Biochemical Characterization of Domains in the Membrane Subunit DrrB That Interact with the ABC Subunit DrrA: Identification of a Conserved Motif†

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ABSTRACT: DrrA and DrrB proteins confer resistance to the commonly used anticancer agents daunorubicin and doxorubicin in the producer organism *Streptomyces peucetius*. The *drrAB* locus has previously been cloned in *Escherichia coli*, and the proteins have been found to be functional in this host. DrrA, a soluble protein, belongs to the ABC family of proteins. It forms a complex with the integral membrane protein DrrB. Previous studies suggest that the function and stability of DrrA and DrrB are biochemically coupled. Thus, DrrA binds ATP only when it is in a complex with DrrB in the membrane. Further, DrrB is completely degraded if DrrA is absent. In the present study, we have characterized domains in DrrB that may be directly involved in interaction with DrrA. Several single-cysteine substitutions in DrrB were made. Interaction between DrrA and DrrB was studied by using a cysteine to amine chemical cross-linker that specifically cross-links a free sulfhydryl group in one protein (DrrB) to an amine in another (DrrA). We show here that DrrA cross-links with both the N- and the C-terminal ends of the DrrB protein, implying that they may be involved in interaction. Furthermore, this study identifies a motif within the N-terminal cytoplasmic tail of DrrB, which is similar to a motif recently shown by crystal structure analysis in BtuC and previously shown by sequence analysis to be also present in exporters, including MDR1. We propose that the motif present in DrrB and other exporters is actually a modified version of the EAA motif, which was originally believed to be present only in the importers of the ABC family. The present work is the first report where domains of interaction in the membrane component of an ABC drug exporter have been biochemically characterized.

Daunorubicin and doxorubicin, two commonly used anticancer drugs, are produced by the soil organism *Streptomyces peucetius*. Self-resistance to these antibiotics in *S. peucetius* is mediated by the action of two proteins, DrrA and DrrB, which are coded by the *drrAB* operon (*1*). Subcloning of the *drrAB* locus has been shown to confer doxorubicin resistance in *Escherichia coli* (*2*). DrrA, a peripheral membrane protein, contains one ABC-type (ATP Binding Cassette) (*3*) consensus nucleotide binding domain (NBD)[1] (*1*, *2*). It forms a complex with DrrB, which is localized to the inner membrane of the *E. coli* cells (*2*). Together, DrrA and DrrB are expected to form an ATP-dependent pump for the efflux of daunorubicin and doxorubicin, resulting in resistance to these antibiotics (*1*, *2*). Interestingly, the DrrAB system bears sequence, structural, and functional similarities to P-glycoprotein (Pgp) found in mammalian tumor cells (*1*, *2*, *4*). Overexpression of Pgp in cancer cells has been implicated in the development of multidrug resistance to a variety of structurally unrelated drugs, including daunorubicin and doxorubicin (*4*, *5*). Thus, both Pgp and DrrAB carry out similar functions, but in two different cell types. An understanding of the bacterial drug transporter DrrAB is thus expected to help in obtaining a better understanding of the function and evolution of the multidrug transporter P-glycoprotein.

Most ABC transporters are made up of four domains—two nucleotide binding domains and two transmembrane (TM) domains. These four domains can be present on the same molecule (Pgp (*6*), CFTR (*7*)), on two half-molecules (LmrA (*8*), Tap1 (*9*), Tap2 (*10*), HlyB (*11*), MsbA (*12*)), or on completely separate polypeptides (DrrAB (*13*), BtuCD (*14*)). In DrrAB, the catalytic and membrane domains are present on separate subunits, which are expected to form a tetramer consisting of two identical subunits of DrrA and two of DrrB (*13*). Although present on separate subunits, the two domains of DrrAB show a strong dependence on each other for stability and function (*13*). Thus, DrrA, the nucleotide binding subunit, binds ATP or GTP in a doxorubicin-dependent manner only if it is in a complex with DrrB (*13*). Further, DrrB is completely degraded if DrrA is not simultaneously expressed (*13*). These characteristics of DrrAB make it an ideal model system to study interactions between the ABC domain and the TM domain of ABC transporters and to identify specific regions of interaction.

How the catalytic ATP-binding domains and the membrane domains in transporters interact with each other, or how energy is transduced between them, is not well understood. An effort is currently underway to better understand these interactions in different ABC transporters,

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[1] Abbreviations: GMBS, (N-[gamma-Maleimidobutyryloxy] succinimide ester); NBD, Nucleotide binding domain; TM, Transmembrane domain; Pgp, P-glycoprotein.

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including medically relevant transporters Pgp (*6*) and CFTR (Cystic Fibrosis Transmembrane Regulator) (*7*, *15*, *16*). As of now, however, not much is known about the mechanism of interaction between membrane domains and ABC domains in these proteins. Both Pgp and CFTR contain all four domains in the same molecule; thus, analysis of interactions between them involves subcloning of the domains (*6*, *7*), which may not be conducive to retaining proper structure and function. Since the domains in the DrrAB system are present on separate subunits, we have taken advantage of this characteristic, in the present study, to study interaction by a heterobifunctional cysteine to amine cross-linking approach. Previous cross-linking studies in this laboratory employed membrane-permeable and -impermeable amine to amine cross-linkers, DSP and DTSSP, and identified a complex of DrrA and DrrB (*13*). However, using DSP and DTSSP, it was not possible to easily determine the regions involved in cross-linking. In the present study, we have used a more direct and specific approach to identify regions in DrrB that are physically close to DrrA and thus may be involved in an interaction with DrrA.

DrrB protein contains a single cysteine at position 260, whereas DrrA protein contains none. A cysteine-less DrrB was first created by substituting a serine at position C260 by site-directed mutagenesis. Twenty single-cysteine substitutions were thus introduced at various positions in the cysteine-less DrrB. The location of the cysteine substitutions in DrrB was based on a recently developed topological model for DrrB (*17*), which suggests that DrrB consists of eight transmembrane domains with both the N-terminal and the C-terminal ends of the protein directed in the cytoplasm. To directly identify the regions of DrrB that interact with DrrA, a heterobifunctional cysteine to amine chemical cross-linking agent, GMBS (*N*-[γ-maleimidobutyryloxy]succinimide ester), was employed. Studies reported here show that DrrA cross-links with both the N-terminal and the C-terminal ends of DrrB, suggesting that these two regions may participate in interaction with DrrA. Furthermore, this study identifies a sequence within the N-terminal cytoplasmic tail of DrrB, which is similar to the "L-loop" motif recently identified in the BtuC protein involved in VitB12 uptake in *E. coli* (*18*). By crystal structure analysis, the L-loop motif has been shown to lie at the interface of BtuC, the membrane component, and BtuD, the ABC component, where it is involved in forming extensive contacts between the two domains. BtuCD is a binding protein-dependent import system. It has previously been shown that permeases belonging to this group contain an EAA motif within their interaction interfaces (*19*). A closer look at the sequence of the L-loop suggested to us that it is actually similar, though not identical, to the "EAA-loop" previously identified in this group of proteins. While the L-loop of BtuC retains several features of the EAA loop, it also shares certain features with the motif present in DrrB (shown in this study) and other exporters (described earlier (*18*)). The sequence analysis and the alignments presented in this article thus allow us to conclude that the exporters, including DrrB, LmrA, MDR1, CFTR, and MsbA, as well as the importer BtuC contain a conserved motif bearing the amino acid sequence $GE_{-1}..A_3R/K..G_7$. We further conclude that this conserved sequence is a modified version of the EAA motif $E_1AA_3RALG_7$— although a significantly modified version with its own distinct features.

The present study is the first report where interacting domains in the membrane component of a drug efflux system have been biochemically analyzed by using a cross-linking approach. The domain identified by cross-linking was found to contain an EAA-like motif previously identified in the interaction interfaces of importers of the ABC family. We propose that the EAA motif or a modified version of the EAA motif may be involved in forming a generalized interface between the membrane component and the ABC component of both uptake and efflux systems. The evolutionary implications of this finding are discussed.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. E. coli TG1 (*20*) and XL1 Blue (Clonetech, BD Biosciences) were used in this study.

