Role of a Conserved Membrane-Embedded Acidic Residue in the Multidrug Transporter MdfA

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ABSTRACT: According to the current topology model of the *Escherichia coli* multidrug transporter MdfA, it contains a membrane-embedded negatively charged residue, Glu26, which was shown to play an important role in substrate recognition. To further elucidate the role of this substrate recognition determinant, various Glu26 replacements were characterized. Surprisingly, studies with neutral MdfA substrates showed that, unlike many enzymatic systems where the size and chemical properties of binding site residues are relatively defined, MdfA tolerates a variety of changes at position 26, including size, hydrophobicity, and charge. Moreover, although efficient transport of positively charged substrates requires a negative charge at position 26 (Glu or Asp), neutralization of this charge does not always abrogate the interaction of MdfA with cationic drugs, thus demonstrating that the negative charge does not play an essential role in the multidrug transport mechanism. Collectively, these results suggest a link between the broad substrate specificity profile of multidrug transporters and the structural and chemical promiscuity at their substrate recognition pockets.

The simultaneous emergence of resistance in eukaryotic and prokaryotic cells to many chemically unrelated drugs is termed multidrug resistance (Mdr).[1] Eukaryotic and prokaryotic Mdr transporters (*1*, *2*), which remove the drugs from the cell cytoplasm or cytoplasmic membrane to the external medium, cause one major form of multidrug resistance. These transporters are usually able to extrude a variety of chemically unrelated lipophilic compounds, many of which are positively charged under physiological conditions. However, many Mdr transporters also interact with neutral and zwitterionic drugs, some of which are relatively hydrophilic. Although interesting features of multidrug recognition have been revealed in the past, it is not yet fully understood how a single transport protein can recognize and transport such an extremely broad spectrum of chemically unrelated molecules.

Often, it has been proposed that cationic drugs interact with membrane-embedded negatively charged residues of Mdr transporters. Mutational analysis demonstrated that, indeed, such acidic residues perform an important role in the transport activity of these transporters (*3*−*7*). Sometimes, the negatively charged membrane-embedded residues have been implicated specifically in the recognition of cationic substrates. For example, a single membrane-embedded acidic residue of the *Escherichia coli* Mdr transporter EmrE is directly involved in the recognition of cationic substrates (*8*). Similarly, the staphylococcal MFS-related Mdr transporter QacA also contains a membrane-embedded acidic residue required for recognition of cationic drugs (*9*), and two membrane-embedded acidic residues in the lactococcal Mdr transporter LmrP from the MFS superfamily are important for multidrug recognition (*10*). Also, with ABC-related Mdr transporters, acidic residues play a role in recognition of cationic substrates, as shown for human MRP1 (*11*). Recent studies on the *E. coli* Mdr transporter, MdfA (*12*), also provided an important insight regarding the role of a membrane-embedded negative charge in multidrug recognition and transport.

MdfA is a 410 amino acid long membrane protein of the MFS family of secondary transporters. Recently, close homologues of MdfA were identified in the following pathogenic bacteria: *Shigella flexneri* (99% homology) (*13*), *Salmonella enterica* serovar Typhi (90% homology) (*14*), and *Yersinia pestis* (73% homology) (*15*). Transport experiments have shown that MdfA is driven by the proton electrochemical gradient and functions as a drug/proton antiporter (*12*, *16*, *17*). One of the most interesting properties of MdfA is its ability to recognize both charged and uncharged compounds (*12*, *18*), and a recent study has demonstrated simultaneous binding of such substrates to the transporter (*19*). As predicted from the hydropathy plot of the protein and gene fusion analyses, the putative 12 transmembrane segments (TMs) of MdfA contain a single membrane-embedded charged amino acid residue, namely, glutamate at position 26, in the middle of the first TM (*20*, *21*) (Figure 1). Previous studies have demonstrated that mutations of Glu26 affect the substrate recognition profile of MdfA (*20*), inferring that Glu26 is an important determinant of drug recognition. In fact, replacement of Glu26 with a lysine residue greatly inhibited the resistance against several positively charged drugs; however, the mutant was active in the efflux of a neutral substrate, chloramphenicol.

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[1] Abbreviations: Mdr, multidrug resistance; TM, transmembrane segment; TPP^+, tetraphenylphosphonium; EtdBr, ethidium bromide; LB, Luria−Bertani medium; DDM, *n*-dodecyl maltoside; NTA, nitriloacetic acid.

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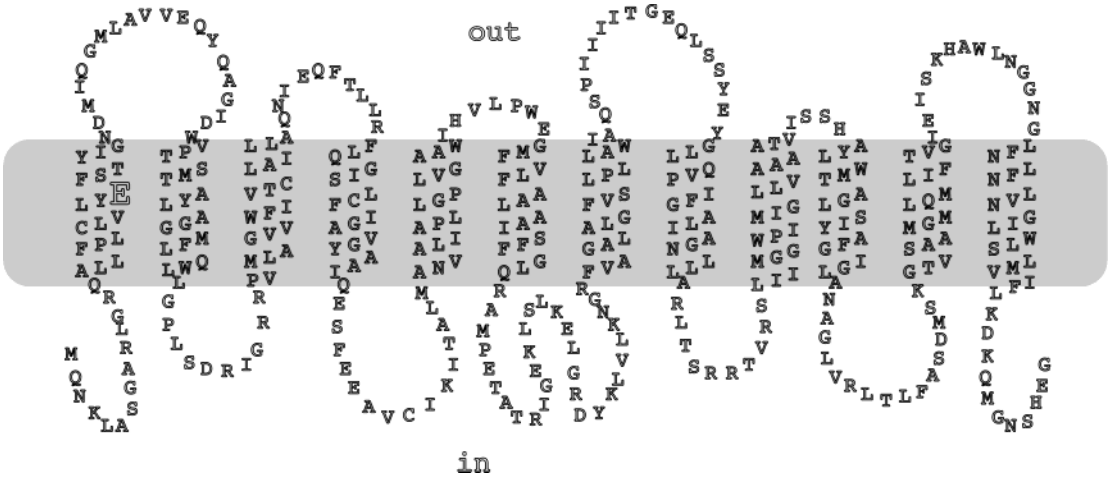


