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PROTEIN INTERACTIONS AND DISEASE PHENOTYPES IN THE ABC TRANSPORTER SUPERFAMILY

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ABC transporter proteins couple the energy of ATP binding and hydrolysis to substrate transport across a membrane. In humans, clinical studies have implicated mutations in 19 of the 48 known ABC transporters in diseases such as cystic fibrosis and adrenoleukodystrophy. Although divergent in sequence space, the overall topology of these proteins, consisting of two transmembrane domains and two ATP-binding cassettes, is likely to be conserved across diverse organisms. We examine known intra-transporter domain interfaces using crystallographic structures of isolated and complexed domains in ABC transporter proteins and find that the nucleotide binding domain interfaces are better conserved than interfaces at the transmembrane domains. We then apply this analysis to identify known disease-associated point and deletion mutants for which disruption of domain-domain interfaces might indicate the mechanism of disease. Finally, we suggest a possible interaction site based on conservation of sequence and disease-association of point mutants.

1. Introduction

ATP-binding cassette (ABC) transporters are membrane-spanning proteins that transport a wide variety of small molecule substrates and ions across cell membranes. Examples include the multidrug transporter P-gp, associated with drug resistance phenotypes in cancer therapy [1], and the cystic fibrosis transmembrane conductance regulator (CFTR) that transports chloride ions [2]. In *Escherischia coli*, the vitamin B12 transporter BtuCD is also an ABC transporter [3], further underscoring the diversity of molecules transported by these proteins.

In humans, the ABC transporters are divided into seven families, labeled ABCA through ABCG. There are half transporters, such as the breast cancer resistance protein BCRP (ABCG2), that consist of one nucleotide-binding domain (NBD) and one transmembrane domain (TMD), and whole transporters, such as the sulfonylurea transporter (ABCC8), that consist of two NBDs and two TMDs [4]. In bacteria and archaea, the NBD and TMD domains are frequently separate genes and the corresponding proteins must associate for

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proper function. One such example is the vitamin B12 transporter BtuCD in *E. coli*, in which the two BtuC proteins and two BtuD proteins associate for transport [3].

Because there are no complete, high-resolution structures of eukaryotic ABC transporters, it is not known how similar their structures and mechanisms are to those of their bacterial and archaeal homologs. However, the striking sequence conservation of domains (*e.g.*, the motif conservation and sequence identity between NBDs of diverse organisms) suggests that, despite differences in gene organization, human ABC transporters are likely to have a quarternary structure similar to those observed in bacteria and archaea [5]. Four crystal structures of NBD dimers (PDB IDs 1L2T, 1XEF, 1L7V and 1Q12) all have a structurally similar NBD/NBD interface, with the Walker A phosphate binding loop of one NBD appearing directly across the interface from the highly conserved 'signature' motif of the opposite NBD [6, 7, 3, 8]. The Cα RMSD (computed with MODELLER's salign feature [17]) between the structures is between 1.7 and 2.7 Å, further demonstrating that the NBD/NBD interface is well conserved among different ABC transporters. An unanswered question about ABC transporter associations is whether the "two NBD / two TMD" model can also include higher-order oligomeric states [9,10].

ABC transporters are also known to interact with a number of other membrane and soluble proteins. The sulfonylurea transporters (ABCC8 and 9) interact with inwardly rectifying (Kir) potassium channels to form ATP-sensitive potassium channels that modulate the electrical activity in cells [11]. The CFTR protein is known to interact with PDZ domains and likely has other binding partners, including adrenergic receptors [12]. Because of the lack of high-resolution structural data, the nature of these interactions at the amino acid residue level is not known.

Point mutations at interfaces can affect the function of ABC transporters in several ways. First, the mutant might destabilize domain folding or association during folding and prevent proper maturation of the protein. A medically relevant example is the deletion mutant ΔF508 in CFTR that is the most common cause of cystic fibrosis. This mutation leads to an immature, lower molecular weight form of the protein that is retained in the endoplasmic reticulum and degraded, which leads to a lack of functional transporters localized to the membrane [2]. Second, the mutant might interfere with the function of an intact transporter by affecting ATP binding and hydrolysis. Third, the mutant might affect allosteric interactions between the domains that are required for substrate binding and transport.

