Functional and Physical Interactions between Partial Molecules of STE6, a Yeast ATP-binding Cassette Protein*

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Carol Berkower‡§, Daniel Taglicht‡, and Susan Michaelis¶

From the Department of Cell Biology and Anatomy, The Johns Hopkins School of Medicine, Baltimore, Maryland 21205

The Saccharomyces cerevisiae a-factor transporter, STE6, is a member of the ATP binding cassette (ABC) transporter superfamily. ABC proteins consist of four modular units that comprise two membrane-spanning domains (MSDs) and two nucleotide-binding domains (NBDs). Like many ABC proteins, STE6 contains these four domains in a single polypeptide; certain other ABC proteins are encoded as pairs of “half-molecules” or are further subdivided. Our previous studies demonstrated that STE6 can be expressed as two half-molecules that are functional when co-expressed. Here we dissect the interactions between modules of STE6 in greater detail. We show by co-immunoprecipitation that STE6 half-molecules interact physically, supporting the view that they co-assemble in vivo to form a functional transporter. We also demonstrate a physical interaction between a STE6 half-molecule and full-length STE6; such complexes appear to be functional, based on the striking finding that the defective activity of full-length STE6 mutated in one of its NBDs can be corrected by co-expression of the corresponding “wild-type” half-molecule. We also show that a quarter-molecule consisting solely of the N-terminal MSD of STE6 can interact physically and functionally with a C-terminal three-quarter molecule of STE6, indicating that information directing the assembly of STE6 from partial molecules is contained, at least in part, within its membrane spans.

The ATP binding cassette (ABC) superfamily consists of structurally related proteins in prokaryotes and eukaryotes, whose major functions are to transport substrates across a membrane (reviewed in Refs. 1 and 2). ABC proteins have a characteristic modular organization; they contain two membrane-spanning domains (MSDs), each with multiple transmembrane spans, and two nucleotide-binding domains (NBDs), which hydrolyze ATP. STE6 is an ABC transporter that mediates export of the a-factor mating pheromone in Saccharomyces cerevisiae; it is one of over 20 ABC proteins known to exist in yeast (3–5). Among yeast ABC proteins, STE6 is the only one for which a native yeast substrate has been identified to date (6, 7).

ABC proteins may be classified by the organization of their four modules. STE6 contains all four modules (two MSDs and two NBDs) on a single polypeptide. This 4-in-1 arrangement is seen in many eukaryotic ABC proteins, including P-glycoprotein, also called the multidrug resistance protein (MDR) (8), and the cystic fibrosis transmembrane conductance regulator (CFTR) (2, 9). Alternatively, many ABC transporters are composed of half-size molecules, which contain one MSD and one NBD on a single polypeptide. Such half-molecules are thought to associate into hetero- or homodimers (2-plus-2 arrangement). The mammalian TAP transporter, which transports peptides into the lumen of the endoplasmic reticulum for antigen presentation, is encoded as two half-molecules (TAP1 and TAP2) that heterodimerize to form the functional peptide transporter (10–12). The Drosophila genes white (w), brown (bw), and scarlet (st) all encode half-molecules that are thought to associate in pairs to transport the pigment precursors responsible for eye color. The products of w and bw are required for guanine transport, while the w and st gene products are required for tryptophan transport (13). The Escherichia coli hemolysin transporter, HlyB, is encoded as a half-molecule that functions as a homodimer (14). Many prokaryotic members of the ABC superfamily are further subdivided, with their MSDs and NBDs on separate polypeptides; examples include the oligopeptide permease (OppBCDF), the histidine permease (HisMPQ), the maltose permease (MalFGK), and a protease exporter (PrtDEF) (15–19). For ABC proteins that consist of more than one polypeptide, the multiple subunits are thought to physically associate with one another in order to form the complete transporter (20).

To begin to define critical sites of contact between modules of an ABC protein, we have examined the interactions between separately expressed domains of STE6 by testing the ability of partial molecules to interact and to restore function to a strain lacking the native STE6 gene. In a previous study, we showed that STE6 half-molecules, when co-expressed, can promote transport of a-factor (21). Here we demonstrate by co-immunoprecipitation that these half-molecules interact physically, supporting the view that they assemble in vivo to form a functional transporter. We also demonstrate a physical interaction between STE6 half-molecules and full-length STE6. Such complexes appear to be functional as well, since we observe strikingly efficient rescue of the defective activity of mutated full-length STE6 by co-expression of the corresponding “wild-type” half-molecule; this type of intragenic complementation may have potential implications for cystic fibrosis gene therapy. We also demonstrate that a STE6 quarter-molecule consisting of the N-terminal MSD of STE6 can interact physically and functionally with the C-terminal three-quarters of STE6. Thus, information capable of driving assembly is contained in the membrane-spanning domains of STE6. Taken together, our
were grown to exponential phase (A)

For preparation of unlabeled extracts for immunoprecipitation, cells and immunoprecipitation of D. Kornitzer and G. Fink).

