The caveolin-binding motif of the pathogen-related yeast protein Pry1, a member of the CAP protein superfamily, is required for in vivo export of cholesteryl acetate

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Abstract Proteins belonging to the CAP superfamily are present in all kingdoms of life and have been implicated in different physiological processes. Their molecular mode of action, however, is poorly understood. Saccharomyces cerevisiae expresses three members of this superfamily, pathogen-related yeast (Pry1), -2, and -3. We have recently shown that Pry function is required for the secretion of cholesteryl acetate and that Pry proteins bind cholesterol and cholesteryl acetate, suggesting that CAP superfamily members may generally act to bind sterols or related small hydrophobic compounds. Here, we analyzed the mode of sterol binding by Pry1. Computational modeling indicates that ligand binding could occur through displacement of a relatively poorly conserved flexible loop, which in some CAP family members displays homology to the caveolin-binding motif. Point mutations within this motif abrogated export of cholesteryl acetate but did not affect binding of cholesterol. Mutations of residues located outside the caveolin-binding motif, or mutations in highly conserved putative catalytic residues had no effect on export of cholesteryl acetate or on lipid binding. These results indicate that the caveolin-binding motif of Pry1, and possibly of other CAP family members, is crucial for selective lipid binding and that lipid binding may occur through displacement of the loop containing this motif. The caveolin-binding motif of the pathogen-related yeast protein Pry1, a member of the CAP protein superfamily, is required for in vivo export of cholesteryl acetate. J. Lipid Res. 2014. 55: 883–894.

Supplementary key words cholesterol • Saccharomyces cerevisiae • pathogen-related yeast

Proteins belonging to the CAP superfamily (pfam PF00188), also known as SCPS (sperm coating proteins), have been implicated in many different physiological contexts, including immune defense in mammals and plants, pathogen virulence, sperm maturation and fertilization, venom toxicity, and prostate and brain cancer. Named after the founding members of this protein superfamily (cysteine-rich secretory protein, antigen 5, and pathogenesis-related 1), the CAP superfamily comprises more than 4,500 members in over 1,500 species, and family members are present in all kingdoms of life. Almost all CAP proteins are secreted glycoproteins and they are stable in the extracellular space over a wide range of conditions. All members of this superfamily share a common CAP domain of approximately 150 amino acids, which adopts a unique α-β-α sandwich fold. The structural conservation of this domain suggests that CAP proteins exert a fundamentally similar function. The molecular mode of action of the CAP proteins, however, has remained enigmatic [for reviews see (1, 2)].

CAP proteins share limited sequence identity with each other and are characterized by two PROSITE-recognized sequence motifs, referred to as the CRISP family signature (shaded boxes in Fig. 1). An early comparison of the structure of the plant pathogenesis-related protein (PR)-1 with that of the human glioma pathogenesis-related protein 1 (GLIPR1) revealed that the small and structurally conserved 17–21 kDa CAP domain adopts a unique α-β-α sandwich fold, in which a three-stranded anti-parallel β-sheet is flanked by three helices on one side, and a fourth helix on the other (3). This three-stacked layer is stabilized by hydrophobic interactions, multiple hydrogen bonds, and by two highly conserved disulfide bonds. These features are thought to provide the thermal, pH, and proteolytic stability required for the extracellular function of these proteins (3, 4). The CAP domain harbors four highly

Abbreviations: DOPE, discrete optimized protein energy; GLIPR1, glioma pathogenesis-related protein 1; PR, pathogenesis-related protein; OD, optical density; PDB, protein data base; Pry, pathogenesis-related yeast; RMSD, root mean square deviation; SC, synthetic complete.
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conserved partially surface-exposed residues, two histidines, and two glutamic acid residues (Fig. 1) (3).

These surface residues were suggested to adopt a conformation similar to the active site histidyl and glutamyl residues of several Zn-dependent proteases and were suggested to form part of a putative active site triad of a CAP protein with a reported in vitro protease activity, Tex31 (5, 6). Apart from a possible catalytic activity of CAP family members, the CAP domain has been suggested to form a stable scaffold for biological interactions with other proteins (7). Although a large number of proteins within the superfamily contain a CAP domain in isolation (e.g., antigen 5 and PR-1), many others contain additional N- or C-terminal extensions (2).