Given the importance of intra- and inter-protein interactions in the ABC transporters, coupled with the large body of data on disease-associated

mutations, we examined known domain interactions in high-resolution crystal structures and used our analysis to suggest possible interface-related mechanisms of human disease. We comprehensively map known disease mutations onto putative nucleotide binding domain interface sites in human ABC transporters. The putative interfacial residues identified in this study can be used to focus efforts in the biochemical identification of functionally important residues.

2. Methods

2.1. *Sequence collection and generation of multiple sequence alignments*

There are 35 structures of ABC transporter proteins in the PDB as of July, 2006. We selected six for study based on the following considerations: diversity of organism representation, diversity of transporter family representation, structural resolution, and completeness of structure as defined by the number of domains crystallized. We selected four structures with definable interfaces. Two are complete ABC transporters with two NBDs and two TMDs: the structure of the vitamin B12 transporter from *E. coli* [3] and the MsbA lipid A exporter from *Salmonella typhimurium* [13]. Two structures are NBD dimers, one from *Methanococcus jannaschii*, and the other from *E. coli* [6, 7]. The final two structures are human transporter NBD monomers [14, 15].

Sequences homologous to each of the proteins in Table 1 were culled by iteratively searching the Uniprot database [16] using the build_profile module of MODELLER [17] with a threshold e-value of 0.01. Build_profile is an iterative database searching method that uses dynamic programming for aligning profiles against sequences and an empirical definition of statistical significance based on the scores collected during the scan of the database [18]. Such automated alignments were also generated for each of the 76 nucleotide binding domains in the 48 human transporters. We used diverse, superfamily-level multiple sequence alignments to examine patterns of conservation across the whole family of ABC transporters rather than dividing the transporters by subfamily.

Table 1. ABC transporter structures used in analysis.

Gene [PDBID]	Organism	Resolution (Å)	Description
CFTR [1XMI]	*H. sapiens*	2.25	Monomeric NBD1 of the cystic fibrosis transmembrane conductance regulator [15]
BtuCD [1L7V]	*E. coli*	3.20	Complete structure (two TMDs and two NBDs) of the Vitamin B12 transporter. Both the TMD and NBD were used in the analysis [3]
Mj0796 [1L2T]	*M. jannaschii*	1.90	Dimeric structure of two NBDs, unknown substrate [6]
HlyB [1XEF]	*E.coli*	2.50	Dimeric structure of the NBDs of the alpha-hemolysin transporter [7]
Tap1 [1JJ7]	*H. sapiens*	2.40	Monomeric NBD of the peptide transporter Tap1 [14]
MsbA [1Z2R]	*S. typhimurium*	4.20	Complete structure of the lipid A exporter MsbA, which is homologous to human multidrug resistance transporters [13]

The CFTR, BtuC (TMD), BtuD (NBD), Mj0796, HlyB, Tap1, MsbA (TMD) and MsbA (NBD) alignments contained 36 199 , 5 444, 36 608, 43 981, 44 134, 28 251, 6 172 and 45 368 sequences, respectively. The alignments are available at http://salilab.org/~libusha/psb2007.

2.2. *Evolutionary conservation: sequence weights and residue position entropies*

We use Shannon entropy to measure the evolutionary conservation at each position (column) in our multiple sequence alignments [19]. Henikoff weighting [20] was used to ensure that entropy calculations were not skewed by large numbers of highly similar sequences which are common in alignments of ABC transporters and which can be a problem with large automatically generated alignments in general. In Henikoff weighting, each column is given an initial weight of 1, which is divided equally between distinct amino acid residues in the column. Within a column, the weight for each amino acid residue is divided equally by the number of times it occurs. Finally, the weight of any given sequence is the sum of the weights of all of the amino acid residues in the sequence. A Shannon entropy:

$$H = -\sum_{aa=1}^{20} P_{aa} \log_2 P_{aa} \quad (1)$$

was calculated for each column where P_{aa} is defined as:

$$P_{aa} = \frac{S_{aa}(i)}{\sum_{aa=1}^{20} S_{aa}(i)} \quad (2)$$

where $S_{aa}(i) = \sum_{i=1}^{n_{aa}} w(i)$ and *w(i)* is the sequence weight and n_{aa} is the number of amino acid residues of a particular type seen in the column. Because of the minus sign in Equation (1), lower numbers indicate greater evolutionary conservation. A MATLAB implementation of Henikoff weighting and the sequence weighting-based Shannon entropy calculation are available on request.