FIGURE 1: Secondary structure model of MdfA. A model for MdfA secondary structure was constructed on the basis of the hydropathy profile, distribution of positively charged residues, and gene fusion analysis (*20*, *21*).

In contrast, when the negative charge was preserved in a Glu26Asp mutant, although chloramphenicol transport decreased, the mutant exhibited almost wild-type multidrug resistance activity against lipophilic cations. These results have suggested that (i) Glu26 is not involved in energy coupling (at least in the chloramphenicol transport cycle), (ii) the negative charge at position 26 may interact electrostatically with the positively charged drugs, and (iii) chloramphenicol recognition is not sensitive to the charge but possibly to other properties of the residue at position 26.

To further examine this model, we constructed additional Glu26 replacements with residues of different size and hydrophobicity. The effect of these mutations on the activity of MdfA with neutral substrates demonstrated that the protein tolerates various structural and chemical changes at position 26. To study the effect of the mutations on the interaction of MdfA with cationic substrates, the ability of the mutants to bind the cationic substrate tetraphenylphosphonium (TPP^+) was analyzed. Surprisingly, some of the mutants were able to bind TPP^+ in the absence of a negative charge at position 26, suggesting that, in addition to the proposed crucial electrostatic interaction, other determinants participate in substrate binding (*22*). The question was whether the negative charge could also be compromised during transport. The results of drug resistance and transport assays showed that, indeed, some of the neutralized Glu26 mutants are able to facilitate low levels of transport and resistance with cationic drugs. Therefore, although the transport of cationic substrates by MdfA is strongly dependent on electrostatic interactions with Glu26, other determinants in the multidrug binding pocket partially preserve transport activity of neutralized Glu26 mutants with cationic substrates. Importantly, the results demonstrate that the negative charge at position 26 does not play an essential role in the multidrug transport mechanism of MdfA.

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]Tetraphenylphosphonium (TPP^+) (30 Ci/mmol) was purchased from Amersham Pharmacia, and [^{3}H]-chloramphenicol (20 Ci/mmol) was bought from ARC. Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), phenylmethanesulfonyl fluoride (PMSF), DNase, ampicillin, kanamycin, ethidium bromide (EtdBr), chloramphenicol, erythromycin, tetracycline, puromycin, and pyronin Y were purchased from Sigma. Tetraphenylphosphonium (TPP^+) was obtained from Fluka, benzalkonium chloride was from Calbiochem, and thiamphenicol was from ICN. The protease inhibitor Pefablock was from Roche. *n*-Dodecyl maltoside (DDM) was purchased from Anatrace. Ni^+-NTA−agarose (Qiagen) was utilized for binding assays. Restriction and modifying enzymes were obtained from New England BioLabs. Oligodeoxynucleotides were synthesized by the scientific services unit at the Weizmann Institute of Science. India HisProbe (Pierce) was used for MdfA-6His detection by Western blotting. Prestained protein molecular weight markers were obtained from New England BioLabs and DNA molecular weight markers from Fermentas. DNA purification kits were obtained from Biological Industries and DNA plasmid prep kits from Qiagen and Promega. All other materials were of reagent grade and were obtained from commercial sources.

Bacterial Strains. E. coli HB101 [*hsdS20* (r_B^- m_B^-), *recA13*, *ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20* (Smr^r), *xyl-5*, *mtl-1*, *supE44*, λ^-/F^-] was used for the propagation and preparation of various plasmid constructs. *E. coli* UT*mdfA::kan* (Edgar and Bibi, unpublished data) or the leaky strain UTL2*mdfA::kan* (*20*) was used in drug resistance and transport experiments.

Site-Directed Mutagenesis. Mutants of Glu26 were constructed using oligonucleotide-directed, site-specific mutagenesis by the two-step PCR method (*23*). The final PCR products were digested with *Sph*I and *Bst*BI and ligated to the *Sph*I−*Bst*BI 3.6 kb fragment of plasmid pT7−5/*mdfA-6His*. Mutants Glu26Val and Glu26Thr were obtained previously (Adler and Bibi, in preparation). The mutations were verified by sequencing the length of the PCR-generated segments through the ligation junctions.