For example, CAP proteins harboring a C-terminal cysteine-rich domain, the defining feature of the CRISP subfamily, have been associated with modulation of the activity of ion channels, which might account for their role in sperm maturation and venom toxicity (6, 8, 9). However, whether the cysteine-rich domain of CRISP proteins acts directly on the ion channel or whether these proteins affect the channel’s activity more indirectly, for example through modulation of the lipid composition of the plasma membrane, remains to be established.

The genome of *Saccharomyces cerevisiae* contains three CAP superfamily members, pathogen-related yeast (Pry)1, -2, and -3, named after their homology to the plant PR-1. We have recently shown that in yeast, these proteins are required for the export of cholesteryl acetate in vivo and that the purified proteins bind sterols in vitro (10). The sterol binding and export function of the yeast Pry1 is confined to the CAP domain, because expression of the CAP domain alone is sufficient to rescue the sterol export phenotype of cells lacking Pry function, and the CAP domain of Pry1 alone binds sterols in vitro. This protein-lipid interaction is specific, as it is lost when a highly conserved cysteine residue that is known to form a disulfide bridge is mutated to serine. The lipid-binding and export function of the Pry proteins appears to be a conserved function of the members of the CAP protein superfamily, because expression of the human CAP protein, CRISP2, relieves the lipid export block of a yeast mutant lacking Pry function, and the purified CRISP2 binds sterols in vitro (10, 11).

The aim of the current study was to perform a structure-function analysis of the yeast Pry1 protein to begin to define the mode of sterol binding of this CAP superfamily member. Based on molecular modeling, site-directed mutagenesis was employed to identify elements within the CAP domain of Pry1 that are important for lipid binding.

**METHODS**

**Yeast strains, growth conditions, and epitope tagging**

Yeast mutant strains were cultivated either in rich media, YPD (containing 1% Bacto yeast extract, 2% Bacto peptone, and 2% glucose; US Biological, Swampscoott, MA) or in minimal defined media (containing 0.67% yeast nitrogen base without amino acids (US Biological), 0.73 g/l amino acids, and 2% glucose). Media supplemented with sterols and fatty acids contained 0.05 mg/ml Tween 80 and 20 μg/ml cholesterol (Sigma Chemical Co., St. Louis, MO). To bypass heme deficiency, cells were grown in media supplemented with 10 μg/ml delta-aminolevulinic acid.

**Site-directed mutagenesis and Western blotting**

Site-directed mutagenesis was performed using PCR fusion cassettes as described previously (12). Protein concentration was...
Lipid labeling and analysis

Acetylation and export of sterols into the culture supernatant were examined as described previously (13). Yeast mutants deficient in heme biosynthesis (hem1Δ) were cultivated in the presence of cholesterol/Tween-80-containing media and labeled with 0.025 μCi/ml [14C]cholesterol (American Radiolabeled Chemicals, Inc., St. Louis, MO). Cells were harvested by centrifugation, washed twice with synthetic complete (SC) media, diluted to an OD600 of 1 into fresh media containing nonradiolabeled cholesterol, and grown overnight. Cells were centrifuged and lipids were extracted from the cell pellet and the culture supernatant using chloroform/methanol (v/v, 1:1). Samples were dried and separated by TLC using silica gel 60 plates (Merck, Darmstadt, Germany) and using the solvent system petroleum ether/diethyl ether/acetate (70:30:2, per vol). Radiolabeled lipids on the TLC were quantified by scanning with a Berthold Tracemaster 40 automatic TLC-linear analyzer (Berthold Technologies, Bad Wildbad, Germany). TLC plates were then exposed to phosphorimagery screens and radiolabeled lipids were visualized using a phosphorimagery Bio-Rad Laboratories, Hercules, CA).

Expression and purification of wild-type and mutant versions of Pry1

DNA encoding wild-type and mutant versions of Pry1 was PCR amplified and subsequently cloned into the NcoI and XhoI sites of pET22b vector (Novagen, Merck, Darmstadt, Germany), which contains a PelB signal sequence to direct the secretion of the expressed protein into the periplasmic space. The vector was transformed into Escherichia coli BL21 and the protein was expressed as a polyhistidine-tagged fusion after lactose induction and overnight growth of the bacteria at 24°C. Cells were harvested, lysed, and incubated with Ni-NTA beads (Qiagen, Hilden, Germany) as per the instructions of the manufacturer. Beads were washed and proteins were eluted with imidazole, concentrated, and quantified.