(25) and the triply iterated myc epitope (26) (from plasmid pKK-1, a gift of A.-S. Ashmore (Boehringer Mannheim).)

was carried out under steady-state conditions, as described previously (21). Immunoprecipitation using the anti-HA 12CA5 monoclonal antibody was done as described (27).

Preparation of Proteins Extracts and Co-immunoprecipitation Assay—For preparation of unlabeled extracts for immunoprecipitation, cells were grown to exponential phase ($A_{600}$ 0.5–1.5) in SC dropout medium. Cells (30 $A_{600}$) were harvested, washed twice in distilled water and once in immunoprecipitation (IP) buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 2 $\mu$g/ml aprotinin, 1 $\mu$g/ml leupeptin, 1 $\mu$g/ml pepstatin, 2 $\mu$g/ml chymotrypsin).

studies with STE6 partial molecules indicate that physical interactions between certain modules of STE6 can occur efficiently and reflect a functionally productive association.

Experimental Procedures

Yeast Strains, Media, and Mating Assays—The yeast strains used in this study are SM1646 (MATa trp1 leu2 ura3 his4 can1 ste6-1/J/A3) (21) and SM2721 (MATa trp1 leu2 ura3 his4 can1 ste6-6/J) in which the STE6 coding 37–1166 or 7–1290 are deleted, respectively. The MATc mating tester strains used are SM1068 (MATa yk1) (22) and KFY112 (MATa ura3-52 leu2-3, 112 trp1 can1 HIS4) (K. Fujimura-Kamada, this laboratory). Quantitative and patch mating assays were performed as described (23).YPD-rich medium and SC synthetic dropout medium were prepared as described previously (22). Yeast strains were grown at 30 °C.

Plasmids—The plasmids used in this study are listed in Table I. The yeast E. coli shuttle vectors are pRS306 (URA3), pRS314 (CEN6 TRP1), pRS315 (CEN6 LEU2), and pRS316 (CEN6 URA3) (24). The epitope tags are the triply iterated HA epitope from influenza hemagglutinin (CEN6 LEU2) and pRS316 (CEN6 URA3).

Table I

| Plasmid | Genotype | Reference |
|--------|----------|-----------|
| pRS306 | URA3     | 24        |
| pRS314 | CEN6 TRP1| 24        |
| pRS315 | CEN6 LEU2| 24        |
| pRS315-1| CEN6 LEU2| 21        |
| pRS316 | CEN6 URA3| 24        |
| pSM192 | CEN6 URA3 STE6 | 21 |
| pSM217 | 2 $\mu$mTR | 24 |
| pSM218 | 2 $\mu$mLEU2 | 21 |
| pSM236 | CEN6 LEU2 STE6 | 21 |
| pSM338 | CEN6 LEU2 ste6-K398R | 21 |
| pSM339 | CEN6 LEU2 ste6-K1093R | 21 |
| pSM402 | CEN6 LEU2 ste6-G509D | 21 |
| pSM403 | CEN6 LEU2 ste6-G1193D | 21 |
| pSM434 | CEN6 TRP1 C-Half | 21 |
| pSM438 | CEN6 LEU2 ste6-G392V | 21 |
| pSM440 | CEN6 LEU2 ste6-G1087V | 21 |
| pSM500 | 2 $\mu$mLEU2 STE6-HA[C-term]$^a$ | 34 |
| pSM521 | CEN6 LEU2 N-1/4 | 28 |
| pSM524 | CEN6 TRP1 C-1/4 | 34 |
| pSM525 | CEN6 TRP1 STE6 (BamHI at codon 337) | 28 |
| pSM526 | CEN6 TRP1 C-3/4 | 34 |
| pSM529 | CEN6 LEU2 N-3/4 | 34 |
| pSM530 | CEN6 TRP1 ste6-K398R | 24 |
| pSM531 | CEN6 TRP1 ste6-G509D | 24 |
| pSM532 | CEN6 TRP1 ste6-G392V | 24 |
| pSM537 | CEN6 TRP1 N-Half-HA[C-term]$^a$ | 28 |
| pSM547 | CEN6 LEU2 STE6 | 26 |
| pSM549 | CEN6 LEU2 N-1/4-HA[N-term]$^a$ | 20 |
| pSM550 | CEN6 LEU2 N-3/4-HA[N-term]$^a$ | 20 |
| pSM671 | 2 $\mu$mLEU2 C-Half-HA[C-term]$^a$ | 34 |
| pSM672 | 2 $\mu$mTR3 STE6-HA[C-term]$^a$ | 34 |
| pSM947 | 2 $\mu$mTR3 N-Half-myc[C-term]$^a$ | 34 |
| pSM967 | 2 $\mu$mTR3 C-Half-myc[C-term]$^a$ | 34 |
| pSM1114 | 2 $\mu$mTR3 ste6-K398R-HA[ecto]$^a$ | This study |
| pSM1115 | 2 $\mu$mTR3 ste6-K1093R-HA[ecto]$^a$ | This study |
| pSM1116 | 2 $\mu$mTR3 ste6-G509D-HA[ecto]$^a$ | This study |
| pSM1117 | 2 $\mu$mTR3 ste6-G1193D-HA[ecto]$^a$ | This study |
| pSM1118 | 2 $\mu$mTR3 ste6-G392V-HA[ecto]$^a$ | This study |
| pSM1119 | 2 $\mu$mTR3 ste6-G1087V-HA[ecto]$^a$ | This study |