Preparation of Membranes. E. coli UTL2*mdfA*::*kan* cells harboring plasmid pUC18/*pARA/mdfA-6His* were grown at 37 °C in LB medium supplemented with carbenicillin (50 μg/mL), ampicillin (50 μg/mL), and kanamycin (30 μg/mL). Overnight cultures were diluted to 0.07 OD_{600} unit, grown in the above medium to 1 OD_{600} unit, and induced with 0.2% arabinose for 1 h. A typical 12 L culture yielded 15−20 g (wet weight) of cells. Cell pellets were washed once in 0.4 L of 50 mM KP_i buffer (K_2HPO_4, KH_2PO_4, pH 7.5)

supplemented with 5 mM $MgSO_4$ and pelleted by centrifugation (30 min, 2000*g*). The cells were resuspended in 60 mL of the same buffer containing also 10 mM β-mercaptoethanol, 30 μg/mL DNase, and 0.5 mM Pefablock and passed three times through a French pressure cell (15000 psi) for disruption. Cell debris was removed by centrifugation (5 min, 8000*g*), and the membranes were collected by ultracentrifugation (1.5 h, 300000*g*). Finally, the membranes were resuspended and homogenized in 20 mL of buffer A (20 mM Tris-HCl, pH 7.9, 0.5 M NaCl, 5 mM imidazole, and 10% glycerol). Aliquots of 2 mL containing about 40 mg/mL total membrane proteins were snap-frozen in liquid nitrogen and stored at −80 °C.

Western Blotting. Overnight cultures of *E. coli* UTL2*mdfA::kan* cells harboring pT7-5 (vector) or pT7-5/*mdfA-6His* constructs were diluted to an OD_{600} of 0.04 in LB supplemented with ampicillin (200 μg/mL) and kanamycin (30 μg/mL) and grown to an OD_{600} of 0.6−0.7. Bacteria were harvested, and membranes were prepared as described previously (*24*). Membrane fractions (15 μg total) were then subjected to SDS−PAGE using 12.5% polyacrylamide in the running gel. Proteins were electroblotted to nitrocellulose membranes, and following incubation with India HisProbe−HRP solution, the membranes were probed by ECL.

Membrane Solubilization and Binding Assay. The binding assays were based on a method developed by Muth and Schuldiner (*8*), with modifications (*19*). For solubilization, two aliquots of membranes were thawed at room temperature and added to 10 mL of buffer A, containing DDM (1.2% final concentration). The mixture was homogenized and agitated gently for 30 min at 4 °C. Insoluble material was discarded by ultracentrifugation (1 h, 250000*g*), and the soluble fraction was mixed in a 15 mL tube with Ni-NTA beads (5 μL per assay). The mixture was agitated gently for 30 min at 4 °C. Unbound material (sup) was discarded after pulse centrifugation (3 min, 700*g*). The beads were then washed once with 2.5 mL of buffer B (20 mM Tris-HCl, pH 7, 0.5 M NaCl, 5 mM imidazole, and 0.1% DDM) and resuspended in buffer C (the same as buffer B but without imidazole). Aliquots (100 μL, containing 5−10 μg of MdfA) of beads were incubated (10 min agitation at 4 °C) with 100 μL of 150 nM [^{3}H]TPP^+ dissolved in buffer C with or without the addition of unlabeled TPP or the indicated test substrate−inhibitor. An aliquot of 180 μL of the reaction mixture was then transferred to a Promega Wizard minicolumn on top of a microfuge tube (1.5 mL) and centrifuged at 10000*g* for 20 s. Unbound (flow-through) material was discarded, and the MdfA-6His-resin was resuspended in 100 μL of buffer D (the same as buffer C but with 350 mM imidazole). The radioactivity of this suspension was measured using liquid scintillation. The amount of MdfA in each experiment was evaluated by comparison with the known purified MdfA standard run on SDS−PAGE and stained by Coomassie Brilliant Blue. The results in Figure 4 are represented as Scatchard plots and used for calculation of K_D values.

Drug Resistance Assays. Resistance of cells harboring the indicated plasmids was assayed in both solid and liquid media. When tested on solid media, drug resistance assays were performed as described by Yerushalmi and Schuldiner (*25*). Specifically, *E. coli* UT*mdfA::kan* cells harboring different pT7-5/*mdfA-6His* plasmids were grown overnight at 37 °C in LB supplemented with the antibiotics ampicillin (200 μg/mL) and kanamycin (30 μg/mL). A series of 10-fold dilutions (10^{-1}−10^{-6}) were prepared for the cultures, and 4 μL of each dilution were spotted on plates containing the antibiotics and different concentrations of the test compound. For assay of TPP^+ resistance in liquid media, overnight cultures of *E. coli* UTL2*mdfA::kan* were diluted into fresh LB containing the antibiotics and grown to an OD_{600} of 1 unit. Cells were then diluted to an OD_{600} of 0.05 unit and aliquoted (50 μL) into 96-well ELISA microplates containing 50 μL of LB with different concentrations of TPP^+. Plates were incubated at 37 °C with shaking, and cell density was monitored continuously by following the absorption at 600 nm in a microplate autoreader.

