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Purification and 3D Structural Analysis of Oligomeric Human Multidrug Transporter ABCG2

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Summary

ABCG2 is a multidrug efflux pump associated with resistance of cancer cells to a plethora of unrelated drugs. ABCG2 is a "half-transporter," and previous studies have indicated that it forms homodimers and higher oligomeric species. In this manuscript, electron microscopic structural analysis directly addressed this issue. An N-terminal hexahistidine-tagged ABCG2^{R482G} isoform was expressed to high levels in insect cells. An extensive detergent screen was employed to effect extraction of ABCG2^{R482G} from membranes and identified only the fos-choline detergents as efficient. Soluble protein was purified to >95% homogeneity by a three-step procedure while retaining the ability to bind substrates. Cryonegative stain electron microscopy of purified ABCG2^{R482G} provided 3D structural data at a resolution of ~18 Å. Single-particle analysis revealed that the complex forms a tetrameric complex (~180 Å in diameter × ~140 Å high) with an aqueous central region. We interpret the tetrameric structure as comprising four homodimeric ABCG2^{R482G} complexes.

Introduction

Resistance to chemotherapy remains an unconquered and considerable barrier to the treatment of cancer. One of the most widespread factors is reduced drug accumulation by active extrusion mechanisms such as the ATP Binding Cassette (ABC) transporters. The first discovered was P-glycoprotein (ABCB1), and it has been implicated most strongly in the resistance of leukemia. In contrast, ABCC1 (MRP1), and not ABCB1, has been shown to confer resistance to chemotherapeutic agents in small-cell lung cancer cell lines. Similarly, investigations with cell lines highly resistant to mitoxantrone or daunomycin discovered the presence of a third multidrug transporter, ABCG2 (BCRP or MXR). These three ABC proteins play a pivotal role in resistance of cancer cells to an extraordinary array of anticancer agents and are known as multidrug transporters due to the promiscuity of their substrate interactions.

ABCG2 has been classified to the G subfamily of ABC transporters, all of which are considered "half-transporters" and comprise one nucleotide-binding domain (NBD) and a single transmembrane domain (TMD) containing six predicted membrane-spanning segments. The most distinguishing sequence feature of the ABCG family is the distinct topology. While the majority of ABC transporters comprise a TMD followed by a C-terminal NBD, the ABCG2 protein has an apparent "reversed structure," with the NBD located N-terminal to the TMD. These structural differences are in stark contrast to the more extensively characterized ABCB1 and ABCC1 multidrug transporters and serve to underlie the importance of ABCG2 structural studies.

ABCG2 has been directly demonstrated to confer resistance to mitoxantrone (Nakagawa et al., 1992), methotrexate (Chen et al., 2003), and camptothecin derivatives (Robey et al., 2001). Additional studies with drug-selected cultured cell lines have identified three different isoforms of ABCG2, with mutations near the third predicted transmembrane segment localized at residue 482 (Mitomo et al., 2003; Ozvegy et al., 2002). The wild-type protein contains an arginine residue, whereas the two most commonly observed mutations are either glycine or threonine. The glycine is a gain-of-function mutation in that resistance to anthracyclines, such as daunomycin and doxorubicin, is conferred and the protein is able to transport Rhodamine 123, although a loss of function with respect to methotrexate is also observed. The reason for the alteration of function has yet to be resolved, and translocated compounds, including the anthracyclines, Rhodamine 123, and camptothecins, are also substrates of ABCB1. However, not all ABCB1 substrates, such as vinblastine and calcein-AM, are recognized by ABCG2. Recent radioligand-binding data have identified the presence of multiple, pharmacologically distinct sites on ABCG2 for drug interaction (Clark et al., 2006). However, the precise nature of these sites and their location within the TMDs has not yet been determined, and there are few selective inhibitors for the protein.

The minimal functional oligomer of ABCG2 has been suggested to be a homodimer, a form necessary if it is to function as an ATP-dependent drug transporter, and several lines of evidence support this hypothesis. First, a large body of evidence from ABC transporters exists and demonstrates that two functional NBDs are required to support substrate translocation. Structural analyses with either isolated NBDs or bacterial ABC proteins, including the MsbA homodimer, have revealed that bound ATP interacts with elements (P loop and