In vitro lipid binding assay

The radioligand binding assay was performed as described previously (10, 14). Purified protein (100 pmol) in binding buffer [20 mM Tris (pH 7.5), 30 mM NaCl, 0.05% Triton X-100] was incubated with [3H]cholesterol (50 pmol) for 1 h at 30°C. The protein was then separated from the unbound ligand by adsorption to Q-Sepharose beads (GE Healthcare, USA); beads were washed and the radioligand was quantified by scintillation counting. For competition binding assays, unlabeled cholesterol or cholesteryl acetate (50 pmol) was included in the binding reaction. To determine nonspecific binding, the ion exchange beads were incubated in the absence of added protein or an excess of unlabeled cholesterol was added into the binding assay. Data were analyzed using PRISM software (GraphPad, San Diego, CA).

RESULTS

As a first step toward an understanding how the yeast CAP family member, Pry1, can bind a hydrophobic ligand such as cholesterol, we modeled the structure of the CAP domain of the yeast Pry1 protein (residues 160-299) using the two closest ortholog proteins with structural data available as templates, human GLIPR2 [protein data base (PDB): 1SMB, X-ray structure at 1.55 Å resolution (15)] and tomato PR1B1 [PDB: 1CFE, NMR structure (4)]. These proteins display 39.5 and 32.9% sequence identity with the CAP domain of Pry1, respectively, and therefore can be used as reliable templates for homology modeling (Fig. 1). Two hundred different models were generated with MODELLER and the one with the lowest value for the discrete optimized energy (DOPE) score was used as a model for Pry1 (16). In this model, the positions of the α helices and β sheet agree with independent secondary structure predictions, and the four conserved cysteine residues appear to form disulfide bridges that help to stabilize the CAP domain, as was observed in the structure of the tomato PR1B1 (Fig. 2A) (4).

Structural analysis of yeast Pry1

Analysis of the model of the yeast protein, however, did not reveal a potential hydrophobic pocket that could accommodate cholesterol, and all reported hydrophobic patches are buried inside the structure and not directly surface accessible, suggesting that cholesterol does not bind by simply sticking to a hydrophobic patch at the protein surface, but that binding of the lipid is likely accompanied by significant structural rearrangement of the protein, as has been observed in other cholesterol binding proteins [e.g., β-elicitin cryptogein (17)]. To gain further insights into the possible mechanism of binding, we first analyzed the experimentally determined structural stability of human of GLIPR2, the closest ortholog of the yeast Pry1. The region displaying the largest b-factors corresponds to a loop (residues 89-101 of GLIPR2, corresponding to residues 236-248 of Pry1), which has homology to the caveolin-binding motif (YNFQQPQG in GLIPR2, YDFSNPGR in Pry1). The human GLIPR2, also known as Golgi-associated PR1 protein, GAPR1, is the only characterized intracellular CAP family member so far. GLIPR2 binds caveolin with a flexible loop that is rich in aromatic side-chains (ωωωωωωω, where ω is any aromatic side-chain), known as the caveolin-binding motif (18, 19). This loop is remarkably conserved in mammals, flies, worms, and fungi, but not in organisms such as plants, wasps, ants, or snakes (Fig. 2B). The conservation of this loop in flies and fungi is particularly striking because caveolin is not present in these organisms. The sequence of the loop harboring the caveolin-binding motif in GLIPR2, however, is poorly conserved in the tomato PR1B1, displays flexibility in the NMR structure of this protein (PDB: 1CFE), and is significantly displaced compared with that of the human GLIPR2 [root mean square deviation (RMSD) = 5.8 Å between residues 89-101 in GLIPR2 and residues 101-115 in PR1B1 for the first NMR conformation]. Moreover, the conformation of this loop in PR1B1 appears to be stabilized by an additional disulfide bridge, which is not present in the yeast or human homologs. The predicted structural flexibility of this loop and its relative low conservation in CAP family members of plants or toxin-producing reptiles and insects.
suggest that this loop may not play an important structural function, and that it could be displaced upon binding to caveolin or other potential ligands such as cholesterol or related small hydrophobic compounds.