Transport Assays. Transport assays were conducted as described by Edgar and Bibi (*12*), with some modifications. For chloramphenicol uptake assays, overnight cultures of *E. coli* UT*mdfA*::*kan* cells with different pT7-5/*mdfA-6His* constructs were diluted to 0.05 OD_{600} unit and grown at 37 °C in LB supplemented with ampicillin (200 μg/mL) and kanamycin (30 μg/mL) to 0.6 OD_{600} unit. The cultures were harvested and washed once with KP_i buffer (50 mM, pH 7.0). The cells were resuspended in the same buffer to an OD_{420} of 10 units and aliquoted (50 μL). Following 2 min recovery at 37 °C in the presence of 0.2% glucose, transport was initiated by the addition of [^{3}H]chloramphenicol (0.2 μM). [^{3}H]TPP^+ uptake was conducted essentially the same as with chloramphenicol. UTL2*mdfA*::*kan* cells were resuspended to an OD_{420} of 7 units. Following 2 min recovery at 37 °C in the presence of 0.2% glucose, transport was initiated by the addition of [^{3}H]TPP^+ (10 μM). Transport was terminated by rapid filtration as previously described (*12*). For efflux assays with ethidium bromide, overnight cultures of *E. coli* UTL2*mdfA*::*kan* cells harboring the pT7-5 vector or the indicated pT7-5/*mdfA-6His* plasmids with *mdfA* constructs were diluted to 0.04 OD_{600} unit, grown at 37 °C in LB supplemented with ampicillin (200 μg/mL) and kanamycin (30 μg/mL) to 0.9−1.0 OD_{600} unit, and kept on ice. Aliquots of cells (0.3 OD_{600} unit) were pelleted, resuspended in 2 mL of KP_i buffer (50 mM, pH 7.0) to 0.3 OD_{600} unit, and loaded with EtdBr (5 μM) at 37 °C for 5 min in the presence of CCCP (100 μM). Loaded cells were then centrifuged, resuspended in the same buffer containing only EtdBr (5 μM), and subjected to fluorescence measurements. After approximately 1 min in the fluoroimeter, glucose was added (final concentration 0.4%). EtdBr efflux was monitored continuously by measuring the fluorescence, using excitation and emission wavelengths of 545 and 610 nm, respectively.

RESULTS

Construction and Preliminary Characterization of MdfA Glu26 Mutants. Our previous studies indicated, based on three Glu26 replacements (Ala, Lys, and Asp), that this residue plays an important role in substrate recognition by MdfA. However, although these studies raised the possibility that substrate recognition might be sensitive to the length or volume of the side chain at position 26, other possibilities could not be ruled out. To further examine the requirements of the side chain at position 26, we investigated both the previous mutants (*20*) and newly constructed mutants, where Glu26 was replaced by residues of different size and

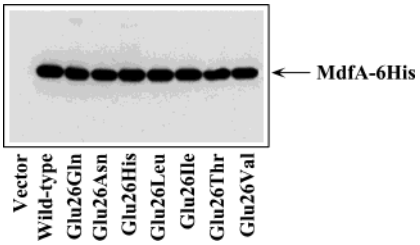
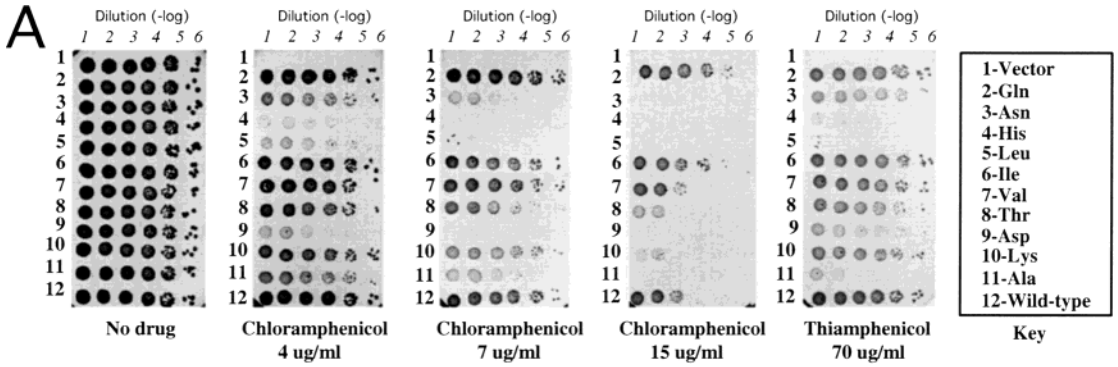


FIGURE 2: Expression of the wild-type MdfA and Glu26 mutants. *E. coli* UTL2*mdfA::kan* cells harboring pT7-5 (vector), pT7-5/*mdfA-6His*, or Glu26 mutants were grown as described in Experimental Procedures. Membrane fractions were analyzed by SDS−PAGE followed by Western blotting using India HisProbe-HRP. Every lane contains 15 μg of total membrane proteins prepared from cells carrying the respective constructs.

hydrophobicity: Gln, Asn, His, Thr, Val, Leu, and Ile. In all cases we used a configuration in which *mdfA* was cloned in plasmid pT7-5 with the wild-type 5′ 231-bp untranslated region of the *mdfA* gene (*12*). In addition, to enable detection and purification of the proteins, a six-histidine tag was fused at the C-termini of the mutants; as shown in Figure 2, a comparable steady-state level of expression was observed with all of the mutants. Initially, to avoid electrostatic considerations, we tested the substrate recognition properties of these mutants with the neutral substrates chloramphenicol and thiamphenicol. Briefly, *E. coli* cells were transformed with plasmids encoding each of the Glu26 mutants and plated onto LB plates containing chloramphenicol or thiamphenicol (Figure 3A). When tested at low concentrations of chloramphenicol (4 μg/mL), all of the transformants were able to grow. However, as the chloramphenicol concentration was increased (7−10 μg/mL), very little or no growth was observed with cells expressing the mutants Glu26His, Glu26Leu, Glu26Asp, Glu26Asn, and Glu26Ala. Surprisingly, at higher concentrations of the antibiotic (15−23 μg/mL), Glu26Gln and Glu26Ile were able to support growth even better than the wild-type MdfA. Similar results were obtained with thiamphenicol (Figure 3A, right panel). Next, the transport of chloramphenicol by the mutants was assayed by rapid filtration as described previously (*12*). Figure 3B shows that the transport activities of the mutants were correlated with the resistance activities (Figure 3A); as expected, cells harboring plain vector accumulated chloramphenicol relatively rapidly (within less than 1 min) to a steady-state level that is about 2.5-fold higher than that observed with cells expressing wild-type MdfA or mutants Glu26Ile, Glu26Gln, and Glu26Val. However, the mutants Glu26Thr, Glu26Asn, and Glu26Leu were less effective in preventing chloramphenicol accumulation, and Glu26His was almost inactive. These results support previous conclusions (*20*) that the negative charge at position 26 of MdfA is not required for transport of neutral substrates and that mutations at Glu26 structurally alter the substrate recognition properties