Media and Growth Conditions. Cells were grown in Luria Broth. Chloramphenicol was added to a final concentration of 20 μg/mL.

DNA Manipulations. The conditions for plasmid isolation, DNA endonuclease restriction analysis, ligation, and sequencing have been described elsewhere (*20*).

Site-Directed Mutagenesis. The cysteine residue at position 260 in DrrB in the plasmid pDx101 was altered to a serine using a Strategene QuikChange multisite-directed mutagenesis kit (La Jolla, CA). The strategy involved the use of complementary primers that incorporated the change at the required position. The sequence of the primers used is

C260S up:
5′-GGCCTGGTCCTGTCCGTGTCGGCAGGG-3′

C260S dn:
5′-CCCTGCCGACACGGACAGGACCAGGCC-3′

These primers were used for polymerase chain reaction amplification using pDx101 as a template (*2*). The amplified product bearing the mutation was then transformed into XL1 blue competent cells (provided by the manufacturer) and plated on medium containing chloramphenicol. Plasmid DNA was isolated from the colonies grown on the selection plates and the desired mutation in the *drrAB* genes was verified by sequencing of DNA. Sequencing was carried out using ABI 377 or 3100 sequencers (Applied Biosystems) in the Core Facilities in the Department of Biology at the Georgia State University. The cysteine-less serine substitution mutant was named C260S in this study. Subsequent single cysteine substitution mutants were then created in the cysteine-less DrrB, C260S. Complementary primers bearing the mutation at the desired location were used for each of these mutations. Primers used for making S23C mutation are shown here as an example:

S23C up:
5′- CGGACGGTGCTGTGCGCGGGTGAACGG-3′

S23C dn:
5′- CCGTTCACCCGCGCACAGCACCGTCCG-3′

Single cysteine substitution mutants were created at amino acid positions 4, 15, 23, 35, 44, 53, 70, 80, 92, 107, 116, 129, 149, 160, 173, 213, 236, 249, 270, and 282 in DrrB. The mutants were named S4C, S15C, S23C, S35C, A44C,

V53C, T70C, S80C, V92C, S107C, V116C, A129C, T149C, A160C, V173C, S213C, S236C, T249C, A270C, and A282C, respectively.

Doxorubicin Resistance Assay. Doxorubicin resistance assays were carried out on M9 (Sambrook 1989) plates supplemented with 0.25% casamino acids. The plates were layered with 4 mL of top agar (0.8% agar in M9 medium) containing the desired concentration of doxorubicin. Briefly 4 mL of top agar containing 0, 4, 6, 8, or 10 μg/mL of doxorubicin and 1 mM Thiamine-HCl was poured on top of M9 plates. The plates were covered with foil to prevent exposure to light. Doxorubicin hydrochloride was purchased from Sigma Chemicals and used with the necessary precautions needed for a light-sensitive chemical. A doxorubicin-sensitive strain of *E. coli*, N43 (*21*), was transformed with the indicated plasmids. N43 cells freshly transformed with the desired plasmid were grown for 8 h in 3 mL of LB with the appropriate antibiotics. Ten microliters of the 8 h old N43 culture from above was inoculated on the plates containing doxorubicin. N43 containing the plasmid pSU2718 was used as a negative control. The plates were then incubated at 37 °C overnight, and growth was recorded after 24 h of incubation.

Fractionation of Cells into Cytosolic and Membrane Fractions. Competent *E. coli* TG1 cells were transformed with the plasmid bearing the mutated gene. A 50 mL portion of cells containing the indicated plasmids was grown to mid-log phase and induced with 0.25 mM IPTG. Growth was continued for an additional 3 h at 37 °C. The cells were spun down and resuspended in 1.5 mL of PBS buffer (20 mM sodium phosphate, pH 7.0) and lysed by a single passage through a French pressure cell at 20 000 psi. The cell lysates were then centrifuged at 10 000*g* for 15 min to remove the unbroken cells. The supernatants were subsequently used to prepare membrane fractions by ultracentrifugation at 100 000*g* for 1 h. The membrane pellets were suspended in cross-linking buffer (20 mM sodium phosphate, 150 mM sodium chloride, 0.5 mM EDTA, pH 7.4) and used for cross-linking of proteins.

Heterobifunctional Cross-Linking Reaction and Western Blot Analysis. A 100 μL reaction volume containing 250 μg of cell membrane protein in the cross-linking buffer was used. GMBS (*N*-[γ-maleimidobutyryloxy]sulfosuccinimide ester; 6.4 Å length), prepared in dimethyl sulfoxide (DMSO), was added to a final concentration of 1 mM. DMSO alone was used in the control samples. Where indicated, 5 mM ATP and/or 35 μM doxorubicin was added into the reaction mix. The reaction was carried out for 1 h at room temperature in a light-protected area. A 25 μL portion of 4 X Laemmli sample buffer was added to stop the reaction. The samples were mixed thoroughly and set aside for 5 min at room temperature. A 25 μL portion (50 μg of membrane protein) of the reaction mixture was then analyzed by SDS-PAGE using a 10% polyacrylamide gel, followed by Western blot analysis using either anti-DrrA or anti-DrrB antibodies. Detection was done by a chemiluminiscent assay using Immun-Star AP, BioRad (Hercules, CA).

RESULTS

Cysteine-less DrrB. Previous studies have suggested that DrrA and DrrB interact with each other in a very specific manner (*13*). This interaction is essential for the maintenance of DrrB and for the function of DrrA, indicating that the two proteins are biochemically coupled. The purpose of the present study was to analyze domains in DrrB that are involved in interaction with DrrA. A model for the membrane topology of DrrB is now available (*17*). This model suggests that DrrB contains eight transmembrane domains with both the N-terminal and the C-terminal ends directed in the cytoplasm (*17*). On the basis of this model, several single-cysteine substitutions in the proposed cytosolic as well as the transmembrane domains of DrrB were made (Figure 1). Cross-linking between a defined cysteine in the DrrB protein and an amine in the DrrA protein was analyzed. The DrrA protein contains 43 primary amines which are spread evenly over the entire length of the protein. Of the 43 amines, 5 are lysines, which may be preferentially involved in cross-linking. The lysines are present at amino acid positions 16, 46, 137, 226, and 327 in DrrA, which contains a total of 329 residues. The cysteine to amine cross-linking approach was possible because DrrB protein contains a single cysteine at position 260, whereas DrrA protein contains none. Using site-directed mutagenesis, the single cysteine in DrrB was altered to a serine residue. To rule out if the C260S mutation has an adverse effect on interaction between DrrA and DrrB, the levels of DrrA and DrrB in the mutant C260S were compared to those in the wild type C260 (Figure 2). Comparable levels of DrrA (Figure 2A) and DrrB (Figure 2B) were seen in the membrane fractions prepared from the wild type and the mutant cells. Since the stability of DrrA and DrrB is a good indicator of interaction (*13*), these results

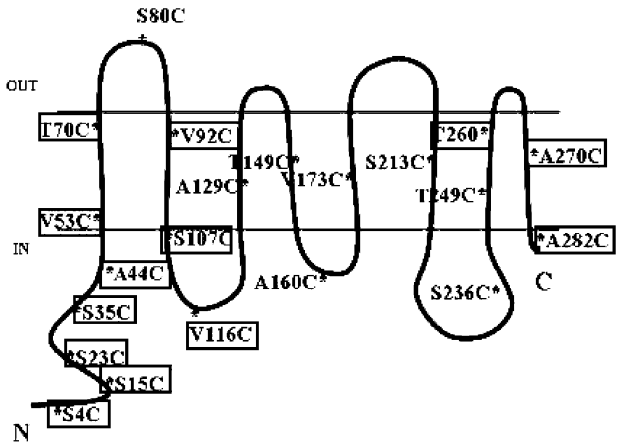

FIGURE 1: Topological depiction of the DrrB protein in the membrane showing the location of various cysteine substitutions. This drawing for the topology of DrrB in the membrane is based on a previously published model of the DrrB protein (*17*). The topological model of DrrB, which is based on gene fusion analysis, suggests that the DrrB protein contains eight transmembrane α helices, with both the N and the C termini in the cytoplasm. To determine domains in DrrB that may be involved in interaction with DrrA, various single-cysteine substitutions were created in the DrrB protein. The location of these cysteines in DrrB is shown. The cysteine substitutions were made in the N- and C-terminal tails, the cytoplasmic loops, and the transmembrane domains of DrrB. S80C is the only cysteine substitution in a periplasmic domain. Since DrrA was found to cross-link with the residues in the N-terminal cytoplasmic tail as well as with residues in TM1 and TM2, S80C was created specifically to map the entire N-terminal domain for its interaction with DrrA. The locations of various cysteines that showed cross-linking with DrrA are marked with rectangles.

