

Short Conceptual Overview

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The CAP protein superfamily: function in sterol export and fungal virulence

Abstract: CAP superfamily proteins, also known as sperm-coating proteins, are found in all kingdoms of life and have been implicated in a variety of physiological contexts, including immune defense in plants and mammals, sperm maturation and fertilization, fungal virulence, and toxicity of insect and reptile venoms as well as prostate and brain cancer. CAP family members are mostly secreted glycoproteins that are highly stable in the extracellular fluid. All members of the superfamily share a common CAP domain of approximately 150 amino acids, which adopts a unique α - β - α sandwich fold. The conserved structure suggests that CAP proteins exert fundamentally similar functions. However, the molecular mode of action of this protein family has remained enigmatic. The budding yeast *Saccharomyces cerevisiae* has three CAP family members designated Pry (pathogen related in yeast), and recent evidence indicates that they act as sterol-binding and export proteins. Expression of the mammalian CAP protein CRISP2, which binds sterols *in vitro*, complements the sterol export defect of a yeast *pry* mutant, suggesting that sterol binding and export is conserved among different CAP family members. Collectively, these observations suggest that CAP family members constitute a novel class of secreted extracellular sterol-binding proteins. A ligand-binding activity of the CAP domain could explain many of the biological activities attributed to these proteins. For example, the strong induction of plant pathogenesis-related 1 protein upon exposure to pathogens may serve to inhibit pathogen proliferation by extracting sterols from the pathogen membrane. Similarly, the presence of these proteins in the venom of toxic insects and reptiles or in the secretome of pathogenic fungi might inflict damage by sequestering sterols or related small hydrophobic compounds from the host tissue.

Keywords: fungal virulence; *Fusarium oxysporum*; mouse infection model; pathogenesis-related (PR-1); sterol detoxification.

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Introduction

The CAP protein superfamily (pfam PF00188) was named after the three founding members cysteine-rich secretory protein, antigen 5, and pathogenesis-related 1, and comprises more than 4500 known members in over 1500 species from all kingdoms of life. CAP proteins have been implicated in a wide variety of biological processes, including immune defense in mammals and plants, pathogen virulence, sperm maturation and fertilization, venom toxicity, and prostate and brain cancer. Almost all CAP proteins are secreted glycoproteins that exhibit a high stability in the extracellular fluid over a wide range of environmental conditions. The overall structural conservation within the CAP superfamily is likely to result in fundamentally similar functions for the CAP domains in the different members, whereas the diversity outside of this core region may alter target specificity and thus modulate physiological responses. For example, mammalian CAP proteins have been classified into nine subfamilies that differ in regions flanking the conserved CAP domain (1). CAP proteins are an intensely studied class of proteins; however, their mode of action has remained elusive [for reviews, see (1, 2)].

The first founding member of the CAP superfamily is the plant pathogenesis-related protein-1 (PR-1), which was identified in 1970 among the proteins induced in tobacco leaves upon viral infection (3). Plant pathogenesis-related (PR) proteins are classified into 17 families according to their molecular mass, PR-1 being the smallest one with 14 kDa. Subsequent studies revealed that other PR proteins, such as glucanase (PR-2), chitinase (PR-3, 4, 8, 11), protease inhibitor (PR-6), peroxidase (PR-9), or ribonuclease activity (PR-10), have different biochemical activities, serving as a direct line of defense against fungal and

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bacterial pathogens (4, 5). Intriguingly, even though it is the most abundant PR protein, PR-1 remains the only member for which no biochemical function is known. Some PR-1 proteins display antimicrobial activity against fungal and oomycete pathogens, but their mechanism of action is unclear (6, 7).

Antigen 5 (Ag5) is the second founding member of the CAP superfamily, an abundant protein present in the venom-secretory ducts of stinging insects that elicits a strong allergenic response. 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 (8). The biochemical activity of Ag5 is unknown, but a related CAP protein from hookworm, neutrophil inhibitory factor (NIF), was shown to abrogate the adhesion of neutrophils to endothelial cells by binding to and thereby blocking integrin action (9).