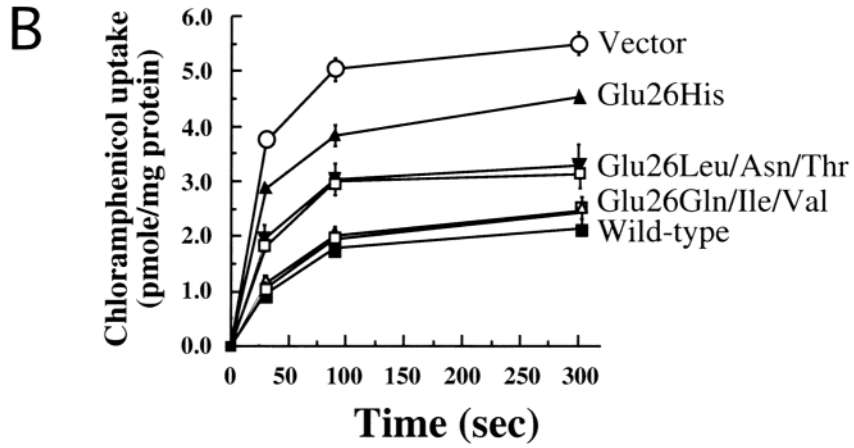


FIGURE 3: Drug resistance and transport by Glu26 mutants of MdfA with neutral substrates. (A) *E. coli* UT*mdfA::kan* cells transformed with pT7-5 (vector), wild-type pT7-5/*mdfA-6His*, or Glu26 mutants were diluted (10^{-1}−10^{-6}), and 4 μL of diluted cultures was spotted on LB agar plates with the selection antibiotics ampicillin (200 μg/mL) and kanamycin (30 μg/mL) (LB-amp-kan), supplemented also with chloramphenicol or thiamphenicol as indicated. (B) MdfA-mediated decreased accumulation of [^{3}H]chloramphenicol (200 nM) in energized UT*mdfA::kan* cells. Uptake of [^{3}H]chloramphenicol was assayed by rapid filtration in cells transformed with the different plasmids, as described in Experimental Procedures. The experiments were performed in triplicate and repeated three times.

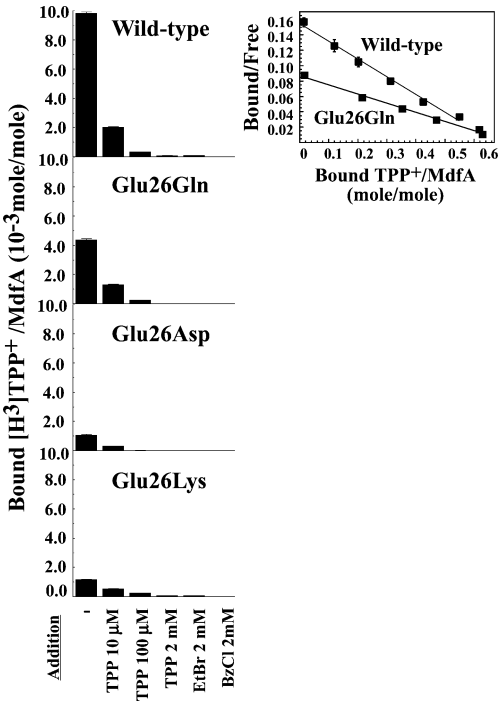


FIGURE 4: TPP^+ binding by purified Glu26 mutants. (Left panel) TPP^+ binding by the purified mutants or wild-type MdfA (as indicated) was measured as described in Experimental Procedures. Ni-NTA beads alone or with solubilized protein were incubated with [^{3}H]TPP^+ (75 nM), with or without the indicated unlabeled competitor. (Right panel) Binding of increasing concentrations of [^{3}H]TPP^+ to MdfA or MdfA-Glu26Gln was measured as described, and K_D's of ~4.9 and ~9 μM, respectively, were derived from the Scatchard plots. The nonspecific component of binding to resin alone was subtracted. The experiments were performed in triplicate and repeated two times. Error bars indicate standard errors of mean.

of MdfA (see Discussion).

TPP^+ Binding by Purified MdfA Glu26 Mutants. The role of the negative charge at position 26 in the recognition of cationic substrates was studied by direct binding assays using selected E26 mutants of MdfA. Three mutants were examined: (i) Glu26Asp, containing a conservative mutation (*20*), (ii) Glu26Lys, which harbors a positive charge at position 26 (*20*), and (iii) Glu26Gln, harboring an uncharged isosteric residue at position 26. The mutants were overexpressed, purified by Ni-NTA affinity chromatography, and subjected to equilibrium binding experiments using [^{3}H]TPP^+ (*19*). Surprisingly, all of the mutants exhibited specific TPP^+ binding, which was inhibited by competitive substrates (Figure 4). As expected, a conservative replacement at position 26 did not abolish TPP^+ binding, and MdfA Glu26Asp retained low TPP^+ binding. However, similar binding activity was also observed with mutant Glu26Lys, suggesting that once Glu26 is replaced by either Asp or Lys, the protein might lose proper recognition of TPP^+, possibly because of structural hindrance, and the observed low binding activity is probably mediated by other determinants in the recognition pocket. This notion is supported by the observation that a mutant harboring the isosteric mutation Glu26Gln, which presumably did not have a major conformational effect on the recognition pocket of MdfA, exhibited high TPP^+ binding activity that is about 50% of that of wild-type MdfA, with a K_D of ~9 μM, that is about two times of that of wild-type MdfA [Figure 4 (*79*)]. These findings further substantiate the notion that Glu26 is an important recognition determinant, but it is not absolutely essential for the TPP^+ binding activity of MdfA.

