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Pathogen-Related Yeast (PRY) proteins and members of the CAP superfamily are secreted sterol-binding proteins

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Sterols and related membrane-perturbing agents are subject to a quality control cycle. Compounds that fail to pass this control are acetylated and secreted into the culture media, whereas lipids that pass the cycle are deacetylated and retained within the cell. Here we describe the identification of a family of conserved proteins, the Pathogen-Related Yeast (PRY) proteins, as a class of sterol-binding proteins. *Saccharomyces cerevisiae* has three members of this family, two of which, Pry1 and Pry2, are secreted, whereas Pry3 is a cell wall–associated protein. Cells lacking both *PRY1* and *PRY2* have a complete block in secretion of the acetylated lipid and Pry1 and Pry2 proteins bind free cholesterol and cholesteryl acetate in vitro. PRY proteins belong to a large protein superfamily of unknown mode of action, the CAP protein superfamily [i.e. cysteine-rich secretory proteins (CRISP), antigen 5, and pathogenesis related 1 proteins]. The conserved CAP domain of Pry1 is necessary and sufficient for lipid export and sterol binding. Expression of a human CAP superfamily member, the cysteine-rich secretory protein 2 (CRISP2), rescues the phenotype of yeast mutants lacking Pry function and purified CRISP2 binds cholesterol in vitro, indicating that lipid binding is a conserved function of the CAP superfamily proteins.

Sterols comprise an essential class of lipids of eukaryotic cells and are found in two major forms that can be reversibly interconverted—free sterols and steryl esters. Whereas free sterols are membrane lipids that are strongly enriched in the plasma membrane, steryl esters belong to the class of neutral lipids and are stored with triacylglycerols in intracellular lipid droplets. Apart from these cell-autonomous functions of sterols, they also serve as precursors to more soluble molecules, some of which harbor signaling functions, such as the steroids, or act as detergents to solubilize dietary lipids, as do the bile acids.

Saccharomyces cerevisiae synthesizes ergosterol as its mature sterol by a set of endoplasmic reticulum (ER)-localized membrane proteins (Erg proteins), which convert triterpene squalene into ergosterol. The first step in the synthesis of ergosterol, the epoxidation of squalene, and the subsequent multistep conversion of lanosterol to ergosterol requires molecular oxygen and cytochrome-dependent desaturation, reduction, and demethylation steps. Baker's yeast, however, is a facultative anaerobic organism that can grow in the absence of oxygen if provided with sterols. Sterol supplementation experiments under anaerobic conditions or in heme-deficient mutants, which phenocopy anaerobic conditions, have been used to determine the structural requirements for sterol function in yeast. Such experiments revealed that yeast can grow in the presence of the major mammalian sterol, cholesterol, if the endogenous ergosterol pool is not depleted below a critical level (1, 2).

We have previously characterized an additional reversible sterol modification in *S. cerevisiae*, the sterol acetylation/deacetylation cycle (3). Acetylation of sterols is catalyzed by an alcohol:O acetyltransferase, Atf2, whereas deacetylation is catalyzed by the cholesteryl acetate hydrolase, Say1 (3, 4). Atf2 and Say1 are both localized to the ER membrane and expose their catalytically important residues into the ER lumen, indicating that acetylation and deacetylation of sterols take place in the ER luminal compartment. The sterol acetylation cycle appears to operate as a lipid proofreading and detoxification cycle because ergosterol precursors as well as nonnatural sterols, such as cholesterol or the steroid precursor pregnenolone, are acetylated by Atf2 but are not efficiently deacetylated by Say1, and, as a consequence, are exported from the cell. Export of cholesteryl acetate requires ongoing vesicular transport from the ER, indicating that acetylated sterols are transported to the cell surface within vesicular carriers (3).

Given that acetylated sterols are even more hydrophobic than the free sterol, we wondered how the acetylated lipid is rendered soluble both en route to the cell surface and in the aqueous culture media. Here we describe the identification of a family of conserved proteins that bind cholesteryl acetate to render it soluble for secretion.

Results

To identify a putative cholesteryl acetate–binding protein in the culture media of cells that acetylate and export this lipid, we enriched for proteins that bind [^{14}C]cholesteryl acetate under native conditions. Therefore, heme-deficient *say1*Δ mutant cells were labeled with [^{14}C]cholesterol, and proteins from the culture supernatant were fractionated by precipitation with ammonium sulfate. Lipids were extracted from individual salt fractions and the presence of the radiolabeled cholesteryl acetate was monitored by TLC. This analysis revealed enrichment of cholesteryl acetate in a 40% ammonium sulfate fraction from the supernatant of *say1*Δ mutant cells (Fig. S1*A*). No cholesteryl acetate was detected in the culture supernatant from heme-deficient wild-type cells. To further enrich for the putative lipid-binding activity, the 40% ammonium sulfate fraction was subfractionated by preparative isoelectric focusing (IEF) and the presence of radiolabeled cholesteryl acetate was monitored (Fig. S1*B*). Individual IEF fractions were separated by SDS/PAGE, a prominent silver-stained band in IEF fraction 3 was excised, and proteins were identified by mass spectrometry (Fig. S1*C*). This analysis revealed the presence of two proteins in the ~50 kDa region, Exg1 and Pry1. *EXG1* encodes for a exo-beta-1,3-glucanase of the cell wall. The yeast Pry1 protein, on the other hand, has not yet been functionally characterized, but based on its sequence homology with the pathogen-related protein 1 (PR-1) family from plants, Pry1 has been assigned as Pathogen-Related Yeast (PRY) (5). PR-1 family members are implicated in

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pathogen defense and belong to a much larger protein superfamily, the CAP proteins [i.e., cysteine-rich secretory proteins (CRISP), antigen 5, and pathogenesis-related 1 proteins] (6, 7).