To examine this possibility, we used the MODELLER software to generate random conformations of the loop (2,000 in total). While complete sampling of the different loop conformations is not feasible due to the astronomical number of degrees of freedom of the problem, geometrical observation of the different conformers suggests that some conformations may open a hydrophobic cavity with a size that could potentially accommodate a molecule such as cholesterol (Fig. 2C). Although we cannot exclude other modes of binding involving remodeling of other parts of the protein (20), the resulting one-to-one binding model is consistent with kinetic data indicating stoichiometric binding of cholesterol to Pry1 (10), as well as our mutagenesis analysis (see below).

Mutations in the caveolin-binding motif affect the in vivo sterol export function of Pry1

The model of sterol binding by Pry1 through displacement of the caveolin-binding motif predicts that hydrophobic residues within this loop (residues 236-248 of Pry1) are important for interaction with the hydrocarbon ring of the sterol. To test the functional significance of these residues, key residues within this loop region were mutated (F239L, F244L, P242C, and A292C) (Fig. 3a). If cholesterol binding is indeed dependent on the loop sequence, the first two mutations are expected to lower the affinity for cholesterol, while not dramatically affecting the overall protein stability, because the Phe to Leu substitution at these two positions maintains the hydrophobic character and possibly hydrophobic interactions at these positions. However, the substitutions may disrupt the conserved caveolin-binding motif (FxFxxxxF) present in the human and yeast sequences. The model therefore predicts that these two substitutions should display reduced cholesterol binding. The last two mutations (P242C and A292C), on the other hand, are expected to strongly affect the conformation of the loop itself and may even prevent the correct folding of the protein by creating nonnative disulfide bridges with other cysteines. Therefore, they are expected to completely abolish sterol binding.

To test these predictions, the mutant versions of Pry1 were expressed as GST-fusions in yeast and their in vivo functionality was assessed. All proteins bearing mutations in this flexible loop were expressed and secreted into the culture supernatant, as is the case for the wild-type protein (Fig. 3B). These results indicate that these mutant versions are properly translocated into the endoplasmic reticulum lumen, packed into secretory vesicles, and secreted out of the cell. To test the in vivo sterol-binding and export function of the mutant proteins, cells were labeled with [14C]cholesterol and Pry1-dependent export of cholesteryl acetate was assessed by extracting lipids from the cell pellet and the culture media of heme-deficient cells lacking the sterol deacetylase Say1 as well as the wild-type versions of Pry2 and Pry3. In these sterol export assays, heme-deficient cells need to be used to allow for uptake of the radiolabeled cholesterol and deficiency of the deacetylase, Say1, is required to monitor accumulation and Pry-dependent export of cholesteryl acetate (10, 13). Lipids were separated
Fig. 3. Mutations within the caveolin-binding motif abrogate the in vivo sterol-export function of Pry1. A: Structural model of Pry1. Residues within the loop region that were substituted are marked in green (F239, P242, F244, A292). B: Loop mutants are secreted. Cells expressing a GST-tagged wild-type or the indicated mutant versions of Pry1 were cultivated, proteins present in the cell pellet (P) and the culture supernatant (S) were TCA precipitated, separated by PAGE, and probed for the presence of the tagged protein and for Wbp1, an integral endoplasmic reticulum membrane protein and subunit of the oligosaccharyl transferase complex. C: Loop mutants are defective in export of cholesteryl acetate. Heme-deficient cells of the indicated genotype expressing a GST-tagged wild-type or mutant versions of Pry1 were radiolabeled with [14C]cholesterol, washed, and chased with media containing unlabeled cholesterol; lipids were extracted from
Mutations in residues outside of the caveolin-binding motif do not affect the in vivo functionality of Pry1

All mutations in the caveolin-binding motif of Pry1 affected its sterol export function in vivo. However, this effect could also arise from a global structural destabilization of the protein. We therefore selected other hydrophobic residues with similar surface exposure as F239 and F244, but located far away from the caveolin-binding motif, to test whether substitutions at these sites would also affect the functionality of the protein in vivo (Fig. 4A). Mutations at these sites (L206V, L219A, F162L) are expected to have similar effects on the global structure of the protein, but will not affect the caveolin-binding motif containing loop. All three of these mutants were properly secreted and complemented the sterol export defect of a mutant lacking Pry function ( pry2Δ pry3Δ), suggesting that these amino acid substitutions did not impact the in vivo sterol-binding activity of Pry1 (Fig. 4).