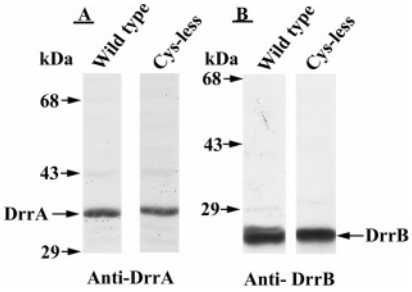

FIGURE 2: Levels of DrrA and DrrB in cells containing wild-type (C260) or cysteine-less DrrB (C260S). *E. coli* cells containing C260 or C260S were induced with IPTG. The lysates were prepared by French press, followed by ultracentrifugation to prepare the membrane fractions, as described in Materials and Methods. Twenty micrograms of membrane protein was analyzed by SDS-PAGE on 10% gels, followed by Western blot analysis using anti-DrrA or anti-DrrB antibodies. A chemiluminescence detection kit was used for detection of the bands. Migration of the standard proteins is shown on the left. (A) Probed with anti-DrrA. (B) Probed with anti-DrrB. Lanes: lane 1, wild-type C260; lane 2, cysteine-less mutant C260S.

suggest that the cysteine-less DrrB is not affected in its interaction with DrrA.

Single Cysteine Substitution Mutants of DrrB. Twenty single cysteine substitution mutants in DrrB were then created using C260S as the starting material (Figure 1). The residues chosen for cysteine substitutions were selected so that they would result in conservative changes. Western blot analysis of the cell membranes showed that both DrrA (Figures 3−5) and DrrB (not shown) are expressed in cells containing cysteine substitution mutants. Some amount of variation in the amounts of DrrA and DrrB was seen in the substitution mutants, as compared to the wild-type cells (Figures 3−5). Experimental variation in the levels of DrrA or DrrB is also routinely seen in the wild-type cells, as expected in a physiological system.

Secondary Structure Analysis of the Cysteine Substitution Mutants. The membrane topology of the DrrB protein has been determined experimentally, and it has been shown to contain eight transmembrane helices with both the N- and the C-terminal tails in the cytoplasm (*17*). The predicted secondary structure of DrrB was determined by the Chou−Fasman method. By this method, the DrrB protein is seen to contain two helical stretches in regions of DrrB that are exposed to the cytoplasm. The first helical region, extending from amino acids 24 to 53, lies within the N-terminal cytoplasmic tail, and the second lies in the third cytoplasmic loop (C3), extending from amino acids 226−248. Since the Chou−Fasman method does not predict transmembrane helices, the secondary structure prediction of DrrB was verified by the PROF method employed by the Predictprotein server (www.predictprotein.org). PROF not only predicted the helices corresponding to the transmembrane domains of DrrB, but more significantly, it predicted the same two helical segments as were shown by the Chou−Fasman method in the cytoplasmic regions of DrrB. These regions lie between amino acids 32 and 49 in the N-terminal domain and between 222 and 245 in the C-terminal domain. Secondary structure prediction was also done for the 20 single-cysteine substitutions and other point mutants used in this study. Three out of 20 cysteine substitution mutants showed some change in the secondary structure of the protein by the Chou−Fasman

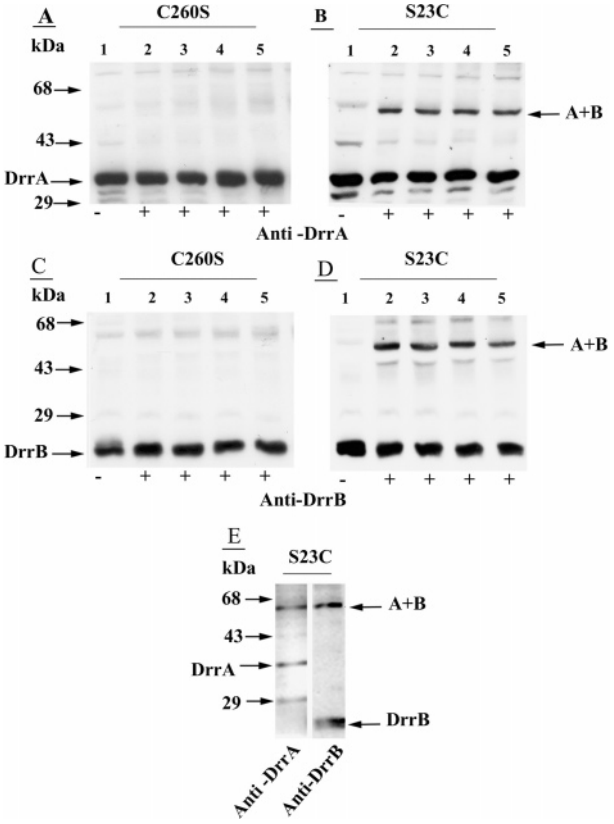

FIGURE 3: Chemical cross-linking between wild-type DrrA and DrrB containing cysteine substitution C260S or S23C. The cell membrane fraction containing the DrrA and DrrB (C260S or S23C) proteins was subjected to chemical cross-linking using different concentrations of GMBS, as described in Materials and Methods. Fifty micrograms of protein was resolved by SDS-PAGE, followed by Western blot analysis using anti-DrrA or anti-DrrB antibodies. The "minus" and "plus" at the bottom of the lanes indicate the absence and presence of the cross-linker in the reaction, respectively. The migration of the protein standards is shown on the left. The location of the cross-linked species is marked as A+B. (A and B) Probed with anti-DrrA. (C and D) Probed with anti-DrrB. Lanes: lane 1, no GMBS; lane 2, 0.2 mM GMBS; lane 3, 0.4 mM GMBS; lane 4, 0.6 mM GMBS; lane 5, 0.8 mM GMBS. (E) A reaction containing the cross-linked S23C sample was analyzed by SDS-PAGE, as described above. The prestained high-molecular-weight protein marker from GibcoBRL was added to the sample before electrophoresis. The resolved proteins were transferred to the nitrocellulose membrane. The lane containing the S23C sample was vertically spliced into halves. Each half was then probed with either the anti-DrrA or anti-DrrB antibodies. Detection was done by the chemiluminescence method. After detection, the vertical halves were aligned using the prestained internal protein marker.

prediction, while the others remained unchanged. Those with the altered secondary structure include S35C, A44C, and S236C. Of these, only S236C was seen to affect doxorubicin resistance phenotype, as described below. The PROF method of prediction showed no significant change in the secondary structure of any of the substitutions or point mutants used in the study.