Pry Function Is Required for Efficient Export of Cholesteryl Acetate. To test for a function of Exg1 and Pry1 in the export of cholesteryl acetate, *say1*Δ heme-deficient cells lacking either Exg1 or Pry1 were labeled with [^{14}C]cholesterol, export of cholesteryl acetate into the media was quantified, and the ratio between the extracellular cholesteryl acetate and the sum of intra- and extracellular cholesteryl acetate was plotted as an export index. This analysis revealed that cells lacking Exg1 exported cholesteryl acetate at approximately the same levels as a *say1*Δ mutant control (Fig. S2*A*). Mutants lacking Pry1, however, displayed an ~40% reduction in levels of extracellular cholesteryl acetate. These data thus indicate that Pry1 is important for the efficient export of acetylated sterols.

The family of yeast Pry proteins contains two additional members—Pry2 and Pry3. All Pry family members share a conserved CAP (sperm-coating glycoprotein [SCP]/PR-1/allergen-like) domain of about 200 amino acids, which constitutes the defining feature of the CAP protein superfamily (6–8). Unlike Pry1 and Pry2, which have this conserved domain in their C-terminal part, Pry3 contains the CAP domain within its N-terminal 200 residues.

To test whether these members of the Pry family may also be important for efficient export of cholesteryl acetate, we examined sterol export in single mutants. This analysis revealed that lack of any one of the Pry family members reduced the efficiency of cholesteryl acetate export but did not completely block lipid export (Fig. S2*B*). To test for a functional redundancy between the Pry family members, we generated combinations of Pry double and triple mutant strains. Analysis of cholesteryl acetate export in these mutants revealed that a *pry1*Δ *pry2*Δ double mutant was completely blocked in cholesteryl acetate export, indicating that Pry1 and Pry2 share a common function in lipid export (Fig. 1 *A* and *B*).

To test whether lack of Pry function would results in intracellular accumulation of cholesteryl acetate, membranes were fractionated over a Renografin density gradient and lipids from individual density fractions were extracted and separated by TLC. This analysis revealed that cells that fail to export cholesteryl acetate accumulate the lipid in a region of the gradient that is enriched for the ER marker proteins, Sec61 and Wbp1 (Fig. 1 *C* and *D*). Free cholesterol, on the other hand, peaked in a region of the gradient, which was enriched in marker proteins of the plasma membrane, Pma1 and Gas1, irrespective of Pry function (Fig. 1*E*). These data thus indicate that Pry function is required to prevent accumulation of cholesteryl acetate in the ER membrane, consistent with a function of Pry proteins in binding of the lipid in the ER and its export along the secretory pathway.

Pry1 and Pry2 Are Secreted Glycoproteins. To further characterize the function of Pry proteins in the export of cholesteryl acetate, we generated epitope-tagged versions of both Pry1 and Pry2. These C-terminally myc- and GST-tagged versions were functional as

