A variety of lipid-binding proteins contain a recently described motif, designated FFAT (two phenylalanines in an acidic tract), which binds to vesicle-associated membrane protein-associated protein (VAP). VAP is a conserved integral membrane protein of the endoplasmic reticulum that contains at its amino terminus a domain related to the major sperm protein of nematode worms. Here we have studied the FFAT-VAP interaction in Saccharomyces cerevisiae, where the VAP homologue Scs2 regulates phospholipid metabolism via an interaction with the FFAT motif of Op1. By introducing mutations at random into Scs2, we found that mutations that abrogated binding to FFAT were clustered in the most highly conserved region. Using site-directed mutagenesis, we identified several critical residues, including two lysines widely separated in the primary sequence. By examining all other conserved basic residues, we identified a third residue that was moderately important for binding FFAT. Modeling VAP on the known structure of major sperm protein showed that the critical residues form a patch on a positively charged face of the protein. In vivo functional studies of SCS2, a second SCS2-like gene in S. cerevisiae, showed that SCS2 was the dominant gene in the regulation of Op1, with a minor contribution from SCS2. We then established that reduction in the affinity of Scs2 mutants for FFAT correlated well with loss of function, indicating the importance of these residues for binding FFAT motifs. Finally, we found that human VAP-A could substitute for Scs2 but that it functioned poorly, suggesting that other factors modulate the binding of Scs2 to proteins with FFAT motifs.

The endoplasmic reticulum (ER) is the major site of lipid metabolism in cells, containing many biosynthetic enzymes as well as lipid sensors that enact homeostatic mechanisms. Cytoplasmic proteins with roles in lipid metabolism must access the ER to perform their specific functions. One mechanism by which a large and diverse group of lipid-binding proteins access the ER is via a short motif called FFAT (two phenylalanines in an acidic tract) that binds directly to the integral ER protein VAP (vesicle-associated membrane protein (VAMP)-associated protein) (1). Sixteen eukaryotic proteins contain perfect FFAT motifs (EFFDAXE with surrounding acidic residues), and divergent FFAT-like motifs have been identified in at least 13 homologues of these proteins. Of the nine human proteins with FFAT motifs, eight are from three different families of lipid transfer proteins, i.e. they contain domains that stabilize hydrophobic lipids in the aqueous environment of the cytoplasm and thus can carry lipid traffic across the cytoplasm (2). Most of these have now been shown to bind VAP (3, 4). Therefore, a large number of lipid traffic steps into/out of the ER are potentially mediated by FFAT-VAP interactions.

VAPs are highly conserved proteins, homologues of which are found in all eukaryotic cells (5–11). Multiple genes have arisen in many species: in human there are three, VAP-A and -B coded on two separate genes, with the latter having a splice variant, VAP-C; in the budding yeast Saccharomyces cerevisiae there are two VAP homologues. The first proposed function for VAP arose from its initial identification as an interactor with the membrane fusion protein synaptobrevin/VAMP in Aplysia (5). A role in membrane traffic was further hinted at by other studies in which VAP was found to bind a variety of other membrane fusion proteins (7, 12) and where antibodies to VAP inhibited Golgi to ER traffic reconstituted in vitro (9). Like many of the fusion proteins, VAP is a type II tail-anchored protein with a globular amino-terminal domain followed by a stalk region containing a coiled-coil (Fig. 1).

Other functions proposed for VAP include its acting as a linker between membranes and microtubules. In Drosophila neurons, VAP is enriched in neuromuscular junctions, which require VAP for normal architecture and normal microtubule organization (11). Recently, a link has been uncovered between VAP function and mammalian neurons, where VAP is enriched on microtubules (10), because a mutation in human VAP-B causes familial amyotrophic lateral sclerosis (ALS) type 8 (13). In contrast, in yeast one of the VAP homologues was originally identified as a multicyclop suppressor of a mutation inducing cholera sensitivity (hence named SCS2) (6). Scs2 was shown to suppress two different mutations causing inositol auxotrophy (6, 14). An additional function of Scs2 is the maintenance of telomeric silencing (15, 16). Overall, these disparate results have not suggested a unified vision of a function for VAPs.

However, there are two hallmarks of VAPs. First, they localize to the ER (10, 14, 17). This plays an important part in the regulation of inositol auxotrophy in yeast, the mechanism of
which has now been described at the molecular level (Fig. 2A) (18). Second, the amino-terminal domain is homologous to the filament-forming major sperm protein 1 (MS1P) of nematode worms, with one particularly conserved 16-amino acid segment (the VAP consensus sequence; Ref. 14 and Fig. 1). MSPs are only found in nematodes, whereas VAP is ubiquitous (10) and is therefore likely to be the primordial membrane protein from which MS1P has evolved. Here, we generated Sec2 mutants in which the interaction with FFAT was specifically inactivated, allowing us to identify conserved residues that form the binding site on VAPs for FFAT motifs.