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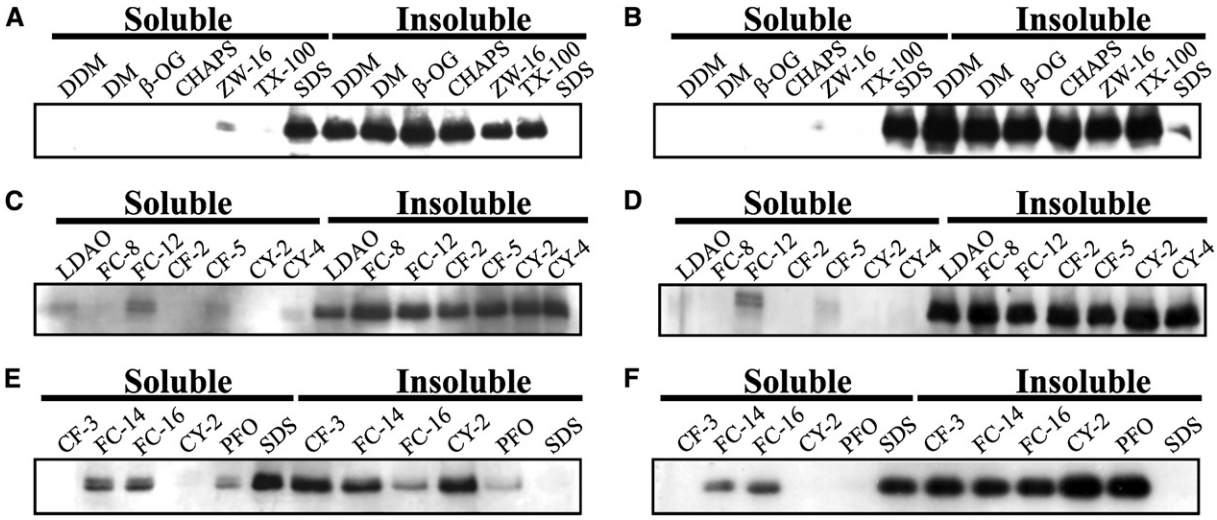

Figure 1. Efficiency of ABCG2^{R482G} Solubilization by a Range of Nonionic and Zwitterionic Detergents

(A–F) Detergent solubilization of ABCG2^{R482G} membranes was performed as described in the Experimental Procedures and was assessed by immunoblotting of the soluble and insoluble material. Detergents are shown above the sample lanes, and each fraction represented 10% of the sample. (A), (C), and (E) were obtained with membranes at a concentration of 1 mg·ml^{-1}, while (B), (D), and (F) correspond to 10 mg·ml^{-1} total membrane protein.

signature sequence) provided by each of the two NBDs (Reyes and Chang, 2005). Also, it has been established that two G subfamily members, ABCG5/ABCG8, heterodimerize to enable cholesterol efflux, and that their interaction is essential for correct localization of the proteins to the plasma membrane compartment (Graf et al., 2003).

Studies characterizing ABCG2 function have pointed to a potential for protein-protein interactions, which have been suggested to be maintained by disulfide bond formation (Kage et al., 2002; Litman et al., 2002). However, other intermolecular interactions, such as those involving the established GXXXG transmembrane helix interaction motif (Russ and Engelman, 2000), have also been implicated (Polgar et al., 2004). Recent evidence gathered from sucrose density gradient sedimentation studies has suggested that while homodimerization is abundant, there is no clear dependence on disulfide bond formation (Xu et al., 2004). Full-length transporters such as ABCB1 (Poruchynsky and Ling, 1994) and ABCC1 (Rosenberg et al., 2001; Soszynski et al., 1998) have also been suggested to form oligomeric structures, although the functional significance has not yet been elucidated, unlike that of SUR1-Kir6.2 oligomerization (Mikhailov et al., 2005), which is critical in mediating potassium conductance.

In the present manuscript, an extraction and purification procedure has been developed to enable further functional and structural analysis of the ABCG2^{R482G} transporter. The protein proved difficult to extract with commonly used detergents and ultimately required the use of fos-choline-16. Drug-binding studies demonstrate that purified protein is capable of specific interaction with transport substrates. An initial structure for ABCG2^{R482G} is presented for the purified protein by using electron microscopy, which indicates that a tetrameric complex with an aqueous central region is formed. We interpret the tetrameric structure as comprising four homodimeric ABCG2^{R482G} complexes.

Results

Solubilization of ABCG2^{R482G} from High Five Insect Cells

Recombinant baculovirus containing either the carboxy-terminal or amino-terminal hexahistidine-tagged constructs for ABCG2^{R482G} were transfected into High Five cells. Immunoblot analysis of expression levels of the two constructs on crude membranes (not shown) demonstrated a significantly higher level of expression of the amino-terminal-tagged ABCG2^{R482G}. Binding studies conducted on crude membranes (Clark et al., 2006) demonstrated functional interaction with published substrates only in the case of the amino-terminally tagged construct.

Solubilization Screen for ABCG2^{R482G}

Crude membrane preparations from the infected insect cells were tested with a range of common nonionic detergents and zwitterionic detergents to investigate the extraction conditions of ABCG2^{R482G}. The initial screen (Figures 1A and 1B) showed no extraction with any detergent other than the strong ionic detergent SDS. Lack of extraction with the glucoside- and maltoside-based detergents indicated that nonionic detergents were not cogent extractors. The zwitterionic detergents, lauryldimethylamine oxide (LDAO), fos-choline (FC), and cyclofos (CF), as well as the nonionic cymal series (CY), were screened. Figures 1C and 1D demonstrate weak extraction of ABCG2^{R482G} by LDAO, FC-12, and CF-5. In contrast to FC-12, FC-8's failure to liberate the protein suggested that the length of the alkyl chain of the detergent may be important in its ability to extract ABCG2^{R482G}. Longer chain length variants of the FC detergents were screened in addition to a number of other conditions (Figures 1E and 1F). Both FC-14 and FC-16 showed extraction irrespective of the protein concentrations used, while pentadecafluoro-octanoic acid (PFO) only showed relatively weak extraction at 1 mg·ml^{-1}.