$^a$ The epitopes were cloned into BamHI sites located either just before the stop codon (C-term), between codons 7 and 8 (N-term), or at codons 68–69 (ecto).

were labeled extracts for immunoprecipitation of STE6 full-length and partial molecules, cells ($A_{600}$) were labeled with 200 $\mu$Ci of EXPRE$^{535}$S (DuPont NEN) for 20 min at 30 °C, and proteins were extracted as above. Following immunoprecipitation and SDS-PAGE, labeled proteins were visualized by autoradiography.

Assay for Absence of Recombination between Full-length Mutant ste6 and a Co-expressed Half-molecule—It was important to confirm that the functional rescue of mutant full-length ste6 molecules by half-molecules is due to complementation rather than to regeneration of a full-length wild-type STE6 gene by DNA recombination. To distinguish between these possibilities, we examined plasmids recovered from 12 independent diploid mating products. If mating were the result of complementation of mutant full-length ste6 by a wild-type half-molecule, then none of the plasmids recovered from the diploid mating products should contain a wild-type STE6 gene. On the other hand, if mating resulted from recombination, we would expect to recover only wild-type full-length STE6-containing plasmids from the diploid mating products.

Two types of tests were carried out. In the first test, individually purified colonies from strain SM1646 transformed with pSM538 (pSM38) (full-length mutant ste6-K398R) plus pSM537 (N-Half) were patch-mated to a MATa mating tester (KFY112), and diploids from 12 independent matings were isolated. Plasmids were recovered from each diploid and transformed into E. coli. DNA was purified from multiple E. coli isolates, and those containing full-length STE6 were analyzed further for the absence or presence of a BglII site, which is diagnostic for the K398R mutation. Plasmids carrying full-length STE6 all contained the BglII site corresponding to the K398R mutation. No plasmids carrying a wild-type STE6 gene were recovered, indicating that mating was not the result of recombination between N-Half and STE6 to regenerate a full-length, wild-type STE6 gene.

In the second test, transformants of SM1646 carrying plasmid pSM434 (ste6-C-Half) together with pSM440 (ste6-G1087V) and transformants carrying plasmid pSM537 (N-Half) together with either pSM338 (ste6-K398R), pSM438 (ste6-G392V), or pSM402 (ste6-G509D) were patch-mated to KFY112. Plasmids were isolated from the resulting diploids and retransformed into strain SM2721 (ste6-A5). Individual transformants displayed either the rare mating phenotype associated with the full-length ste6 mutant or the nonmating phenotype associated with expression of a lone STE6 half-molecule. No transformant displayed the confluent mating phenotype associated with complementation of a full-length ste6 mutant by co-expression of a half-molecule. This test provided independent evidence that the increase in mating observed when a half-molecule is co-expressed with full-length mutant ste6 is due to complementation and not to recombination and generation of a wild-type STE6 gene.
INTERACTIONS BETWEEN PARTIAL MOLECULES OF STE6

Fig. 1. STE6 partial molecules used in this study. STE6 full-length and partial molecules are shown schematically. The two NBDs of STE6 are represented by spheres and the MSDs by wavy lines. Positions marked on the bar below the constructs designate starting and ending amino acids for the partial molecules. For example, C-3/4 contains amino acids 337 to 1290 of STE6. In addition, all partial molecules contain the first seven amino acids of STE6 and are expressed from the STE6 promoter.