EXPERIMENTAL PROCEDURES

Yeast Strains—Yeast genotypes and strain names were as follows: wild types BY4741 and BY4742 (19) for inositol auxotrophy assays; RS453B for localization studies (20); spo1-TLY501 (18), galactose inducible Ssc2-TLY251 (1). Other strains carrying gene deletions were: Δsec2 (BY4742 YER120W;konMX4), obtained from Euroscarf (Frankfurt, Germany); and Δsec2-TLY521 (RS453B YBL091C-A: S. pombe HIS5), made by the PCR method with a heterologously selectable marker, as described previously (20). Double mutant Δsec2Δsec22 strains were made by mating Δsec2 and Δsec22 strains, sporulating this diploid, and selecting HIS5 "KAN" haploids, one of which was used for this study (TLY522).

Plasmids—Plasmids for bacterial expression were either based on pGEX-4T-3 (Amersham Biosciences) for glutathione S-transferase (GST) fusions (1) or pTrcHis-A (Invitrogen) for green fluorescent protein (GFP) fusions to FFAT Opi1 (ENLDDEEFFDASE) scrambled FFAT (GST) fusions (1) or human VAP-A (242 residues from IMAGE clone 4706502). Site-directed mutagenesis was performed by the QuikChange method (Stratagene). All constructs were checked by sequencing.

In Vitro FFAT Binding Assay—GGF-FFAT Opi1/scrambled FFAT/FFAT OSBP and GST-Sec2/VAP-A fusion proteins were expressed and purified from E. coli as described (1). Binding was carried out with GST-Sec2/VAP-A bound to glutathione beads in a 100-μl reaction for 30 min at room temperature in phosphate-buffered saline (pH 7.4) with 0.5 mg/ml soybean trypsin inhibitor. GST-Sec2/VAP-A beads and bound GFF-tagged fusion proteins were removed by centrifugation, and unbound GFF was detected by fluorometry of the supernatant using an LS50 spectrophotometer (excitation 485 nm, emission 515 nm, slit widths 10 nm). Approximate dissociation constants (Kd) were estimated by curve fitting and Lineweaver-Burke analysis.

Microscopy-based Mutagenic Screen—Cells with the SCS2 promoter replaced with that of GAL1/10 (TLY251) were transformed with an integrating plasmid that uniformly expressed GFF-FFAT (see above) and checked for localization of this construct to the ER on induction of Sec2 expression (growth in galactose). These cells were then transformed with a mixture of (a) the RFP-Myc-Sec2 plasmid digested to remove residues 1–130 of Sec2 and (b) a 596-base pair DNA molecule spanning this region synthesized by 60 cycles of amplification with Mutazyne (Stratagene), with a mutation frequency of ~2/100 base pairs and overlapping the plasmid (67 nucleotides across the Myc tag and 410 nucleotides across residues 130–176). Cell transformation indicated that the PCR product had been used to repair the digested plasmid, as no colonies grew when it was omitted. Cells were picked directly from small colonies grown in glucose, to repress endogenous Sec2, and scored for localization of red and green fluorescence. For sequencing gap-repaired Sec2, the plasmid coding region was amplified by PCR.

Live Cell Imaging—Yeast cells growing in log phase at 30 °C were visualized as described previously (1).

Inositol Auxotrophy Growth Assays—Cells growing in synthetic defined medium lacking inositol (Bio101), or the same medium supplemented with 100 μM inositol for 48 h at 37 °C unless otherwise stated, were assayed as described (14, 15). A minimum of three colonies was used for each sample, and expression levels were checked by Western blot analysis with anti-Myc antibodies (data not shown). Serial dilutions of cells were calculated to achieve patches of 5–15 cells at the highest dilution.

RESULTS

The Sec2 Homologue Scs22 Plays a Minor Role in Regulation of Phospholipid Metabolism—Prior to testing mutant Sec2 alleles in vivo, we investigated whether these assays would be affected by the second VAP homologue in S. cerevisiae at locus YBL091C-A. Because of an amino-terminal intron (23, 24), automated curtailment of the yeast genome omitted the VAP consensus sequence and wrongly predicted an open reading frame of just the carboxyl-terminal 99 residues (Fig. 1, arrow). This gene, a second form of SCS2, hence named SCS22, was first identified in 1995 (6), and subsequently its deletion was found not to induce one phenotype of Δsec2 (15). Despite its obscurity, SCS22 is likely to be more primitive than SCS2, because related budding yeast (Ashbya gossypii and Kluveromyces waltii) contain single VAP homologues with introns similar to SCS22 (25, 26).

To analyze the function of SCS22, we examined a Δscs22 strain for inositol auxotrophy at an elevated temperature, this being a phenotype of Δsec2, where lack of Sec2 activates the transcription factor Opi1 (Fig. 2A). Accordingly, and as shown previously (18), Opi1 was activated by a point mutation in its FFAT motif that inhibited binding to Sec2 (Fig. 2B). In contrast to Δsec2, Δsec22 cells showed no inositol auxotrophy (Fig. 2C). However, Δsec2Δsec22 double mutant cells displayed a more severe phenotype than Δsec2 alone (Fig. 2C). This indicates that Sec22 functions in parallel to Sec2 (although the latter dominates the former) and that Δsec2Δsec22 cells provide the most sensitive background to test the function of Sec22 variants.

