswick MJ and Gay NJ (1982) Distantly related sequences in the α - and β -subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J* 1: 945–951.
- Zaman GJR, Flens MJ, van Leusden MR De Haas M, Mulder HS, Lankelma J, Pinedo HM, Scheper RJ, Baas F, Broxterman HJ and Borst P (1994) The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump. *Proc Natl Acad Sci USA* 91: 8822–8826.
- Zhu Q, Sun H and Center MS (1997) Functional analysis of the nucleotide binding domains of the multidrug resistance protein MRP. *Oncol Res* 9: 229–236.

Address for correspondence: Melvin S. Center, Division of Biology, Kansas State University, Manhattan, KS 66506, U.S.A.