Structural and functional characterization of the CAP domain of pathogen-related yeast 1 (Pry1) protein

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The production, crystal structure, and functional characterization of the C-terminal cysteine-rich secretory protein/antigen 5/pathogenesis related-1 (CAP) domain of pathogen-related yeast protein-1 (Pry1) from Saccharomyces cerevisiae is presented. The CAP domain of Pry1 (Pry1CAP) is functional in vivo as its expression restores cholesterol export to yeast mutants lacking endogenous Pry1 and Pry2. Recombinant Pry1CAP forms dimers in solution, is sufficient for in vitro cholesterol binding, and has comparable binding properties as full-length Pry1. Two crystal structures of Pry1CAP are reported, one with Mg2+ coordinated to the conserved CAP tetrad (His208, Glu215, Glu233 and His250) in spacegroup I41 and the other without divalent cations in spacegroup P6122. The latter structure contains four 1,4-dioxane molecules from the crystallization solution, one of which sits in the cholesterol binding site. Both structures reveal that the divalent cation and cholesterol binding sites are connected upon dimerization, providing a structural basis for the observed Mg2+-dependent sterol binding by Pry1.

Members of the eukaryotic CAP (cysteine-rich secretory protein/antigen 5/pathogenesis related-1) or SCP/TAPS (Sperm-coating protein/Tpx/antigen 5/pathogenesis related-1/Sc7) superfamily have a wide range of physiological activities including sperm maturation, fertilization, fungal virulence, cellular defense, and immune evasion1–6. However, the underlying mechanisms by which CAP proteins perform these seemingly unrelated functions remain poorly understood. CAP proteins contain a CAP or SCP domain, named after mammalian CAP proteins characterized in semen and seminal fluid7–10. The CAP domain (NCBI domain cd00168 or Pfam PF00188) is an ~15 kDa cysteine-rich domain with limited (<38%) sequence identity1–4. Several CAP protein structures have been reported and they all contain a conserved alpha-beta-alpha sandwich domain8,11–20. The CAP domains differ in the lengths of their strands and helices and ~47% of the domain is composed of loops, making it difficult to accurately predict their structure11–13. The prototypical CAP domain has a central cavity characterized by the cavity tetrad, formed by four key residues from the four CAP motifs: His from CAP1, Glu from CAP2, His from CAP3, and Glu from CAP4 and these tetrad residues bind divalent cations including zinc and magnesium8,13,21,22. CAP1 and CAP2 are defined in the PROSITE database (http://www.expasy.ch/prosite) as CRISP motifs, while CAP3 and CAP4 are additional CAP motifs defined by Gibbs and colleagues8.

CAP proteins bind lipids; for example Tablysin-15 from a blood-feeding arthropod is an anti-inflammatory scavenger of eicosanoids with a hydrophobic channel that binds leukotrienes with sub-micromolar affinities19. Structures revealing how palmitate and other lipids bind to this hydrophobic channel have been reported19. Additionally, GLIPR2/GAPR-1 which is highly over-expressed in glioblastoma multiforme, binds to the surface of liposomes containing negatively charged lipids23,24. Structures showing how GAPR-1 binds up to three phosphati-dylinositol molecules strongly enough to resist denaturation or organic solvent extraction have been reported23,24. Our previous studies revealed that Saccharomyces cerevisiae CAP proteins are required for the export of sterols in vivo and they bind cholesterol in vitro25,26.

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The *Saccharomyces cerevisiae* genome encodes three CAP proteins known as Pathogen Related in Yeast (Pry1–3). Pry1 and Pry2 are secreted glycoproteins and Pry3 is associated with the yeast cell wall. The sterol binding and export properties of these proteins are localized to the CAP domain, which is sufficient to rescue sterol export properties of cells lacking endogenous Pry proteins. Computational modeling suggests that sterol binding to Pry1 occurs through displacement of a flexible loop, the caveolin binding motif (CBM). While point mutations within the CBM abrogated sterol binding and export, mutations of residues located outside the CBM including highly conserved putative catalytic residues have minimal effect on lipid binding and sterol export. These studies defined the CBM as a crucial motif for lipid binding in vitro and sterol export in vivo. Furthermore, the expression of heterologous CAP proteins rescues the block in sterol export of yeast mutants lacking Pry function, indicating that sterol export is a conserved function of CAP proteins from different species. As part of our ongoing efforts to characterize the lipid binding properties of this ubiquitous superfamily of proteins, we present the structural and functional characterization of the CAP domain of Pry1 (Pry1CAP) from *Saccharomyces cerevisiae*.

