Mapping of Interdomain Interfaces Required for the Functional Architecture of Yor1p, a Eukaryotic ATP-binding Cassette (ABC) Transporter

Silvere Pagant, Ethan Y. Brovman, John J. Halliday, and Elizabeth A. Miller
From the Department of Biological Sciences, Columbia University, New York, New York 10027

ATP-binding cassette (ABC) transporters are a large superfamily of proteins that mediate substrate translocation across biological membranes. Our goal was to define the intramolecular interactions that contribute to quaternary assembly of a eukaryotic ABC transporter and determine how the architecture of this protein influences its biogenesis within the secretory pathway. We used chemical cross-linking approaches to map interdomain interactions in the yeast ABC transporter, Yor1p, which functions as a pleiotropic drug pump at the plasma membrane. We have identified interactions between the two nucleotide-binding domains (NBDs) and between the NBDs and specific intracellular loops (ICLs) that are consistent with current structural models of bacterial ABC exporters. Furthermore, we detected relatively weak NBD-NBD and ICL-ICL interactions that may correspond to transient sites of cross-talk between domains required for coupling of ATP hydrolysis with substrate translocation. Mutation of a key residue in ICL2 caused misassembly of the altered protein, leading to increased sensitivity to the mitochondrial poison, oligomycin. We identified intragenic suppressing mutations that rescued the oligomycin resistance associated with this aberrant protein and demonstrated that the suppressing mutations restored multiple interdomain interfaces. Together, our biochemical and genetic approaches contribute to a greater understanding of the architecture of this important class of proteins and provide insight into the quality control surveillance that regulates their biogenesis and deployment within the eukaryotic cell.

ATP-binding cassette (ABC) transporters are integral membrane proteins that translocate substrates across biological membranes using a mechanism that couples ATP binding/hydrolysis with transport. Full ABC transporters, from prokaryote to human, share a global structure composed of two cytoplasmic nucleotide-binding domains (NBDs) that contain conserved regions involved in ATP binding/hydrolysis and at least two membrane-spanning domains (MSDs). Despite the remarkable conservation of architecture across kingdoms, the physiology of membrane transport mediated by ABC transporters is extremely diverse. ABC transporters can act as either exporters or importers and handle a vast array of molecules, including ions, polysaccharides, vitamins, lipids, and peptides, with each transporter relatively specific for a given set of ligands. The quaternary organization of ABC transporters is of great interest to gain further insight into both the shared molecular mechanisms governing the function of ABC transporters and the specificity and directionality of substrate transport.

The architecture of ABC transporters also has important implications for human health because ABC transporters play roles in drug resistance, ion transport, and other fundamental physiological processes. Mutations in a single ABC transporter, the cystic fibrosis transmembrane regulator (CFTR) cause cystic fibrosis in humans as a result of inappropriate chloride transport across epithelial membranes (1). Many disease-related mutations in CFTR cause the protein to improperly assemble within the endoplasmic reticulum (ER) (2), resulting in engagement of an ER quality control process that prevents deployment of misfolded proteins. However, the nature of the aberrant assembly that triggers the quality control checkpoint remains poorly understood (3–5). Thus, a comprehensive understanding of the quaternary structure of ABC transporters may provide insight into the folding defect(s) induced by disease-related mutations and might facilitate the design of novel therapeutic strategies.

A large body of biochemical data, complemented more recently by crystal structures of several prokaryotic ABC transporters, reveals that several important domain-domain interfaces govern the quaternary structure of ABC transporters (6). Interaction of the two MSDs within the lipid bilayer creates the substrate-binding pocket and the translocation channel. Similarly, the two cytoplasmic NBDs interact with each other to create two composite ATP-binding pockets (7–13). The recent crystal structures of full prokaryotic transporters (14–19) suggest that intracellular loops (ICLs) that extend from the MSDs to interact with the NBDs may be responsible for the communication between domains that couples ATP binding/hydrolysis to substrate transport. ICLs present a tripartite design of 2 long α-helices, which extend the helical structure of the adja-
cent TMHs into the cytoplasm that are separated by short “coupling” helices oriented parallel to the membrane plane and interact with the NBDs. The bacterial transporters, Sav1866 and MsbA, are formed by dimerization of two half-transporters, with the ICLs of one polypeptide interacting predominantly with the NBD of the opposite half-transporter (17, 19). This ICL-NBD architecture, referred to as domain-swapping, may be responsible for the coordinated action of the two halves of the transporter. Interestingly, biochemical studies informed by the Sav1866 structure, have demonstrated that similar domain swapping is also involved in the quaternary structure of the eukaryotic transporters, P-glycoprotein and CFTR (20, 21). Conversely, the crystal structure of the bacterial importers, BtuCD and ModBC, are distinct in that the ICLs only contact the NBD of the same half-transporter (14, 16).