Cysteine-rich secretory proteins (CRISPs) constitute the third founding members of the CAP superfamily. They 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 containing an N-terminal CAP domain and a C-terminal cysteine-rich domain (CRD) with a conserved spacing of up to 16 Cys residues. This modular structure is likely to result in a dual-function protein with distinct activities associated with the N-terminal CAP domain and the C-terminal CRD domain (1, 10). Recent data suggest that the CRD adopts a fold similar to potassium channel inhibitors and may thus modulate the activity of cyclic nucleotide-gated ion channels (see the following) (11).

Structure of CAP proteins

CAP proteins share only limited sequence identity, including two signature PROSITE-recognized motifs referred to as CRISP motifs (<http://expasy.ch/prosite>) (Figure 1A). The NMR structure of plant PR-1 protein and the crystal structures of several CAP proteins revealed that the conserved CAP sequence motifs are present in a small and structurally conserved 17- to 21-kDa CAP domain, which adopts a unique α - β - α 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 two highly conserved disulfide bonds (Figure 1B). These features are thought to provide the high thermal, pH, and proteolytic stability reported for CAP proteins, consistent

with the structural requirements of an extracellular function (11–14).

A seminal study used the NMR structure of plant PR-1 as template to model the structure of the human glioma pathogenesis-related protein 1 (GLIPR1), revealing the presence of four conserved partially surface exposed residues, two histidines, and two glutamic acids. The high structural conservation of the CAP domain immediately suggested a common mode of action of these proteins in plant pathogen defense and brain immune cells (13). These surface residues were later suggested to form part of a putative active-site triad of a CAP protein with a reported *in vitro* protease activity, Tex31 (15). However, although a large number of proteins within the superfamily contain a CAP domain in isolation (e.g., Ag5 and PR-1), many others contain additional N- or C-terminal extensions (1).

Proposed functions of CAP proteins

A 28-kDa CAP family member, Tex 31, was purified from the venom duct of the marine cone snail *Conus textile* and found to have proteolytic activity *in vitro*. This activity was abolished by serine protease inhibitors and stimulated by addition of Ca^{2+} (15). Modeling of the Tex31 sequence to the structure of PR-1 and Ag5 was consistent with a possible catalytic role of the conserved surface-exposed histidine and glutamic acid residues (15). The lack of a conserved serine in this putative active-site triad led others to propose that dimerization of CAP proteins is required to complete the formation of the active site (14). However, subsequent 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 is still lacking (11, 16, 17). Apart from a possible catalytic activity, the CAP domain has been suggested to form a stable scaffold for biological interactions with other proteins (18), although no interactions of plant PR-1 with other proteins have been detected (19).

CRISP subfamily CAP proteins harboring a C-terminal CRD have been associated with modulation of the activity of ion channels, which might account for their role in sperm maturation and venom toxicity (11, 20, 21). However, whether the CRD acts directly on the ion channel or whether CRISP proteins affect the channel activity indirectly, for example, through concomitant alterations of the lipid composition of the plasma membrane, remains to be established.