**Results**

**Pry1CAP forms dimers in solution.** Pry1CAP was overexpressed in *Pichia pastoris* with a yield of approximately 500 mg of ~99% pure protein from a 1 L shake flask culture. Pry1CAP migrates on a reducing Coomassie stained SDS PAGE gel at ~16 kDa (Fig. 1A). As was observed for some CAP proteins including Glipr-1, GAPR-1 and Na-ASP-2, Pry1CAP crystallizes with a monomer in the assymetric unit while dimerizing in solution, eluting from a size exclusion column as a single sharp peak, with a molecular mass of 30.8 kDa, which is twice the theoretical molecular weight of 15.9 kDa (Fig. 1B).

**Pry1CAP binds and exports cholesterol.** Pry1CAP used in these studies is functional for cholesteryl acetate export in vivo because a plasmid encoding Pry1CAP complemented the cholesteryl acetate export defect of mutant yeast cells that lacked endogenous Pry1 and Pry2 (Fig. 2A). The efficiency of cholesteryl acetate export by Pry1CAP was comparable to that of full-length Pry1 as indicated by the similarity of the export indices (Fig. 2B). Addition of an increasing amount of [3H]-cholesterol resulted in a concentration dependent and saturable binding of cholesterol to Pry1CAP protein. In vitro, Pry1CAP displayed saturation binding kinetics with an apparent K_d of 2.08 ± 0.07 μM, which is comparable to cholesterol binding by full-length Pry1 (K_d of 1.25 ± 0.42 μM) (Fig. 2C). The saturable binding observed by titration of the ligand is not due to limited solubility of cholesterol, as indicated by the control experiment in which the in vitro affinity of Pry1 to cholesterol was measured by increasing the concentrations of the purified protein (0–500 pmol) rather than increasing the concentration of the radioligand. This results in a saturable binding curve with an apparent K_d of 0.87 ± 0.18, which is very similar to the K_d obtained by titrating the ligand (K_d of 1.25 ± 0.42 μM). Furthermore, cholesterol binding by Pry1CAP is inhibited by EDTA and adding magnesium ions restores sterol binding, indicating that magnesium is important for sterol binding by Pry1CAP (Fig. 2D).

**Overall Structure of Pry1CAP.** Two Pry1CAP structures solved by molecular replacement with a monomer in the assymetric unit are reported and their atomic coordinate and structure factors have been deposited in the protein data bank under accession number 5ETE and 5JYS, for the 1,4-dioxane and Mg^{2+} complex, respectively. Both Pry1CAP structures are very similar with an rmsd of all atoms of 0.318 Å. In both structures, the monomer is a dimer with 512 amino acids residues Ser151 through Ser157, which contains two predicted O-glycosylated sites, are disordered. The overall monomer surface area for Pry1CAP is 7125 Å^2 and its topology is an alpha-beta-alpha sandwich made up of 6 helices, 2 disulfide bonds, 12 beta turns and 1 beta bulge (Fig. 3B,C). The overall structure of Pry1CAP is 16.9% strand, 33.1% alpha-helix, 2.1% 3-10 helix and 47.9% loop with longer loops than predicted...
The 3–10 helix is involved in the alpha-beta-alpha sandwich and was previously observed in SmV AL4 but not in any of the other representative CAP protein structures.

Divalent cation binding. Pry1CAP has a large central (1638 Å³ volume) cavity, which contains the tetrad that binds divalent cation other CAP protein11,14,19,21,23,29, and this cavity is distinct from the CBM cavity (Figs 3C and 4A). The CAP cavity tetrad residues of Pry1CAP (His208, Glu215, Glu233 and His247) superpose well with the corresponding residues from representative CAP proteins8,11,13,29 (Fig. 4B). In one Pry1CAP structure, a magnesium ion from the crystallization solution is coordinated by the tetrad which results in a slight shift in the histidines when compared to the structure without the divalent cation (Fig. 4C).