The Binding Site for FFAT Motifs Is in the MSP-VAP Domain—We have previously shown that both Sec2 and human VAP-A bind FFAT motifs (1), implying that elements conserved between these proteins are responsible for the binding. By far the largest conserved element is the MSP-VAP domain (elsewhere called the MSP domain, for example in Ref. 27), which is therefore a good candidate to contain the binding site. To test this proposition formally, fusion proteins of GST and the MSP-VAP domain alone, terminating at two different carboxyl-ter-
minal prolines (P142 or P154; see Fig. 1), were expressed. Both bound specifically to a FFAT motif coupled to GFP (GFP-FFAT) but not to the same peptide in scrambled order (GFP-SCRAM) (Fig. 3, and data not shown). The affinity of binding by GST-VAP 1–142 \( (K_D \approx 13 \mu M) \) was very similar to GST-VAP 1–224 \( (K_D \approx 11 \mu M) \).

A Mutagenic Screen Identifies Residues Critical for MSP-VAP Domain-FFAT Motif Interaction—The VAP consensus sequence has been implicated in binding to FFAT motifs, in agreement with a recent study (3). A visual screen of Ssc2 mutants identifies residues critical for FFAT binding. A, basis for microscopy-based screen. Constant levels of GFP-FFAT and variable levels of RFP-Scs2 were expressed in a strain depleted for endogenous Ssc2p. The uniformly expressed, soluble GFP-FFAT reporter localized to the ER only in cells expressing wild-type Scs2p control (WT) or mutant Opi1p (D203A) fused to GFP. Dilutions of cells were patched onto agar with either no inositol (−Ino) or 100 \( \mu M \) inositol (+Ino) and grown at 37 °C for 48 h. As shown previously (18), Opi1 gained activity (i.e. repressed INO1, causing inositol auxotrophy) in parallel with loss of binding to Ssc2. C, SCS2 contributes to the regulation of inositol auxotrophy in the absence of SCS2. Inositol auxotrophy assay for wild-type, Δsacs2, Δsacs22, or Δsacs2Δsacs22 yeast transformed with either empty vector (ø) or Scs2. Strains were assayed for inositol auxotrophy at 37 °C as described in B. Both wild-type and Δsacs2 strains grown without inositol showed reduced numbers of similarly sized colonies, indicating that Δsacs22 shows no phenotype. Although Δsacs2 cells grew poorly without inositol, Δsacs2Δsacs22 double mutants did not grow at all. This deficit was rectified by the introduction of plasmid-borne Ssc2.

FIG. 3. The MSP-VAP domain of VAP-A binds a FFAT motif. In vitro binding of the MSP-VAP domain of human VAP-A (GST-VAP 1–142, open circles) expressed and purified from bacteria was directly compared with the whole soluble region of human VAP-A (GST-VAP 1–224, closed circles). GST-VAP 1–142 bound GFP-FFAT specifically without any binding to the scrambled FFAT sequence (closed squares). The affinity of binding by GST-VAP 1–142 \( (K_D \approx 13 \mu M) \) was very similar to GST-VAP 1–224 \( (K_D \approx 11 \mu M) \).

FIG. 4. A visual screen of Ssc2 mutants identifies residues critical for FFAT binding. A, basis for microscopy-based screen.Constant levels of GFP-FFAT and variable levels of RFP-Ssc2 were expressed in a strain depleted for endogenous Ssc2p. The uniformly expressed, soluble GFP-FFAT reporter localized to the ER only in cells with high levels of RFP-Ssc2. B, analysis of RFP-Ssc2 mutants that abrogated GFP-FFAT localization to the ER. Of 192 Ssc2 mutants screened, 13 were found in which GFP-FFAT was diffuse. The sequences of these contained 46 mutations (3.5 per sequence), the location and frequency of which are plotted above the axis (red). 30 control Ssc2 sequences with unaffected FFAT targeting contained 60 mutations (2.0 per sequence) were plotted below the axis (blue). Only four residues (Lys-40, Thr-41, Thr-42, Lys-120) in which mutation caused loss of FFAT targeting, were also highly conserved. C, site-directed mutagenesis of Ssc2 and recruitment of GFP-FFAT to the ER. Images show GFP-FFAT in cells expressing wild-type Ssc2p control (WT), K40A and K40N single mutations, and T41A/T42A double mutation.