We use Yor1p, a yeast ABC transporter, as a model to study the biogenesis of this important family of proteins in Saccharomyces cerevisiae, which affords both genetic and biochemical approaches (22). Yor1p is a plasma membrane protein that acts as a drug pump to clear toxic substances from the cytosol and is required for cellular resistance to the mitochondrial poison, oligomycin. Yor1p shares the same domain arrangement and topology as CFTR and P-glycoprotein, and we have previously shown that the MSD-MSD interaction between TMH6 and TMH12 is analogous to that observed in the two human transporters. Deletion of a phenylalanine residue within NBD1, equivalent to the CFTR-ΔF508 deletion, causes Yor1p-ΔF670 to be retained in the ER and degraded by ER-associated degradation (ERAD). Moreover, this mutation of Yor1p destabilized the interface between TMH6 and TMH12, similar to that described for CFTR-ΔF (23). In this present study, we probe the quaternary structure of Yor1p, using biochemical and genetic approaches to demonstrate that Yor1p presents a Sav1866-like architecture with respect to ICL-NBD interfaces. We demonstrate that interactions between specific ICLs are influential in shaping the active quaternary structure of ABC transporters. Furthermore, we exploit the in vivo phenotype associated with perturbation of Yor1p folding to characterize a new allele equivalent to a disease-causing mutation in CFTR and identify intragenic suppressors that rescue the folding of this aberrant protein.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—Cultures were grown at 30°C in standard rich medium (YPD: 1% yeast extract, 2% peptone, and 2% glucose) or synthetic complete medium (SC: 0.67% yeast nitrogen base and 2% glucose, supplemented with amino acids appropriate for auxotrophic growth). For testing sensitivity to oligomycin, strains were grown to saturation, and then 10-fold serial dilutions were applied to YPEG plates (1% yeast extract, 2% peptone, 3% ethanol, and 3% glycerol), that were supplemented with oligomycin (Sigma-Aldrich).

Plasmids—The plasmids used in this study are listed in supplemental Table S1. pEAE83 bearing YOR1-HA in pRS316 was a gift from Scott Moye-Rowley (University of Iowa). This plasmid was the basis for site-directed mutagenesis using QuickChange mutagenesis (Stratagene, La Jolla, CA) to obtain various hemagglutinin (HA)-tagged Yor1p cysteine mutants used in cross-linking experiments and to create the R387G mutant. pEAE93 was a gift from Scott Moye-Rowley and contains an in-frame fusion of green fluorescent protein (GFP) to the C terminus of Yor1p; this plasmid was the basis for site-directed mutagenesis to introduce the R387G mutation. Expression of the YOR1-GFP fusions was enhanced by co-expressing a dominant active form of the transcription factor, pdr1-3, also a gift from Scott Moye-Rowley.

Cross-linking—Cells expressing cysteine-substituted forms of Yor1p-HA were grown to mid-log phase, harvested, and converted to spheroplasts (22). Spheroplasts were washed twice in 20 mM HEPES, pH 7.4, and incubated with 1,2-ethanediyl bis-methanethiosulfonate (M2M), 1,5-pentanediyl bis-methanethiosulfonate (M5M), or 3,6-dioxaoctane-1,8-diyli bismethanethiosulfonate (M8M) (Toronto Research Chemicals, North York, ON), prepared as a 100× stock in dimethyl sulfoxide. Cells were cross-linked for 15 min at room temperature, then collected by centrifugation, and resuspended in 100 μL of 1% SDS prior to addition of 50 μL of 3× SDS sample buffer without reducing agent. Cells were disrupted by glass bead lysis (15 min, 4°C), heated to 55°C for 5 min, and proteins were separated by non-reducing SDS-PAGE, transferred to polyvinylidene difluoride and analyzed by immunoblot analysis using anti-HA antibodies (Covance, Princeton, NJ).