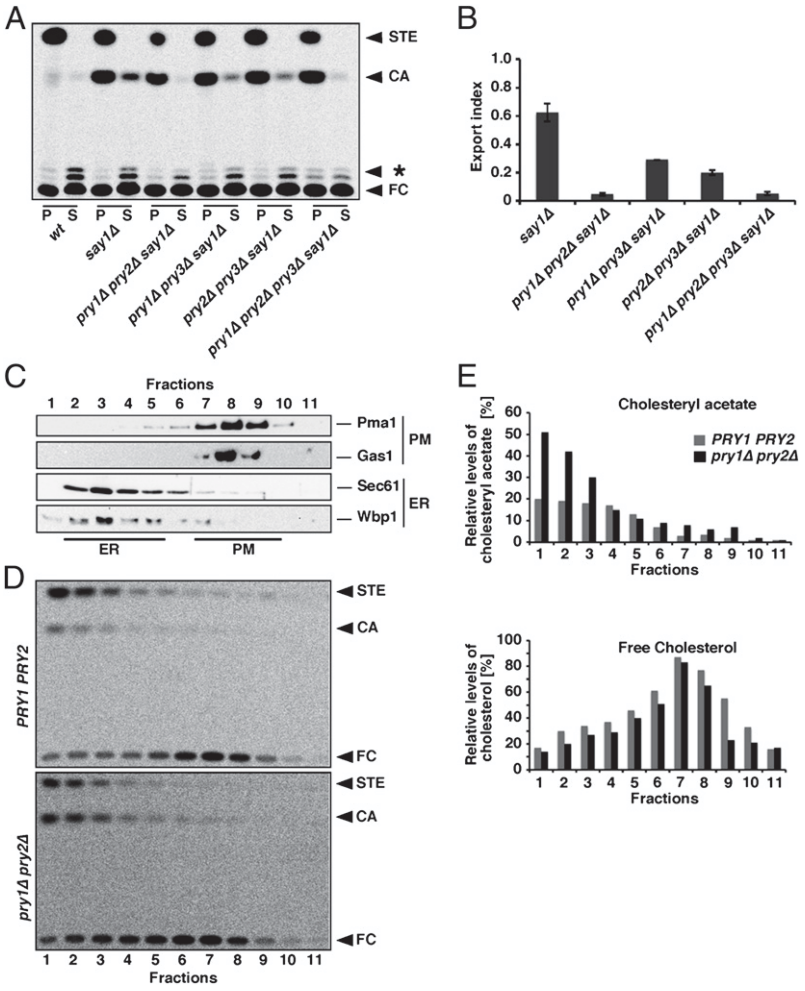

Fig. 1. Pry function is required for ER export of cholesteryl acetate. (*A*, *B*) Export of cholesteryl acetate is blocked in a *pry1*Δ *pry2*Δ double mutant. Pry double- and triple-mutant strains of the indicated genotypes were tested for export of cholesteryl acetate. Heme-deficient cells were cultivated in the presence of radiolabeled cholesterol. Lipids were extracted from the cell pellet (P) and culture supernatant (S), separated by TLC, and visualized by phosphorimaging. The level of cholesteryl acetate was quantified by radioscanning, and the ratio between extracellular and intracellular cholesteryl acetate (CA) is plotted as an export index (exported CA/total CA). (*C*) Distribution of ER and plasma membrane proteins in a Renografin density gradient. Membranes were fractionated on a Renografin gradient and proteins were precipitated and probed for the plasma membrane markers, the proton pumping ATPase, Pma1, and the GPI-anchored Gas1. The distribution of the translocon subunit, Sec61, and that of the oligosaccharyl transferase, Wbp1, is shown to indicate the location of ER membranes. (*D*, *E*) Accumulation of cholesteryl acetate in cells lacking Pry function. Heme-deficient *say1*Δ mutant cells harboring or lacking Pry function were cultivated in the presence of radiolabeled cholesterol, and membranes were fractionated over a Renografin gradient. Lipids were extracted from individual fractions, resolved by TLC, and quantified by radioscanning. (*B*) Mean ± SD of three independent determinations; (*C*–*E*) representative data of two independent experiments. *, position of an unidentified lipid; FC, free cholesterol; PM, plasma membrane; STE, steryl esters; wt, wild type.

indicated by their ability to efficiently export cholesteryl acetate in the absence of the other Pry family member (i.e., Pry1-myc was functional when tested in a *pry2Δ pry3Δ say1Δ hem1Δ* mutant background and Pry2-myc was tested in a *pry1Δ pry3Δ say1Δ hem1Δ* background) (Fig. 2*A*). If Pry1 and Pry2 were directly required for solubilization and export of cholesteryl acetate, one would expect both proteins to be secreted. To test whether this was the case, cells expressing tagged versions of Pry proteins were cultivated and the presence of the proteins in the cell pellet and the culture supernatant was examined by Western blotting. This analysis revealed that both Pry1 and Pry2 are soluble proteins that are enriched in the culture medium, as is Hsp150, a secreted heat-shock protein (Fig. 2*B*). Pry3, however, was only detected in the cell pellet, consistent with its association with the cell wall (9).

In Western blots with a tagged version of Pry proteins, we noted that Pry1 and Pry2 migrate much slower (~116 kDa) than predicted from their sequence (~31 kDa). Pry1 and Pry2 contain no predicted *N*-glycosylation sites, and treatment with endoglycosidase H revealed no differences in migration between treated and nontreated samples, indicating that Pry1 and Pry2 are not *N*-glycosylated (Fig. S3). To examine whether Pry proteins are O-glycosylated, protein extracts were treated with trifluoromethane sulfonic acid, which cleaves the glycosidic bond, proteins were separated by SDS/PAGE, and examined by Western blotting (10). This analysis revealed that Pry1 and Pry2 migrated much faster after the chemical treatment, indicating that both proteins are O-glycosylated (Fig. 2*C*).

Pry1 and Pry2 Bind Cholesteryl Acetate in Vivo. To test whether the Pry proteins directly bind cholesteryl acetate, GST-tagged versions of Pry1 and Pry2 were purified from the supernatant of cells that export radiolabeled cholesteryl acetate. Lipids were then extracted from the glutathione-eluted material and resolved by TLC. This analysis revealed that cholesteryl acetate copurified with both of the Pry proteins, indicating that Pry1 and Pry2 are cholesteryl acetate–binding proteins (Fig. 3*A*). The presence of only one of the two secreted Pry proteins appears to be sufficient for cholesteryl acetate binding because lipid export and binding to either Pry1 or Pry2 was observed in the absence of other Pry family members.