RESULTS

Interaction of STE6 Half-molecules in Vivo—We showed previously that co-expression of the N-Half and C-Half of STE6 leads to efficient export of a-factor (21). Here, we sought to determine whether the functional complementation of the two half-molecules reflects their physical interaction. STE6 half-molecules and other partial constructs used in this study are depicted schematically in Fig. 1. We constructed epitope-tagged versions of each half of STE6, using different epitopes, and asked whether antibodies specific to an epitope present on one half could immunoprecipitate the other, when the two halves were co-expressed in the same cell. The N-Half of STE6 was tagged with the myc epitope to generate N-Half-myc, while the C-Half of STE6 was tagged with the influenza hemagglutinin epitope to generate C-Half-HA.

The two halves of STE6 were expressed from separate vectors in a Δste6 strain. Extracts were prepared in buffer containing 1% Triton X-100, and proteins were immunoprecipitated with mouse monoclonal anti-myc antibodies. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunodetection was carried out with HRP-conjugated mouse monoclonal anti-HA antibodies. The anti-myc immunoprecipitates do not contain a specific band recognized by anti-HA antibodies when either N-Half-myc or C-Half-HA is expressed alone (Fig. 2, lanes 1 and 2). However, when both halves are co-expressed in the same cell, a 60–65-kDa band corresponding to C-Half-HA is apparent (Fig. 2, lane 3), indicating that C-Half-HA co-immunoprecipitates with N-Half-myc, and suggesting that they can form a complex in vivo. (It should be noted that C-Half-HA exhibits a greater mobility than its predicted molecular mass of 72 kDa, as does untagged C-Half (21).) The interaction between the two halves must occur in vivo prior to cell lysis, since co-immunoprecipitation is not observed when lysates from two strains expressing each half separately are mixed (Fig. 2, lane 4). The efficiency of the interaction between the two halves can be roughly quantitated by comparison of the amount of C-Half-HA obtained by co-immunoprecipitation with N-Half-myc (lane 3) to that obtained when C-Half-HA is immunoprecipitated directly with anti-HA antibodies (lanes 5–8). The amount of C-Half-HA that co-immunoprecipitates with N-Half-myc is about 10% of the total amount of immunoprecipitable C-Half-HA.

We have previously shown that STE6 half-molecules when expressed independently exhibit the same stability as one another and as full-length STE6 (27). To ensure that the level of N-Half-myc is the same in the absence and presence of the C-Half-HA, the membrane in the upper left panel of Fig. 2 was reprobed with HRP-conjugated anti-myc antibodies (Fig. 2, lower panel). Comparison of lanes 2 and 3 indicates that N-Half-myc is present at an equivalent level whether or not C-Half-HA is present.

Interaction of Half-molecules with Full-length STE6—To test if STE6 half-molecules can also associate with full-length STE6, we tagged each STE6 half-molecule with the myc epitope and expressed it alone or in combination with full-length STE6-HA. As shown in Fig. 3, full-length STE6-HA can co-immunoprecipitate both with N-Half-myc (lane 3) and with C-Half-myc (lane 11). The efficiency of this interaction, as quantitated by comparison to the sample directly immunoprecipitated with anti-HA, is about 20% (lanes 5–8 and 13–16), roughly similar to the interaction between the two halves themselves.

Functional Rescue of ste6 NBD Mutants by Co-expression of a Half-molecule—To examine whether the observed interaction between full-length and half-molecules of STE6 is a functional one, we asked if a wild-type half-molecule could rescue the mating defect of a full-length ste6 mutant. We expressed six ste6 substitution mutants, three altered in NBD1 and three in NBD2, either alone or in combination with a corresponding wild-type half-molecule (containing a wild-type version of the mutated NBD). The three NBD1 point mutants, G392V, K398R, and G509D, have mating efficiencies ≤1% of the wild-type level (21) and Table I). When N-Half is expressed in strains bearing these ste6 mutants, mating is increased dramatically (Table II and Fig. 4). When C-Half is expressed

Fig. 2. Co-immunoprecipitation of N-Half-STE6 and C-Half-STE6. To examine if the N-Half and C-Half of STE6 interact, detergent extracts were prepared from a Δste6 strain (SM2721) containing a plasmid expressing either N-Half-myc (lane 2; pSM947) or C-Half-HA (lane 1; pSM671), or containing both plasmids together (lanes 3 and 5–8; pSM671 + pSM947). Proteins were immunoprecipitated with either anti-myc (lanes 1–4) or anti-HA antibodies (lanes 5–8). Immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with either anti-HA (top panel) or anti-myc (lower panel) HRP-conjugated antibodies. For the mixed sample (lane 4), portions of the extracts used for lanes 1 and 2 were mixed just prior to immunoprecipitation. The same extract was immunoprecipitated with either anti-myc or anti-HA to generate the samples run in lanes 3 and 5, respectively. In lanes 6, 7, and 8, different percentages (20%, 4%, and 0.8%) of the sample loaded in lane 5 (100%) were run, in order to estimate the portion of total C-Half-HA that could be co-immunoprecipitated with N-Half-myc (lane 3).