Doxorubicin Resistance. To determine if cysteine substitutions in DrrB have an effect on the function of the transporter, doxorubicin resistance assays were carried out. Most of the strains containing single-cysteine substitutions showed levels of doxorubicin resistance comparable to that of the wild-type strain (Table 1), suggesting that the function of the complex is not significantly affected by the single-cysteine substitutions constructed in this study. However, a few

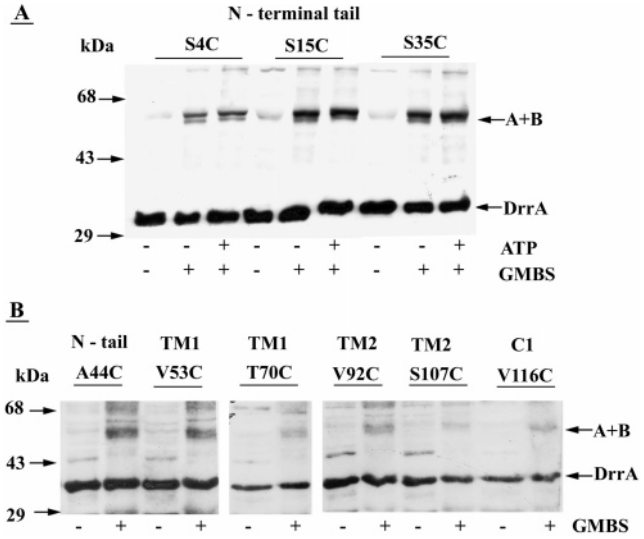

FIGURE 4: Chemical cross-linking between wild-type DrrA and DrrB containing cysteine substitutions in the N-terminal cytoplasmic tail, the transmembrane domains TM1 and TM2, and the cytoplasmic loop C1. The conditions used for chemical cross-linking between DrrA and DrrB were same as described in the legend to Figure 3. The proteins were resolved by SDS-PAGE, followed by Western blot analysis using anti-DrrA antibodies. The "minus" and "plus" at the bottom of the lanes indicate the absence and presence, respectively, of the cross-linker or ATP in the reaction. The migration of the protein standards is shown on the left. The location of the cross-linked species is marked as A+B. (A) S4C, S15C, S35C. (B) A44C, V53C, T70C, V92C, S107C, and V116C.

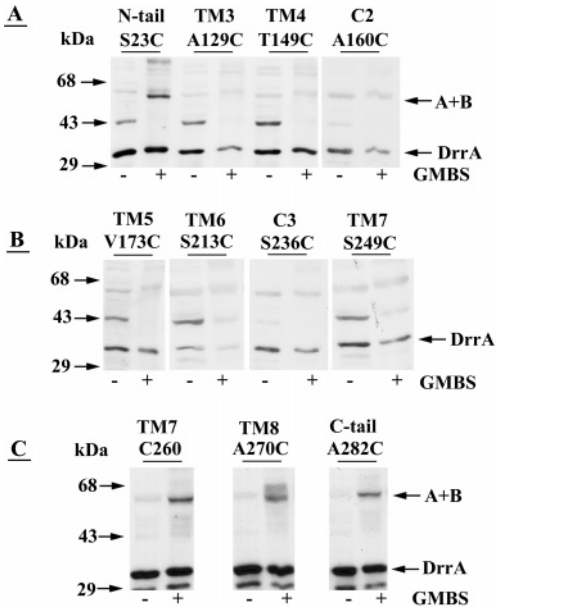

FIGURE 5: Chemical cross-linking between wild-type DrrA and DrrB containing cysteine substitutions in various transmembrane domains (TM3−TM8) or cytoplasmic loops C2 and C3. The conditions used for chemical cross-linking between DrrA and DrrB were same as described in the legend to Figure 3. The proteins were resolved by SDS-PAGE, followed by Western blot analysis using anti-DrrA antibodies. The "minus" and "plus" at the bottom of the lanes indicate the absence and presence of the cross-linker in the reaction, respectively. The migration of the protein standards is shown on the left. The location of the cross-linked species is marked as A+B. (A) S23C (positive control), A129C, T149C, A160C. (B) V173C, S213C, S236C, S249C. (C) C260 (wild type), A270C, A282C.

substitutions, including V53C, T70C, V92C, A129C, S236C, and S249C, showed a decrease in resistance to doxorubicin (Table 1). The possible significance of these observations is discussed later.

Table 1: Doxorubicin Resistance of *E. coli* N43 Cells Expressing Wild Type DrrA with DrrB Containing Different Cysteine Substitutions[a]

domain of DrrB	location of cysteine	amt of dox, μg/mL			
		0	4	6	8
(wild type)	C260	+++	+++	+++	++
N-terminus	S15C	+++	+++	+++	++
N-terminus	S23C	+++	+++	+++	++
N-terminus	S35C	+++	+++	++	++
N-terminus	A44C	+++	+++	++	+
TM 1	V53C	+++	++	++	−
TM 1	T70C	+++	+	+	+
P 1	S80C	+++	++	+	−
TM 2	V92C	+++	±	−	−
TM 2	S107C	+++	+++	++	++
C 1	V116C	+++	+++	+++	++
TM 3	A129C	+++	++	+	−
TM 4	T149C	+++	++	++	+
C 2	A160C	+++	+++	++	+
TM 5	V173C	+++	+++	++	+
TM 6	S213C	+++	+++	++	+
C 3	S236C	+++	++	+	−
TM 7	S249C	+++	+	+	−
TM 8	A270C	+++	+++	++	++
C-terminus	A282C	+++	+++	++	++
cysteine-less DrrB	C260S	+++	+++	+++	+++
vector only	pSU2718	++++	±	−	−

[a] Legend: +++, very good growth; ++, good growth; +, some growth; −, no growth.

Heterobifunctional Cross-Linking. Heterobifunctional cross-linking using GMBS was performed between the 20 single cysteine substitution mutants of DrrB (as well as the wild-type C260) and DrrA. GMBS is an NHS-ester cross-linker. It interacts with a free sulfhydryl group, provided by a cysteine residue on one arm and an amine residue on the other, thus forming a cross-linked product. The N-terminal mutant S23C was the first mutant where a cross-linked product of DrrA and DrrB was identified (Figure 3). Western blot analysis of the cross-linked sample identified a species of about 60 kDa when probed with either anti-DrrA (Figure 3B, lanes 2−5) or anti-DrrB (Figure 3D, lanes 2−5) antibodies. On the basis of its size, this species would correspond to one subunit of DrrA and one subunit of DrrB. Cross-linking could be seen at GMBS concentrations as low as 0.2 mM (Figure 3B,D, lane 2). No significant increase in the cross-linking efficiency was observed when higher concentrations of GMBS were used (Figure 3B,D, lanes 2−5); however, at concentrations higher than 2 mM, a significant drop in the intensity of the 60 kDa species with a concomitant increase in the higher species with molecular mass greater than 200 kDa was seen (not shown). Since high concentrations of the cross-linker can result in nonspecific aggregates, the maximum concentration of GMBS used in this study was 1 mM.

The 60 kDa cross-linked species did not appear in the cysteine-less (C260S) sample on addition of the cross-linker (Figure 3A,C) or in the S23C control sample where DMSO alone was added instead of the cross-linker (Figure 3B,D, lane 1). To further verify if the same cross-linked species was detected by both anti-DrrA and anti-DrrB antibodies, a lane containing the cross-linked S23C sample was spliced vertically into halves. Each half was probed with either the

anti-DrrA or the anti-DrrB antibodies. The reaction was developed by the chemiluminescence assay, and the halves of the nitrocellulose membrane were then aligned with the help of internal protein markers. Results in Figure 3E show that the same cross-linked species reacted with both antibodies, suggesting that it consists of both DrrA and DrrB.

Both anti-DrrA and anti-DrrB antibodies were also seen to cross-react with some other proteins nonspecifically (Figure 3). This is not unexpected, since the cross-linking experiments reported here are carried out with the cell membrane preparations containing many other proteins in addition to DrrA and DrrB. Furthermore, the antibodies used for detection of the cross-linked species are polyclonal in nature, thus resulting in cross-reactivity with certain other epitopes (*2*). The addition of the cross-linker also appeared to change the mobility of some of these unrelated proteins. For example, a 43 kDa protein picked up nonspecifically with the anti-DrrA antibody (Figure 3B, lane 1) is decreased or is not seen in the cross-linked samples (Figure 3B, lanes 2−5). It should be pointed out that the disappearance of the 43 kDa band happens in all the samples, irrespective of the presence (S23C) or absence (C260S) of a cysteine in DrrB (Figure 3A). Since the 60 kDa cross-linked species is not seen in C260S (even though the 43 kDa band disappears), it clearly indicates that the cross-linked species seen in S23C is not the result of cross-linking between DrrB and this 43 kDa unrelated protein. Thus, the 43 kDa protein cross-links with another unknown protein in the membrane. Since our focus in this study is on specific cross-linked species of DrrA and DrrB, further analysis of the 43 kDa protein was not carried out.

