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# The function of yeast CAP family proteins in lipid export, mating, and pathogen defense

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**In their natural habitat, yeast cells are constantly challenged by changing environmental conditions and a fierce competition for limiting resources. To thrive under such conditions, cells need to adapt and divide quickly, and be able to neutralize the toxic compounds secreted by their neighbors. Proteins like the pathogen-related yeast, Pry proteins, which belong to the large CAP/SCP/TAPS superfamily, may have an important role in this function. CAP proteins are conserved from yeast to man and are characterized by a unique αβα sandwich fold. They are mostly secreted glycoproteins and have been implicated in many different physiological processes including pathogen defense, virulence, venom toxicity, and sperm maturation. Yeast members of this family bind and export sterols as well as fatty acids, and they render cells resistant to eugenol, an antimicrobial compound present in clove oil. CAP family members might thus exert their various physiological functions through binding, sequestration, and neutralization of such small hydrophobic compounds.**

**Keywords:** cholesterol; detoxification; fatty acids; lipid-binding proteins; PR-1 homologs; SCP/TAPS family proteins



The CAP proteins constitute a large protein superfamily with members found in all kingdoms of life. The family is named after three founding members: **C**ysteine-rich secretory proteins (CRISP), antigen 5 (Ag5), and pathogenesis-related protein 1 (PR-1), and is also known as SCP (sperm coating protein) or TAPS (Tpx-1/Ag5/PR-1/Sc7). It presently is constituted of 13 584 members in 2863 species (Pfam PF00188). CAP proteins are implicated in many fundamental biological processes, ranging from immune defense in mammals and plants, sperm maturation and fertilization, prostate and brain cancer, pathogen virulence and venom toxicity. CAP family members are mostly secreted glycoproteins that are very stable in the extracellular fluid. Even though CAP proteins are intensely studied, their mode of action has remained elusive (for review see ref. [1–3]).

Using yeast as a model organism, we could recently show that CAP proteins bind sterols as well as fatty acids at two independent binding sites. Through binding to these secreted proteins, these hydrophobic compounds are solubilized and transported out of the cell. This lipid export function of the CAP proteins is essential under specific growth conditions, for example, in cells that accumulate high levels of free fatty acids. On the other hand, cells lacking these proteins are hypersensitive to eugenol, an antimicrobial compound present in clove oil. Eugenol directly competes with cholesterol for binding to at least one of the yeast CAP protein, Pry1, *in vitro*, indicating that eugenol

**Abbreviations**
Ag5, antigen 5; CAP, cysteine-rich secretory proteins (CRISP), antigen 5 (Ag5), and pathogenesis-related protein 1 (PR-1); CRD, cysteine-rich domain; CRISP, cysteine-rich secretory proteins; FABP, fatty acid-binding proteins; GLIPR1, glioma pathogenesis-related protein 1; GPI, glycosylphosphatidyl inositol; PR-1, pathogenesis-related protein 1.

and possibly other structurally related small hydrophobic compounds bind to the same site on the protein as cholesterol. These results suggest that CAP proteins may function both intra- as well as extracellularly to bind and thereby neutralize potential membrane perturbing hydrophobic compounds in yeast. In mammalian cells, these proteins may bind and neutralize immune modulating lipid signals, such as eicosanoids, thereby allowing insects to prolong their blood feed and parasites to evade the immune reaction of their host. Additionally, in plants, the sterol sequestering function of these proteins suppresses growth of sterol-auxotrophic pathogens. Thus, CAP proteins may exert a variety of physiological functions through a common mode of action, the sequestration of small hydrophobic compounds.

## An example of the functional diversity within the CAP superfamily: the founding members

The pioneer of the three founding members of the CAP superfamily was identified in 1970 when proteins induced upon viral infection of tobacco leaves were first identified and characterized [4]. These pathogenesis-related (PR) proteins were initially numbered according to their molecular mass upon SDS gel electrophoresis and have subsequently been classified into 17 families, with PR-1 being the smallest with 14 kD. Some of these PR proteins have glucanase activity (PR-2), whereas others have chitinase (PR-3,4,8,11), protease inhibitor (PR-6), peroxidase (PR-9), or ribonuclease activity (PR-10), and thus serve as a direct line of defense against fungal and bacterial pathogens [5,6]. The only PR family for which no biochemical function was known was PR-1, even though specific members of the tobacco and tomato family displayed antimicrobial activity against oomycete pathogens and their overexpression conferred pathogen resistance. Yet, their mechanism of action remained to be characterized [7–9].