The CAP superfamily is characterized by two CRISP family signature motifs, one of which contains a strongly conserved cysteine residue known to be involved in a disulfide bridge in the antigen 5 allergen (Prosite database, PDOC00772). To determine whether the observed protein–lipid interaction is specific, we generated a mutant version of Pry1 in which this highly conserved cysteine residue was replaced by a serine (C279S) (Fig. 3*B*). This mutant version of Pry1 is still secreted, suggesting that the point mutation does not affect folding and forward transport of the protein (Fig. 3*C*). Cells expressing this Pry1^{C279S} mutant version still exported cholesteryl acetate, most likely because of the presence of wild-type Pry2, but the radiolabeled lipid was not enriched when the mutant protein was affinity purified on glutathione sepharose, indicating that lipid binding of Pry1 is lost on mutating this conserved cysteine residue (Fig. 3*D*). Pry proteins thus function as lipid-binding and export proteins.

Pry1 and Pry2 Bind Sterols in Vitro. To assess direct binding of sterols to Pry1 and Pry2 proteins, we expressed hexa-histidine-tagged versions of these proteins in *Escherichia coli* and purified them by affinity chromatography on nickel-sepharose beads (Fig. S4). Binding of radiolabeled free cholesterol to Pry proteins was then monitored using an in vitro binding assay. This analysis revealed that both Pry1 and Pry2 display saturable binding to free cholesterol with a K_d of ~0.7 μM (Fig. 4*A*). Binding of cholesteryl acetate was also saturable with a K_d of ~0.3 μM (Fig. 4*B*). The binding of cholesterol to Pry proteins appears to be stoichiometric, whereas that of cholesteryl acetate is substoichiometric. The apparent substoichiometric binding of cholesteryl acetate to Pry proteins observed in this assay may be explained by the more hydrophobic nature of the ligand, requiring much longer periods to equilibrate between the protein-bound and -unbound state. Consistent with the observation that the Pry1^{C279S} mutant version does not export and bind cholesterol in vivo, the protein exhibited a more than threefold reduced binding affinity toward free cholesterol and cholesteryl acetate when tested in the in vitro binding assay (Fig. 4*C*). The binding specificity of Pry1 to various steroids was assessed in a competitive binding assay (Fig. 4*D*). This revealed that both the fungal sterol ergosterol and the plant sterol sitosterol competed efficiently with the radiolabeled cholesterol for binding to Pry1. Sterols with a more hydrophilic aliphatic side chain, such as 25-hydroxycholesterol, however, failed to compete with cholesterol for binding to Pry1. Unrelated lipids, such as free fatty acids, triacylglycerol, or phosphatidylcholine, however, also failed to bind to Pry1, as assessed by a radioligand binding assay (Fig. S5). These results indicate that Pry proteins bind sterols with an affinity that is comparable to that of other sterol-binding proteins

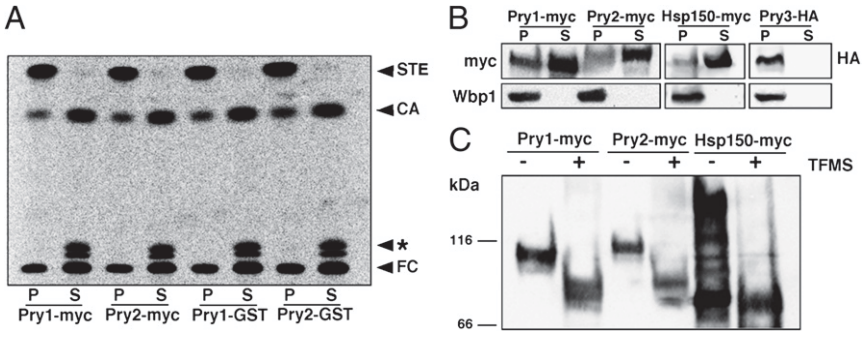

Fig. 2. Pry1 and Pry2 proteins are secreted and O-glycosylated. (*A*) C-terminally epitope-tagged versions of Pry1 and Pry2 are functional. Export of cholesteryl acetate in cells expressing a chromosomally tagged versions of either Pry1 or Pry2 in a *pryΔ hem1Δ say1Δ*–deficient background was analyzed by radiolabeling with [^{14}C]cholesterol. (*B*) Pry1 and Pry2 are secreted proteins. Cells expressing myc-tagged versions of Pry1, Pry2, Hsp150, or an HA-tagged version of Pry3 were cultivated. Proteins were extracted from the cell pellet (P) or TCA precipitated from the culture supernatant (S) and analyzed by Western blotting. Presence of the ER membrane protein Wbp1 in the cell P is shown as a loading control for intracellular proteins. (*C*) Pry1 and Pry2 are O-glycosylated. Proteins were extracted from cells expressing epitope-tagged versions of Pry proteins. They were left untreated (–) or were treated with trifluoromethane sulfonic acid (TFMS) to remove O-linked glycans. Proteins were separated by SDS/PAGE and detected with antibodies against the respective epitope. The O-glycosylated Hsp150 serves as control for the efficiency of the treatment. Data are representative of two independent experiments. *, position of an unidentified lipid; CA, cholesteryl acetate; PM, plasma membrane; STE, steryl esters; TCA, trichloroacetic acid.