Limited Proteolysis—Cells expressing different alleles of Yor1p-HA were grown to mid-log phase, harvested, and converted to spheroplasts. Spheroplasts were washed twice in 20 mM HEPES, pH 7.4, resuspended in 100 μL of 20 mM HEPES, pH 7.4 and divided into four 25-μl reactions (2.5 μg/mL reaction). Each reaction was treated with a final concentration of 0, 25, 50, or 100 ng/μl trypsin (Sigma-Aldrich) for 10 min on ice. Digestion was terminated by addition of 0.2 μg/ml (final concentration) soybean trypsin inhibitor (Sigma-Aldrich) to all reactions and incubated on ice for 15 min. Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride, and the pattern of Yor1p fragments analyzed by immunoblot using an anti-HA antibody.

Live Cell Imaging—Strains expressing Yor1p-GFP or Yor1pR387G-GFP were grown in selective medium to mid-log phase and were imaged using a Nikon TE300 inverted microscope (Melville, NY) with 100X.N.A. 1.4 PlanApo optics and a Hamamatsu Orca-ERG charge-coupled device camera. Images were collected with the Openlab 5.0 (Improvision, Waltham, MA) software system and analyzed using Adobe Photoshop (Adobe Systems, Mountain View, CA).

RESULTS

Cysteine cross-linking has proven to be a powerful tool for probing in vivo the proximity of different domains in a variety of ABC transporters (12–13, 20–23). We have previously used this approach to define interactions between MSDs in Yor1p (22). We wanted to extend this strategy to define additional interdomain interactions that govern the quaternary structure of Yor1p to determine the global architecture of a eukaryotic ABC exporter. We used the various crystal structures of dimeric bacterial transporters, coupled with sequence alignment of Yor1p and SAV1866 or CFTR as a guide to inform the positioning of specific cysteine residues within both the NBDs.
and ICLs (supplemental Fig. S1). Yor1p contains 17 endogenous cysteine residues, which do not contribute to significant cross-linking in the context of the wild-type protein (22). To rule out nonspecific interactions, we subjected individual cysteine substitutions to cross-linking, which demonstrated that single substitutions did not give cross-linked products with endogenous cysteines, with the exception of C303 discussed below (supplemental Fig. S2). Furthermore, introduction of single and double cysteine substitutions did not impair the function of Yor1p, as determined by in vivo oligogomycine resistance (supplemental Fig. S3).

**NBD1-NBD2 Interface**—Structural and biochemical data from several ABC transporters show that the two NBDs of a full transporter complex are oriented in a head to tail configuration and that they engage, at least temporarily, to create an interface with two composite ATP-binding pockets formed by the Walker motifs of one NBD and an “ABC signature” (LSGGQ) sequence of the opposing NBD (7–13). To gain structural insight into the organization of the NBDs in Yor1p, we introduced cysteine residues at specific positions in these domains, equivalent to the sites used to describe the interactions forming the NBD2 composite site and the so-called central region of CFTR (13). Cells expressing HA-tagged wild-type Yor1p that contained different combinations of cysteine substitutions at S711 (signature sequence of NBD1), A1251 (Walker A of NBD2), H767 (central region of NBD1), and S1395 (central region of NBD2) were exposed to a methanethiosulfonate cross-linker. Membranes were solubilized with detergent, and the mobility of Yor1p was monitored by non-reducing SDS-PAGE and anti-HA immunoblotting: cross-linked products migrate more slowly under non-reducing SDS-PAGE and were readily distinguished from the native protein. On exposure to increasing concentrations of a cross-linker with a 13-Å spacer arm (M8M), a species with reduced mobility was detected for the NBD1/NBD2 cysteine pairs S711C/A1251C and H767C/S1395C (Fig. 1A and B). Thus the NBDs of Yor1p make similar contacts to those observed for CFTR (13). Furthermore, residue S711C of the signature sequence of NBD1 also showed robust cross-linking with S1395C in the central region of NBD2 (Fig. 1C). The specificity of these interactions is illustrated by the fact that no cross-linked species were detected between H767 and A1251 (Fig. 1D) as predicted by the structural model of the NBD dimer of MJ0796 (Fig. 1E) (8).

To gain more information about the proximity of residues involved in the NBD-NBD interface of Yor1p, we tested the ability to form cross-linked species in the presence of reagents with shorter spacer arms, M5M (9.1 Å) and M2M (5.2 Å). Cross-linking between the residues shaping the NBD2 composite site was reduced when the S711C/A1251C pair was exposed to the shorter cross-linkers, M2M and M5M, compared with that observed using M8M (Fig. 1A). Interestingly, the S711C/S1395C pair showed robust cross-linking even in the presence of the shortest spacer arm (5.2 Å). Furthermore, we detected some marginal cross-linking in the absence of cross-linker, which likely corresponds to spontaneous disulfide bond formation during cell lysis. These data suggest that the NBD1 signature sequence domain and the central region of NBD2 form a relatively close interface that are readily cross-linked (Fig. 1C).