Substitutions of putative active site residues of Pry1 do not affect sterol-binding and export

A CAP family member from the venom duct of the cone snail, Tex 31, was shown to have proteolytic activity in vitro. The activity was inhibited by serine protease inhibitors and stimulated by Ca²⁺ (5). Computational modeling of the Tex31 sequence to the structure of PR-1 and antigen 5 was consistent with a possible catalytic role of the conserved surface-exposed histidine and glutamic acid residues (5). The lack of a conserved serine in this putative active site led to the proposition that dimerization is required to complete the formation of an active site triad, characteristic for enzymes belonging of the α/β hydrolase fold superfamily (15). However, other studies failed to detect protease activity with purified CAP family members, and a conclusive demonstration of the protease activity for a mammalian, fungal, or plant CAP protein still remains to be provided (8, 21).

To examine whether these surface-exposed putative active site residues, which are conserved in Pry1, are functionally important for the sterol-binding activity of Pry1, we substituted H170, H250, and E233 to alanine (Fig. 5A). The resulting mutant versions were properly synthesized and secreted from the cells and all three mutations complemented the sterol export defect of a yeast mutant lacking Pry function, indicating that these putative active site residues are not crucial for the functionality of the protein in vivo (Fig. 5).

Mutations in the caveolin-binding motif of Pry1 affect binding of cholesteryl acetate but not that of cholesterol

To examine whether the mutations in the caveolin-binding domain, in unrelated hydrophobic residues, or those in putative active site residues affect the lipid binding properties of Pry1 in vitro, hexahistidine-tagged versions of these proteins were expressed in bacteria and purified by affinity chromatography. SDS-PAGE analysis of the purified proteins revealed a single Coomassie-stained band with an apparent molecular mass of approximately 37 kDa (data not shown).

The sterol-binding capacity of the mutant versions was assessed by in vitro binding assays and plotted relative to that of wild-type Pry1 (Fig. 6A). This analysis revealed that all the mutations retained their ability to bind free cholesterol. To examine why the mutations in the caveolin-binding motif failed to complement the defect in export of cholesteryl acetate in vivo, we performed competition binding experiments in which cold cholesteryl acetate competes with [3H]cholesterol for binding to the protein. These binding assays revealed that only the mutations in the caveolin-binding site affected in vitro binding of cholesteryl acetate (Fig. 6B). To confirm these results we performed titration experiments to determine the relative affinity of some of the mutant proteins to cholesteryl acetate. The results of these experiments indicate that mutations in the caveolin-binding site selectively affected binding of cholesteryl acetate, but not that of free cholesterol (Fig. 7). Taken together, the results presented here are consistent with a role of the caveolin-binding motif in selective lipid binding of Pry1 in vitro and in vivo.