FIG. 2. A role for Ssc2, a second VAP homologue in S. cerevisiae, in the regulation of phospholipid metabolism. A, relationship between Ssc2 and inositol metabolism in yeast. Opi1 is one of four yeast proteins with a FFAT motif, and a fraction of Opi1 is held inactive outside of the nucleus by interacting with both Ssc2 and phosphatidic acid (black ovals) on the ER. Inhibition of binding between Opi1 and FFAT motif on Opi1 reduces membrane anchoring of Opi1, allowing recognition of its nuclear localization signal (filled arrow) and translocation to the nucleus, where it represses the transcriptional activators Ino2 and Ino4 (boxed) acting at multiple phospholipid biosynthesis genes, including INO1, which codes for the rate-limiting enzyme in inositol synthesis (18). B, mutation of the FFAT motif results in the activation of Opi1. Δopi1 cells were transformed with GFP alone (empty) and wild-type (WT) or mutant Opi1p (D203A) fused to GFP. Dilutions of cells were patched onto agar with either no inositol (−Ino) or 100 \( \mu M \) inositol (+Ino) and grown at 37 °C for 48 h. As shown previously (18), Opi1 gained activity (i.e. repressed INO1, causing inositol auxotrophy) in parallel with loss of binding to Ssc2. C, SCS2 contributes to the regulation of inositol auxotrophy in the absence of SCS2. Inositol auxotrophy assay for wild-type, Δsacs2, Δsacs22, or Δsacs2Δsacs22 yeast transformed with either empty vector (ø) or Scs2. Strains were assayed for inositol auxotrophy at 37 °C as described in B. Both wild-type and Δsacs2 strains grown without inositol showed reduced numbers of similarly sized colonies, indicating that Δsacs22 shows no phenotype. Although Δsacs2 cells grew poorly without inositol, Δsacs2Δsacs22 double mutants did not grow at all. This deficit was rectified by the introduction of plasmid-borne Ssc2.

minal prolines (P142 or P154; see Fig. 1), were expressed. Both bound specifically to a FFAT motif coupled to GFP (GFP-FFAT) but not to the same peptide in scrambled order (GFP-SCRAM) (Fig. 3, and data not shown). The affinity of binding by GST-VAP 1–142 was similar to that of full-length VAP just missing the transmembrane domain (13 and 11 \( \mu M \), respectively). These results indicate that the MSP-VAP domain is solely responsible for binding FFAT motifs, in agreement with a recent study (3).
TABLE I
Mutations in 13 nonbinding and 30 binding Scs2 sequences

| Sequence no. | Mutations |
|--------------|-----------|
| Nonbinding   |           |
| 1            | K38N K40M T41A P132S (V146D) (P168T) |
| 2            | V43A E60A A76T A110S E111D K120R Q137P |
| 3            | T41A P19S P95N K120R |
| 4            | S21L V122D N136S Q137P Q140R |
| 5            | K38N V39H (V161M) |
| 6            | K40R T41H |
| 7            | K40N A76T |
| 8            | K40N Q63L A116G |
| 9            | V10M T42A |
| 10           | A36T T41I L71M A110T |
| 11           | I25S R50I |
| 12           | K40T K413N (A160V) (E166V) |
| 13           | T42I T72N |
| Binding      |           |
| 14           | V64I A102V (E147D) (D152H) |
| 15           | I82P Q114H |
| 16           | Y13F |
| 17           | E74V T89A |
| 18           | K45R |
| 19           | I67V K119I N124Y |
| 20           | I82N S129C |
| 21           | S15C R50I |
| 22           | G98S P112L |
| 23           | A76T D113V |
| 24           | V4L |
| 25           | F68V |
| 26           | L61I |
| 27           | S21T Q115H |
| 28           | S31L K38N V49M E141G |
| 29           | V42F |
| 30           | P8L A57S |
| 31           | Y122H |
| 32           | I6F V10G H132Y |
| 33           | D103N H132Y |
| 34           | P91S A100P |
| 35           | I5T D92E L90W |
| 36           | T29 M L90S |
| 37           | T22S |
| 38           | R50I |
| 39           | P51S |
| 40           | D32Y V39I K45N F79Y |
| 41           | Q137F |
| 42           | E19V N97H Q137P Q140R |
| 43           | L90F A116V |

Mutations found in 43 Scs2 variants were scored for binding to a FFAT motif consisting of 13 nonbinding and 30 binding sequences. Underlining indicates that the same residue is mutated in both the nonbinding and binding clones: double underlining, precisely the same mutation was found in both sections; single underlining, differing mutations in each section. Mutations occurring carboxyl-terminally to the MSP-VAP domain are shown in parentheses. Mutations in residues that we propose to form the conserved core of the binding site for FFAT motifs are shown in bold.

| Mutations |
|-----------|
| V43A E60A A76T A110S E111D K120R Q137P |
| S21L V122D N136S Q137P Q140R |
| K38N V39H (V161M) |
| K40R T41H |
| K40N A76T |
| K40N Q63L A116G |
| V10M T42A |
| A36T T41I L71M A110T |
| I25S R50I |
| K40T K413N (A160V) (E166V) |
| V64I A102V (E147D) (D152H) |
| I82P Q114H |
| Y13F |
| E74V T89A |
| K45R |
| I67V K119I N124Y |
| I82N S129C |
| S15C R50I |
| G98S P112L |
| A76T D113V |
| V4L |
| F68V |
| L61I |
| S21T Q115H |
| S31L K38N V49M E141G |
| V42F |
| P8L A57S |
| Y122H |
| I6F V10G H132Y |
| D103N H132Y |
| P91S A100P |
| I5T D92E L90W |
| T29 M L90S |
| T22S |
| R50I |
| P51S |
| D32Y V39I K45N F79Y |
| Q137F |
| E19V N97H Q137P Q140R |
| L90F A116V |

with red fluorescence at the ER, indicating that the PCR product had no frameshift or nonsense mutations, were examined for the distribution of green fluorescence: GFP-FFAT on the ER was scored as "binding"; diffuse GFP-FFAT was scored as "nonbinding." From 192 colonies with ER-localized RFP-Scs2, 13 nonbinders detected. These plasmid-borne copies of Scs2 were sequenced, as well as 30 from binding controls. In the 13 non-binding clones, there were 46 mutations in 30 different residues (Table I). When plotted in histogram format, the mutations clearly clustered around the first half of the VAP consensus sequence, indicating that this portion of the protein is the most critical (Fig. 4B). Thus, scanning the whole MSP-VAP domain in an unbiased manner strongly implicated the first half of the VAP consensus sequence as a major component of the FFAT binding site.