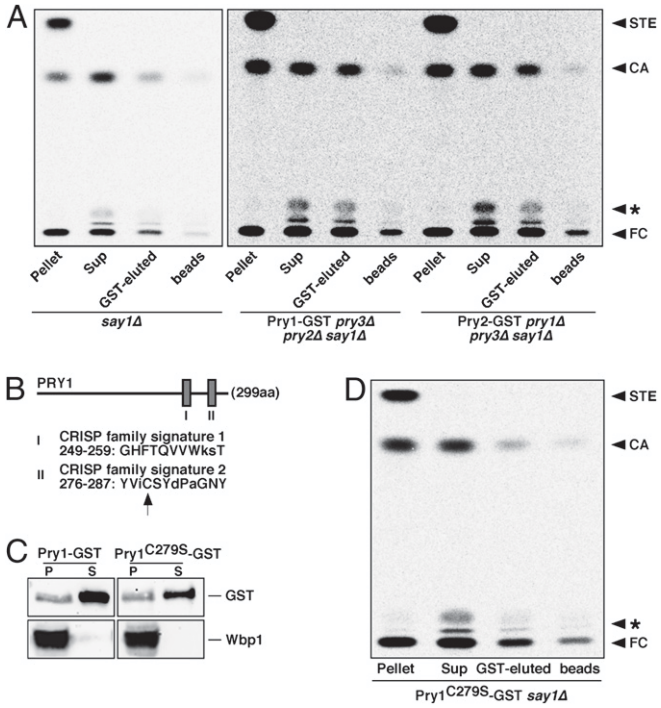

Fig. 3. Pry1 and Pry2 bind cholesteryl acetate in vivo, and lipid binding requires a conserved cysteine residue. (*A*) Pry1 and Pry2 bind cholesteryl acetate. Heme-deficient *say1*Δ mutant cells expressing GST-tagged versions of Pry1 or Pry2 proteins were cultivated in the presence of radiolabeled cholesterol. Proteins from the culture supernatant were concentrated by microfiltration and affinity purified on glutathione sepharose beads. Proteins were eluted by glutathione (10 mM). Lipids from the cell pellet (P), culture supernatant (Sup), the GST-bound material, and the Sepharose beads after elution (beads) were extracted and analyzed by TLC. (*B*) CAP superfamily members have a conserved cysteine residue in the CRISP family signature 2. Illustration of the position of the two conserved CRISP family signature motifs within Pry1. The conserved cysteine at position 279 of Pry1, indicated by an arrow, was mutated to serine. (*C*) Pry1^{C279S} is secreted. Cells expressing GST-tagged versions of Pry1 or Pry1^{C279S} were cultivated and proteins were extracted from the cell P or the culture supernatant (S) and analyzed by Western blotting. Presence of the ER membrane protein Wbp1 in the cell P is shown as a loading control. (*D*) Lipid binding of Pry1 requires the conserved cysteine residue. Cells expressing a GST-tagged version of Pry1^{C279S} were radiolabeled with [^{14}C]cholesterol, the proteins from the culture supernatant were concentrated, and affinity purified on glutathione beads. Lipids from the cells, culture supernatant, the GST-bound material, and the Sepharose beads after glutathione elution were extracted and analyzed by TLC. Data are representative of two independent experiments. *, position of an unidentified lipid; CA, cholesteryl acetate; FC, free cholesterol; STE, steryl esters; Sup, culture supernatant.

such as the oxysterol-binding protein, Osh4 (K_d of ~0.3 μM), the steroidogenic acute regulatory protein StAR/STARD1 (K_d of ~0.03 μM), or NPC2 (K_d of ~0.1 μM) (11–13).

To examine the structure of the Pry proteins, CD spectra of the proteins purified from bacteria were recorded. These indicate that both proteins contain a high proportion of β-strand elements (28% for Pry1 and 40% for Pry2) and a lower proportion of α-helical structures (12% for Pry1 and 8% for Pry2). These data also indicate that the Pry1^{C279S} mutant version has secondary structure elements that are similar to that of the nonmutant Pry1 and thus is likely folded properly (Fig. S6).

CAP Domain of Pry1 Is Necessary and Sufficient for Lipid Export and Sterol Binding. To determine which part of the Pry1 protein is required for sterol binding, we tested a set of N-terminal truncations for complementation of the export block of a *pry1*Δ *pry2*Δ double mutant. This analysis revealed that deletion of the N-terminal Ser/Thr-rich domain of Pry1 up to position 150 did not affect lipid export (Fig. S7 *A* and *B*). Deletions into the CAP domain, however, impaired the lipid export function of the protein. These results indicate that the CAP domain is required for lipid export in vivo. To test whether the CAP domain itself is sufficient for sterol binding in vitro, the domain was expressed in bacteria, purified, and sterol binding was measured. The results of these experiments indicate that the CAP domain itself is necessary and sufficient for sterol binding (Fig. S7*C*).