Cationic Drug Resistance Profile of the Different Glu26 Mutants. Previously, on the basis of chloramphenicol resistance and transport assays, we proposed that the negative charge of Glu26 is not involved in the transport mechanism of MdfA. However, we could not rule out the possibility that, with other substrates (e.g., cationic drugs), this negative charge plays a role in both recognition and transport (*26*). Since, as shown above, charge neutralization at position 26 did not abolish TPP^+ binding, we reasoned that some of the neutral Glu26 mutants might also be able to mediate transport of cationic substrates. This possibility was tested initially by drug resistance assays using a broad range of positively charged MdfA substrates. *E. coli* UT5600 cells or the outer membrane permeability mutant UTL2 (*27*), both deleted of the chromosomal *mdfA* gene (UT*mdfA::kan* or UTL2*mdfA::kan*) (*20*), was transformed with pT7-5/*mdfA-6His* encoding various Glu26 mutants and plated onto LB plates containing the indicated cationic drugs. With TPP^+ and EtdBr, the resistance was examined by monitoring the growth of cells in liquid media supplemented with 150 μM TPP^+ (Figure 5B) or increasing concentrations of EtdBr (Figure 5C). Figure 5 shows that, although the mutations had drastic effects on multidrug resistance, in agreement with the proposed role of Glu26 in cationic substrate recognition, the multidrug resistance activity was not completely abrogated. More specifically, several Glu26 mutants were able to confer low resistance to the cationic drugs EtdBr, benzalkonium, puromycin, erythromycin (Figure 5A,C), TPP^+ (Figure 5B), and tetracycline (data not shown). Each of these functional mutants (Glu26Gln, His, Ile, Lys, or Val) exhibited a distinct multidrug resistance profile. With EtdBr, the active mutants were Glu26Ile, Glu26Lys, and Glu26Val; with benzalkonium, they were Glu26Gln, Glu26Ile, and Glu26Val; with puromycin, they were Glu26Ile and Glu26Gln; and with tetracycline (data not shown) and erythromycin, the active mutant was Glu26Lys. In the presence of TPP^+, cells expressing the mutants Glu26Gln, Glu26His, Glu26Ile, and Glu26Lys grew better than the cells harboring mutants Glu26Leu and Glu26Asn, which did not confer TPP^+ resistance. As expected, the apparent mutant-mediated levels of resistance were always considerably lower than with the wild-type MdfA. However, the observed activities are significant and reproducible. Therefore, the negative charge at position 26 might be compromised not only in drug binding (Figure 4) but also in the drug resistance activity of MdfA. This notion was further tested directly by drug transport assays.

Cationic Drug Transport Activity of the Different Glu26 Mutants. Figure 6 shows the results of transport assays where we measured the transport of the positively charged substrates EtdBr and TPP^+ by various MdfA Glu26 mutants. The transport assays used (see Experimental Procedures) enabled reproducible detection of low transport activities. With EtdBr (Figure 6A), a rapid efflux was observed in cells expressing the wild-type transporter. In accordance with the drug resistance data, cells expressing MdfA Glu26Ile exhibited low but significant EtdBr efflux activity, compared to cells expressing the inactive mutant MdfA Glu26Asn or compared to control cells harboring the plain vector. With TPP^+ (Figure 6B), cells expressing the mutants MdfA Glu26Asn or

