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Characterization of a novel domain 'GATE' in the ABC protein DrrA and its role in drug efflux by the DrrAB complex

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ABSTRACT

A novel domain, GATE (Glycine-loop And Transducer Element), is identified in the ABC protein DrrA. This domain shows sequence and structural conservation among close homologs of DrrA as well as distantly-related ABC proteins. Among the highly conserved residues in this domain are three glycines, G215, G221 and G231, of which G215 was found to be critical for stable expression of the DrrAB complex. Other conserved residues, including E201, G221, K227 and G231, were found to be critical for the catalytic and transport functions of the DrrAB transporter. Structural analysis of both the previously published crystal structure of the DrrA homolog MalK and the modeled structure of DrrA showed that G215 makes close contacts with residues in and around the Walker A motif, suggesting that these interactions may be critical for maintaining the integrity of the ATP binding pocket as well as the complex. It is also shown that G215A or K227R mutation diminishes some of the atomic interactions essential for ATP catalysis and overall transport function. Therefore, based on both the biochemical and structural analyses, it is proposed that the GATE domain, located outside of the previously identified ATP binding and hydrolysis motifs, is an additional element involved in ATP catalysis.

1. Introduction

ATP-binding cassette (ABC) superfamily of proteins play pivotal roles in multiple biological processes, including transport of various molecules and drugs [1]. ABC proteins typically consist of two nucleotide binding domains (NBDs) and two transmembrane domains (TMDs) which are either found on separate subunits or within the same polypeptide [2]. This investigation focuses on the bacterial ABC transporter DrrAB that carries out efflux of the anti-cancer antibiotics doxorubicin (Dox) and daunorubicin (Dnr) in the producer organism *Streptomyces peucetius* [3]. This system belongs to the DRA family of ABC proteins to which eukaryotic proteins of the ABCA sub-family also belong [4]. In this system, DrrA (containing the NBD) and DrrB (the TMD) together form a tetrameric complex in the membrane [5]. Proper association of the two proteins is essential for both proteins to achieve stability and active conformation and therefore the overall function of the transporter complex [5,6].

ABC proteins typically consist of a 200 amino acid-long ABC cassette normally located within the N-terminal domain (NTD) of the NBD. It contains all the conserved motifs required for ATP binding and hydrolysis, including Walker A, Q-loop, Signature motif, Walker B, and the Switch motif [7–9] (Fig. 1A). While the function of the ABC cassette has been the subject of intense investigation, the role of the C-terminal domain (CTD) of the NBD has remained largely unexplored. This is possibly due to the fact that the sequence of CTD is highly variable except in closely related ABC proteins. Recent studies have, however, shown that this additional sequence (when present) at the C-terminus of the NBD may be associated with specialized functions [10,11]. The crystal structures of many of these ABC proteins reveal that despite the diversity present in their amino acid sequence, the CTDs contain a common β-sheet fold indicating that this structure may be critical for the function [10–12]. Previously developed DrrA homology model using MalK structure as the template showed that the CTD of DrrA also contains a β-sheet-rich structure similar to the one seen in other ABC proteins [13]. Within the CTD of DrrA we identified three novel motifs/domains [13]. Two of these motifs, DEF (previously referred to as LDEVFL, [13]) and CREEM, present in the extreme C terminus of DrrA, are conserved among close prokaryotic and eukaryotic homologs belonging to the DRA family of ABC

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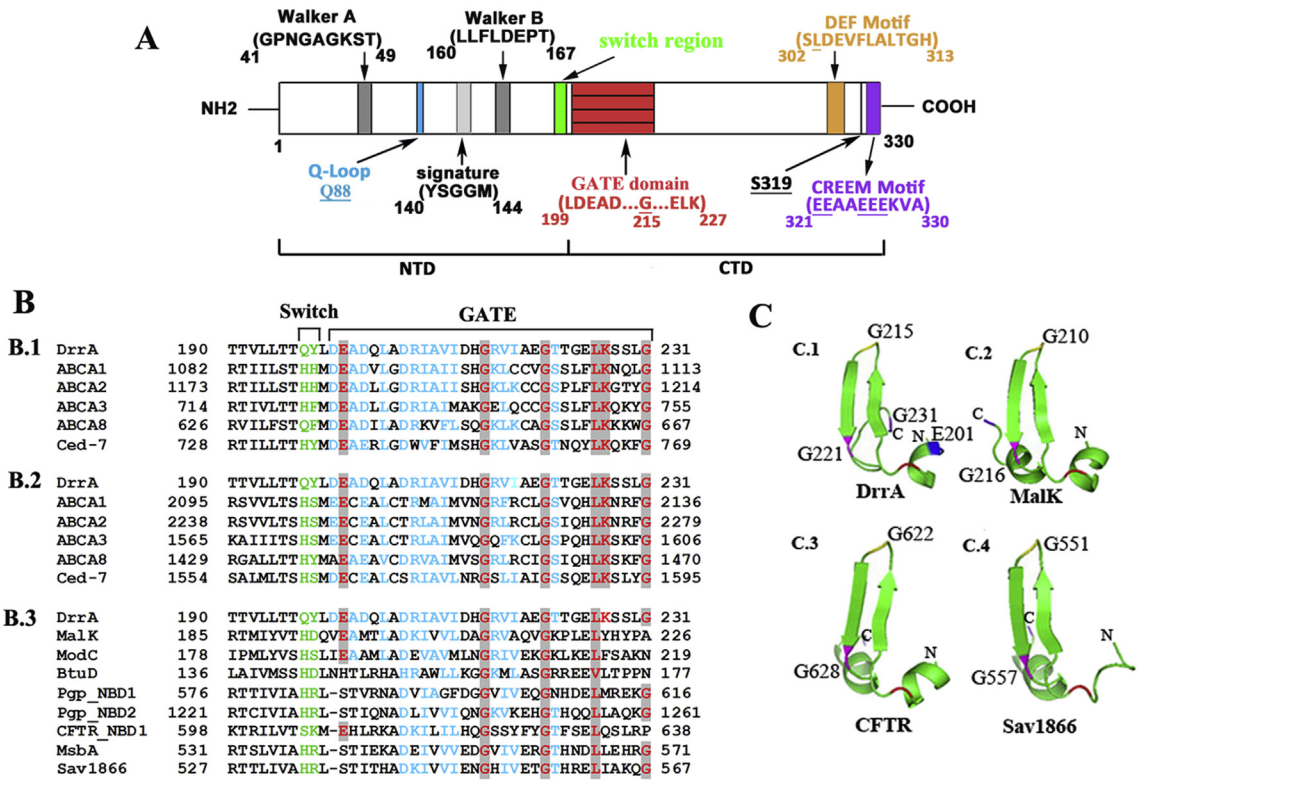