Among the 30 residues implicated in the screen, several were also mutated in binding clones, where we found 60 mutations in 48 different residues (Table I and Fig. 4B). Eight mutations were reproduced exactly in a nonbinding clone and a binding clone (Table I, double underlines). Since these mutations appear not to compromise FFAT binding, only the remaining 22 residues were candidates for the binding site for FFAT motifs (Table I). We therefore examined their position in an alignment of multiple VAP sequences across eukaryotic evolution (Fig. 5), which showed that only four of the 22 candidates residues (Lys-40, Thr-41, Thr-42, Lys-120) are highly conserved in all VAPs, with mutations in these four residues alone accounting for 10 of the 13 nonbinders (Table I). This indicates that Lys-40, Thr-41, Thr-42, and Lys-120 may be critical for the interaction with FFAT motifs.

The error-prone PCR screen generated clones with multiple mutations, so we next decided to test the effect of introducing point mutations at the critical residues identified in the VAP consensus sequence. For Lys-40, a neutral substitution (K40A) had no discernible effect (Fig. 4C). In contrast, a less conservative mutation such as that identified in the screen (K40N) severely diminished targeting of GFP-FFAT to the ER (Fig. 4C). Therefore, Lys-40 is important but not essential for binding. Mutation of both Thr-41 and Thr-42 to alanines (T41A/T42A) completely abolished the ability of Scs2 to localize GFP-FFAT (Fig. 4C), implying that one or both of these threonines are critical for the FFAT-VAP interaction as well (Fig. 5).

The Residues in Scs2 Required for FFAT Binding in Vitro Are Also Required for the Regulation of Opi1 in Vivo—In addition to Lys-40, Thr-41, and Thr-42, the only other highly conserved residue identified by the screen was Lys-120. The finding that two lysines are important for binding FFAT motifs, which are highly acidic, led us to look at all eight highly conserved bases in the MSP-VAP domain of Scs2 (Lys-38, Lys-45, Arg-50, Arg-82, Lys-84, and Lys-122, as well as Lys-40 and Lys-120) (Fig. 5, asterisks). Interestingly, all of these are predicted to lie on the same face of the protein, except for Arg-50, which is in an adjacent strand (28). Nonconservative mutations in three of these (K38N, K45N, and R50I) occurred in FFAT-binding mutants of Scs2 (Table I), so these are unlikely to play a critical role in the binding site. To test the role of the other five, we mutated them singly to asparagine (Fig. 6). K40N had the strongest effect on in vitro binding, followed by K120N and then K84N, whereas R82N and K122N had only a slight effect (Fig. 6).

To determine the functional significance in vivo of these basic residues, we examined the ability of the Scs2 mutants to rescue the inositol auxotrophy of Δscs2Δscs22 cells (Fig. 6). Cells expressing the K40N mutant (low affinity for FFAT) failed to grow in the absence of inositol. The K120N mutant (intermediate affinity) produced a minor growth defect. K84N (small reduction in affinity) had a marginal growth defect. By comparison, both R82N and K122N (hardly altered affinity) grew as wild type. Extending this analysis to Thr-41 and Thr-42, we found that Thr-42 was critical for FFAT binding, with T42A producing similar loss of binding and inositol auxotrophy as K40N, whereas T41A produced a barely noticeable effect, less than K84N (Fig. 6). Overall, these results indicate that inositol auxotrophy resulting from scs2 mutations is caused by a specific disruption of a binding site that includes the conserved residues Lys-40, Thr-42, and Lys-120, with a marginal contribution from Lys-84.

Human VAP-A Has Different Properties than Scs2 in Yeast and Only Partially Rescues the Inositol Auxotrophic Phenotype—We next examined whether regulation of proteins containing FFAT motifs was entirely a product of the FFAT-VAP interaction or whether additional layers of regulation are important. To accomplish this, we determined whether human VAP-A could carry out the function of yeast VAPs, because...
Although the FFAT-VAP interaction is well conserved, other interactions are less likely to be conserved. We transformed $\text{Scs2}_{\text{H9004}}$ yeast with plasmids expressing human VAP-A. Human VAP-A rescued the inositol auxotrophy to some extent, this being best detected under less stringent conditions than those used to test Scs2 (at 35 °C rather than 37 °C). Rescue was not seen with human VAP-A (T46A/T47A) (Fig. 7A), which is presumably defective in FFAT binding. However, direct comparison of human VAP-A with Scs2 showed that the human protein was only partially active (Fig. 7B), even though it has essentially the same affinity for FFAT (see Figs. 3 and 6 and Ref. 1).