Conversely, the central regions of the two different NBDs, represented by the H767C/S1395C pair, seem more distant because cross-linking was only observed in the presence of high concentrations of M8M and was undetectable with the shorter reagents (Fig. 1B).

**ICL-NBD Interactions**—The structure of prokaryotic Sav1866 revealed that the quaternary architecture of ABC transporters involves specific interactions between the four ICLs and the two NBDs (17) (Fig. 2B). The first ICL of each half of the transporter is predicted to engage a very small interface comprised of residues located above the P-loop (or WalkerA) of its own NBD. The same coupling helix also seems to interact with a small loop preceding the signature sequence of the opposing NBD. Conversely, the coupling helix of the second ICL interacts exclusively with the opposing NBD through a relatively large interface. The details of these interactions seem to be conserved in eukaryotic transporters; interactions between ICL4 and NBD1, and ICL2 and NBD2 have been described for P-glycoprotein and CFTR (20, 21).

We sought to describe additional interdomain interactions necessary for the quaternary structure of Yor1p, and therefore introduced cysteine substitutions into residues predicted by sequence alignment to correspond to the ICL/NBD contact sites of Sav1866 (supplemental Fig. S1). We monitored the proximity of these residues by in vivo sulfhydryl-specific cross-linking as described above. As negative controls, we tested the cross-linking of single cysteine point mutants, which largely failed to show any cross-linked species when incubated with M8M (supplemental Fig. S2), with the single exception of E1362C (Fig. 2C, middle panel). This residue in NBD2 was expected to engage with the coupling loop of ICL1, which contains an endogenous cysteine, Cys-303; when this residue was...
altered to alanine in the context of the E1362C substitution the cross-linked product was no longer detected (Fig. 2C, left panel). This suggests that E1362C interacts with the endogenous Cys-303 and that the cross-linking species detected in the single E1362C illustrates an ICL1-NBD2 boundary. We suggest that this interface reflects an intermediate proximity of the two domains because incubation with shorter cross-linkers (M2M and M5M) dramatically reduced the efficiency of the cross-linking reaction in comparison with that obtained with M8M (Fig. 2C, right panel).

In addition to the ICL1-NBD2 interaction, we also detected the predicted ICL4-NBD1 interaction, with a cross-linked species formed between E646 (upstream of the Q loop of NBD1) and A1086 (in the coupling loop of ICL4) (Fig. 2D). This interface was also specific for the M8M cross-linker, and was only detected at the highest concentrations of M8M, consistent with an intermediate proximity of these domains. Furthermore, this interface may be spatially restricted, because we were unable to detect cross-links between E646 and D1082, another residue of the ICL4 coupling loop (data not shown). This absence of interaction is unlikely to result from the general inaccessibility of these residues to the cross-linker, because both residues were cross-linked independently to additional sites. Conversely, the interaction between ICL2 and NBD2 likely represents a more robust interface: P1294C, upstream of the Q-loop of NBD2, was cross-linked to two different cysteines, K399C and Y403C, introduced independently into the coupling loop of ICL2 (Fig. 2E, left panels and data not shown). Moreover, Y403C was also cross-linked to N1264C, located immediately adjacent to the ICL3 contact site on NBD2 (Fig. 2E, right panels). Finally, strong cross-linked products were detected for both Y403C/P1294C and Y403C/N1264C even at low concentrations of M8M and with the shorter cross-linkers, M2M and M5M (Fig. 2E). We also attempted to characterize the predicted ICL3-NBD1 interaction, using a D984C E705C variant, but were unable to detect any cross-linked species. Taken together, these data demonstrate that ICL1, ICL2, and ICL4 interact with their opposite NBDs, likely mediating cross-talk between the two halves of the protein.