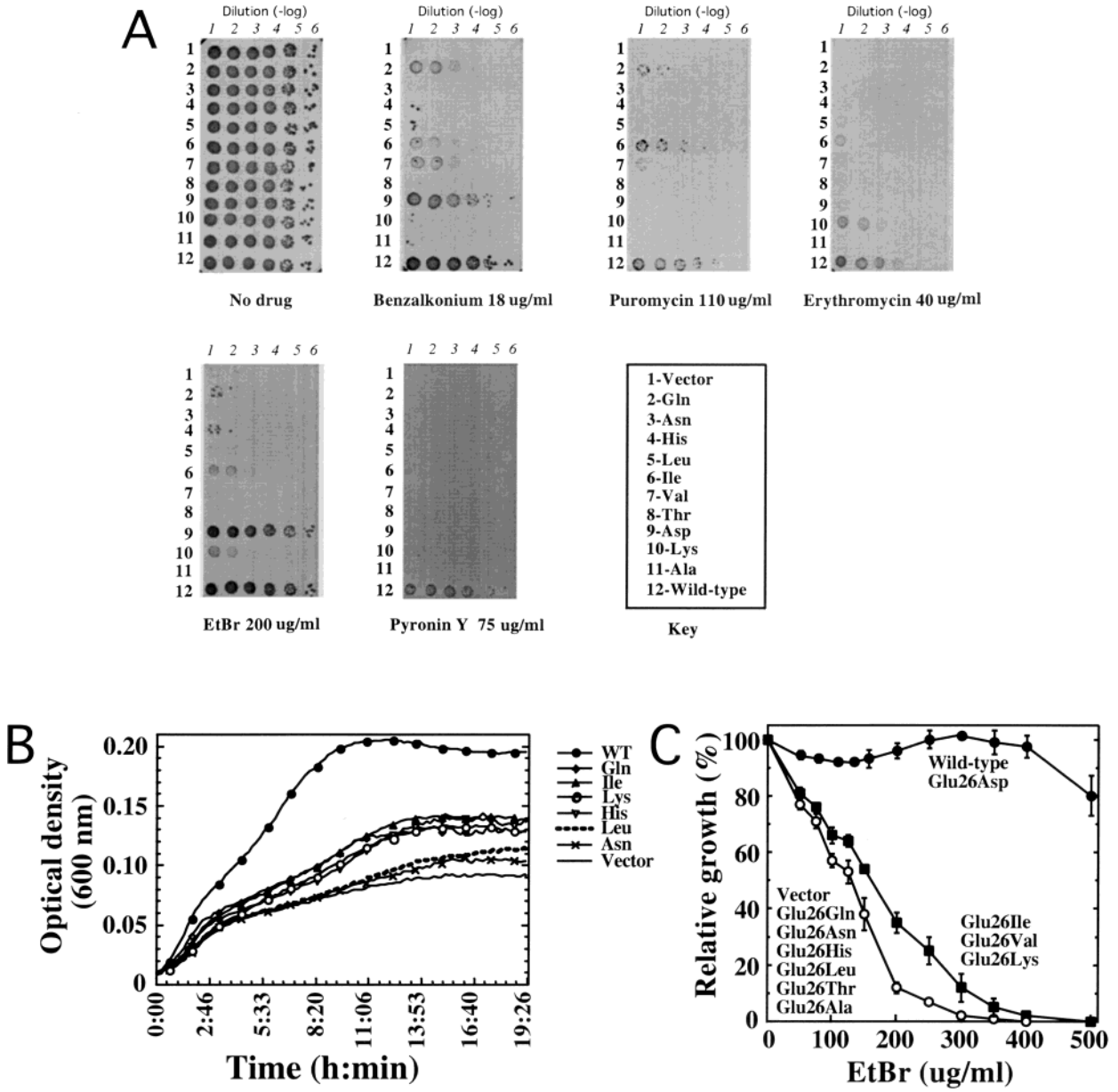


FIGURE 5: Drug resistance of the wild-type and Glu26 mutants of MdfA. (A) *E. coli* UT*mdfA::kan* cells transformed with pT7-5 (vector), wild-type pT7-5/*mdfA-6His*, or Glu26 mutants were diluted (10^{-1}−10^{-6}), and 4 μL of diluted cultures was spotted on LB agar plates with the selection antibiotics ampicillin (200 μg/mL) and kanamycin (30 μg/mL) (LB-amp-kan), supplemented also with tested cationic substrates. (B, C) Relative growth in 96-well plates (as monitored by continuously measuring the absorption at 600 nm, optical distance ~2 mm) of *E. coli* UTL2*mdfA::kan* cells harboring the above plasmids in LB-amp-kan broth containing TPP^+ (150 μM; panel B) or EtdBr (increasing concentrations, panel C). The experiments were performed three times, and the results shown are representative.

Glu26Leu behaved like cells harboring the vector alone, and they accumulated TPP^+ rapidly (within 30 s) to a steady-state level that was at least 7-fold higher than that observed in cells expressing wild-type MdfA. In contrast, mutant Glu26Lys, which was able to confer low resistance to TPP^+ (Figure 5), demonstrated low but reproducible transport activities, resulting in a reduced accumulation of TPP^+ in the cells.

These results, combined with previous findings (*20*), demonstrated that, indeed, Glu26 plays an important role in multidrug recognition by MdfA. However, although the negative charge at this position plays a specific role in electrostatic interaction with cationic substrates, it is not absolutely essential, indicating, as suggested in other studies (*22*, *28*), that the multidrug recognition site is formed by multiple interactions, in addition to the proposed electrostatic one. Moreover, these results support the proposal that the negative charge at position 26 is not essential for the transport mechanism of MdfA (e.g., proton translocation), regardless of the chemical nature of the test substrates.

DISCUSSION

Recent progress has been made in our understanding of the promiscuous nature of Mdr transporters that interact with structurally dissimilar compounds. The prevailing view favors a direct mechanism by which Mdr transporters physically interact with a variety of compounds and actively export them across or out of the membrane. The multidrug recognition pocket in multidrug binding proteins is believed to encompass a large volume (*28*, *29*) and allows binding of various substrates by both different and shared binding determinants (*19*, *28*, *29*). In the RND-related Mdr trans-