To understand why human VAP-A rescued Scs2 function poorly, we tested a series of chimeras between human VAP-A and Scs2 (Fig. 7C). Only chimeras containing the MSP-VAP domain from Scs2 rescued well, whereas this domain from human VAP-A functioned poorly, indicating an additional function of the MSP-VAP domain of Scs2. One possibility is that this additional function relates to intracellular targeting, because human VAP-A has been reported to localize to membrane compartments outside of the ER (9, 11, 29, 30). We therefore examined the intracellular targeting of human VAP-A and found it not only in the ER but also in the vacuole (Fig. 8A). In contrast, GFP-Scs2 was restricted to the ER. To determine the source of this difference, we examined targeting by VAP-A/Scs2 chimeras. This indicated that the major determinant of targeting was the transmembrane domain; all chimeras containing this domain from Scs2 targeted solely to the ER, whereas the transmembrane domain of human VAP-A was necessary but not sufficient for exit from the ER (compare chimeras SVV and SSV, Fig. 8B). Mistargeting did not correlate with reduced function (Fig. 7C). This implies that the additional function of the MSP-VAP domain of Scs2 is not related to targeting.
FIG. 7. Human VAP-A partially rescues Scs2 function. A, Δscs2Δscs2 cells were transformed with plasmids expressing human VAP-A and the mutant T46A/T47A, and inositol auxotrophy was assayed as described for Fig. 3 but at 35 °C, at which temperature the double deleted cells showed marginal growth. Ino, inositol. B, same as in A, except at 37 °C and including a plasmid expressing Scs2. At this higher temperature, rescue by human VAP-A was partial, in comparison with complete rescue by Scs2. C, reduced function of human VAP-A maps to its MSP-VAP domain. VAP-A/Scs2 chimeras as shown in the diagram on the right (domains labeled as in Fig. 1) were assayed in the Δscs2Δscs2 strain as described in B. Efficient rescue correlated with the presence of the MSP-VAP domain from Scs2, whereas constructs with the MSP-VAP domain of human VAP-A rescued poorly.

DISCUSSION

We have studied an evolutionarily conserved interaction by which VAP acts as an ER receptor for many different proteins containing FFAT motifs: lipid binding proteins in yeast (18) and mammalian cells (3, 4, 17, 31) and other proteins of unknown function (32). We have identified a cluster of residues that define a binding site for FFAT motifs on the MSP-VAP domain of Scs2. In addition, we have shown that these residues are important for one function of Scs2, i.e. binding Opi1 to prevent the repression of phospholipid metabolism. Inositol auxotrophy, as with Δscs2, was seen with variants of Scs2 that failed to bind a FFAT motif in vitro, defining the conserved residues Lys-40, Thr-42, Lys-120, and to a lesser extent Lys-84 as key for binding FFAT. This core of the FFAT binding site is highly conserved, so the findings are likely to be applicable to VAPs in widely divergent species.

To further demonstrate the role of these conserved residues, we modeled the human VAP-A sequence onto the tertiary structure of MSP1α (Fig. 9) (33). The sequence conservation between VAPs and MSP1 (20% identical, 20% homologous) is largely confined to the predicted core structural components (the β-sheets, Fig. 5), and VAPs are predicted to fold in the same immunoglobulin-like seven-stranded β-sandwich as MSP1 (28). Our model shows that the key residues for FFAT binding cluster on one face of the molecule, their side-chains are predicted to point outward into solution, and the critical threonine might hold this region together (Fig. 9 A). A surface plot predicts a single positively charged band across the protein, with the critical residues at its core and the other five conserved basic residues at the periphery (Fig. 9 B). The remainder of the surface is predicted to be neutral or acidic (data not shown). The model also predicts the presence of two shallow pits on either side of a critical lysine (Lys-45 in human VAP-A, Lys-40 in Scs2), which are partly lined by the conserved aromatic side chains of Tyr-52 and Phe-88 (Tyr-47 and Phe-85 in Scs2). It is possible that each of these accommodates one of the central phenylalanines of the FFAT motif (EFFDAXE). Note that the residues in MSP1 required for its multimerization are poorly conserved in VAPs (Fig. 5), which are therefore unlikely to form filaments (34).

Based on our results, it might be possible to predict which other proteins with MSP-VAP domains can bind FFAT motifs. In addition to other VAP homologues, there are two families of proteins, widespread throughout the Metazoa, that contain MSP-VAP domains and transmembrane domains. One family is entirely unstudied and consists of VAP extended at the amino terminus with a lipid transfer domain of the CRAL/TRIO/Sec14 type. Interestingly, the MSP-VAP domains in these proteins have all four of the most critical residues for FFAT binding (data not shown), which leads us to predict that these proteins, with their own lipid transfer domain in cis, can bind FFAT motifs and hence access a variety of other lipid-binding proteins. The second family of proteins with MSP-VAP domains is known as MOSPDs (motile sperm domain-containing proteins), one of which is important for normal heart de-
predicted to lie on either side of Lys-45 (Lys-40 in Scs2). Also note two shallow pits that are critical threonine (Thr-46) points inward away from the binding site. The side chain of which points outward into solution and forms hydrogen bonds (dashed lines) to both Lys-85 and Lys-87 on strand f. The non-critical threonine (Thr-42 in Scs2) is uniquely variant in Scs22 (serine), which may signify that this protein plays a smaller part in lipid metabolism and is more active in some of the other possible interactions of Scs2 (15, 16, 36). Nevertheless, residual binding of Scs22 to FFAT motifs may explain how Opi1 is slightly targeted to the nuclear envelope in ∆scs2 strains (1, 37).