Antigen 5, the second founding member of the CAP superfamily is one of the most abundant and immunogenic protein present in the venom-secretory ducts of stinging insects. Ag5 proteins form part of a cocktail of salivary proteins that are believed to function either in suppression of the host immune system or in prevention of blood clotting to prolong feeding [10]. The biochemical activity of Ag5, however, is not known.

Cysteine-rich secretory proteins, the third and last founding members of the CAP superfamily are highly enriched in the mammalian reproductive tract and in the venom-secretory ducts of snakes, lizards, and other vertebrates. CRISPs are two-domain proteins with an N-terminal CAP domain and a C-terminal cysteine-rich domain (CRD) containing a conserved spacing of up to 16 Cys residues, which adopts a fold that is similar to potassium channel inhibitors and thus may modulate the activity of ion channels [11]. CRISPs are likely to be dual function proteins with an activity associated with both the N-terminal CAP domain and the C-terminal CRD domain [1,12].

Cysteine-rich secretory proteins 3 transcription is dramatically upregulated in prostate cancer, for which it serves as a biomarker [13]. Upregulation of other CAP family members in malignant cells indicates a possible role of these proteins in cancer progression. For example, glioma pathogenesis-related protein 1 (GLIPR1) and GLIPR2 are among the most highly induced transcripts in human gliomas. These astrocyte-derived brain tumors account for the majority (65%) of all brain tumors [14]. These observations raise the possibility that CAP proteins may serve as diagnostic tools and ultimately perhaps even as therapeutic targets [1].

## CAP family proteins adopt a unique αβα sandwich fold

The CAP proteins share limited sequence identity with each other but they share two signature PROSITE-recognized motifs, referred to as CRISP motifs (http://prosite.expasy.org/) (Fig. 1). The NMR structure of plant PR-1 protein and the crystal structures of several CAP proteins have been determined. They revealed that these conserved sequence motifs are present in a small and structurally conserved 17–21 kD CAP domain, which adopts a unique αβα sandwich fold. The tight packing of the α-helices on both sides of the central β-sheet results in a compact, bipartite molecular core, which 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 reported for CAP proteins, consistent with the structural requirements of an extracellular function of these proteins. In addition, the surface of the CAP domain displays a large cavity that might be important for their interaction with other proteins (Fig. 2) [11,15–18].

The strong structural conservation of the CAP domain early on suggested a common mode of action of these proteins in plant pathogen defense and brain immune cells [16]. Although a large number of proteins within this superfamily contain a CAP domain in isolation, such as the venom allergen Ag5 or the plant PR-1, many other family members contain additional