2.3. *Interface definition*

Domain interfaces were defined according to PiBase, a database of domain interactions from x-ray crystal structures in PDB that uses a 5.5Å cutoff for heavy atom interatomic distances to define residues at an interface [21]. The functional unit of ABC transporters is two transmembrane domains (TMD) complexed with two nucleotide-binding domains (NBD) [3, 13, 5]. For the complete ABC transporter structure BtuCD, we define three interfaces: NBD/TMD, NBD/NBD and TMD/TMD. For the dimeric structures we define only the NBD/NBD interfaces. The structure of each domain was aligned to the BtuCD structure (for TMD/NBD interactions) and the Mj0796 structure (for NBD/NBD interactions) with the salign routine in MODELLER [17]. All residues that aligned to interface residues in BtuCD or Mj0796 were predicted to also be interface residues.

2.4. *Homology transfer annotations*

We used the multiple sequence alignments to predict the locations of interface amino acid residues in each of the human ABC transporter NBDs. We assume that if a residue aligns to a known interface residue in the Mj0796 structure (for NBD/NBD alignments) or the BtuCD structure (for NBD/TMD alignments) that it is also at an interface in homologous family members. Residues that aligned to defined interface residues (Interface definition) were examined for disease associations as annotated in the VARIANT records of the Uniprot database [16].

2.5. *Surface conservation*

We used the molecular graphics visualization program Chimera [22] to identify sites of putative binding interactions that have not yet been functionally characterized, by locating surface regions of medium to high conservation (excluding defined interface sites). Medium conservation is defined as no greater than half of the highest column entropy found in a given alignment (Figure 2B).

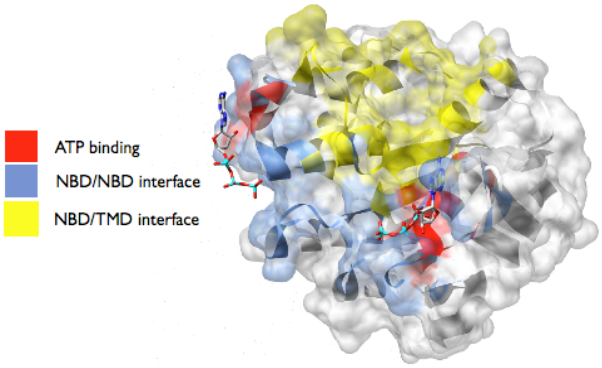

Figure 1. **ABC transporter interdomain interfaces.** Interfaces are defined according to PiBase [21]. Interfaces are mapped on to the representative structure of the Mj0796 ABC transporter nucleotide binding domain (NBD) dimer structure from *M. jannaschii* (PDB ID: 1L2T) [6].

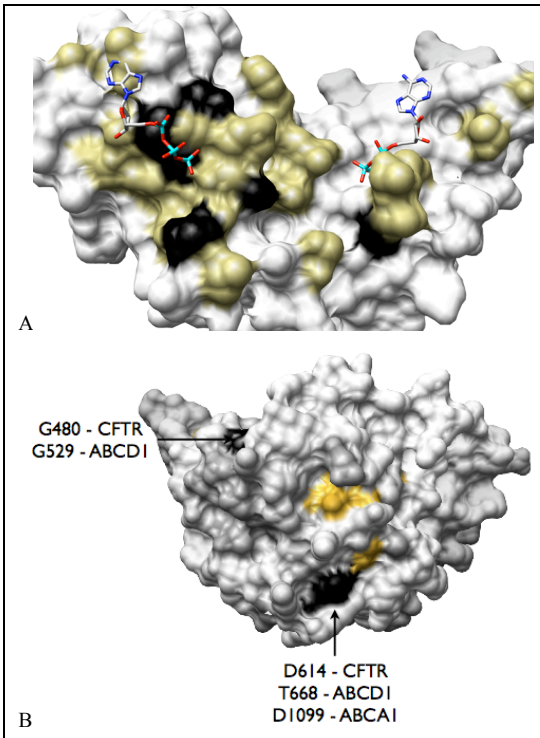

Figure 2. **Disease-associated residues at putative ABC transporter interfaces.**