Sterol Binding Is a Conserved Function of CAP Superfamily Members. Because the yeast Pry proteins belong to the CAP superfamily of proteins, which are conserved from prokaryotes to human, we wondered whether lipid binding is a conserved function of these proteins. Therefore, we tested whether expression of the human CAP family member CRISP2 would rescue the lipid export phenotype of a *pry1*Δ *pry2*Δ double mutant yeast. CRISP2 is expressed in the testis and epididymis and participates in sperm–egg interaction during fertilization (7). Expression of human CRISP2 cDNA was placed under the control of a galactose inducible promoter, and the protein was targeted to the secretory pathway by replacing its endogenous signal sequence by one derived from the yeast pre-pro-alpha factor (amino acids 1–89). Cells were cultivated in either glucose or galactose-containing media, labeled with [^{14}C]cholesterol, and export of cholesteryl acetate into the culture supernatant was monitored. This analysis revealed that expression of human CRISP2 protein complemented the lipid export phenotype of a Pry double mutant, indicating that these CAP superfamily members are structurally and functionally conserved (Fig. 5*A*). To assess direct binding of CRISP2 to lipids, the protein was expressed and purified from *E. coli* and its in vitro binding to cholesterol was assessed. CRISP2 binds free cholesterol with a K_d of 0.5 μM (Fig. 5*B*). These data thus indicate that lipid binding is a conserved function of members of the CAP superfamily.

Pry Proteins Act in Detoxification of Hydrophobic Compounds. We have previously shown that mutants in the lipid acetylation cycle are sensitive to small hydrophobic compounds, which potentially affect the function and integrity of cellular membranes, such as eugenol, a member of the alkylbenzene class of compounds present in clove oil, nutmeg, cinnamon, and bay leaf, and is used as local antiseptic and anesthetic (3). To examine whether Pry proteins are important for cells to grow in the presence of eugenol, we tested whether deletions of *PRY* genes results in eugenol hypersensitivity. This analysis revealed that deletion of any one of the *PRY* genes results in hypersensitivity of the mutants toward eugenol, indicating that Pry function is required for cells to resist eugenol toxicity (Fig. 6*A*). The eugenol hypersensitivity of pry mutants, however, was rescued by expression of the human CRISP2, indicating that the detoxification function of the CAP superfamily members is conserved (Fig. 6*B*). To test whether Pry proteins would directly bind eugenol, we performed a competitive binding assay to purified Pry1 and Pry2. The results of these in vitro–binding experiments indicate that eugenol efficiently competes with [^{14}C]cholesterol for binding to Pry proteins, indicating that Pry proteins not only bind free sterols and short-chain steryl esters but also that these proteins bind small hydrophobic compounds and thus may directly protect the cells from the potential harmful action of such compounds (Fig. 6*C*). To examine a possibly direct function of Pry proteins in eugenol detoxification, we placed expression of Pry under control of a galactose-inducible promoter and tested eugenol sensitivity as a function of galactose concentration. These experiments revealed a dose-dependent resistance against eugenol, suggesting that Pry proteins detoxify by mass action rather than through a catalytic mechanism (Fig. 6*D*).