**ICL-ICL Interactions**—In addition to the interdomain communication between ICLs and NBDs, the Sav1866 structure indicates that ICLs might also engage in lateral contacts with the ICLs of the opposite half of the transporter (17) (Fig. 2A and B). More precisely, contact between the coupling helices of ICL1 and ICL4, which both engage an interface with the surface of NBD1, is predicted, and a similar interface may form between ICL2 and ICL3, which both engage NBD2. To investigate the proximity of the coupling helices of the different ICLs, we examined their ability to form cross-linked products in response to thiol-sensitive probes of different length using combinations of cysteine pairs between the ICLs. Specifically, we examined various permutations of the R301C (ICL1), K399C (ICL2), M980C (ICL3), and D1082C (ICL4) substitutions. No productive cross-linking with M8M could be detected for the R301C/K399C and R301C/M980C pairs, which would correspond to ICL1-ICL2 or ICL1-ICL3 interactions, respectively (Fig. 3A). Inefficient cross-linking was observed with the highest concentration of M8M for the M980C/D1082C pair, suggesting a relatively weak association, if any, between the coupling helices of ICL3 and ICL4 (Fig. 3B). Conversely, the R301C/D1082C and K399C/M980C pairs resulted in a robust cross-linking product, detected even in the absence of cross-linker and enhanced by cross-linker of any length (Fig. 3, C and D). These data suggest close contacts between the coupling helices of ICL1 and ICL4 and of ICL2 and ICL3, respectively.

**FIGURE 2.** ICL-NBD interfaces of Yor1p. A, schematic diagram of Yor1p domain arrangement and topology. B, schematic representation of the predicted ICL-NBD interfaces of Yor1p based on the structure of the bacterial protein Sav1866 (17). The NBD dimer is represented from the perspective of the membrane plane and TMHs have been omitted to reveal ICL-coupling helices. Interdomain interaction between (C) ICL1 and NBD2 (D) ICL4 and NBD1, and (E) ICL2 and NBD2 were detected as described in the legend to Fig. 1.
which may mediate cross-talk between the N-terminal and C-terminal halves of Yor1p. All of these different interactions are in accord with predictions based on the Sav1866 structure. However, we also observed cross-linking between K399C and D1082C, indicating a potential interaction between the coupling helices of ICL2 and ICL4 (Fig. 2E). This is somewhat surprising as these domains are predicted to interact exclusively with different NBDs. However, the positioning of these two loops along a central groove of each respective NBD and in a perpendicular orientation to the NBD-NBD interface (Fig. 2F) is suggestive of a transient proximity between the two loops during conformational change associated with substrate binding/transport and/or ATP binding/hydrolysis. Consistent with a transient interaction between ICL2 and ICL4, cross-linking was less robust than the ICL1-ICL4 and ICL2-ICL3 interactions in that it was only detected in presence of the longest cross-linker, M8M.

A CF-related Mutation in ICL2 of Yor1p Causes Impaired Function—The hypothesis that ICLs play a critical role in the domain-domain interfaces that contribute to quaternary assembly of ABC transporters is supported by the observation that numerous CF-associated mutations map to the four ICLs of CFTR. Heterologous expression of 30 of these mutants showed that the majority were retained in the endoplasmic reticulum (3–5). These mutations likely result in defective assemblies that are recognized by the ER quality control process and destroyed, similar to the fate of the most common CF-related mutation, CFTR-ΔF508. We have previously developed a number of genetic and biochemical tools to examine the biogenesis of wild-type and mutant forms of Yor1p (22). We made use of these assays to characterize mutant forms of Yor1p that contained alterations in the ICLs equivalent to the CF-related mutants of CFTR. Impaired function or trafficking of Yor1p results in sensitivity to the mitochondrial poison, oligomycin (24). A plasmid bearing an allele of YOR1 that contained a mutation in ICL2 equivalent to the R285G allele of CFTR, yor1-R387G, was introduced into a strain that contained a chromosomal deletion of YOR1. Like yor1-ΔF670, expression of yor1-R387G was unable to confer viability at very low concentrations of oligomycin (0.1 mg/ml), whereas wild-type YOR1 conferred strong resistance to the drug (Fig. 4A). These data suggest that the R387G mutation impairs the function and/or the trafficking of Yor1p.