Fig. 3. Interactions between full-length and half-molecules of STE6. N-Half and C-Half of STE6 were co-expressed in the same cell. The N-Half of STE6 was tagged with the myc epitope to generate N-Half-myc, while the C-Half of STE6 was tagged with the influenza hemagglutinin epitope to generate C-Half-HA.
together with the NBD2 mutants, G1087V, K1093R, and G1193D, mating efficiency is also dramatically increased (Table II and Fig. 4 for G1087V). Therefore, the physical interaction between full-length STE6 and half-molecules appears to reflect a functionally significant interaction. In contrast, co-expression of C-Half with an NBD1 mutant does not significantly improve mating, nor does co-expression of N-Half with an NBD2 mutant (Fig. 4).

**Fig. 3.** Co-immunoprecipitation of full-length STE6 with N-Half-STE6 and C-Half-STE6. To examine whether full-length STE6 interacts with STE6 half-molecules, proteins were extracted from cells (SM2721) harboring either a single plasmid containing full-length STE6-HA (lanes 1, pSM500, and lane 9, pSM672), N-Half-myC (lane 2, pSM947), or C-Half-myC (lane 10, pSM967), or harboring a pair of plasmids, one containing full-length STE6-HA combined with another containing either N-Half-myC (lanes 3 and 5–8, pSM500 and pSM947) or C-Half-myC (lanes 11 and 13–16, pSM672 and pSM967). Lanes 4 and 12 represent mixtures of portions of the extracts used to generate lanes 1 and 2 or lanes 9 and 10, respectively; extracts were mixed prior to immunoprecipitation. Immunoprecipitations were performed with the indicated antibodies. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with HRP-conjugated anti-HA antibodies. The same extract was immunoprecipitated with either anti-myC or anti-HA to generate the samples run in lanes 3 and 8. In lanes 5, 6, and 7, different percentages (0.8%, 4%, and 20%) of the amount loaded in lane 8 (100%) were run to estimate the portion of total full-length STE6-HA that could be co-immunoprecipitated with N-Half-myC (lane 3). Likewise, samples run in lanes 11 and 16 are derived from the same extract, and lanes 13–15 are used for quantitation.

**Table II**

Rescue of full-length ste6 mutants by co-expression of a half-molecule

| STE6 mutation | Mating efficiency (%) |
|---------------|------------------------|
|               | alone + N-Half (pSM537) |
| pSM438 G392V  | 0.3  27                 |
| pSM338 K398R  | 0.3  9                  |
| pSM402 G509D  | 0.6  13                 |
| pSM440 G1087V | 0.1  47                 |
| pSM339 K1093R | 7    61                  |
| pSM403 G1193D | 6    55                  |

* The mating efficiencies of strains bearing the indicated mutant STE6 plasmids were measured by quantitative mating assays and are expressed as percentages of the mating efficiency of a strain containing a wild-type STE6 plasmid (pSM322). Each value is averaged from three experiments. The mating efficiency of a Δste6 strain carrying a single plasmid that contains only the N-Half or C-Half of STE6 is < 0.001%.

**Fig. 4.** The mating defect of strains bearing mutant full-length ste6 is rescued by co-expression of the corresponding N-Half or C-Half molecule. Patches of MATα strains bearing a TRP1 or LEU2 plasmid, or both, were replica-plated onto a lawn of MATα cells (SM1068) on SD medium to select for mating. Only prototrophs which result from mating can grow. Plasmids harbor wild-type STE6, a half-molecule, or mutant full-length ste6, as indicated. Each strain contains one of the TRP1 plasmids: pSM537 (N-Half), pSM434 (C-Half), pSM525 (Full-length), or a LEU2 plasmid with full-length mutant ste6: pSM438 (G392V), pSM338 (K398R), pSM402 (G509D), pSM440 (G1087V), or a combination of both a TRP1 plasmid and a LEU2 plasmid, as indicated.