Discussion

1,4-Dioxane binds to the cholesterol binding site. Electron density for four 1,4-dioxane molecules were observed in the initial molecular replacement 2F₀ − F₂ maps when dioxane was present in the crystallization solution; this density is absent in the Mg²⁺ structure, which does not have 1,4-dioxane in the crystallization.

Based on other CAP structures (Fig. 4A). The 3–10 helix is involved in the alpha-beta-alpha sandwich and was previously observed in SmV AL4 but not in any of the other representative CAP protein structures.

Divalent cation binding. Pry1CAP has a large central (1638 Å³ volume) cavity, which contains the tetrad that binds divalent cation other CAP protein11,14,19,21,23,29, and this cavity is distinct from the CBM cavity (Figs 3C and 4A). The CAP cavity tetrad residues of Pry1CAP (His208, Glu215, Glu233 and His247) superpose well with the corresponding residues from representative CAP proteins8,11,13,29 (Fig. 4B). In one Pry1CAP structure, a magnesium ion from the crystallization solution is coordinated by the tetrad which results in a slight shift in the histidines when compared to the structure without the divalent cation (Fig. 4C).
solution. Density corresponding to one 1,4-dioxane molecule was observed in the 194 Å³ volume cavity created by the CBM, confirming for the first time the ability of the cholesterol binding site of Pry1 to bind a ligand. Since a 1,4-dioxane molecule binds to the CBM cavity, and the CBM is verified to be important for cholesterol binding, the effect of 1,4-dioxane on cholesterol binding was tested. 1,4-dioxane inhibits in vitro binding of [3H]-cholesterol to Pry1CAP in a dose dependent manner (Figure S.1). However, the inhibition was only observed at relatively high concentrations of 1,4-dioxane, and is possibly due to the increased hydrophobicity of the 1,4-dioxane containing solvent compared to the aqueous buffer. Interestingly, 1,4-dioxane does not bind to other lipid binding sites notably the palmitate binding site of Tablysint-1519 or phosphatidylinositol binding sites of GAPR-123,24. The presence of dioxane in the crystallization solution was incompatible with the formation of complexes of Pry1CAP with cholesterol or palmitate and all co-crystallization and soaking experiments yielded crystals with dioxane only. While cholesterol is virtually insoluble in water, it is soluble in 1,4-dioxane, and 1,4-dioxane is a major component of the CryoSol kit that is used for co-crystallizing proteins with hydrophobic ligands30–32. Efforts are underway to identify either a sterol solubilizing agent that is suitable for co-crystallization or a crystallization condition that is compatible with sterol binding.

Comparison of Pry1CAP with other CAP proteins. Using PDBFold, GAPR-1 with bound inositol hexakisphosphate (PDB entry 4aiw)23, and the apo structure of the same protein (PDB entry 1smb)44 were identified as the most similar structures to that of Pry1CAP. The second best score was that of VAL4 from Schistosoma mansoni (SmVAL4), which lacks the prototypical CAP cavity (PDB entry 4p27)23. This is followed by the NMR structure of a plant CAP protein (P14a, PDB entry 1cfe)33; crystal structures of the hookworm CAP protein Na-ASP-2, (PDB entries 4ly5, 4nui, 4nuo, 4nuk)11,22; the structures of human glioma pathogenesis related protein (sGLIPR1 PDB entry 3q2r)13; and structures of snake venom CRISPs notably pseudochetoxin (PDB entry 2dda)29. All these proteins share under 35% sequence identity with Pry1CAP and the greatest differences between representative CAP protein structures are in loop regions as well as in the length of helices and strands (Fig. 4A). The flexible regions in CAP proteins are important for ligand binding and make up ~47% of the structure. Therefore, each new CAP protein structure offers information that cannot be generated simply by homology modeling.