We investigated whether the increased oligomycin sensitivity conferred by the Yor1p-R387G mutant was the result of protein misfolding by probing the MSD-MSD interface using cysteine cross-linking between the 6th (Leu-479) and 12th (Leu-1162) TM domains of Yor1p. Compared with wild-type Yor1p with the L479C/L1162C substitutions, which forms cross-links to generate a discrete species in non-reducing SDS-PAGE following exposure to cross-linker (M8M), no cross-linked products were detected for Yor1p-R387G or Yor1p-ΔF670 that contained the same L479C/L1162C pair. Instead, the native protein was converted to a very high molecular weight aggregate that largely failed to enter the resolving gel (Fig. 4B). The introduction of R387G or ΔF670 in the cysteine-substituted constructs that probe the various interfaces between NBDs and ICLs lead to similar results (data not shown), illustrating that the ΔF670 and R387G alleles of Yor1p share a defective conformation prone to aggregate in the presence of thiol-sensitive cross-linker. This higher order aggrega-
Intragenic suppressors of Yor1-R387G—We sought to understand more about the nature of the R387G mutation and how it affects the quaternary structure of Yor1p by identifying intragenic suppressors of the oligomycin sensitivity associated with this allele. We reasoned that a second mutation in YOR1-R387G might correct the folding deficiency of the mutant by inducing a compensatory structural change. We anticipated that the position of these compensatory mutations would confirm our biochemical characterization of the Yor1p quaternary structure and/or reveal the contribution of novel domain-domain interactions. A plasmid bearing yor1-R387G was mutagenized and introduced into a Δyor1 strain, and transformants that conferred oligomycin resistance were selected. Plasmids from oligomycin-resistant transformants were recovered, and YOR1 was sequenced to identify the suppressing mutation. As a final control, any suppressing mutations would confirm our biochemical characterization of the Δyor1 strain to confirm the oligomycin-resistant phenotype.

After screening ∼300,000 independent transformants, we isolated nine substitutions (N413K, K424Q, A958V, M962T, N968S, A1180V, V1184I, I1293T, and G1372S) that rescued the oligomycin sensitivity of yor1-R387G (Fig. 5A). These mutations clustered in three different regions of Yor1p (Fig. 5B): ICL2, the site of the original mutation, ICL3 and NBD2, which are the two domains that showed robust interactions with ICL2 in the cross-linking experiments described above. The positioning of these suppressing mutations is suggestive of a restor-
ration of interdomain interactions that are perturbed by the R387G mutation.

To test this model, we first examined the ICL2-NBD2 interface, predicted to be directly impaired by the R387G substitution, by introducing the Y403C/N1264C cysteine pair into the suppressed alleles of Yor1p-R387G. In each case, the suppressing mutation was able to rescue the ICL2-NBD2 interaction, as detected by the appearance of cross-linked products (Fig. 6A and data not shown). This observation confirms that suppressing mutations can rescue folding and assembly defects associated with specific alleles of aberrant ABC transporters. Furthermore, the capacity of the suppressing mutations located in ICL3 to restore an ICL2-NBD2 interface strengthens our biochemical observations of robust interaction between these three domains. To assess if the ICL2-NBD2 correction could also restore the MSD-MSD interface, we introduced the suppressing mutations into the R387G L479C/L1162C variant. We detected correctly cross-linked species for every suppressing mutation isolated, indicative of a widespread ability to rescue the native interaction between TMH6 and TMH12 (Fig. 6B and data not shown). We note that these suppressing mutations confer some degree of cross-linking between L479C and L1162C even in the absence of cross-linker, a phenomenon that is also seen in the context of wild-type Yor1p (Fig. 4B). Taken together, these data demonstrate that the specific changes created by these second-site mutations result in the restoration of the general conformation of Yor1-R387G.

**DISCUSSION**

A large body of biochemical work has aimed at better understanding how ABC transporters couple ATP binding/hydrolysis and substrate translocation across the lipid bilayer. Recent structural characterization of several full prokaryotic ABC transporters has provided new insight by revealing details of various interdomain interactions that likely govern the quaternary architecture of these proteins. To test the participation of these diverse interfaces in the biogenesis and function of the yeast ABC transporter Yor1p, we introduced specific cysteine substitutions and conducted thiol-sensitive cross-linking experiments to analyze the in vivo proximity of key residues. We have previously used this strategy to examine the interface between the MSDs, thought to be responsible for substrate binding and translocation. We have now described the dimerization interface of the two NBDs of Yor1p, which appear to form two composite ATP-binding pockets similar to that observed from the NBD dimer structure of the bacterial protein MJ0796 (8). This architecture has also been described for CFTR through a similar approach of molecular modeling and chemical cross-linking (13). Furthermore, we have demonstrated that the second ICL of each MSD (ICL2 and ICL4) engages with the opposite NBD (NBD2 and NBD1, respectively), as suggested by the Sav1866 structure (17). These ICLs also interact with the first ICLs of the opposite MSD (ICL3 and ICL1 respectively). The combined effect of these interactions is to create an MSD-NBD interface composed of two symmetrical modules: an ICL1-ICL4 unit that contacts NBD1 and an ICL2-ICL3 unit that interacts with NBD2 (Fig. 2, A and B).