**Fig. 5.** Immunoprecipitation of a-factor secreted from ste6 mutant strains. Cells (SM1646) carrying plasmids with wild-type or mutant full-length ste6 (with the indicated mutant alleles), in the absence (−) or presence (+) of a complementing half-molecule, were radiolabeled with [35S]cysteine under steady-state conditions. Cell-associated and extracellular fractions were immunoprecipitated with a-factor antiserum, separated on a 16% SDS-polyacrylamide gel, and visualized by fluorography and autoradiography. The mature extracellular species of a-factor is shown. Immunoprecipitation of intracellular a-factor (not shown) indicates that the cell-associated level of a-factor is comparable for all mutants examined. Plasmids are as follows: pSM322 (lane 1), pRS315 (lane 2), pSM338 (lanes 3 and 4), pSM402 (lanes 5 and 6), and pSM438 (lanes 7 and 8), either with pSM537 (lanes 4, 6, and 8) or without it (lanes 3, 5, and 7), and plasmids pSM339 (lanes 9 and 10), pSM403 (lanes 11 and 12), or pSM440 (lanes 13 and 14), either with pSM434 (lanes 10, 12, and 14) or without it (lanes 9, 11, and 13).

ste6 NBBD mutants that impair mating efficiency do so because the a-factor pheromone is exported from the cell at reduced levels (21). We therefore asked whether the functional complementation observed when a half-molecule is co-expressed with a full-length ste6 NBBD mutant is also manifested as an increased level of a-factor export. Cells were metabolically labeled, and secreted a-factor was immunoprecipitated from the medium. As shown in Fig. 5, lanes 3–8, for each NBBD mutant that we tested, co-expression of N-Half causes a dramatic increase in the amount of a-factor exported. Similarly, co-expression of C-Half causes increased a-factor export from each NBBD2 mutant (Fig. 5, lanes 9–14). Therefore, complementation of a full-length ste6 NBBD mutant by a half-molecule occurs by improving the ability of mutant ste6 to transport its substrate, a-factor, and not by circumventing the normal dependence of mating on a-factor export.

To ensure that rescue of a full-length ste6 point mutant by a half-molecule is achieved by complementation and not by recombination between the two plasmids to regenerate a wild-type STE6 gene, we analyzed DNA from diploids produced when the MATα Δste6 strain co-expressing both constructs was mated to a MATα partner. DNA was obtained from over 15 independent diploid mating products and analyzed, as described under “Experimental Procedures.” In no case was a wild-type STE6 gene recovered, indicating that the observed increase in mating was due to complementation resulting from the interaction of two separately encoded molecules, rather than DNA recombination.

The reduced activity of the ste6 NBBD mutants used in this study could in principle result either from impaired function or from mislocalization in the ER as has been observed for certain MDR1 and CFTR mutants (28, 29). To distinguish between these two possibilities, we compared the cellular localization of
the six NBD mutants with that of wild-type STE6 by immunofluorescence. It should be noted that although STE6 is present at the plasma membrane in an endocytosis-defective mutant, due to its rapid turnover rate in a wild-type strain STE6 exists in a non-localized state (27). As shown in Fig. 6, in all of the mutants used in this study, the staining pattern is indistinguishable from that of wild-type STE6 (compare panels e–h to panel b). For reference, staining with the ER marker Kar2 is also shown (Fig. 6, panels i–j). These results indicate that the reduced activity of the mutants cannot be attributed to retention of STE6 in the ER, in contrast to certain STE6 mutant alleles isolated in this laboratory that are retained in the ER and show a staining pattern coincident with that of Kar2.2

Co-expression of STE6 Quarter and Three-quarter Molecules—We wished to determine which domain(s) of STE6 mediate the interaction between its two halves. To do so, we tested whether modular portions of STE6, such as an isolated MSD or NBD, could associate with the rest of the molecule and if such an association could regenerate a functional transporter.

We generated STE6 partial (quarter and three-quarter) molecules consisting of N-1/4-HA and C-3/4, and also N-3/4-HA and C-1/4, as shown schematically in Fig. 1. We chose to tag only the N-terminal partial molecule of each pair, since we expected from the relatively high efficiency of previous co-immunoprecipitations (see above, Fig. 2 and Fig. 3) that any co-precipitation of one-quarter and three-quarter partial molecules would be detectable by direct visualization of labeled proteins in the immunoprecipitate. C-terminal partial molecules can also be identified by antibodies against NBD2. Cells carrying plasmids encoding pairs of 1/4 and 3/4 partial molecules were metabolically labeled, proteins were solubilized in sample buffer and immunoprecipitated with a mixture of both 12CA5 and C12-JH210 antibodies prior to SDS-PAGE and autoradiography.