The CAP tetrad and CBM cavities are connected in the dimer. The observation that EDTA inhibits cholesterol binding and that addition of Mg²⁺ restores cholesterol binding by Pry1 is surprising since the cholesterol binding CBM and the CAP tetrad that binds Mg²⁺ are located at distinct sites in the Pry1CAP monomer. Furthermore, our previous studies identified SmVAL4, a CAP protein lacking the CAP tetrad as an effective sterol binder and exporter. Mg²⁺ dependent sterol binding by Pry1CAP requires the interaction of both binding sites, likely occurring via dimerization, because Pry1CAP forms dimers in solution, and both crystal structures have
Figure 4. CAP protein motifs. (A) Structural features of Pry1CAP and primary sequence alignment with selected representative CAP proteins. This figure was generated with ESPript48. The different secondary structure elements shown are alpha helices as large squiggles labelled (α), 3_10-helices as small squiggles labelled (η), beta strands as arrows (β), and beta turns (TT). Identical residues are shown in white on red background, and conserved residues in red. The locations of the cysteine residues involved in disulfide bonds are numbered in green. CAP motifs are highlighted in gold. The representative CAP structures are Na-ASP-2 (PDB entry 1u53), SmVAL4 (PDB entry 4p27), GAPR-1 (PDB entry 1smb), and sGLIPR1 (PDB entry 3q2r). (B) The CAP tetrad residues of Pry1CAP (PDB entry 5jys) superimpose with other CAP structures. CAP structures are colored as follows Pry1CAP (orange), SmVAL4 (magenta), GAPR-1 (cyan), sGLIPR1 (gray) and Na-ASP-2 (yellow). (C) The superposed divalent cation binding site of Pry1CAP with Mg^{2+} (PDB entry 5jys, orange) and without Mg^{2+} (PDB entry 5ete, blue). The numbers correspond to those of Pry1CAP and the magnesium ion is shown as green sphere.
a crystallographic dimer in which the CBM and CAP tetrad are connected within a large 7063 Å³ volume cleft (Fig. 5). The same dimer was observed in two different structures of Pry1CAP, that were obtained from different crystallization conditions with different spacegroups and crystal morphologies. Incidentally, the cavities of the two covalently linked CAP domains of Na-ASP-112 are connected by a large cleft and prior to this study a dimer that allowed the connection of the CAP tetrad and lipid binding cavities of single CAP domain proteins had not been identified8,11,12. Beyond affecting sterol binding, the implications of having such a large interconnected cleft are undefined; however, the cavity volume is large enough to bind other ligands, such as peptides, which may be relevant for some observed functions of other eukaryotic CAP proteins, for example, the serine protease activity of the cone snail CAP protein Tex3133 and the ability of the hookworm NIF to inhibit neutrophils34.

Conclusions
Two crystal structures of the CAP domain of pathogen-related yeast protein from Saccharomyces cerevisiae, Pry1CAP, are presented. Both structures reveal that the cholesterol binding CBM is large enough for cholesterol binding. In one structure a 1,4-dioxane molecule from the crystallization mixture occupies the CBM cavity confirming its ability to bind hydrophobic ligands. In the second structure Mg²⁺ is coordinated by the CAP cavity tetrad residues. Pry1CAP is functional in cholesterol export in vivo and binds cholesterol in vitro with comparable affinity to full length Pry1. Interestingly, cholesterol binding by Pry1CAP is inhibited by EDTA and restored by the addition of Mg²⁺ indicating that presence of the divalent cation is important for sterol binding. The cholesterol and Mg²⁺ binding sites are distinct and unconnected in the Pry1CAP monomer. Pry1CAP is a dimer in solution and the cholesterol and Mg²⁺ binding sites are connected by a large cleft in a crystallographic dimer, providing a structural basis for Mg²⁺-dependent sterol binding by Pry1CAP.

Methods
Recombinant protein expression and purification of Pry1CAP. The carboxyl terminal CAP domain (corresponding to amino acid residues Ser151 through Ala296) of Pry1 from Saccharomyces cerevisiae was amplified by PCR and ligated into pPICZαA vector using the XhoI and XbaI sites. After linearization, the vector was transformed into Pichia pastoris strain X33. The transformants were selected on zeocin-resistant YPD plates and verified by PCR amplification using pPICZαA vector flanking primers (α-factor and 3′AOX1). Ten colonies with the right insert were picked and screened for induction of recombinant Pry1CAP protein with 0.5% methanol at 30 °C for 72 hours. The highest expressing colony was chosen for recombinant protein expression and purification as previously described for SmVAL427. Full length Pry1 was produced similarly to Pry1CAP.