S23C, which showed a specific 60 kDa cross-linked species of DrrA and DrrB (described above), was used as a positive control in further cross-linking experiments. The remaining 19 cysteine substitution mutants and the wild-type C260 were analyzed for the cross-linked product of DrrA and DrrB by Western blot analysis using both antibodies; however, only the blots obtained with the anti-DrrA antibody are shown (Figures 4 and 5). As seen with S23C, all the other cysteine substitution mutants tested in the N-terminal tail, S4C, S15C, S35C, and S44C, also showed the appearance of the 60 kDa species on cross-linking with GMBS by Western blot analysis (Figure 4A,B). The effect of addition of ATP (Figure 4A) or doxorubicin on cross-linking was also determined. No significant effect of addition of either substrate alone or together on cross-linking was observed (not shown). Analysis of the mutants in TM1 (V53C and T70C) and TM2 (V92C and S107C) also showed the 60 kDa cross-linked species containing DrrA and DrrB (Figure 4B). However, S80C, a cysteine substitution in the first periplasmic loop (P1), did not show cross-linking with DrrA (not shown). V116C located in the first cytoplasmic loop also showed cross-linking with DrrA (Figure 4B). Analysis of mutants in TM's 3, 4, 5, and 6 and the intervening cytoplasmic loops C2 and C3 did not show the 60 kDa cross-linked species (Figure 5A,B). Wild-type DrrB, which according to the topological model contains residue C260 in the seventh transmembrane domain, cross-linked with DrrA (Figure 5C), although another substitution mutant, S249C, also in the seventh transmembrane domain, did not cross-link with DrrA (Figure 5B). Finally, both A270C in the eighth transmembrane domain (Figure 5C) and A282C at the C-terminal end of DrrB showed cross-linked products with DrrA (Figure 5C).

Together, the chemical cross-linking data presented above show that DrrA cross-links with both the N-terminal and the C-terminal domains of the DrrB protein, suggesting that these regions may be involved in interaction. At the N terminus, the interaction involves the first 50 amino acids of the N-terminal cytoplasmic tail and includes the first and second transmembrane domains extending up to the first cytoplasmic loop of the DrrB protein. At the C-terminal end, the interaction between DrrA and DrrB involves the seventh and the eighth transmembrane helices of DrrB and it extends to the short cytosolic C-terminal tail. The intensity of the cross-linked species varies, depending on the location of the cysteine in the DrrB protein. The best cross-linking was seen with cysteines in the N-terminal cytosolic tail, suggesting that this may be the major site of interaction with DrrA.

Identification of a Conserved Motif in DrrB. As described above, the Chou−Fasman and the PROF methods of secondary structure prediction showed that the DrrB protein contains two helical stretches that are present in regions exposed to the cytoplasm. The first helical region, extending from amino acids 24 to 53, lies within the N-terminal cytoplasmic tail and is part of the N-terminal domain that cross-links with DrrA; the second lies in the third cytoplasmic loop (C3), extending from amino acids 226 to 248. On further analysis, it was found that the helical region present in the N-terminal cytoplasmic tail of DrrB contains an amino acid sequence similar to the L-loop motif present in BtuC (*18*), which is the membrane component of the BtuCD system involved in VitB12 uptake in *E. coli* (Figure 6). The L-loop in BtuC is present in a helical stretch of the cytoplasmic loop between TM6 and TM7. On the basis of the crystal structure of the BtuCD complex, the L-loop of BtuC forms extensive contacts with the ABC component, BtuD. It has also been previously suggested that the L-loop of BtuC shows similarity to a certain sequence in the first cytoplasmic loop of exporters, including the drug exporters MDR1 and LmrA, the lipid exporter MsbA, and the chloride channel CFTR (*18*), all members of the ABC superfamily.

An alignment of the N-terminal region of DrrB with the L-loop in BtuC and several other export and import proteins is shown in Figure 6. This alignment is different from that reported earlier by Locher et al. (*18*); thus, a clarification is needed at this point about the basis used for the alignment shown in Figure 6 in this study as well as about the relationship between the sequence of the L-loop (*18*) and the EAA-loop (*19*). Even though Locher et al. stated that there is a limited similarity of the L-loop of BtuC with the EAA-loop (*18*) of the binding protein-dependent importers (*19*), their sequence alignments did not reflect such a similarity. The alignment shown in Figure 6 in the present study uses the sequence analysis carried out by Saurin et al. (*22*) as the basis for the identification of the EAA-loop motif. In the analysis carried out by Saurin et al., 61 integral membrane proteins belonging to the binding protein dependent importers were classified on the basis of the similarities between their EAA motifs. It was shown that these proteins can be classified into eight clusters. The sequences belonging to the main cluster were called the "EAA cluster". Members of this cluster contain conserved sequences almost identical with the $E_1AA_3...G_7...I.LP$ motif; however, other clusters

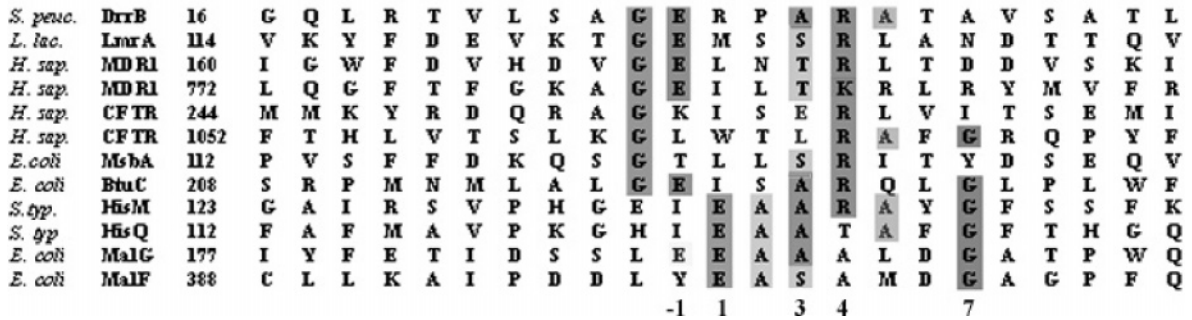

FIGURE 6: Alignment of the amino acid sequence of regions of DrrB, LmrA, MDR1 (N domain), MDR1 (C domain), CFTR (N domain), CFTR (C domain), MsbA, BtuC, HisM, HisQ, MalG, and MalF predicted to interact with their ABC domains/subunits. The dark gray regions show residues that are highly conserved, whereas the residues in light gray are less conserved. The first amino acid in the sequence of each protein is shown. The position of certain residues of the EAA domain is marked at the bottom. The E of the EAA loop is marked as position 1, and the E of the EAA loop in BtuC is marked as position −1.

show some differences compared to the original EAA motif. Within the EAA motif, the central part A_3RALG_7 is the most conserved—of these, the flanking A_3 and G_7 being highly conserved and the middle residues RAL less conserved (*22*). According to this classification (*22*), BtuC is assigned to the cluster of iron−siderophore transporter proteins, which contain the central $A_3...G_7$ sequence; however, they lack the E residue at position 1 and instead contain an E or a D at position −1 (Figure 6). We have based our alignment shown in Figure 6 on the information described above. Since BtuC contains a well-defined $A_3...G_7$ central domain, we have placed the A of the central domain A...G of BtuC at position 3 as described in (*22*). Thus, the E in BtuC falls in the −1 position, as for the members of the iron−siderophore cluster. Once BtuC was thus properly aligned with the EAA domain of other importers, DrrB and other exporters were then aligned with BtuC to obtain the alignment in Figure 6.

As seen in BtuC, the drug exporters, including DrrB, LmrA, MDR1 (N-terminal domain), and MDR1 (C-terminal domain), and other exporters shown in Figure 6 also contain the E residue at the −1 position and a somewhat less conserved $A_3...G_7$ central domain. The G at position 7 in the exporters does not seem to be very highly conserved; however, this group of proteins, along with BtuC, contains a highly conserved G at the −2 position and a positively charged residue R/K at position 4, which may be derived from the central domain A_3RALG_7 of the original EAA motif. Thus, it appears that the conserved sequence in DrrB and other exporters also bears a similarity to the EAA domain. From the sequence analysis described above and the alignment shown in Figure 6, we conclude that DrrB and other exporters contain a conserved motif bearing the sequence $GE_{-1}..A_3R/K..G_7$, which appears to be a modified version of the original EAA motif ($E_1AA_3RALG_7$). Furthermore, the motif present in the BtuC protein, a binding protein dependent importer, has characteristics of both the EAA motif of importers and the motif present in the exporters.