(A). A close-up of the first nucleotide binding domain of the human CFTR (PDB ID: 1XMI) [15]. Interface residues were defined using homology transfer annotation based on the structure of an NBD dimer from *M. jannaschii* (PDB ID: 1L2T) [6] and are shown in gold. Residues with known cystic fibrosis-associations at the NBD/NBD interface are shown in black (Table 2). An N-terminal helix in the CFTR structure is hidden to show the complete interface as defined by the 1L2T structure.

(B). The exposed, non-NBD surface of the 1L2T structure. Residue positions in yellow have entropies of no more than 2.1 bits. Residue positions in black are associated with cystic fibrosis (CFTR), adrenoleukodystrophy (ALD) and high-density lipoprotein deficiency type 2 (ABCA1).

3. Results

3.1. *Differential conservation of interfaces in ABC transporters*

We examined evolutionary conservation at the amino acid residue level for three different interfaces in ABC transporter structures (*Interface definition*).

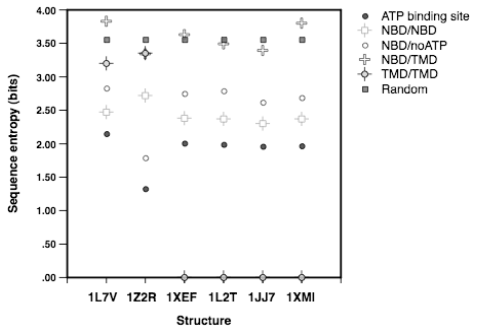

Figure 3. **Evolutionary conservation at binding and interface sites in six ABC transporter structures.** The ATP binding site, which forms part of the interface between the nucleotide binding domains (NBDs) has the lowest entropy due to highly conserved residues in the Walker A, B and 'signature' motifs. The NBD/NBD interface is well conserved even when the ATP-binding residues are removed from consideration (NBD/noATP). The TMD interfaces, both with the cognate NBD and the cognate TMD (only definable for 1Z2R and 1L7V) are not highly conserved.

We were only able to define the TMD/TMD interface for the two complete structures, 1Z2R and 1L7V. We found that the NBD/NBD interface was consistently more conserved than either the NBD/TMD and TMD/TMD interfaces (Figure 3, *Discussion*).

3.2. *Disease associated mutations at ABC transporter interfaces*

We found a total of 68 disease-associated positions at PiBase-defined interfaces in 10 transporters (Table 2, Figure 2A). Of these positions, 65 were single residue mutations and three were deletions. Thirty-eight mutations were at the NBD/NBD interface and 30 at the NBD/TMD interface. We also found conserved surface residues that included two positions associated with disease in several ABC transporters. These residues correspond to the 1L2T residues 1, 2, 31, 60, 164, and 213. There are 587 total known disease mutations in the 10 transporters, of which 504 are found in ABCC7, ABCD1 or ABCA4 [16].

Table 2. **Disease associated mutations at putative ABC transporter interfaces.** Human protein residues that aligned with the NBD/NBD or NBD/TMD interface were examined for disease association using Uniprot [16]. The two interfaces overlap by two residues.