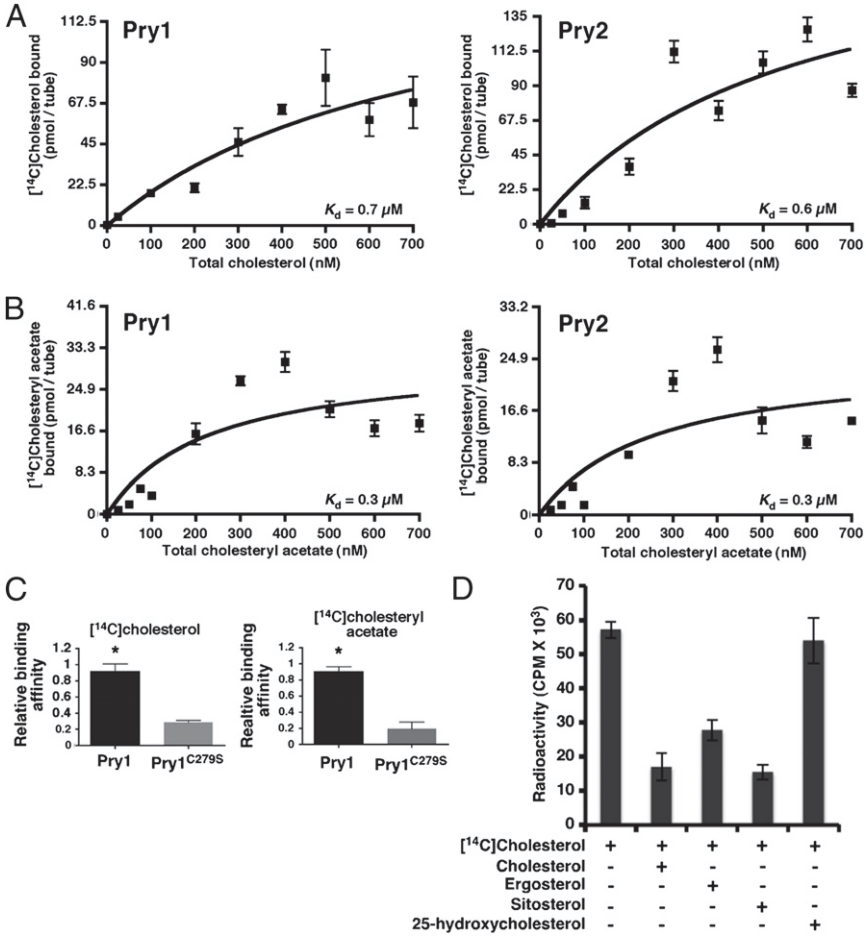

Fig. 4. In vitro binding of Pry proteins to cholesterol and cholesteryl acetate. (*A*) Pry1 and Pry2 bind free cholesterol in vitro. Purified Pry1 or Pry2 protein (100 pmol) was incubated with increasing concentrations of radiolabeled cholesterol and binding of the radioligand to the protein was determined by scintillation counting. (*B*) Binding of Pry1 and Pry2 to cholesteryl acetate. Purified Pry1 or Pry2 protein was incubated with increasing concentrations of cholesteryl acetate and ligand binding was assayed as described for *A*. (*C*) The Pry1^{C279S} mutant has reduced lipid-binding affinity. Purified Pry1 wild-type and the Pry1^{C279S} mutant version were incubated with radiolabeled free cholesterol or with cholesteryl acetate at a concentration of 25 nmol; ligand binding was quantified by scintillation counting and is plotted in relation to lipid binding of the wild-type protein set to 100%. Error bars are SEMs, asterisks denote $P < 0.05$ (two-sided *t* test). (*D*) Sterol-binding specificity of Pry1. Purified Pry1 protein was incubated with 25 nmol [^{14}C]cholesterol and binding of the radioligand was competed by addition of an equal concentration of the unlabeled sterol indicated. Data are mean ± SD of three independent experiments.

Discussion

In this study, we report the identification of yeast members of the CAP superfamily, Pry1 and Pry2, as proteins that bind and export cholesteryl acetate. Binding of cholesteryl acetate to Pry is likely to occur in the ER lumen where cholesteryl acetate is being formed and where Pry proteins are likely to enter due to their signal sequence, become O-glycosylated, and form disulfide bridges. Consistent with this notion, lack of Pry1,2 function results in accumulation of cholesteryl acetate in the ER membrane. Pry1,2 proteins are then transported to the cell surface via the secretory pathway and released into the aqueous environment, where they keep the bound lipid soluble. The function of Pry3 in lipid export is less clear. Pry3 is a cell wall–associated protein and contains a signal for attachment of a Glycosylphosphatidylinositol (GPI) anchor (9). The observation that deletion of Pry3 alone results in a decreased lipid export and renders the mutant eugenol sensitive indicates that the protein participates in the detoxification pathway even in the presence of functional Pry1 and Pry2, possibly by protecting the plasma membrane.

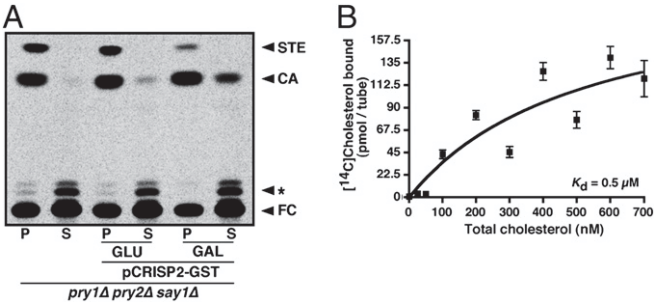

Fig. 5. Human CRISP2 complements for yeast Pry function and binds cholesterol. (*A*) Expression of human CRISP2 complements the cholesteryl acetate export defect of Pry mutant cells. Heme-deficient *pry1*Δ *pry2*Δ *say1*Δ mutant cells containing a plasmid-bearing human CRISP2 cDNA were cultivated in media containing either glucose (GLU) or galactose (GAL) and cells were radiolabeled with cholesterol. Lipids were extracted from the cell pellet (P) and culture supernatant (S) and analyzed by TLC. (*B*) Purified CRISP2 binds cholesterol in vitro. Purified CRISP2 protein (100 pmol) was incubated with increasing concentrations of radiolabeled cholesterol and binding of the radioligand to the protein was determined by scintillation counting. Each data point is the average of two independent measurements ± SD. *, position of an unidentified lipid; CA, cholesteryl acetate; FC, free cholesterol; STE, steryl esters.

The lipid-binding and export function of the Pry proteins observed in yeast appears to be a conserved function of members of this protein superfamily because expression of a human Pry homolog, CRISP2, relieves the lipid export block of a mutant strain lacking both *PRY1* and *PRY2*. Deletion mapping revealed that CAP domain of Pry1 is required for lipid export in vivo and the CAP domain itself is sufficient for lipid binding in vitro. Consistent with a lipid-binding function of the CAP domain, all CAP superfamily members tested in this study—Pry1, Pry2, and CRISP2—bind free cholesterol and cholesteryl acetate in vitro. These results suggest that the members of this protein superfamily exert their physiological functions thorough lipid binding by their CAP domain.