Site-Directed Mutagenesis of Residues in Domains of DrrB That Cross-Link with DrrA. To determine if residues within the interaction domains, identified by chemical cross-linking, are important for the function of the DrrB protein, conservative changes in certain residues in each domain were made. These changes include S23A, G25A, E26D, and S35A in the N-terminal domain and C260A and A270S in the C-terminal domain. Doxorubicin resistance assays showed that S23A, G25A, and E26D mutations in the N-terminal domain confer sensitivity to between 4 and 6 μg/mL of doxorubicin while the cells containing the wild-type DrrA and DrrB grow up to 8−10 μg/mL of doxorubicin (Table 2). Since S35A mutation in the N-terminal domain and C260A and A270S in the C-terminal domain showed no change in the doxorubicin resistance phenotype as compared to the wild type, nonconservative mutations of these three residues, including S35I, C260E, and A270Y, were created. S35I mutation conferred doxorubicin sensitivity, while cells containing C260E and A270Y still remained relatively unaffected (Table 2). Protein expression analysis by SDS-PAGE showed that most mutations, except S23A, S35A, and A270Y show varying degrees of reduction in the levels of DrrA and DrrB. The most severe reduction in the levels of both DrrA and DrrB is seen with S35I mutation (Figure 7). G25A and E26D also showed significantly reduced levels of DrrA and DrrB, although these are less affected as compared to S35I. No significant change in the predicted secondary structure of any of the mutated proteins was seen by the Chou−Fasman or by the PROF method of secondary structure prediction.

Table 2: Doxorubicin Resistance of *E. coli* N43 Cells Expressing Wild-Type DrrA with DrrB Containing Mutations in the N-Terminal Cytoplasmic Tail or the C-Terminal End[a]

domain of DrrB	location of cysteine	amt of dox, μg/mL			
		0	4	6	8
(wild type)	C260	+++	+++	+++	++
N-terminus	S23A	+++	++	−	−
N-terminus	G25A	+++	±	−	−
N-terminus	E26D	+++	±	−	−
N-terminus	S35A	+++	+++	+++	+++
N-terminus	S35I	+++	±	−	−
C-terminus	C260A	+++	+++	+++	−
C-terminus	C260E	+++	+++	+++	−
C-terminus	A270S	+++	+++	+++	+
C-terminus	A270Y	+++	+++	++	++
vector only	pSU2718	+++	±	−	−

[a] Legend: +++, very good growth; ++ good growth; + some growth; −, no growth.

DISCUSSION

The aim of the present study was to characterize domains in the DrrB protein that are involved in interaction with the ABC component DrrA. It has previously been shown that the DrrA and DrrB proteins are biochemically coupled for their function and stability, although they form separate

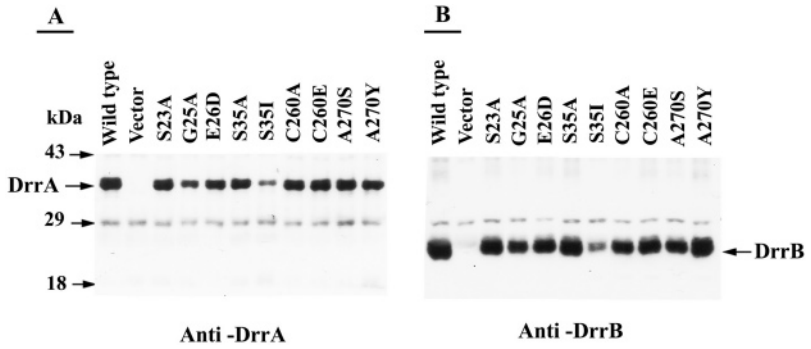

FIGURE 7: Levels of DrrA and DrrB in cells containing point mutations in the N-terminal cytoplasmic tail or the C-terminal end of DrrB. *E. coli* cells containing the indicated plasmids were induced with IPTG. The lysates were prepared by French press, followed by ultracentrifugation to prepare the membrane fractions. Twenty micrograms of membrane protein was analyzed by SDS-PAGE on 10% gels, followed by Western blot analysis using anti-DrrA or anti-DrrB antibodies. A chemiluminescence detection kit was used for detection of the bands. Migration of the standard proteins is shown on the left. (A) anti-DrrA. (B) anti-DrrB.

subunits in the transporter (*13*). To understand how these two proteins regulate the stability and function of each other and result in the formation of a functional transporter, it is necessary to understand their molecular interactions. The strategy employed to study interaction between DrrA and DrrB made use of a cysteine to amine cross-linking approach. This study was facilitated by the availability of a model for the membrane topology of DrrB (*17*). Thus, it was possible to introduce cysteine residues in DrrB at the potential sites of interaction with DrrA. Since DrrA is a soluble protein, the sites of interaction are expected to lie in the domains of DrrB exposed in the cytoplasm. Cysteine-less DrrB was first created by replacing the single cysteine in DrrB at position 260 with a serine residue. Twenty single cysteine substitution mutants were then created at desired locations in the cysteine-less DrrB (Figure 1). Cross-linking between DrrA and DrrB was then carried out by using the chemical cross-linker GMBS, which enables the formation of a cross-link between a primary amine in one protein (DrrA) and a free sulfhydryl group in another (DrrB) (*23*, *24*).

Doxorubicin resistance assays showed that the cysteine-less DrrB C260S as well as most of the single cysteine substitutions in DrrB did not affect the function of the transporter. Six (V53C, T70C, V92C, A129C, S236C, S249C) out of 20 substitutions created in this study, however, showed varying levels of doxorubicin-sensitive phenotype (Table 1). All of the affected residues, except S236C, lie in the predicted membrane spanning helices of DrrB. Both DrrA (Figures 4 and 5) and DrrB (not shown) are still expressed in all of the six strains. Secondary structure analysis by the Chou−Fasman method showed that five (V53C, T70C, V92C, A129C, S249C) of these six mutants, which resulted in doxorubicin sensitivity, showed no change at all in their predicted secondary structure, the exception being S236C, which showed a split in the helical stretch predicted between residues 226 and 248 in the C terminus of DrrB. Thus, we can conclude that doxorubicin sensitivity in V53, T70, V92, A129, and S249C is not the result of a change in the secondary structure, whereas doxorubicin sensitivity in S236C may possibly be due to such a change. Since most of the affected residues, except S236C, occur in the membrane domains, it is possible that these residues may be involved in substrate recognition and thus may be crucial for the transport function of the DrrB protein. Studies to determine the role of these residues in the function of the transporter will be carried out in the future.

From the chemical cross-linking studies presented in this paper, we can conclude that DrrA cross-links with cysteines introduced in both the N-terminal and the C-terminal ends of DrrB. At the N-terminal end, the best cross-linking is seen with residues in the N-terminal cytoplasmic tail, even though it extends to residues in the first and second transmembrane domains and the first cytoplasmic loop. Interaction at the C-terminal end includes the seventh and the eighth transmembrane domain as well as the short C-terminal cytoplasmic tail of DrrB. It is intriguing that DrrA also cross-links with residues in the transmembrane domains of DrrB. This might indicate that DrrA embeds itself into the membrane during its interaction with DrrB. A similar phenomenon has been observed with other peripheral membrane proteins involved in transport, most significantly with SecA (*25*). It has been shown that some part of SecA is permanently embedded in the membrane (*26*−*28*) and that it may also play a significant role in the formation of the channel for the secretion of proteins (*25*).