Size-exclusion chromatography (SEC). For SEC experiments, 20 µg of Pry1CAP was injected onto a Yarra SEC-2000 column (Phenomenex, Torrance, CA) at flow-rate of 0.5 ml/min with a Shimadzu Prominance series HPLC (Kyoto, Japan) using PBS pH 7.4 as the mobile phase. The elution was monitored with a photo diode array detector (Shimadzu). The system was calibrated using Bio-Rad gel filtration standard (Hercules, CA) consisting...
of proteins with molecular weights of 670, 158, 44, 17 and 1.35 kDa. Data analysis was performed on the 280 nm wavelength extracted chromatograph using Shimadzu LCsolution version 1.25. Experiments were conducted in triplicate.

**Crystallization.** Crystallization conditions were identified and optimized after screening for crystals using the following screens, Qiagen Cryos, PEGS and Hampton Crystal Screen I. The largest crystals were obtained at 298 K by vapor diffusion in sitting drops by mixing 4 μL of protein solution (15 mg/ml in PBS 7.4.) with 1.5 μL of the reservoir solution (1.6 M ammonium sulfate, 10% (v/v) 1,4-dioxane, and 0.1 M MES pH 6.5) and equilibrating against 350 μL of reservoir solution. Crystals of dimension 0.2 mm X 0.3 mm X 0.5 mm grew after 3 months. The crystallization process was shortened to 5 days by increasing the protein concentration to 50 mg/ml and the

| Data Collection | PDB entry 5ete | PDB entry 5jys |
|-----------------|----------------|----------------|
| X-ray Source    | Rigaku FR-E+   | Rigaku FR-E+   |
| Detector        | Rigaku HTC     | Rigaku HTC     |
| Wavelength      | 0.15418 nm     | 0.15418 nm     |
| Space group     | P6_22          | I4              |
| Cell dimensions | a = b = 124.74 Å, c = 59.06 Å, α = β = γ = 90.00° | a = b = 69.11 Å, c = 97.73 Å, α = β = γ = 90.00° |
| Resolution (Å)  | 30.8–2.1 (2.16–2.10) | 34.5–1.89 (1.99–1.90) |
| Number of total reflections | 230544 (17841) | 65957 (4185) |
| Number of unique reflections | 16255 (1304) | 18355 (1200) |
| Rmerge          | 9.7 (62.4)     | 8.2 (42.8)     |
| Compleness (%)  | 99.8 (100)     | 100 (100)      |
| Redundancy      | 14.2 (13.7)    | 3.6 (3.5)      |
| Min(I) half-set correlation CC(1/2) | 0.998 (0.963) | 0.995 (0.902) |
| Average Mosaicity | 1.8 | 1.01 |
| Resolution (Å)  | 30.8–2.01 (2.10–2.01) | 34.5–1.79 (1.83–1.79) |
| Number of total reflections | 77208 (4323) | 69597 (4185) |
| Number of unique reflections | 18511 (1324) | 21607 (1254) |
| Rmerge          | 0.105 (1.296)  | 0.073 (0.629)  |
| Compleness (%)  | 99.7 (98.9)    | 100 (100)      |
| Redundancy      | 14.1 (13.0)    | 3.6 (3.4)      |

**Table 1. Statistics for data collection and model refinement.**
temperature to 37 °C, without compromising the diffraction quality of the crystals. A second crystal form was obtained with 100 mM Tris pH 8.5 and 200 mM magnesium chloride as the crystallization buffer. These crystals required higher protein concentrations (150 mg/ml Pry1CAP in 0.1 M Tris pH 8.0) and took up to 3 weeks to grow. No crystallization hits were observed when Pry1CAP was screened in 50 mM sodium acetate pH 4.5, 50 mM sodium HEPES pH 7.5, or sodium citrate pH 6.5, whereas weakly diffracting plate-like crystals were obtained with 50 mM sodium cacodylate as protein storage buffer.