The cell pellet (P) and the culture supernatant (S), and separated by TLC. The position of free cholesterol (FC), cholesteryl acetate (CA), and steryl esters (STE) is indicated on the right. Stars mark positions of unidentified cholesterol-derivatives. D: Quantification of the export of cholesteryl acetate. The export index indicates the relative percentages of cholesteryl acetate that are exported by the cells (ratio between the extracellular cholesteryl acetate and the sum of intra- and extracellular cholesteryl acetate). Data represent mean ± SD of two independent experiments.
Fig. 4. Mutations in other hydrophobic residues do not affect export of cholesteryl acetate in vivo. 
A: Structural model of Pry1. The two residues within a common loop (L206, L219) and the one residue at the base of helix 1 (F162) that were substituted are indicated in blue. B: The mutants are all secreted. Cells expressing a GST-tagged wild-type or the indicated mutant versions of Pry1 were cultivated, proteins present in the cell pellet (P) and the culture supernatant (S) were TCA precipitated, separated by PAGE, and probed for the presence of the tagged protein and for Wbp1. C: Mutants are functional for export of cholesteryl acetate. Heme-deficient cells of the indicated genotype expressing a GST-tagged wild-type or mutant versions of Pry1 were radiolabeled with [14C]cholesterol, washed, and chased with media containing unlabeled cholesterol; lipids were extracted from the cell pellet (P) and the culture supernatant (S), and separated by TLC. The position of free cholesterol (FC), cholesteryl acetate (CA), and steryl esters (STE) is indicated on the right. Stars mark positions of unidentified cholesterol-derivatives. D: Quantification of the export of cholesteryl acetate. The export index indicates the relative percentages of cholesteryl acetate that is exported. Data represent mean ± SD of two independent experiments.
Fig. 5. Mutations in putative active-site residues are functional for cholesteryl acetate export in vivo. A: Structural model of Pry1. Residues proposed to form part of a putative active site are indicated in blue (H170, E233, H250). B: Mutants in putative active site residues are secreted. Cells expressing a GST-tagged wild-type or the indicated mutant versions of Pry1 were cultivated, proteins present in the cell pellet (P) and the culture supernatant (S) were TCA precipitated, separated by PAGE, and probed for the presence of the tagged protein and for Wbp1. C: Mutants in putative active site residues are functional in export of cholesteryl acetate. Heme-deficient cells of the indicated genotype expressing a GST-tagged wild-type or mutant versions of Pry1 were radiolabeled with $^{14}$C]cholesterol, washed, and chased with media containing unlabeled cholesterol; lipids were extracted from the cell pellet (P) and the culture supernatant (S), and separated by TLC. The position of free cholesterol (FC), cholesteryl acetate (CA), and sterol esters (STE) is indicated on the right. Stars mark positions of unidentified cholesterol-derivatives. D: Quantification of the export of cholesteryl acetate. The export index indicates the relative percentages of cholesteryl acetate that is exported. Data represent mean ± SD of two independent experiments.

DISCUSSION

The data presented in this study indicate that the caveolin-binding motif that is present in the yeast CAP superfamily member, Pry1, is important for the in vivo as well as the in vitro sterol binding activity of the protein. Analysis of known structures and homology modeling indicate that the loop region harboring this motif is one of the most flexible parts of the protein and may be displaced upon lipid binding. Point mutations within this motif selectively
Lipid binding by the CAP family member, Pry1

...their aromatic residues are buried within the protein and thus are unlikely to interact directly with caveolin (23). On the other hand, the caveolin-binding motif is unlikely of relevance for a direct interaction between Pry1 and caveolin, because a homolog of caveolin is not detectable in the yeast genome and caveolae are not present in the plasma membrane of this fungus. Thus, we consider it more likely that the loop harboring this sequence motif in Pry1 requires the characteristic spacing of the aromatic side chains to exert its function in selective lipid binding, which, according to our computational modeling experiments, requires displacement of the loop and exposure of a hydrophobic cleft. Given that the substitutions of two of the aromatic residues within this motif by leucine (F239 and F244), an at least equally

abrogate cholesteryl acetate binding without affecting binding of free cholesterol. Mutations outside the caveolin-binding motif, either in distant hydrophobic amino acids with a similar role in protein stability or in putative active site residues, on the other hand, did not affect the functionality of the protein in vivo, because cells expressing these mutant versions were still capable of exporting cholesteryl acetate, nor did they affect binding of free cholesterol or cholesteryl acetate in vitro.

The caveolin-binding motif was originally uncovered by screening phage display peptides that bind to the caveolin-scaffolding domain of caveolin, a coat protein required for formation of caveolae, small flask-shaped pits of the plasma membrane of metazoan (19, 22). However, the caveolin-binding motif is a widespread sequence motif rich in aromatic residues, which is present in about 30% of human proteins (23). Recent analysis of the solvent accessible area of putative caveolin-binding motifs in proteins of known structure indicates that, the majority of...
considerably more hydrophobic than cholesterol, as indicated by their different migration in the TLC systems in which cholesteryl acetate migrates much closer to the long-chain steryl esters (mostly cholesteryl palmitoleate) than to the free cholesterol. It thus diffuses more slowly through the aqueous space and requires a much longer time to reach binding equilibrium compared with free cholesterol, explaining why cholesteryl acetate does not compete for binding as efficiently as does a sterol with a free 3′ hydroxyl group. The fact that mutations in the caveolin-binding site selectively affect binding of cholesteryl acetate is hydrophobic amino acid, abrogates binding of cholesteryl acetate indicates that the cleft does not only need to be hydrophobic but also that the aromatic character of the side chain is important.