Two alternate explanations (shown in the model in parts A and B of Figure 8) can account for the observation that DrrA cross-links simultaneously with both the N- and C-terminal ends of DrrB. The models shown in Figure 8 depict the complex of DrrA and DrrB as a tetramer. On the basis of the cross-linking studies carried out earlier, it has been suggested that the stoichiometry of the functional complex of DrrAB may be A_2B_2 (*13*). Tetramers containing DrrA and DrrB were not seen in the present study and are not expected to form in this situation, because cross-linking with GMBS specifically requires a cysteine. Each subunit of DrrB has only one cysteine; thus, it can form a cross-link with only one subunit of DrrA. The possible mechanism of interaction between DrrA and DrrB by each of these models can be explained as follows. (1) According to the model in Figure 8A, one subunit of DrrA binds to the N terminus of one subunit of DrrB and to the C terminus of the other subunit. (2) According to the model in Figure 8B, the halves of a subunit of DrrB fold upon each other such that the N- and C-terminal ends of DrrB are close to each other, and one subunit of DrrA is then able to interact with both ends simultaneously. We currently favor the second model (Figure 8B), because suppressor analyses presently being carried out in this laboratory suggest that the N- and the C-terminal ends of DrrB may be on the same interface (unpublished results). However, further biochemical and genetic analyses are required to clearly determine whether the interaction between

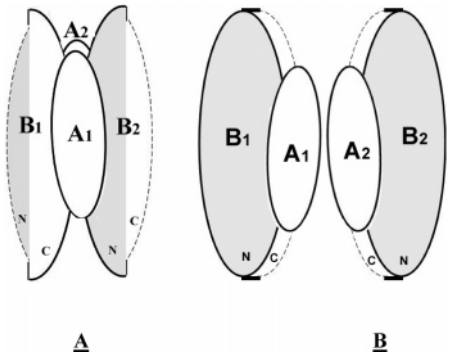

FIGURE 8: Models showing two possible mechanisms of interaction between DrrA and DrrB. The complex of DrrA and DrrB is shown as a tetramer consisting of two subunits of DrrA (A_1 and A_2) and two subunits of DrrB (B_1 and B_2). Further, each subunit of DrrB is shown as containing the N half and the C half (B_1N, B_1C and B_2N, B_2C). On the basis of the observation that DrrA interacts with both the N- and C-terminal ends of DrrB, two alternate models for interaction are proposed. (A) One subunit of DrrA interacts with two subunits of DrrB simultaneously. Thus, $DrrA_1$ contacts B_1C and B_2N. Similarly, $DrrA_2$ contacts B_1N and B_2C. (B) One subunit of DrrA interacts with both the N- and C-termini of the same subunit of DrrB. This is possible if the halves of DrrB fold upon each other, allowing the N- and C-termini to come together on an interface. Thus, $DrrA_1$ binds to the interface of B_1N and B_1C. Similarly, $DrrA_2$ binds to the interface of B_2N and B_2C.

DrrA and DrrB occurs by the mechanism depicted in model A or B in Figure 8. It will also be important in future studies to determine if a single region in DrrA interacts with both termini in DrrB or whether the interaction with DrrB occurs to two separate domains in DrrA. Even though the cross-linking seen in this study is likely to occur between subunits within a tetramer, the possibility of cross-linking between tetramers cannot be ruled out. Both intra- and intermolecular cross-linking between cysteines in Pgp, where all four domains are present in a single molecule, has been seen (*29*). However, DrrA and DrrB form separate subunits that are held together by noncovalent interactions within a tetramer of $DrrA_2B_2$; thus, at this time it is not possible to distinguish between cross-links within a tetramer vs those between tetramers.

Crystal structure information for three ABC transporters, including *E. coli* BtuCD, *E. coli* MsbA, and *V. cholera* MsbA, has recently become available (*18*, *30*, *31*). This information provides valuable insights into the structure of the complex and into the domains involved in interaction between the membrane component and the ABC component in these systems. Interestingly, we find that present within the N-terminal domain of DrrB, which shows cross-linking with DrrA, is a sequence which has significant similarity to a sequence in the "L-loop" motif recently identified in BtuC by crystal structure analysis (*18*) (Figure 6). According to the crystal structure of the BtuCD complex, the L-loop in BtuC forms extensive contacts with the ABC component BtuD. It has also been reported that the sequence of the L loop bears local similarity to a sequence present in the first cytoplasmic loop of drug exporters, MDR1 and LmrA, and the lipid exporter, MsbA, and to the fourth cytoplasmic loop in CFTR (*18*). In the present study, we find similarity of the L-loop to a sequence in the N-terminal cytoplasmic tail of DrrB and biochemically demonstrate that this region may be involved in interaction with DrrA. In both DrrB and BtuC, this sequence lies in a helical stretch in a cytoplasmic domain of the protein; in DrrB, it is present in the N-terminal cytoplasmic tail, while in BtuC, it is present in the cytoplasmic loop between TM6 and TM7 (*18*).

From the analysis carried out in this study (Figure 6), we conclude that the L-loop of BtuC is actually similar to the EAA loop of other importers. We further conclude that DrrB and other exporters (as well as the importer BtuC) contain a conserved motif with the sequence $GE_{-1}..A_3R/K..G_7$ and that it is actually a modified version of the original EAA motif ($E_1AA_3RALG_7$) present in the binding protein dependent permeases. Our results thus indicate for the first time that the EAA motif or a modified version of this motif may be present at the interaction interfaces in both uptake and efflux systems, suggesting that the sequences involved in interaction are derived from an ancestor before the importers and exporters of the ABC family might have diverged.

A conserved motif, similar to the one identified in the N-terminal domain, was not identified in the C-terminal interacting domain of DrrB in this study. It should be mentioned, however, that a second helical region was identified in the third cytoplasmic loop, between residues 226 and 248, of DrrB. It is possible that, in the three-dimensional structure, this region (extending up to the C-terminal end of DrrB) may actually lie close to the conserved motif present in the N-terminal domain of DrrB and thus may contribute to forming the interface with DrrA, as is indicated by the cross-linking data (Figure 5C). Such architecture for DrrB would also be suggested by the model in Figure 8B. However, further biochemical and genetic studies are needed to answer some of these questions.

Mutagenesis of certain residues in and around the conserved motif in the N terminus of DrrB, including S23A, G25A, E26D, and S35I, resulted in sensitivity to doxorubicin, although mutagenesis of C260 and A270 in the C terminus did not confer doxorubicin sensitivity significantly. These findings indicate that the motif in the N terminus is required for the function of the DrrB protein, whereas the domain in the C terminus may not be directly involved in function but may be at the same interface of interaction, as was suggested above. G25A, E26D, and S35I mutations also resulted in reduced levels of DrrA and DrrB. Since the stability of DrrB is known to be dependent on its interaction with DrrA, such a phenotype might indicate a direct role for these residues in interaction between DrrA and DrrB. Second-site suppressor analysis of the S23A, G25A, E26D, and S35I mutations will be carried out in the future to verify if these residues are indeed directly involved in interaction with DrrA, as is suggested by the cysteine cross-linking studies reported here. Mutagenesis of certain residues in the conserved motif in MDR1 was also previously shown to result in loss of drug resistance (*32*); however, these residues were never biochemically shown to be directly involved in interaction with the ABC domain of MDR1. The only system where biochemical characterization has previously been carried out is the maltose uptake permease, where a typical EAA-loop (*33*), present in the membrane subunits MalF and MalG, has been shown to be involved in interaction with MalK by chemical cross-linking studies (*34*).

In summation, this study identifies two domains in DrrB that may be involved in interaction with DrrA and identifies a motif in the N-terminal cytoplasmic tail of DrrB. This motif has similarities to the EAA motif of binding protein dependent importers and the L-loop motif of BtuC. Other interactions between DrrA and DrrB, if present, could not

be identified by the strategy employed in this study. Further studies, including the crystal structure analysis of the complex, would be required to identify other interactions and to determine the overall structure of the complex. Not only is the present study important for understanding the mechanism of interaction and function of the DrrAB complex, but it is also a necessary step toward elucidation of the temporal sequence of events leading to the biogenesis of the complex. Since the specific binding of DrrA to DrrB protects DrrB from proteolysis, we have previously suggested that DrrA may act like a chaperone to facilitate the proper assembly of the complex in the membrane (*13*). Furthermore, DrrB regulates the catalytic activity of DrrA via precise, but not yet understood, conformational changes. Future studies in this laboratory will identify the interacting domains in DrrA and will also try to unravel the concomitant conformational changes that are essential for the function of the complex as a transporter.