**Data collection, structure determination and analysis.** Crystals were transferred into a cryo-protecting solution containing 75% precipitant solution and 25% glycerol for ~30 seconds, and flash-cooled directly in a stream of N₂ gas at 113 K prior to collecting diffraction data. X-ray diffraction data were collected at the Baylor College of Medicine core facility (Rigaku HTC detector, Rigaku FR-E+ SuperBright microfocus rotating anode generator, with VariMax HF optics) using the Crystal Clear (d₄trek) package.25 Data was integrated using MosFLM and scaled with SCALA.26 Pry1CAP structures were solved after multiple molecular replacement attempts (MR) using different search models with PHASER.27,28 The optimal model that resulted in the lowest Rfree was a polyalanine model of GAPR-1 (PDB entry 1smb).14 The MR solution indicated a monomer per asymmetric unit giving a Matthews’ coefficient of 4.3 Å³/Da with 72% solvent content.29 Molecular replacement was followed by automatic model building using ARP/wARP.30,31 The final models were obtained by iterative manual model building cycles using the program Coot32 followed by structure refinement with REFMAC5.33,34 and PHENIX.35 Structural figures were generated using PyMOL.36 Structures were analyzed and Fig. 3B was generated using PDBSumm (https://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html). Details of the quality of the structure as well as data collection are shown in Table 1. Protein structures most similar to Pry1CAP were identified using PDBeFold’s structure similarity option (http://www.ebi.ac.uk/msd-srv/ssm/), which allows a 3-D structural alignment taking both the alignment length and rmsd into account.

**In vitro lipid binding.** The radioligand binding assay was performed as described previously.25,47 100 pmol of purified protein in binding buffer (20 mM Tris, pH 7.5, 30 mM NaCl, 0.05% Triton X-100) was incubated with 0–400 pmol of [3H]-cholesterol (American Radiolabeled Chemicals Inc., St Louis, Missouri, USA) 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. The effect of 1,4-dioxane was determined by performing the in vitro assay in the presence of 1,4-dioxane (0–4%v/v). The effect of divalent cations on cholesterol binding was measured by performing the in vitro binding reaction in the presence of different concentrations of EDTA and magnesium chloride. At least two independent experiments were performed under each experimental condition and data is reported as the mean ± standard deviation. Calculation of the Kᵦ value and curve fitting was performed using the statistical software GraphPad Prism, La Jolla, CA.

**In vivo yeast sterol export assay.** Acetylation and export of sterols into the culture supernatant was examined as described elsewhere.26 In this sterol export assay, the export of radiolabeled cholesterol acetate by hem1ΔΔ say1Δ cells upon complementation with plasmid containing genes of interest is monitored. Yeast cells (say1Δ hem1ΔΔ) were cultivated in the presence of cholesterol/Tween 80 and were labeled with 0.025μCi/ml [3H]-cholesterol (American Radiolabeled Chemicals Inc., St Louis, Missouri, USA). Cells were harvested by centrifugation, washed twice with synthetic complete (SC) media, and grown overnight in fresh medium containing non-radiolabeled cholesterol. Cells were centrifuged and lipids were extracted from the cell pellet and the culture supernatant using chloroform/methanol [1:1, (v/v)]. Samples were dried and separated by thin-layer chromatography on silica gel 60 plates (TLC; Merck, Darmstadt, Germany) using the solvent system petroleum ether/diethyl ether/acetic acid [70:30:2, (v/v)]. TLCs were exposed to phosphorimager screens and radiolabeled lipids were visualized and quantified using a phosphorimager (Bio-Rad Laboratories, Hercules, California, USA). The export index is the relative levels of CA that is exported by the cells, and calculated as the ratio of extracellular CA to the sum of intracellular and extracellular CA. Export experiments were performed in triplicate and reported as the mean ± standard deviation of three independent experiments.

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Author Contributions
O.A.A. and R.S. conceived the studies and interpreted the results, A.K. produced and crystallized Pry1CAP, R.D. performed in vivo sterol export and in vitro binding studies, E.M.H. performed SEC analysis, O.A.A. collected crystallographic data, and solved crystal structure. All authors contributed to the writing and editing of the final manuscript.

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