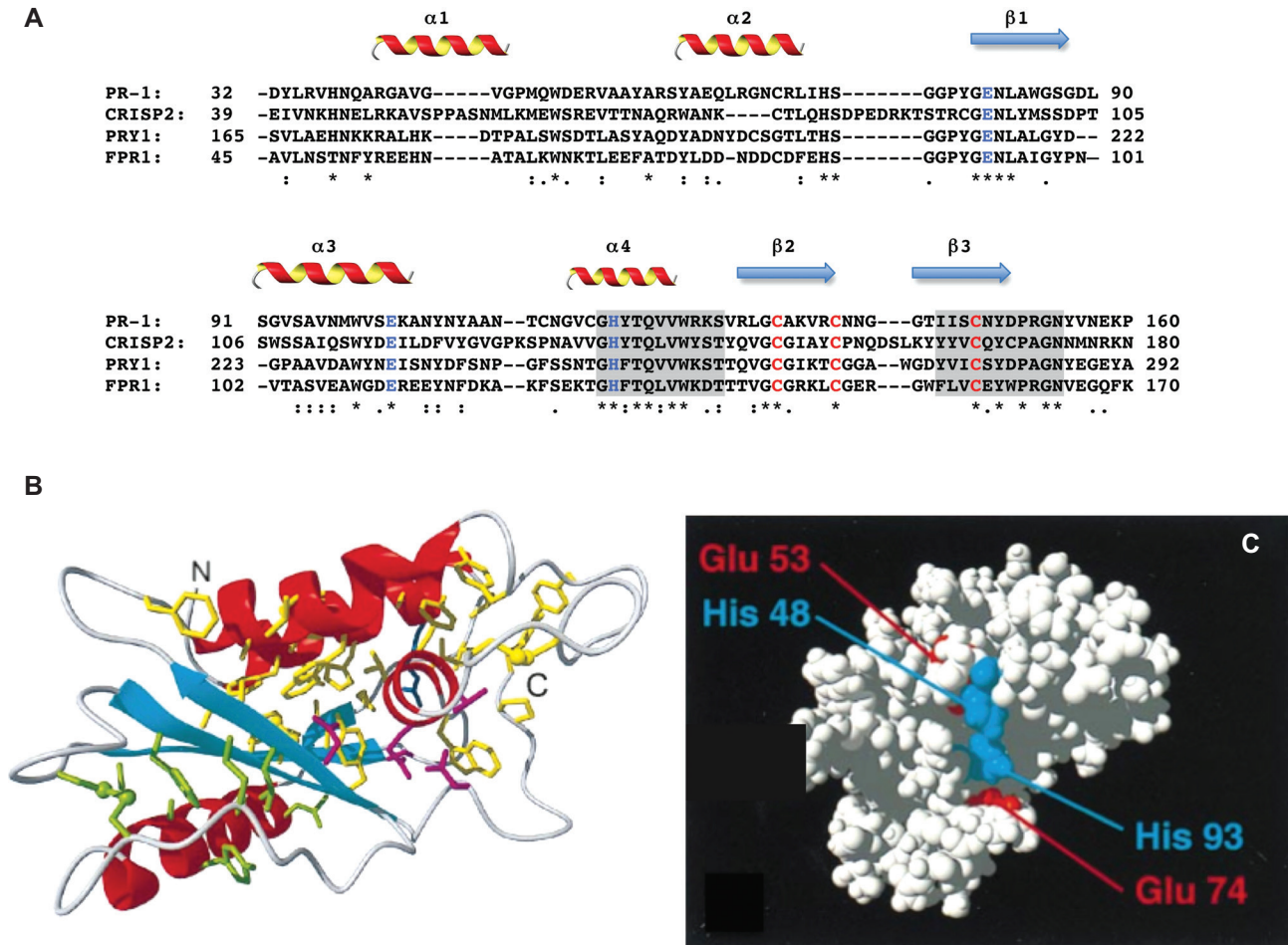


Figure 1 Conservation and structure of the CAP domain.

(A) Partial sequence alignment of a core set of CAP proteins, PR-1 (*Arabidopsis thaliana*), CRISP2 (*Homo sapiens*), Pry1 (*S. cerevisiae*), Fpr1 (*F. oxysporum*). Secondary-structure elements are indicated and conserved residues of a putative active site are in blue; conserved cysteines are in red. The two CRISP motifs are shaded. (B) Ribbon drawing of the CAP domain of PR-1. α -Helices are shown in red, β -strands in cyan. Amino acid side chains that form a large hydrophobic cluster are shown in yellow; those forming a smaller hydrophobic cluster are green. Disulfide bonds are shown as ball-and-stick models. (C) Space-filling representation of PR-1 with the partially surface exposed histidyl and glutamyl residues depicted in blue and red, respectively. (B) and (C) are adapted from (13) (© 1998, National Academy of Sciences, USA).

The role of CAP proteins in humans and their implication in cancer

Transcriptional deregulation of two CAP superfamily members in malignant cells suggested a possible function of these proteins in cancer development or progression. GLIPR1 and GLIPR2 (also known as RTVP1/GAPR1) are among the most highly up-regulated transcripts in human gliomas, i.e., astrocyte-derived brain tumors, which account for over 65% of all human primary brain tumors (22). Interestingly, GLIPR1 has pro-apoptotic activity and acts as a tumor suppressor in prostate cancer (23). On the other hand, the CRISP family member CRISP3 is expressed in different cell types of the immune system including

pre-B cells, and is believed to function in innate immunity. Transcription of CRISP3 is dramatically increased in prostate cancer and serves as a potential biomarker (24). These observations raise the possibility that CAPs may be used as diagnostic agents and perhaps, ultimately, as therapeutic targets (1).