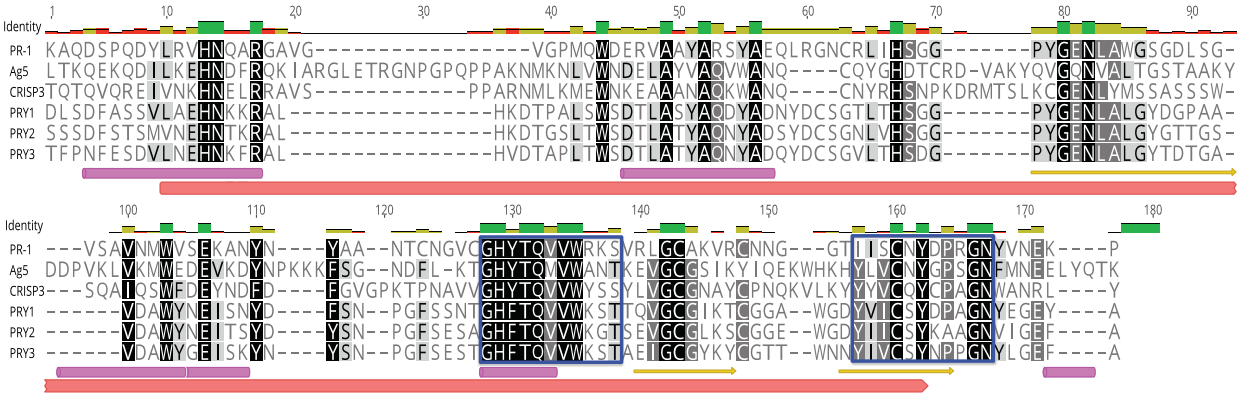


**Fig. 1.** Sequence alignment of yeast CAP proteins Pry1, 2, and 3 with the founding members of the CAP superfamily, CRISP3, Ag5, and PR-1. Partial sequence alignment of the core set of CAP proteins around the CAP domain, PR-1 (*Arabidopsis thaliana*), Ag5 (*Vespula vulgaris*), CRISP3 (*Homo sapiens*), Pry1, 2, and 3 (*Saccharomyces cerevisiae*). The degree of amino acid conservation is indicated by the bars in the line on top. Secondary structure elements are indicated by the colored lines below the sequence alignment; magenta, α-helix; yellow, β-sheet. The extent of the CAP domain is indicated by the red bar. The two signature PROSITE-recognized motifs, the CRISP motifs, are boxed in blue. The alignment was generated using MAFFT, and illustrated using GENEIOUS 10.2.3 (Biomatters software, Auckland, New Zealand).

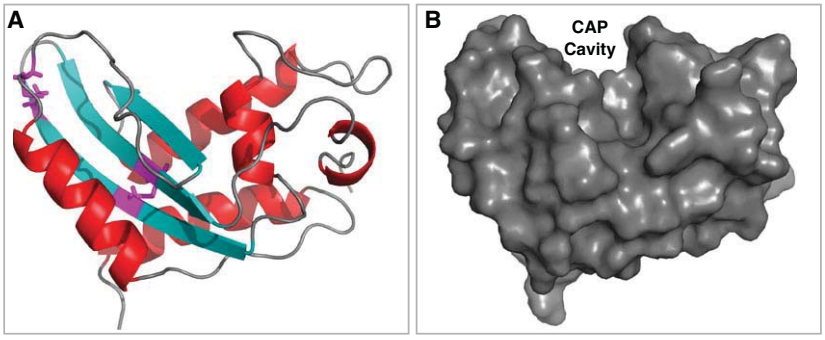


**Fig. 2.** Structure of the CAP domain of yeast Pry1. (A) Ribbon drawing of the CAP domain of Pry1 (Pdb: 5JYS). α-helices are shown in red, β-strands in cyan. Disulfide bonds are shown as stick models in magenta. (B) Space-filling representation of the CAP domain of Pry1 showing the CAP cavity. Generated using PYMOL (Molecular Graphics System, version 1.3r1; DeLano Scientific, Palo Alto, CA, USA).

N- or C-terminal extensions, which are thought to modulate the function of the CAP domain and thereby may alter target specificity and thus the physiological response [1].

## Pathogen-related in yeast, Pry proteins bind and export sterols

We have previously shown that sterols, sterol precursors, and other small hydrophobic compounds are subject to an acetylation/deacetylation cycle and have proposed that this cycle acts as a lipid detoxification pathway because acetylated sterols and sterol precursors are excreted from the cells [19]. At that time, we also proposed that the secretion of such hydrophobic compounds is likely to require proteins that bind and solubilize these lipids. These proteins could then be identified as the Pathogen Related in Yeast, Pry proteins, that is, the yeast CAP family members [20]. The yeast genome codes for three different Pry proteins. Pry1 and Pry2 are secreted glycoproteins, whereas Pry3 encodes a glycosylphosphatidyl inositol (GPI)-anchored cell wall protein [21]. These three Pry proteins share a redundant function in secretion of cholesteryl acetate, but a *pry1*Δ *pry2*Δ double mutant has an almost complete block in sterol secretion. Purified Pry1 and Pry2 bind free and acetylated sterols *in vitro*. Sterol binding is saturable with a dissociation constant in the low micromolar range ($K_d$ of ~ 0.7 μM), and the CAP domain of Pry1 is necessary and sufficient for sterol binding *in vitro* and *in vivo* [20,22]. Pry proteins thus bind sterols with an affinity that is comparable to that of other sterol-binding proteins such as the yeast member of the oxysterol-binding protein family, Osh4 ($K_d$ of ~ 0.3 μM), the steroidogenic acute regulatory protein StAR/STARD1

($K_d$ of ~ 0.03 μM), or Niemann–Pick Disease Type C2 protein NPC2 ($K_d$ of ~ 0.1 μM) [23–25].