Our cross-linking data demonstrating proximity of ICL2 with ICL3 and NBD2 were validated by in vivo genetic approaches that identified second-site suppressing mutations that rescued a specific lesion in ICL2. This mutant protein, Yor1p-R387G, shows an impaired MSD-MSD interaction, which is rescued by the intragenic suppressing mutations, perhaps through a compensatory change that rescues the ICL2/ICL3 and/or ICL2/NBD2 interface. These genetic results support the model that a structural module is formed by interactions between ICL2, ICL3, and NBD2, and highlight the utility of genetic screening approaches in the unbiased identification of candidate residues involved in these interfaces.

The ICL-NBD interface, found in structures of both importers and exporters, is predicted to be involved in the transmission of conformational changes between MSDs and NBDs, a fundamental aspect of substrate transport that remains poorly understood. The observation that the second ICL of one MSD contacts the opposite NBD, a structural feature known as domain-swapping, has given rise to the suggestion that cross-domain interactions may be important for the harmonized response of the two halves of the protein to such conformational changes. However, domain-swapped interactions do not appear to be universal among ABC transporters; the structures of bacterial ABC importers reveal that the dimer interface forms from the two separate monomers and likely involves a distinct mechanism of transport. One limitation of these structural studies is that the full transporters have been crystallized in the absence of substrate, making the interpretation of how substrate binding influences interdomain interaction and transport difficult. Another outstanding question is how nucleotide binding and/or hydrolysis influences domain arrangement and facilitates transport. Although a number of different crystal structures have been solved for transporters stabilized by the presence of different nucleotides, three structures of ABC exporters in different states, MsbA-AMP-PNP, MsbA-ADP.Vi, and Sav1866-ADP do not show any striking conformational differences (17–19). Moreover, in vitro characterization

---

**FIGURE 6. Supressing mutations correct folding defects of Yor1p-R382G.** The effect of the suppressing mutations on the folding lesions presented by Yor1p-R382G was analyzed by cross-linking. Suppressing mutations isolated, indicative of a widespread ability to rescue the interfaces (A) between ICL2 and NBD2, and (B) between TMH6 and TMH12.
of the domain-domain interactions in CFTR suggests that the ICL4-NBD1 and ICL2-NBD2 interfaces do not change in the presence of ATP, ADP, or AMP-PNP, suggesting that the formation of these two modules is not influenced by ATP binding/hydrolysis (21). Conversely, experiments examining the nucleotide dependence of substrate binding to ICL1 of the human transporter associated with antigen presentation were suggestive of structural rearrangements during ATP hydrolysis (25). Interestingly, we detected a weak association between ICL2 and the transporter associated with antigen presentation were suggested to perturb interactions between NBD1 and the polypeptide backbone, but may instead alter the presentation of residues localized on the protein surface (26). This has been suggested to perturb interactions between NBD1 and the MSDs, which are mediated by the ICLs, thereby indirectly affecting the association between TMH6 and TMH12. Indeed, recent cross-linking experiments suggest that F508 in CFTR interacts directly with different residues of ICL4, supporting the model that domain-domain interfaces are the primary cause of misassembly of CFTR-ΔF508 (21).

ER retention is a feature shared by other disease-related CFTR mutants, many of which contain alterations in the various ICLs, and therefore also likely possess interdomain assembly defects. However, the molecular determinants that govern ER retention and quality control remain largely unknown, despite their relevance as potential therapeutic targets. The combined analysis of the impact of various disease-related mutations on the conformation of the protein and on its trafficking within the cell should lead to a better understanding of the folding lesions that trigger ER quality control. For example, Yor1p-R387G is delivered to the plasma membrane, suggesting that this protein is able to escape the ER quality control despite a defect in the MSD-MSD interaction that resembles the defect shown by Yor1p-ΔF670. However, the aberrant conformations of Yor1p-R387G and Yor1p-ΔF670 are likely to be distinct: Yor1p-ΔF670 presents greater sensitivity to trypsin and the second-site mutations isolated for their capacity to correct the assembly of Yor1p-R387G were not able to rescue the oligomycin sensitivity of Yor1p-ΔF670 (data not shown). These data suggest that the ER quality control checkpoint is able to discriminate differences in the folding properties between Yor1p-R387G and Yor1p-ΔF670. The continued application of the genetic and biochemical tools described here will not only inform our understanding of the interactions that drive quaternary assembly of ABC transporters but also holds great promise in elucidating the mechanisms of ER quality control.