Yeast Pry proteins function in sterol binding and export

New insight into the function of the CAP family of proteins comes from recent studies in budding yeast. The *S. cerevisiae* genome codes for three members of this

protein superfamily, termed Pry. Pry1 and Pry2 are secreted glycoproteins, whereas Pry3 is associated with the cell wall and contains a signal for attachment of a glycosylphosphatidylinositol anchor (25, 26). Pry1 and Pry2 share a redundant function in the export of acetylated cholesterol, a lipid intermediate that accumulates in cells lacking a corresponding lipid deacetylase (27). Double mutants lacking both Pry1 and Pry2 accumulate cholesteryl acetate in the endoplasmic reticulum membrane, whereas in the presence of a wild-type copy of either Pry1 or Pry2 cholesteryl acetate is secreted from the cells and accumulates in the culture media (26, 27). Purified Pry1 and Pry2 bind both free cholesterol and cholesteryl acetate in a dose-dependent manner, consistent with a role of Pry proteins in binding and solubilizing sterols and possibly other small hydrophobic compounds.

Importantly, the sterol binding and export function of the yeast CAP proteins Pry1 is confined to the CAP domain because expression of the CAP domain alone is sufficient to rescue the sterol export phenotype of a *pry1Δ pry2Δ* double mutant, and the CAP domain of Pry1 alone binds sterols *in vitro*. Evidence for the specificity of the protein-lipid interaction comes from the finding that it is prevented by mutation of a highly conserved cysteine residue known to form a disulfide bridge. Most importantly, the lipid-binding and export function of the Pry proteins appears to be conserved among the members of the CAP protein superfamily because expression of the human CAP protein CRISP2 relieves the lipid export block of a yeast *pry1Δ pry2Δ* double mutant, and purified CRISP2 binds sterols *in vitro* (26).

Cells lacking *PRY1* and *PRY2* are hypersensitive to the plant oil eugenol, and this phenotype is also rescued by expression of human CRISP2. Eugenol is a member of the allylbenzene class of compounds present in clove oil, nutmeg, cinnamon, and bay leaf that is used as local antiseptic and anesthetic and has antifungal and bacteriostatic activity (28). Pry proteins bind eugenol and confer dose-dependent resistance against this potential membrane-perturbing compound (26, 27). Thus, members of the CAP protein superfamily may exert a wide variety of physiological functions through binding to lipids and related small hydrophobic compounds that affect the integrity of cellular membranes.

In the light of these results, it is conceivable that the function of plant PR-1 proteins in pathogen defense involves a sterol binding and sequestration mechanism. Capturing sterols from the surface membrane of pathogens could exert a growth inhibitory effect or even kill the invaders. Sterols are not only an essential lipid constituent

in eukaryotic plasma membranes, but also bacteria synthesize hopanoid and tetrahymanol compounds that are structurally and functionally related to sterols (29–31). Drugs that target ergosterol, the fungus-specific membrane sterol, or products like azoles that inhibit specific steps in ergosterol biosynthesis, are widely used in antifungal treatments of plants, animals, and humans (32). The sterol-binding mechanism of CAP proteins could therefore be exploited as a novel mode of action to target invasive microbial infections.

CAP proteins act as fungal virulence determinants

CAP proteins have recently emerged as novel virulence factors in pathogenic fungi. The *RBT4* gene encodes a predicted secreted CAP protein from the human pathogen *Candida albicans* that was originally identified in a search for transcripts under control of a morphogenetic regulator, Tup1. *Candida* mutants lacking *RBT4* had reduced virulence both in a rabbit cornea and in a systemic mouse infection model (33). *Rbt4*, together with a close homolog, *Rbe1*, were recently detected in the fungal secretome and shown to be part of a family of five CaPRY proteins in *C. albicans*. Whereas single deletions of *RBE1* or *RBT4* resulted in a moderate attenuation in a mouse model for disseminated candidiasis, the *rbe1Δ rbt4Δ* double mutant was dramatically reduced, suggesting that different CAP family proteins have partially redundant roles in virulence (34).