Most importantly, the block in sterol secretion of a *pry1*Δ *pry2*Δ double mutant is rescued by expression of CRISP2, a mammalian CAP superfamily member that is expressed in the testis and epididymis and participates in sperm–egg interaction during fertilization, indicating that sterol binding is a conserved feature of CAP superfamily members [1,20,26]. Similarly, expression of SmVAL4, a CAP superfamily member from the human parasite, *Schistosoma mansoni*, in yeast cells lacking Pry function rescues their block in sterol export and purified SmVal4 binds cholesterol *in vitro* [27]. *Schistosoma* is a pathogenic flatworm, causing snail fever (bilharzia), which, after malaria, is the second most devastating parasitic disease, affecting more than 200 million people worldwide. The CAP proteins of these organisms are potential vaccine candidates [28,29].

To characterize the mode of sterol binding of these proteins in more detail, we performed computational modeling studies. These homology models revealed the presence of a flexible loop within the CAP domain of Pry1, which could potentially displace to bind cholesterol. This loop harbors a so called caveolin-binding motif, that is, a motif containing a short stretch of aromatic amino acids [30]. The caveolin-binding motif in the human CAP superfamily member, GLIPR2/GAPR1, had previously been shown to interact with caveolin by immunoprecipitation [31]. Mutations in the flexible loop region of Pry1 abrogate binding of acetylated sterols *in vitro* as well as *in vivo*, indicating that this region is indeed important for ligand binding [32] (Fig. 3A,B). Consistent with this notion, CAP family member from the cacao pathogen *Moniliophthora perniciosa* that do not bind cholesterol can be converted into sterol binders by a single point mutation in their caveolin-binding motif [33]. The mode of sterol binding by Pry1 is thus different from that of other cytosolic sterol-binding proteins such as Osh4, StAR/STARD1, or NPC2, which all bind the lipid in a deep hydrophobic tunnel buried inside the protein [23,34–36].

The CAP domain of Pry1 forms dimers in solution and the structure revealed the presence of 1,4-dioxane in the putative cholesterol-binding site [22]. However, so far, no crystals grown in the presence of cholesterol