REFERENCES

1. Riordan, J. R., Rommens, J. M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielinski, J., Løk, S., Plavsic, N., Chou, J. L., Drumm, M. L., Ianuzzini, M. C., Collins, F. S., and Tsui, L.-C. (1989) Science 245, 1066–1073
2. Kopito, R. R. (1999) Physiol. Rev. 79, (Suppl. 1), S167–S173
3. Seibert, F. S., Jia, Y., Mathews, C. J., Hanrahan, J. W., Riordan, J. R., Loo, T. W., and Clarke, D. M. (1997) Biochemistry 36, 11966–11974
4. Seibert, F. S., Lindsell, P., Loo, T. W., Hanrahan, J. W., Clarke, D. M., and Riordan, J. R. (1996) J. Biol. Chem. 271, 15139–15145
5. Seibert, F. S., Lindsell, P., Loo, T. W., Hanrahan, J. W., Riordan, J. R., and Clarke, D. M. (1996) J. Biol. Chem. 271, 27493–27499
6. Linton, K. J. (2007) Physiology (Bethesda) 22, 122–130
7. Hopflner, K. P., Karcher, A., Shin, D. S., Craig, L., Arthur, L. M., Carney, I. P., and Tainer, J. A. (2000) Cell 101, 789–800
8. Smith, P. C., Karpowich, N., Millen, L., Moody, J. E., Rosen, J., Thomas, P. J., and Hunt, J. F. (2002) Mol. Cell 10, 139–149
9. Chen, J., Lu, G., Lin, J., Davidson, A. L., and Quiocho, F. A. (2003) Mol. Cell 12, 651–661
10. Zaitseva, J., Jenewein, S., Wiedenmann, A., Benabdelhak, H., Holland, I. B., and Schmitt, L. (2005) Biochemistry 44, 9680–9690
11. Fetsch, E. S., and Davidson, A. L. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 9685–9690
12. Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2002) J. Biol. Chem. 277, 41303–41306
13. Mense, M., Vergani, P., White, D. M., Alberg, G., Nairn, A. C., and Gadsby, D. C. (2006) EMBO J. 25, 4728–4739
14. Locher, K. P., Lee, A. T., and Rees, D. C. (2002) Science 296, 1091–1098
15. Pinkett, H. W., Lee, A. T., Lum, P., Locher, K. P., and Rees, D. C. (2007) Science 315, 373–377
16. Hollenstein, K., Frei, D. C., and Locher, K. P. (2007) Nature 446, 213–216
17. Dawson, R. J., and Locher, K. P. (2007) Nature 443, 180–185
18. Dawson, R. J., and Locher, K. P. (2007) FEBS Lett. 581, 935–938
19. Ward, A., Reyes, C. L., Yu, J., Roth, C. B., and Chang, G. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 19005–19010
20. Zolnerckis, J. K., Woooding, C., and Linton, K. J. (2007) Faseb. J. 21, 3937–3948
21. Serohijos, A. W., Hegedus, T., Aleksandrov, A. A., He, L., Cui, L., Dokholyan, N. V., and Riordan, J. R. (2008) Proc. Natl. Acad. Sci. U. S. A. 105, 3256–3261
22. Pagant, S., Kung, L., Dorrington, M., Lee, M. C., and Miller, E. A. (2007) Mol. Biol. Cell 18, 3398–3413
23. Chen, E. Y., Bartlett, M. C., and Clarke, D. M. (2004) J. Biol. Chem. 279, 39620–39627
24. Katzmann, D. J., Hallstrom, T. C., Voet, M., Wysock, W., Golin, J., Volckaert, G., and Mayoe-Rowley, W. S. (1995) Mol. Cell. Biol. 15, 6875–6883
25. Herget, M., Oancea, G., Schrodt, S., Karas, M., Tampe, R., and Abele, R. (2007) J. Biol. Chem. 282, 3871–3880
26. Lewis, H. A., Zhao, X., Wang, C., Sauder, J. M., Rooney, I., Nolan, B. W., Lorimer, D., Kearins, M. C., Conners, K., Condon, B., Maloney, P. C., Guggino, W. B., Hunt, J. F., and Emtage, S. (2005) J. Biol. Chem. 280, 1346–1353