Another CAP family protein, *Fpr1*, was reported in the ascomycete *Fusarium oxysporum*, a ubiquitous fungal pathogen that causes vascular wilt disease on a wide range of plant species and can produce life-threatening infections in immunocompromised humans (17). *Fpr1* is an extracellular protein with an N-terminal secretion signal, a serine/proline-rich region of unknown function, and a C-terminal CAP domain including the highly conserved His and Glu residues. Western blot analysis of culture supernatants revealed that *Fpr1* is secreted by *F. oxysporum* as a precursor migrating at 40 kDa and rapidly converted by proteolytic cleavage into the major 30-kDa form. Strikingly, transcription of the *fpr1* gene was rapidly induced during growth in human blood, and immunodepressed mice infected with *fpr1Δ* mutants exhibited significantly lower mortality rates than those infected with the wild-type strain. Importantly, the CAP domain was required for the function of *Fpr1* in virulence because complementation with

a native *fpr1* allele restored virulence of the *fpr1* Δ mutant to wild-type levels, whereas an allele in which two of the predicted active-site residues had been mutated (*fpr1*^{H170A,E177A}) did not (17).

Evidence for expansion of CAP proteins in fungal pathogens

A survey of predicted CAP proteins in sequenced fungal genomes revealed that most fungi contain two members of the protein family that cluster into well-separated clades. The presence of these ‘core’ CAP proteins in non-pathogens including yeast, suggests they must fulfill functions unrelated to pathogenicity, such as binding and export of sterols and related small hydrophobic compounds (26). However, some fungal pathogens display a remarkable increase in the number of CAP family proteins. *C. albicans* has five members, all of which cluster within a separate Hemiascomycete-specific clade, suggesting that this CAP gene family has expanded recently during evolution. Even more strikingly, the plant pathogens *F. oxysporum*, *Fusarium graminearum*, and *Magnaporthe grisea* contain a new family of CAP proteins that appears phylogenetically closer to plant PR-1 proteins than to the fungus-specific clades, raising the intriguing possibility that their ancestor might have originated from a horizontal gene transfer event. Interestingly, the new clade also includes the *F. oxysporum* virulence determinant Fpr1 (17). Similarly, the genome of *Monilophthora perniciosa*, a basidiomycete fungus that causes witches’ broom disease in cacao, was shown to contain 11 CAP family genes some of which were highly and specifically expressed during the interaction with the host plant (35). Collectively, these findings suggest a possible link between the evolutionary expansion of fungal CAP proteins and pathogenicity.

Possible function of CAP proteins during fungal infection

Although the role of CAP proteins in fungal virulence remains unclear, the capacity of the yeast homologs to bind sterols and related small hydrophobic compounds suggests a parallel between the ligand-binding activity and their function during infection. This question is of particular interest because some CAP proteins from the

pathogen-specific clades lack several of the conserved cysteine residues involved in intramolecular disulfide bridges, suggesting distinct ligand-binding properties and/or protein stabilities (17).

Fungal infections, particularly in humans, are difficult to control because, as eukaryotes, their metabolism closely resembles ours. As a result, most current fungicides and medical antifungals are based on only a handful of active principles. Sterol metabolism is a known Achilles heel of fungi and acts as a major fungicide target in medicine and agriculture (36). Intriguingly, plants have exploited ergosterol binding in the fungal membrane as a mechanism of antifungal phytoalexins such as the tomato glycoalkaloid tomatine (37). The finding that secreted CAP proteins are conserved fungal virulence factors suggests that they could serve as potential targets to reduce fungal infection.

Outlook

The breakthrough finding that the yeast CAP family members Pry1 and Pry2 are required for exporting cholesterol acetate and that these proteins directly bind the lipid has provided a new lead to address the molecular function of CAP family members in other organisms. It is conceivable that CAP proteins exert their multitude of biological tasks through a common mechanism of binding/sequestering sterols and/or related small hydrophobic compounds. Thus, sterol sequestration from the host tissue by fungal pathogens might facilitate tissue penetration, consistent with the role of CAP proteins in virulence. Meanwhile, targeting of sterols from microbial invaders by host CAP proteins could account for their function in innate immunity of plants and mammals. Finally, interaction with lipids might also contribute to the multiple roles of human CAP proteins associated with cancer development.

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