REFERENCES

1. Guilfoile, P. G., and Hutchinson, C. R. (1991) A bacterial analogue of the mdr gene of mammalian tumor cells is present in Streptomyces peucetius, the producer of daunorubicin and doxorubicin, *Proc. Natl. Acad. Sci. USA 88*, 8553−7.
2. Kaur, P. (1997) Expression and characterization of DrrA and DrrB proteins of Streptomyces peucetius in *Escherichia coli*: DrrA is an ATP binding protein, *J. Bacteriol. 179*, 569−75.
3. Fath, M. J., and Kolter, R. (1993) ABC transporters: bacterial exporters, *Microbiol. Rev. 57*, 995−1017.
4. Gottesman, M. M., and Pastan, I. (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter, *Annu. Rev. Biochem. 62*, 385−427.
5. Ambudkar, S. V., Lelong, I. H., Zhang, J., Cardarelli, C. O., Gottesman, M. M., 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.
6. Loo, T. W., and Clarke, D. M. (1995) P-glycoprotein. Associations between domains and between domains and molecular chaperones, *J. Biol. Chem. 270*, 21839−21844.
7. Ostedgaard, L. S., Rich, D. P., DeBerg, L. G., and Welsh, M. J. (1997) Association of domains within the cystic fibrosis transmembrane conductance regulator, *Biochemistry 36*, 1287−1294.
8. van Veen, H. W., Venema, K., Bolhuis, H., Oussenko, I., Kok, J., Poolman, B., Driessen, A. J., and Konings, W. N. (1996) Multidrug resistance mediated by a bacterial homologue of the human multidrug transporter MDR1, *Proc. Natl. Acad. Sci. USA 93*, 10668−10672.
9. Gileadi, U., and Higgins, C. F. (1997) Membrane topology of the ATP-binding cassette transporter associated with antigen presentation (Tap1) expressed in *Escherichia coli*, *J. Biol. Chem. 272*, 11103−11108.
10. Klein, I., Sarkadi, B., and Varadi, A. (1999) An inventory of the human ABC proteins, *Biochim. Biophys. Acta 1461*, 237−262.
11. Gentschev, I., and Goebel, W. (1992) Topological and functional studies on HlyB of *Escherichia coli*, *Mol. Gen. Genet. 232*, 40−48.
12. Dean, M., Hamon, Y., and Chimini, G. (2001) The human ATP-binding cassette (ABC) transporter superfamily, *J. Lipid Res. 42*, 1007−1017.
13. Kaur, P., and Russell, J. (1998) Biochemical coupling between the DrrA and DrrB proteins of the doxorubicin efflux pump of Streptomyces peucetius, *J. Biol. Chem. 273*, 17933−17939.
14. Bass, R. B., Locher, K. P., Borths, E., Poon, Y., Strop, P., Lee, A., and Rees, D. C. (2003) The structures of BtuCD and MscS and their implications for transporter and channel function, *FEBS Lett. 555*, 111−115.
15. Welsh, M. J., Anderson, M. P., Rich, D. P., Berger, H. A., Denning, G. M., Ostedgaard, L. S., Sheppard, D. N., Cheng, S. H., Gregory, R. J., and Smith, A. E. (1992) Cystic fibrosis transmembrane conductance regulator: a chloride channel with novel regulation, *Neuron 8*, 821−829.
16. Wang, W., He, Z., O'Shaughnessy, T. J., Rux, J., and Reenstra, W. W. (2002) Domain-domain associations in cystic fibrosis transmembrane conductance regulator, *Am. J. Physiol. Cell Physiol. 282*, C1170−C1180.
17. Gandlur, S. M., Wei, L., Levine, J., Russell, J., and Kaur, P. (2004) Membrane topology of the DrrB protein of the doxorubicin transporter of streptomyces peucetius, *J. Biol. Chem. 279*, 27799−27806.
18. Locher, K. P., Lee, A. T., and Rees, D. C. (2002) The E. coli BtuCD structure: a framework for ABC transporter architecture and mechanism, *Science 296*, 1091−1098.
19. Dassa, E., and Hofnung, M. (1985) Sequence of gene malG in E. coli K12: homologies between integral membrane components from binding protein-dependent transport systems, *Embo J. 4*, 2287−2293.
20. Sambrook, J., Fritsch, E. F., Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, New York.
21. Nakamura, H., Tojo, T., and Greenberg, J. (1975) Interaction of the expression of two membrane genes, acrA and plsA, in *Escherichia coli* K-12, *J. Bacteriol. 122*, 874−879.
22. Saurin, W., Koster, W., and Dassa, E. (1994) Bacterial binding protein-dependent permeases: characterization of distinctive signatures for functionally related integral cytoplasmic membrane proteins, *Mol. Microbiol. 12*, 993−1004.
23. Brinkley, M. (1992) A brief survey of methods for preparing protein conjugates with dyes, haptens, and cross-linking reagents, *Bioconjug. Chem. 3*, 2−13.
24. Peeters, J. M., Hazendonk, T. G., Beuvery, E. C., and Tesser, G. I. (1989) Comparison of four bifunctional reagents for coupling peptides to proteins and the effect of the three moieties on the immunogenicity of the conjugates, *J. Immunol. Methods 120*, 133−143.
25. Wang, H. W., Chen, Y., Yang, H., Chen, X., Duan, M. X., Tai, P. C., and Sui, S. F. (2003) Ring-like pore structures of SecA: implication for bacterial protein-conducting channels, *Proc. Natl. Acad. Sci. USA 100*, 4221−4226.
26. Eichler, J., and Wickner, W. (1998) The SecA subunit of *Escherichia coli* preprotein translocase is exposed to the periplasm, *J. Bacteriol. 180*, 5776−5779.
27. Chen, X., Brown, T., and Tai, P. C. (1998) Identification and characterization of protease-resistant SecA fragments: secA has two membrane-integral forms, *J. Bacteriol. 180*, 527−537.
28. Ramamurthy, V., and Oliver, D. (1997) Topology of the integral membrane form of *Escherichia coli* SecA protein reveals multiple periplasmically exposed regions and modulation by ATP binding, *J. Biol. Chem. 272*, 23239−23246.
29. Urbatsch, I. L., Gimi, K., Wilke-Mounts, S., Lerner-Marmarosh, N., Rousseau, M. E., Gros, P., and Senior, A. E. (2001) Cysteines 431 and 1074 are responsible for inhibitory disulfide cross-linking between the two nucleotide-binding sites in human P-glycoprotein, *J. Biol. Chem. 276*, 26980−26987.
30. Chang, G., and Roth, C. B. (2001) Structure of MsbA from E. coli: a homologue of the multidrug resistance ATP binding cassette (ABC) transporters, *Science 293*, 1793−1800.
31. Chang, G. (2003) Structure of MsbA from Vibrio cholera: a multidrug resistance ABC transporter homologue in a closed conformation, *J. Mol. Biol. 330*, 419−430.
32. Currier, S. J., Kane, S. E., Willingham, M. C., Cardarelli, C. O., Pastan, I., and Gottesman, M. M. (1992) Identification of residues in the first cytoplasmic loop of P-glycoprotein involved in the function of chimeric human MDR1-MDR2 transporters, *J. Biol. Chem. 267*, 25153−25159.
33. Mourez, M., Hofnung, M., and Dassa, E. (1997) Subunit interactions in ABC transporters: a conserved sequence in hydrophobic membrane proteins of periplasmic permeases defines an important site of interaction with the ATPase subunits, *Embo J. 16*, 3066−3077.
34. Hunke, S., Mourez, M., Jehanno, M., Dassa, E., and Schneider, E. (2000) ATP modulates subunit-subunit interactions in an ATP-binding cassette transporter (MalFGK2) determined by site-directed chemical cross-linking, *J. Biol. Chem. 275*, 15526−15534.

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