CAP superfamily members are found in both prokaryotes and eukaryotes (7). The first representative of this type of proteins, PR-1, was detected in tobacco leaves after infection with tobacco

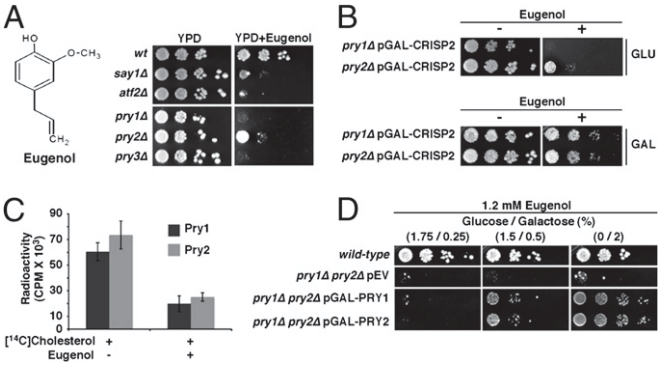

Fig. 6. Pry function is required for lipid detoxification. (*A*) Cells lacking Pry function are hypersensitive to eugenol. Cells of the indicated genotype were serially diluted 10-fold and spotted onto plates containing or lacking eugenol. (*B*) Expression of human CRISP2 rescues the eugenol sensitivity of *pry* mutant cells. Cells were diluted and spotted on glucose (GLU)- or galactose (GAL)-containing plates with or without eugenol. (*C*) Pry proteins bind eugenol. Purified Pry protein (100 pmol) was incubated with radiolabeled cholesterol (25 nmol) in the presence or absence of an equal amount of eugenol and binding of the radioligand to the protein was determined. (*D*) Dose dependence of eugenol resistance. Cells of the indicated genotype expressing Pry proteins from a galactose inducible promoter (pGAL-PRY) were serially diluted and spotted on plates containing different ratios of glucose and galactose). Growth tests are representative of two independent experiments; (*C*) means ± SD of two determinations. wt, wild type; YPD, yeast extract, peptone, dextrose.

mosaic virus. The plant PR-1 proteins now constitute one of nine subfamilies of the CAP protein superfamily, also known as the SCP protein family, which covers more than 3,400 proteins from 1,189 species (Pfam PF00188) (6, 7).

Mammals encode several putative CAP family members; there are 36 homologs in the human and 21 in mouse genome. Many of these genes are expressed in the reproductive tract (particularly the CRISP subfamily members), in immune cells and organs, and tumors, or during embryogenesis (7). Most CAP superfamily members are secreted proteins and thus may function in autocrine or paracrine signaling, possibly by regulating the activity of ion channels or as proteases or protease inhibitors (7).

The structure of several CAP proteins has been resolved and revealed that the structurally conserved CAP domain adopts a unique α–β–α sandwich fold that is stabilized by disulfide bridges between the central β-sheet and the C-terminal helical elements (8, 14). The conservation of this domain is likely to reflect a common basic mode of action of these proteins, which may be modulated by elements that are flanking the conserved domain, such as the cysteine repeats present in the CRISP subfamily (7). The role of some of the CRISP proteins in sperm maturation and egg fertilization may be compatible with a possible sterol-binding function of these proteins because these processes are known to depend on the sequestration of cholesterol from the plasma membrane. For in vitro fertilization, sterol sequestration from the plasma membrane of sperms is achieved using a cholesterol acceptor such as serum albumin or cyclodextrin (15).

That yeast Pry1 and Pry2 proteins are required for exporting cholesteryl acetate in vivo, that these proteins directly bind the lipid in vitro, that this lipid-binding activity maps to the CAP domain of Pry1 and is functionally conserved in the human CRISP2 provides a lead to address the molecular function of this important protein superfamily.

Materials and Methods

Yeast Strains, Growth Conditions, Epitope-Tagging, and Western Blotting. See *SI Materials and Methods* for details.

Lipid Labeling and Analysis. Sterol acetylation and export into the culture media was examined as described previously (3). To determine the subcellular distribution of sterols, heme-deficient mutants were labeled with [^{14}C]cholesterol and membranes from 20 OD units of cells were separated on a renografin-76 density gradient as described (16).

Enrichment for Cholesteryl Acetate Binding Activity. For salt precipitation, the culture supernatant corresponding to ~2,000 OD_{600} of heme-deficient cells that were labeled with [^{14}C]cholesterol, as described in the text for the cholesteryl acetate export assay, was fractionated by the addition of ammonium sulfate to the indicated concentration. Samples were stirred at 4 °C for 30 min and then centrifuged at 20,000 × *g* for 30 min. The precipitate was resuspended in 5 mL of 10 mM Tris, pH 7.5, and an aliquot was used to extract and analyze cofractionating lipids.

For preparative isoelectric focusing, salt-fractionated samples were loaded on a MicroRotofor liquid-phase isoelectric focusing device (Bio-Rad Laboratories). Individual Rotofor fractions were trichloroaceticacid (TCA) precipitated and separated by SDS/PAGE. Silver-stained bands were excised and peptides were analyzed by tandem mass spectrometry using an ABI 4700 MALDI-TOF/TOF (Applied Biosystems) at the Lausanne Protein Analysis Facility (Lausanne, Switzerland).

Expression and Purification of CAP Proteins from *E. coli*. See *SI Materials and Methods* for details.

In Vitro Lipid Binding and Competition Assay. See *SI Materials and Methods* for details.

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