FIG. 6. ste6 NBD mutants are normally localized. Indirect immunofluorescence of STE6 (panels a–h) and of Kar2 (panels i–j) was carried out as described under “Experimental Procedures.” The yeast strain used (SM724) carried either HA-tagged wild-type STE6 (b and i) or the HA-tagged ste6 mutants: ste6-G392V (c), ste6-G1087V (d), ste6-K398R (e and i), ste6-K1093R (f), ste6-G509D (g), and ste6-G1193D. As a control for the specificity of anti-HA staining, a strain carrying a plasmid with untagged STE6 was used (a). Plasmids bearing the mutants are pSM329 (a) and pSM1114-pSM1119 (panels b–h, respectively). To demonstrate the difference between the punctate staining pattern of STE6 and that of an ER-localized protein, Kar2, anti-Kar2 antibodies (from M. Rose) were used for immunofluorescence in a strain bearing wild-type STE6 (i) and one of the mutants (ste6-K398R) (j).

FIG. 7. Association between individual domains of STE6 as judged by co-immunoprecipitation of one-quarter and three-quarter partial molecules. Cells were metabolically labeled with EXPRE35S35S. In A, strains contain either C-3/4 (lanes 1 and 4, pSM528), N-1/4-HA (lanes 2 and 5, pSM549), or both (lane 3). In B, strains contain either C-1/4 (lanes 1 and 4, pSM524), N-3/4-HA (lanes 2 and 5, pSM550), or both (lane 3). Proteins were detergent-extracted and immunoprecipitated with either monoclonal antibody 12CA5, which recognizes the HA epitope (lanes 1–3 in both panels), or polyclonal antibody C12-JH210, which recognizes STE6-NBD2 (lanes 4 and 5 in both panels). For improved clarity, immunoprecipitated proteins were solubilized in sample buffer and reimmunoprecipitated with a mixture of both 12CA5 and C12-JH210 antibodies prior to SDS-PAGE and autoradiography.

2 D. Loayza, A. Tam, and S. Michaelis, unpublished observations.
corresponding to C-1/4 is detectable (Fig. 7B, lane 3). Indeed, localization studies of the C-1/4 molecule indicate that it is completely soluble and does not associate with membranes (data not shown).

When these three-quarter or one-quarter partial molecules are expressed individually in a Δste6 strain, no STE6 function is observed, as assayed by mating (Fig. 8 and data not shown). However, co-expression of N-1/4 and C-3/4 in the same strain efficiently restores its ability to mate (Fig. 8). In contrast, co-expression of N-3/4 together with C-1/4 does not give rise to any functional STE6 (Fig. 8). This lack of function is not due to low levels of C-1/4 production (see Fig. 7B, lane 4), nor is it due to instability of the C-1/4 partial molecule, which is actually much more stable than full-length STE6 and other partial molecules (27). These results indicate that MSD1 of STE6, when expressed independently, can form a productive association with the rest of the molecule, whereas a separately encoded NBD2 cannot associate with the rest of the molecule and fails to form a functional transporter.

DISCUSSION

A distinguishing feature shared by members of the ABC superfamily is their modular design. Each transporter consists of four domains, including two MSDs and two NBDs, which may be encoded either as a single polypeptide (referred to here as a full-length protein), as two half-molecules, or as three or four separate polypeptides. We showed previously that STE6 can be severed into half-molecules, and when these two halves are co-expressed, a nearly wild-type level of STE6 function is observed (21). Two models could account for this result: 1) either the two halves must interact physically to form a single functional complex or 2) they may carry out completely independent activities, and thus do not require a direct physical interaction in order to function. The co-immunoprecipitation experiments reported here demonstrate that the two separately encoded halves of STE6 can physically interact with each other and support the model that association between STE6 half-molecules is necessary for function. The interaction between STE6 half-molecules takes place only in vitro and not in vivo after protein solubilization, suggesting that some cellular component, for instance a chaperone, may be required to mediate the assembly of the two halves.

A physical association between separately encoded modules has also been demonstrated for several other ABC proteins. For instance, the mammalian TAP transporter is composed of two “half-molecules,” TAP1 and TAP2. Both subunits are required for peptide transport (30), and interaction between the subunits has been demonstrated by co-immunoprecipitation (12).

Other ABC proteins for which a physical interaction between modules has been detected include the bacterial maltose and histidine permeases, whose individual domains are encoded as separate polypeptides (20, 31), and mammalian MDR1 which, like STE6, is encoded as a full-length ABC protein. When artificially severed, the two halves of MDR1 were shown to interact by co-immunoprecipitation and to exhibit in vitro drug-stimulated ATPase activity that relied on the presence of both half-molecules (32, 33). While these studies with MDR1 examined function in vitro, the experiments presented here for STE6 partial molecules provide in vivo evidence for both physical and functional interactions between modules of STE6.