Despite their obvious overall homology, Scs2 and human VAP-A have some structural differences (Fig. 1) that may account for their differing localizations and functions in yeast. One of these differences was responsible for the mammalian protein being less well confined to the ER. This altered targeting mapped to the transmembrane domain and may be associated with the ability of this region of most VAPs to dimerize, as they contain a GXXG motif, whereas it is unlikely that the transmembrane domain of Scs2 dimerizes (17, 38). The exit of human VAP-A from the yeast ER is consistent with previous reports of fly and vertebrate VAPs outside of the ER (9, 11, 29, 30). The traffic of human VAP-A to the yeast vacuole, once it has escaped from the ER, may result from recognition of its transmembrane domain by a default degradation pathway (39). Another difference, despite the similar affinity for FFAT motifs, is that human VAP-A only substituted partially for Scs2 function, suggesting that Scs2 has a stronger interaction with full-length Opi1 in vivo. This also suggests that the MSP-VAP domain of Scs2 interacts with another protein to achieve tight binding to full-length Opi1, and shows that the FFAT-VAP interaction may be regulated in vivo.

The role of VAP outside of the ER may be important in motor neurons, because mutant VAP-B in type 8 familial ALS targets a punctate non-ER compartment (13). Quite fortuitously, one of the Scs2 mutants in our screen reproduced the exact same mutation (sequence 39: P51S, equivalent to P56S in human VAP-B; see Table I). Although the proline in question is conserved in most MSP-VAP domains, the MOSPD family has an acidic residue at this position, implying that a serine is unlikely to destabilize the overall structure. Scs2 with the P51S mutation was a FFAT binder; therefore we predict that the mutant VAP-B in type 8 familial ALS is not grossly misfolded and can recruit proteins with FFAT motifs. Overall, our results indicate how VAP binds FFAT motifs and can be used in different model systems to specifically inactivate this interaction of VAP.

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REFERENCES

1. Loewen, C. J., Roy, A., and Levine, T. P. (2003) EMBO J. 22, 2025–2035
2. Wirtz, K. W. (1991) Annu. Rev. Biochem. 60, 73–99
3. Wyles, J. P., and Ridgway, N. D. (2004) Exp. Cell Res. 297, 533–547
4. Amarillo, R., Ramschandran, S., Sabanay, H., and Lev, S. (2005) J. Biol. Chem. 280, 5934–5944
5. Skelch, P. A., Martin, K. C., Kandel, E. R., and Bartsch, D. (1995) Science 269, 1580–1583
6. Nikawa, J., Murakami, A., Esumi, E., and Hosaka, K. (1995) J. Biochem. (Tokyo) 118, 39–45
7. Weir, M. L., Klip, A., and Trimble, W. S. (1998) Biochem. J. 333, 247–251
8. Nishimura, Y., Hayashi, M., Inada, H., and Tanaka, T. (1999) Biochem. Biophys. Res. Commun. 254, 21–26
9. Soussan, L., Burakov, D., Daniels, M. P., Toister-Achituv, M., Porat, A., Yarden, Y., and Elazar, Z. (1999) J. Cell Biol. 146, 301–311
10. Skelch, P. A., Fabian-Fine, R., and Kandel, E. R. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 1101–1106
11. Pennetta, G., Hiesinger, P., Fabian-Fine, R., Meinertzhagen, I., and Bellen, H. (2002) Neuron 35, 291–306
12. Weir, M. L., Xie, H., Klip, A., and Trimble, W. S. (2001) Biochem. Biophys. Res. Commun. 286, 616–621
13. Nishimura, A. L., Mitne-Neto, M., Silva, H. C., Richieri-Costa, A., Middleton, S., Casiceio, D., Kok, P., Oliveira, J. R., Gillingwater, T., Webb, J., Skelch, P., and Zatz, M. (2004) Am. J. Hum. Genet. 75, 822–831
14. Kagiwada, S., Hosaka, K., Murata, M., Nikawa, J., and Takatsuki, A. (1998) J. Bacteriol. 180, 1700–1708
15. Craven, R. J., and Petes, T. D. (2001) Genetics 158, 145–154
16. Cuperus, G., and Shore, D. (2002) Genetics 162, 633–645
17. Wyles, J. P., McMaster, C. R., and Ridgway, N. D. (2002) J. Biol. Chem. 277, 28906–28918