The observation that mutations in the caveolin-binding site selectively affect binding of cholesteryl acetate is remarkable. The fact that cholesteryl acetate competes with free cholesterol for binding to wild-type Pry1 indicates that the binding sites of these two ligands at least partially overlap and that only one of the two ligands can be stably bound to the protein at a given time. Cholesteryl acetate is considerably more hydrophobic than cholesterol, as indicated by their different migration in the TLC systems in which cholesteryl acetate migrates much closer to the long-chain steryl esters (mostly cholesteryl palmitoleate) than to the free cholesterol. It thus diffuses more slowly through the aqueous space and requires a much longer time to reach binding equilibrium compared with free cholesterol, explaining why cholesteryl acetate does not compete for binding as efficiently as does a sterol with a free 3′ hydroxyl group. The fact that mutations in the caveolin-binding site selectively affect binding of the
cholesteryl acetate thus may indicate that the accommodation of the ester group of the ligand requires a critical flexibility of the loop region, which is lost if key residues are mutated, as is the case in the four mutations tested here (F239L, F244L, P242C, and A292C). From the binding model shown in Fig. 2C, one could thus imagine that the loop mutations would still provide sufficient flexibility to allow binding of cholesterol but not enough to allow binding of cholesteryl acetate, which, due to its more hydrophobic nature, may also require deeper penetration into the hydrophobic cleft that is opened by the displacement of the loop.

CAP superfamily members are mostly secreted proteins exerting a number of different physiological functions (1, 2). Our observation that the yeast family members are required for the export of sterols in vivo, that the exported sterol copurifies with Pry proteins, and that the proteins bind cholesterol in vitro, indicates that the CAP domain acts as a lipid-binding domain. In agreement with this proposition, the CAP domain of Pry1 itself is necessary and sufficient for sterol export in vivo and for cholesterol binding in vitro (10). Moreover, expression of the human superfamily member, CRISP2, rescues the sterol export defect of yeast cells lacking Pry function and purified CRISP2 binds cholesterol in vitro, suggesting that CAP superfamily members may generally act as lipid-binding proteins (10, 11).

The function of a human member of the CAP superfamily, CRISP1, is consistent with the proposition that these proteins may act by binding lipids. CRISP1 function is required during sperm capacitation, a process that occurs in the female reproductive tract and results in an increase in sperm motility, changes in the properties of the sperm plasma membrane, including changes in protein phosphorylation and acquisition of the ability to undergo the acrosome reaction. In all mammalian species examined, capacitation requires removal of cholesterol from the sperm plasma membrane. During in vitro fertilization, capacitation is achieved by coincubation with a cholesterol acceptor such as serum albumin or cyclodextrin (24). In vivo, CRISP1 is present on the sperm during epididymal maturation, but the protein is lost during capacitation. Addition of exogenous CRISP1 to mature sperm in vitro, however, inhibits capacitation, suggesting that CRISP1 may block cholesterol efflux and thereby prevent premature capacitation (25).

Functions of other CAP family proteins in lipid binding are indicated by studies of tabbsin-15 from a blood-feeding arthropod, and those of glioma pathogenesis-related-2 protein (GLIPR2/RTVP1/GAPR1). Structural analysis of tabbsin-15 revealed a hydrophobic channel that binds leukotrienes with sub-micromolar affinities, indicating that the protein functions as an anti-inflammatory scavenger of eicosanoids (20). GLIPR2, on the other hand, is the smallest of the mammalian CAP proteins, and the one most closely related to yeast Pry1 (26). It is highly expressed in the tumor glioblastoma multiform, which arises from brain immune cells and accounts for over 65% of all human primary brain tumors (27–29). GLIPR2 does not contain a predicted signal sequence or conserved disulfide bonds, but the protein is myristoylated and associates with the cytosolic surface of the Golgi membrane (18). The non-myristoylated protein binds to the surface of liposomes containing negatively charged lipids and this interaction is regulated by dimerization of the protein (30, 31). Interestingly, the protein can bind up to three molecules of phosphatidylinositol tightly enough to resist denaturation or organic solvent extraction, which has led to the speculation that it may form covalent bridges with the lipid (30).

While these data are all consistent with a function of CAP superfamily proteins in lipid binding, structural data to resolve the sterol binding site is now required to further understand the mode of action of these proteins.

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