To begin to identify regions of interaction between modules of the STE6 polypeptide, we further subdivided STE6 into quarter and three-quarter molecules. We expressed these partial molecules either alone or together and assessed their physical interactions by co-immunoprecipitation and their functional interactions by mating tests. Our results demonstrate both a physical and a functional interaction in vivo between the N-terminal quarter molecule consisting solely of MSD1 of STE6 and a C-terminal three-quarter molecule containing the remainder of STE6 (NBD1-MSD2-NBD2). This finding indicates that the association between the two halves of STE6 occurs at least in part via membrane-spanning domains. Conversely, we did not find evidence for interaction between the C-terminal quarter molecule (NBD2) and the N-terminal three-quarters of STE6 (MSD1-NBD1-MSD2), suggesting that the NBDs, or at least NBD2, may provide little or no contribution to the interaction between the two halves of STE6. Alternatively, it is possible that when the C-terminal quarter molecule is expressed separately, it is improperly folded such that it interacts with a chaperone, at the expense of its interaction with the N-terminal three-quarters of STE6. Interestingly, a study that tested the association of domains within MDR1 (33) produced results somewhat different from those reported here for STE6. That study found that two independently encoded NBDs of MDR1 can interact with each other, as can two MSDs. In addition, it was found that an NBD and its corresponding MSD can also interact, suggesting that a significant degree of association between the two halves of MDR1 occurs through both the MSDs and NBDs. However, the in vitro functional significance of these MDR1 partial-molecule interactions was not tested. Our studies indicate that for STE6, the combined use of the co-immunoprecipitation assay, which tests physical interactions between separate domains, together with assays that measure STE6 function in vivo (mating and a-factor export), can provide an effective method for analyzing specific regions involved in the association of domains of STE6. For instance, by studying the effects of MSD1 mutations on the interaction and function of partial molecules, we expect to locate more precisely the regions within MSD1 that are important for its association with the rest of STE6.

We have shown here by co-immunoprecipitation that a STE6 half-molecule, which is inactive by itself, can associate with full-length STE6. Furthermore, we have demonstrated that this interaction is functionally productive. Strikingly, when full-length STE6 containing an NBD mutation is co-expressed with the corresponding wild-type half-molecule, STE6 activity is dramatically restored, despite the fact that neither molecule alone can support mating to a significant extent. This “half-molecule complementation” phenomenon occurs for mutations in both halves of STE6. We showed that restoration of STE6 function is not due to the regeneration of a wild-type STE6 gene by DNA recombination. Instead, it is likely that a complex is formed between mutant full-length STE6 and the wild-type half-molecule. Wild-type STE6 may itself form an oligomeric
complex consisting of two or more full-length molecules. The level at which the half-molecules complement the mutant full-length protein is most likely at the functional level, since none of the mutants tested here show any evidence of mislocalization; it remains to be tested whether a half-molecule could complement a mislocalized full-length version of STE6. It should be noted that we were unable to demonstrate complementation between two full-length ste6 mutants, one containing a mutation in NBD1, the other in NBD2 (data not shown). It is not clear why two NBD mutants fail to complement each other, while a half-molecule can complement mutant full-length STE6. Perhaps there are significantly greater sterical limitations on interactions between the halves of STE6 when they are contained within two separate full-length STE6 proteins than between a lone half-molecule and full-length STE6. It is also possible that when a STE6 oligomer is formed that contains more then one mutant NBD, the entire oligomer is rendered nonfunctional.

The phenomenon of rescue of the defective function of a full-length ste6 mutant by co-expression of a half-molecule has potentially interesting implications for the treatment of disorders involving mutant ABC proteins, notably cystic fibrosis (CF). Many CF-causing mutations result in a single-residue alteration in one of the two NBDs of CFTR (9). It is attractive to speculate that the expression of a CFTR half-molecule via gene therapy might rescue the mutant defect and restore a high level of function. Furthermore, expression of half-molecules might minimize possible problems associated with ectopic expression of full-length CFTR, such as the potential side effects of producing functional CFTR in cell types that do not normally make the protein, or the difficulty of packaging such a large gene into a viral particle. It was reported that the C-terminal half of CFTR can physically associate with its N-terminal half when both are co-expressed in HeLa cells (34). It would be interesting to determine whether such interactions produce a functional CFTR-like complex and whether a CFTR half-molecule could rescue function when co-expressed with mutant versions of full-length CFTR.

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Carol Berkower, Daniel Taglicht and Susan Michaelis

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