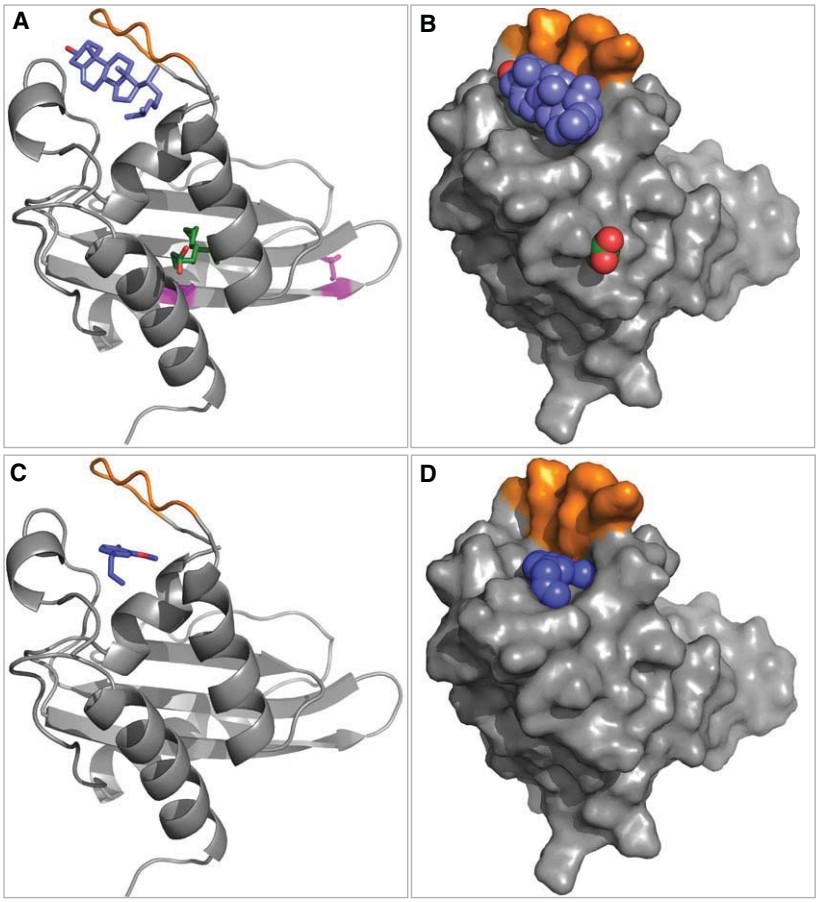


**Fig. 3.** Proposed mode of ligand binding by the CAP domain. (A) Ribbon drawing of the CAP domain of Pry1 showing binding of cholesterol (blue) and palmitate (green), the caveolin-binding motif is indicated in orange. The two disulfide bonds are shown as stick models in magenta. For illustration purposes, the cholesterol molecule was manually positioned so as to fill optimally the open cavity, adapted from [32]. The position of palmitate corresponds to that observed in the crystal structure of tablysin-15 [38]. Oxygen atoms on the two ligands are indicated in red. (B) Space-filling representation of the Pry1 CAP domain with the two bound ligands. Same color code as in panel A. Generated using PYMOL. (C, D) Binding of eugenol (blue) to the cholesterol-binding site of Pry1.

could be obtained to confirm the putative mode of action at the structural level.

Mapping the structural requirements on the part of the ligand for binding to Pry1 revealed that the aliphatic side chain of cholesterol and related sterols is important for binding, whereas the 3-hydroxyl group of the sterol appears dispensable [37]. The CAP domain thus binds the mammalian cholesterol, the fungal ergosterol and the plant stigmasterol, but it does not bind steroids, which lack the aliphatic side chain.

Pry1 and Pry2 bind eugenol and possibly other related small hydrophobic compounds. Eugenol is a member of the allylbenzene class of compounds that is present in clove oil, nutmeg, cinnamon, and bay leaf, and is used as a local antiseptic and anesthetic. Yeast mutants lacking Pry function are eugenol hypersensitive, whereas the overexpression of Pry1, Pry2 or the mammalian CRISP2 renders them eugenol resistant [20]. Eugenol competes with cholesterol for binding to Pry1 and Pry2, indicating that the binding sites for the two lipids are overlapping, which is consistent with molecular modeling studies (Fig. 3C,D) [20,37]. These data thus indicate that CAP proteins might not only promote the secretion of potentially harmful hydrophobic compounds, such as, for example, intermediates in sterol biosynthesis, but they might also sequester such compounds when present in the extracellular space [19].

## Pry proteins bind and export fatty acids

The saliva of blood-feeding arthropods contains proteins, peptides, and small molecules that inhibit host systems of inflammation and hemostasis. One of these proteins, a CAP protein from the horsefly *Tabanus yao*, tablysin-15, is a very potent inhibitor of platelet and endothelial cell function. Interestingly, the structure of the CAP domain of tablysin-15 revealed a hydrophobic channel that was occupied by a fatty acid and the delipidated protein binds proinflammatory leukotrienes with submicromolar affinity, suggesting that tablysin-15 acts as a scavenger of eicosanoids [38]. Eicosanoids are released in the skin by activated mast cells, causing pain and itching, and they elicit changes in the vascular permeability at the site of an insect bite.

Computational modeling and sequence comparison revealed that the hydrophobic channel described for tablysin-15 is conserved in Pry1, suggesting that Pry1 may also be able to bind fatty acids (Fig. 3A,B). *In vitro* binding assays with purified Pry1 confirmed that the protein binds [$^3$H]-palmitic acid with a saturable binding kinetics and an apparent $K_d$ of 112 μM. Interestingly, the two lipid-binding sites on Pry1, the sterol- and the fatty acid-binding sites, are distinct and nonoverlapping because binding to [$^3$H]-palmitic acid cannot be competed for by addition of unlabeled cholesterol and *vice versa* [39].

To test whether Pry1 also binds fatty acids *in vivo* and to determine whether this lipid-binding function is physiologically relevant, we took advantage of the observation that mutant cells lacking two major acyl-CoA synthases, Faa1 and Faa4, secrete high levels of fatty acids into the culture medium [40]. This secretion of fatty acids required Pry function since transcriptional shut off of *PRY1* in a *pry2*Δ *pry3*Δ *faa1*Δ *faa4*Δ mutant background reduced the levels of exported fatty acids and instead resulted in their intracellular accumulation [39].