[Transporter] Disease(s)	NBD/NBD	NBD/TMD
[ABCA1] High density lipoprotein deficiency type 2	N935S	
[ABCA3] Respiratory distress syndrome	N568D	
[ABCA4] Stargardt disease (STGD), Fundus flavimaculatus (FFM), Age-related macular degeneration 2 (ARMD2)	R943W (STGD/FFM) N965S (STGD) S1063P (STGD) E1087D/K (STGD) G1091E (FFM) G1975R (STGD) E2096K (STGD) H2128R (STGD)	L1014R (STGD) T1019A (STGD) K1031E (STGD) E1036K (STGD) V1072A (STGD) L2027F (STGD/FFM) R2030Q (STGD/FFM) L2035P (STGD)
[ABCA12] Lamellar icthyosis	N1380S G1381E E1539K	
[ABCC2] Dubin-Johnson syndrome	R768W	Q1382R ΔM1393
[ABCC6] Autosomal recessive pseudoxanthoma elasticum	T1301I G1302R Q1347H	R1314Q Q1347H D1361N
[ABCC7/CFTR] Cystic fibrosis	G458V S549I/N/R G551S R553Q D579G G1244E	S492F E504Q ΔF507 ΔF508 W1282R R1283M F1286S N1303H
[ABCC8] Persistent hyperinsulinemic hypoglycemia of infancy	G715V V1359M G1377R G1380S R1435Q E1505K	R1392H R1419C R1435Q
[ABCD1] Adrenoleukodystrophy	G507V S552P S606P/L G608D E609G/K E630G S633I V635M S636I	P543L S552P Q556R P560R M566K
[ABCG5] Sitosterolemia		E146Q

4. Discussion

We have comprehensively mapped known disease-associated mutations to putative interfaces and found that 68 disease-associated positions in 10 transporters fall at putative interfaces. This indicates that a majority of disease-associated ABC transporters (10/17) have mutations at interface regions. Single residue point mutations were the most common and accounted for 65 of the disease-associated positions; the other three were single residue deletions. Thirty-eight mutations were at the NBD/NBD interface and 30 at the NBD/TMD interface. We hypothesize that many disease-association mutations involving ABC transporters may be due to disruption of domain-domain binding interactions.

Proper function of ABC transporters involves cycles of substrate binding and release which are currently thought to be governed by an 'ATP switch'-type mechanism with ATP binding and hydrolysis causing formation and dissociation of an NBD/NBD dimer. The switch, between open and closed dimer states, causes conformational changes in the TMDs that enable substrate transport [5]. While large conformational changes have been seen in mammalian ABC transporters using electron microscopy [23, 24], the specific residue interactions at both the NBD/NBD interface and the TMD/NBD interface that are involved in these interactions in human transporters is lacking.

As noted earlier, interface mutations can disrupt ABC transporter domain interactions in several ways: by interfering with ATP binding or hydrolysis, by destabilizing or preventing proper folding and association of the domains, or by interfering with allosteric communication between domains that is suggested by the large conformational changes seen during the transport cycle. Defining residues at these interfaces is useful to experimentalists interested in examining specific residue interactions that stabilize or abrogate interface interactions. For example, in CFTR, a hypothesized hydrogen bond between R555 in NBD1 and T1246 in NBD2 stabilizes the open, chloride-transporting state of the protein [27]. The high conservation of the NBD/NBD interface at the superfamily level suggests that there are likely additional residue interactions that stabilize dimer formation and facilitate transport.

The relative lack of conservation at the TMD/NBD interface and the large number of disease-associated mutations at this interface (Table 2) might indicate that NBD/TMD mutations might lead to defects in folding and maturation rather than directly affecting the function of a properly processed, intact transporter. The ΔF508 mutant falls at the TMD/NBD interface and leads to an immature protein that is tagged for degradation and does not localize properly to the cell

membrane [2]. A recent study showed that mutating the analogous residue in P-glycoprotein (MDR1), Y409, also led to an immature form of the protein with an altered NBD/NBD interface. This observation indicates a misfolded protein with improper or incomplete domain associations [25]. Another predicted TMD/NBD interface mutant, R1435Q mutant in ABCC8, could not form functional KATP channels and showed 10-fold reduced expression compared to wild-type ABCC8. Either protein instability or defective transport to the cell membrane could cause this phenotype [26]. Alternatively, the lack of conservation at this interface might suggest TMD/NBD interactions are subfamily specific, in contrast to the overall high conservation of residues at the NBD/NBD interface.