FIG. 9. Three-dimensional model of FFAT binding site of human VAP-A. A, ribbon diagram of strands c/f/g of human VAP-A. Coordinates from C. elegans MSP1α were modeled onto human VAP-A (33) using Swiss Model and DeepView. The image was generated in PyMOL. Conserved side chains discussed in the text are highlighted (lysines/arginines, blue; threonines, yellow; aromatics, magenta). Note that all of the noncritical basic residues Lys-43, Arg-50, Arg-55, Lys-85, and Arg-120 (black labels; equivalent to Lys-38, Lys-45, Arg-50, Arg-82, and Lys-122 in Scs2) are located peripherally around a core that includes the critical bases Lys-45, Lys-87, and Lys-118 (red labels; equivalent to Lys-40, Lys-84, and Lys-120 in Scs2) and the critical threonine (Thr-47), the side chain of which points outward into solution and forms hydrogen bonds (dashed lines) to both Lys-85 and Lys-87 on strand f. The noncritical threonine (Thr-46) points inward away from the binding site. B, surface charge plot (PyMOL) of the predicted FFAT binding surface of human VAP-A. Colors indicate basic (blue), acidic (red), and neutral (white) areas. Note that all eight conserved lysine/arginines contribute to a single contiguous cationic band. Also note two shallow pits that are predicted to lie on either side of Lys-45 (Lys-40 in Scs2).
18. Loewen, C. J., Gaspar, M. L., Jesch, S. A., Delon, C., Kiistakos, N. T., Henry, S. A., and Levine, T. P. (2004) *Science* **304**, 1644–1647
19. Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P., and Boeke, J. D. (1998) *Yeast* **14**, 115–132
20. Levine, T. P., and Munro, S. (2001) *Mol. Biol. Cell* **12**, 1633–1644
21. Sikorski, R. S., and Hieter, P. (1989) *Genetics* **122**, 19–27
22. Campbell, R. E., Tour, O., Palmer, A. E., Steinbach, P. A., Baird, G. S., Zacharias, D. A., and Tsien, R. Y. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 7877–7882
23. Spingola, M., grate, L., Haussler, D., and Ares, M., Jr. (1999) *RNA* **5**, 221–234
24. Blandin, G., Durrens, P., Tekai, F., Aigle, M., Boletin-Fukuhara, M., Bon, E., Casaregola, S., de Montigny, J., Gaillardin, C., Lepingle, A., Llurente, S., Malpertuy, A., Nevegilde, C., Ozer-Kalogeropoulos, O., Perrin, A., Potier, S., Souciet, J., Talla, E., Toffano-Noche, C., Wesolowski-Louvel, M., Marck, C., and Dujon, B. (2000) *FEBS Lett.* **487**, 31–36
25. Kellis, M., Birren, B. W., and Lander, E. S. (2004) *Nature* **428**, 617–624
26. Dietrich, F. S., Voegeli, S., Brachat, S., Lerch, A., Gates, K., Steiner, S., Mohr, C., Pohmann, R., Luedi, P., Choi, S., Wing, R. A., Flavier, A., Gaffney, T. D., and Philipp, P. (2004) *Science* **304**, 304–307
27. Pall, G. S., Wallis, J., Aston, R., Brownstein, D. G., Gautier, P., Buerger, K., Mulford, C., Mullins, J. J., and Forrester, L. M. (2004) *Genomics* **84**, 1051–1059
28. Bullock, T. L., Roberts, T. M., and Stewart, M. (1996) *J. Mol. Biol.* **263**, 284–296
29. Lapiere, L. A., Tuma, P. L., Navarre, J., Goldenring, J. R., and Anderson, J. M. (1999) *J. Cell Sci.* **112**, 3723–3732
30. Foster, L. J., Weir, M. L., Lim, D. Y., Liu, Z., Trimble, W. S., and Klip, A. (2000) *Traffic* **1**, 512–521
31. Hanada, K., Kumagai, K., Yasuda, S., Nishimatsu, M., and Nishijima, M. (2005) *Nature* **436**, 803–809
32. Ettayebi, K., and Hardy, M. E. (2003) *J. Virol.* **77**, 11790–11797
33. Baker, A. M., Roberts, T. M., and Stewart, M. (2002) *J. Mol. Biol.* **320**, 491–499
34. Smith, H. E., and Ward, S. (1998) *J. Mol. Biol.* **279**, 605–619
35. Veleculecu, V. E., Zhang, L., Zhou, W., Vogelstein, J., Breslai, M. A., Bassett, D. E., Jr., Hieter, P., Vogelstein, B., and Kinzel, K. W. (1997) *Cell* **88**, 243–251
36. Gavrin, A. C., Bosche, M., Krause, R., Grandi, P., Marzich, M., Bauer, A., Schultz, J., Rick, J. M., Michon, A. M., Cruce, C. M., Remor, M., Hofert, C., Scheler, M., Bajenovic, M., Ruffner, H., Merino, A., Klein, K., Haidak, M., Dickson, D., Rudi, T., Nau, V., Bask, A., Bastuck, S., Huhse, B., Leutwein, C., Heurtier, M. A., Copley, R. R., Edelman, A., Querfurth, E., Kyb, V., Dreses, G., Rada, M., Bouwmeestet, T., Bork, P., Seraphin, S., Kuster, B., Neubauer, G., and Superti-Furga, G. (2002) *Nature* **415**, 141–147
37. Brickner, J. H., and Walter, P. (2004) *J. Virol.* **77**, 11790–11797