The function of Pry to export fatty acids under these conditions is essential, since a complete deletion of all three *PRY* genes in an *faa1*Δ *faa4*Δ mutant background renders cells inviable [39]. This system thus allows for an *in vivo* assay to test various CAP family members for their ability to bind and export fatty acids. Pry proteins, to our knowledge, are the first proteins identified to promote the secretion of free fatty acids. They thus differ from the family of abundantly expressed intracellular fatty acid-binding proteins (FABP), which reversibly bind hydrophobic ligand, such as saturated and unsaturated long-chain fatty acids, eicosanoids and other lipids, with high affinity [41,42]. Albumins, on the other hand, constitute a class of well-characterized secreted FABP, but these proteins are generally believed to bind and solubilize fatty acids only once they are secreted into the circulation [43,44].

## Pry3 affects the yeast mating reaction

Among the three members of the Pathogen-Related Yeast proteins, Pry3 stands out because: (a) it is more than twice as long as either Pry1 or Pry2, (b) it has its CAP domain in its N-terminal part followed by a long Ser/Thr-rich region, whereas Pry1 and Pry2 have the CAP domain in the C-terminal part of the protein, and (c) Pry3 not only has an N-terminal signal sequence (SS) but also an ω-site that directs attachment of a C-terminal GPI anchor [45] (Fig. 4). The post-translationally modified Pry3 is retained in the yeast cell wall by attachment to β-1,6-glucans via its GPI remnant [21,45]. Interestingly, this extracellular protein has been implicated in the mating reaction, both at the transcriptional and the phenotypic levels.


**Fig. 4.** Structural organization of the yeast CAP family members. Schematic drawing of the overall structural organization of the yeast CAP family members. The CAP domain is indicated in red, Ser/Thr-rich sequences are shown in green, the N-terminal SS is indicated as well as the C-terminal ω-site directing GPI attachment to Pry3 (blue box).

In response to α-factor, full-length *PRY3* mRNA declines and concomitantly a shorter transcript appears, which has 5′ start 452 nucleotides inside the open reading frame [46]. The repression of the full-length transcript depends on the pheromone-induced transcription factor Ste12, which binds the promoter of *PRY3* close to the TATA box thereby impeding its proper recognition [46]. Expression of both the short and the long transcripts rely on the daughter cell-specific transcription factor Ace2, hence the gene is specifically expressed in daughter cells and may be important for mother–daughter separation [47,48]. Phenotypically, overexpression of Pry3 renders cells more resistant to organic solvents and they display a strongly reduced mating efficiency [46,49]. The precise function of Pry3 in the mating reaction and the role of the CAP domain in that function, however, remains to be defined. However, given that this complex order of events requires remodeling of the cell wall and fusion of the two plasma membranes, the hypothesis that the lipid-binding function of the CAP domain of Pry3 may be involved in some of these events is test worthy.

## CAP proteins in pathogen defense

The plant PR-1, one of the three founding members of the CAP superfamily is synthesized in response to infections with pathogens [5,6]. Expression of PR-1 in yeast mutants lacking their endogenous CAP proteins rescues their block in sterol export and purified PR-1 binds sterols *in vitro*. Remarkably, this sterol-binding activity is important to protect plants from oomycetes, an important class of sterol-auxotrophic plant pathogens [50]. Purified PR-1 inhibits growth of the sterol auxotroph *Phytophthora brassicae in vitro*. This growth inhibition was released if either a mutant version of PR-1 that cannot bind sterols was used in the assay or when the growth media was supplemented with an excess of sterols, thus indicating that the growth inhibition of oomycetes is likely due to sterol sequestration by PR-1 [50,51].

Taken together available data on the mode of action of members of this huge family of proteins support a model in which these proteins act to bind and sequester small hydrophobic compounds such as sterols, fatty acids, and even eicosanoid-derived immune modulatory signals to suppress the immune response of the host. They neutralize the action of membrane perturbing antimicrobial compounds, such as eugenol, or sequester sterols from sterol-auxotrophic pathogens. In any case, CAP proteins are secreted and produced to which might either be (infective agents, cancer cells, or plants under attack). promote proliferation and well-being of the producer, infective agents, cancer cells or plants under attack. The challenge now will be to actually identify the chemical nature of the ligand that these proteins bind and sequester under their specific physiological conditions.

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