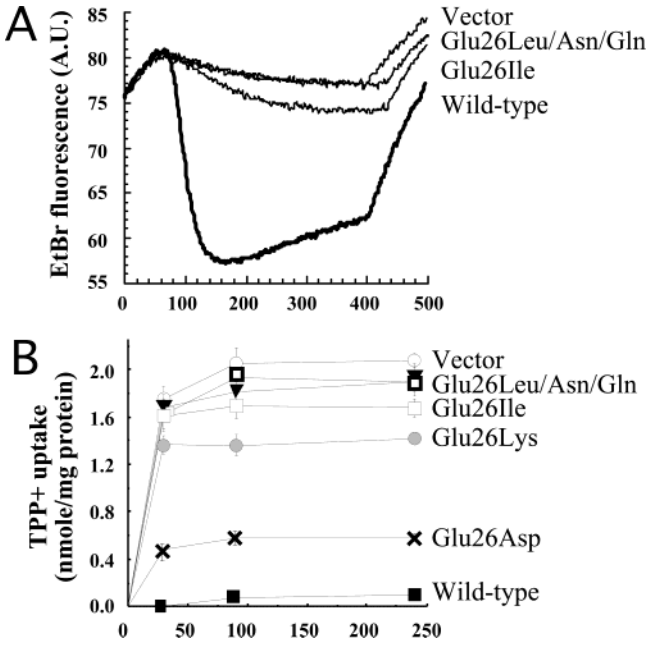


FIGURE 6: Transport activity of the wild-type MdfA and Glu26 mutants. (A) Efflux of EtdBr (5 μM) was monitored continuously by following its fluorescence (which is increased upon interaction of the compound with nucleic acids) in UTL2*mdfA::kan* cells as described in Experimental Procedures. Glucose (0.4%) was added to energize transport. Efflux of EtdBr is represented by a decrease in fluorescence. CCCP (100 μM) was added after 400 s to dissipate the proton electrochemical potential. (B) MdfA-mediated decreased accumulation of [^{3}H]TPP$^+$ (10 μM) in energized UTL2*mdfA::kan* cells. Uptake of [^{3}H]TPP$^+$ was assayed by rapid filtration in cells transformed with different plasmids, as described in Experimental Procedures. The experiments were performed three times, and the results shown are representative.

porter, AcrB, it was proposed that cationic substrates may interact with the acidic headgroups of lipids (*30*). However, in other instances, a role for electrostatic interactions between cationic drugs and acidic residues in the multidrug binding pocket has been proposed (*8*, *9*, *20*, *28*, *31*, *32*).

Here we have further examined the proposed role of a membrane-embedded glutamate residue (Glu26) in drug recognition and transport by the *E. coli* Mdr transporter MdfA. Initially, we tested the effects of various mutations at this position on the recognition of neutral substrates by MdfA and observed that the resistance to chloramphenicol or its derivative, thiamphenicol (also uncharged), was differentially affected by the mutations. Briefly, replacements of Glu26 with Ala, Asn, His, Leu, and Asp dramatically reduced or abolished the resistance activity, whereas mutants harboring Gln, Ile, Val, Thr, and Lys retained their high resistance activities. Interestingly, the mutants Glu26Gln and Glu26Ile exhibited higher resistance activities than wild-type MdfA with the neutral substrates. As expected, the chloramphenicol resistance activities of the mutants correlated well with their chloramphenicol transport activities. However, despite the clear, reproducible, functional differences between the mutants, as presented here and previously (*20*), no obvious structural or chemical requirement at position 26 could be deduced from these mutations, except for a possibility that a nonbranched γ-carbon might facilitate chloramphenicol resistance. This is supported by the fact that mutant Glu26Met exhibits a relatively high chloramphenicol resistance activity (data not shown). Therefore, we propose that the multidrug recognition pocket of MdfA is able to tolerate many structural changes at position 26 by conformational adaptability and interplay with other substrate recognition determinants (Adler and Bibi, in preparation).

Previously, an attempt has been made to distinguish between the involvement of the charge (Glu26) in the process of substrate recognition and/or transport by MdfA (*20*). In this study we proposed that the negative charge is required only for drug recognition and not for transport activity. However, this conclusion was based on the assumption that MdfA utilizes the same mechanism for transport of cationic substrates and neutral ones, despite recent studies which have shown discrete transport reactions for differentially charged substrates that differ in their electrogenicity (*17*). To better understand the role of this important site in MdfA function, we characterized the multidrug recognition and transport activities of the mutants described above using an expanded spectrum of positively charged MdfA substrates.

Initially, we tested whether charge replacements at position 26 abrogated the binding of the cationic substrate TPP$^+$. The results showed that MdfA mutants retain measurable TPP$^+$ binding activities also in the absence of a negative charge. These outcome is in agreement with previous studies performed on the multidrug binding pocket of the soluble multidrug binding protein Bmr, which demonstrated that the negative charge is essential for binding of some, but not all, of the cationic substrates tested (*22*). Similarly, other Mdr transporters also seem to employ a combination of electrostatic interactions and other determinants of recognition (*9*−*11*, *31*, *33*).

Next, we investigated the question of whether the negative charge at position 26 participates in the transport mechanism of cationic substrates, in light of previous studies which have shown that it is not essential for the transport of chloramphenicol. To this end, we tested the hypothesis that since their TPP$^+$ binding was not abolished, some of the nonacidic Glu26 mutants might also be able to confer resistance and mediate transport with cationic substrates. The results showed that replacing Glu26 always had a dramatic effect on the activity of MdfA. Surprisingly, however, several nonacidic Glu26 mutants exhibited low but appreciable levels of resistance and transport with positively charged compounds. These observations indicate that MdfA tolerates, to some extent, many substitutions at position 26, including those that eliminate the negative charge, thus confirming the suggestion that, although important, the negative charge at position 26 does not play an essential mechanistic role in the transport of neutral and cationic substrates.

In conclusion, we have demonstrated that mutations at position 26 of MdfA affect the substrate recognition profile in general and that although the negative charge at this position is important for efficient interaction of the transporter with cationic substrates, it is not an essential factor in the transport mechanism of MdfA. Interestingly, the results showed that MdfA tolerates a variety of changes at position 26, including size, hydrophobicity, and charge, in an unpredictable manner, thus revealing yet another important aspect of multidrug recognition. Therefore, we propose a link between the broad substrate specificity profile of multidrug transporters and the structural and chemical promiscuity at their substrate recognition pocket.

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