Fig. 1. Sequence and structure analysis of the GATE domain. ***A***, schematic representation of the conserved domains in DrrA. Numbers indicate the location of specific amino acid residues. ***B***, amino acid alignment of GATE with homologs of the ABC superfamily. *B.1 and B.2*, alignment of GATE with the corresponding region of NBD 1 and 2 of close homologs of the DRA family. *B.3*, alignment of GATE with diverse ABC proteins. Switch motif, green; highly conversed residues, red; similar residues, blue. ***C***, tertiary structure of GATE domain. C.1, structure of the GATE domain derived from an established model of DrrA [13]. Key residues E201, G215, G221, and G231 are marked in blue, yellow, pink and purple, respectively. Equivalent residues in the structures of ABC homologs (C.2, C.3 and C.4) have the same labels. The PDB accession numbers and source of each protein are provided in supplementary data. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

proteins and were previously shown to be critical for catalytic function and assembly of the DrrAB transporter [13].

This study focuses on the third conserved domain, GATE (Glycine-loop And Transducer Element) (previously described as LDEAD, [13]) whose function remains completely unknown. This 33 amino acid region (residues 199–231) is located immediately downstream of the Switch motif and shows high sequence and structural conservation among both close and distant homologs from ABC superfamily. Based on the biochemical and structural analyses shown in this article, we propose that the GATE domain is an additional element that plays a critical role in the catalytic function of the DrrAB complex.

2. Materials and methods

2.1. Bacterial strains, plasmids, and antibodies

The bacterial strains used in this study include *Escherichia coli* TG1, N43, LE392Δ*uncIC*, CC118, and XL1-Blue. The plasmids used in this study were constructed previously in this lab and include pDx101 (*drrAB* in pSU2718, [5]), pLA330 (*drrA*::*lacZ* fusion in pMLB1069, pLAB15 (*drrA* and the first 45 base pairs of *drrB*::*lacZ* fusion in pMLB1069), and pLAB283 (full length *drrA-drrB*::*lacZ* fusion in pMLB1069 [5,14], and the resulted fusion proteins were LA330, LAB15 and LAB283, respectively. For Western blot analysis, rabbit polyclonal antibodies against DrrA and DrrB proteins were used [15]. Chloramphenicol was added to 20 μg/ml for all pDX101-containing cell cultures and ampicillin to 75 μg/ml for all pMLB1069-containing cultures.

2.2. Site-directed mutagenesis

Mutations were introduced using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), as described [13]. Plasmids pDX101, pLA330, pLAB15 or pLAB283 were used as templates to generate indicated mutations in GATE domain of DrrA.

2.3. Preparation of inside-out vesicles (IOVs)

E. coli cells containing indicated plasmids were grown in LB at 37 °C until mid-log phase and induced with 0.25 mM IPTG for 3 h. The cell pellet was resuspended in 1xPBS buffer, pH 7.4, and lysed with French press at 16,000 p.s.i. twice. The membrane fraction was prepared according to the previously published protocol [5] and washed twice with 20 ml of PBS buffer.

2.4. Dox efflux assay

Dox efflux was analyzed in IOVs prepared from LE392Δ*uncIC* cells, as described previously [16]. Briefly, 250 μg of IOVs were resuspended in 3 ml of 1 × PBS buffer, pH 7.4, with 0.1 mg/ml creatine kinase and 5 mm creatine phosphate. Dox was added to a final concentration of 1.0 μm. The fluorescence spectra were recorded on an Alphascan-2 spectrofluorometer (excitation, 480 nm and emission, 590 nm). After 100 s, 1 mM Mg^{2+} and 1 mM ATP (pH 7.5) were added to start the reaction and detection continued for additional 400 s. The rate of transport was determined from the slope of the initial linear range between 100 and 200 s.