Given the 30 disease mutants at this interface, the lack of conservation at the NBD/TMD interface does not indicate that this region is unimportant for ABC transporter function. However, it suggests that instead of a larger conserved interaction footprint as seen in the NBD/NBD interface, perhaps a small number of conserved residues form the necessary contacts for communication between the domains. In the TMD of BtuCD, the A221 in the L2-loop is one of only three moderate to highly conserved residues in the TMD. In MsbA, the residues G122 and E208 are well conserved, and contribute to the TMD/NBD interface.

We also suggest a possible interaction site distinct from those observed in the crystallographic structures based on conserved surface residues and disease-association in human ABC transporters (Figure 2B). Surface residues not at defined interfaces are generally not well conserved (Appendix Figure A1) in our analysis. However, a moderate to highly conserved region on the surface of the 1L2T structure includes the aligned human mutations: D1099Y, in ABCA1 associated with high density lipoprotein deficiency type 2, D614G in CFTR associated with cystic fibrosis and T668I in ABCD1 associated with adrenoleukodystrophy.

Observing three different transporters with disease-associated mutations at the same solvent-exposed position suggests that this position is conserved for a functional reason. If the residues indeed form part of an interaction site with an unknown partner, that partner might also be conserved in multiple transporters. Alternatively, these residues could indicate a region that stabilizes oligomerization of complete ABC transporters. This example also demonstrates the utility of homology transfer annotation for locating functionally important residues.

There is little experimental data available defining the specific effect of disease-associated mutations on ABC transporters. A recent review noted that the majority of CFTR mutants have not been experimentally characterized [2].

The difficulty of working with these large membrane proteins underscores the need for computational analysis that provides hypotheses for the mechanism of domain interactions in ABC transporters that can be verified experimentally. We used this analysis to prioritize residues selected to experimentally probe domain interactions in the human multidrug ABC transporter P-gp. We will apply our method to new ABC transporter structures as they become available, and we intend to explore using other measures of residue conservation, including determining site-specific mutation rates and locating coevolving residues, in the future [28, 29].

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Appendix

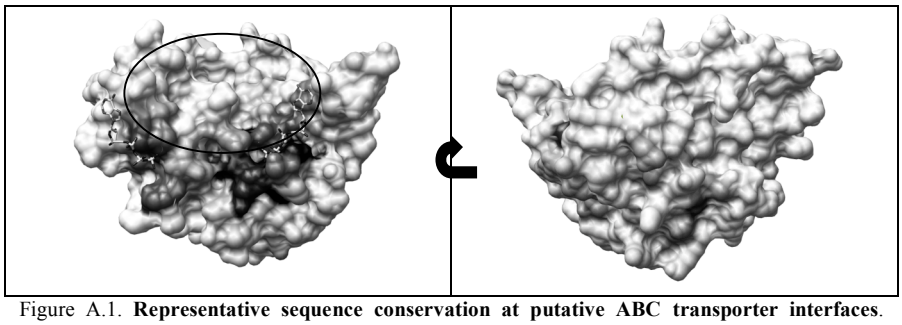

Figure A.1. **Representative sequence conservation at putative ABC transporter interfaces**. Residue conservation was mapped on to the structure of an NBD dimer from *M. jannaschii* (PDB ID: 1L2T) [6]. Conservation is colored from black to white, with black indicating high conservation and white indicating low conservation. The TMD/NBD interface region (left panel) defined by alignment to the BtuCD structure is circled, and shows low conservation. The NBD interface is visible as a curve of high conservation extending from one ATP molecule (shown in stick) to the other. The right panel is rotated 180 degrees horizontally and shows some solvent-exposed regions of higher conservation.

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1. Addendum

As of Friday, December 22, 2006, Scripps researcher Geoff Chang retracted five membrane protein structures he had published and deposited in the PDB due to errors in his calculation of the structures [1].

One of the retracted structures, the ABC transporter MsbA (PDB accession: 1Z2R) [2], was used to calculate domain interface sequence entropy in our PSB publication "Protein Interactions and Disease Phenotypes in the ABC transporter Superfamily."

The retraction of this structure does not alter any of the main conclusions in our paper. This structure was one of six ABC transporter structures used to calculate evolutionary conservation at binding and interface sites in ABC transporters shown in Figure 3 of our paper.

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