Proline-rich antimicrobial peptides targeting protein synthesis

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The innate immune system employs a broad array of antimicrobial peptides (AMPs) to attack invading microorganisms. While most AMPs act by permeabilizing the bacterial membrane, specific subclasses of AMPs have been identified that pass through membranes and inhibit bacterial growth by targeting fundamental intracellular processes. One such subclass is the proline-rich antimicrobial peptides (PrAMPs) that bind to the ribosome and interfere with the process of protein synthesis. A diverse range of PrAMPs have been identified in insects, such as bees, wasps and beetles, and crustaceans, such as crabs, as well as in mammals, such as cows, sheep, goats and pigs. Mechanistically, the best-characterized PrAMPs are the insect oncocins, such as Onc112, and bovine bactenecins, such as Bac7. Biochemical and structural studies have revealed that these PrAMPs bind within the ribosomal exit tunnel with a reverse orientation compared to a nascent polypeptide chain. The PrAMPs allow initiation but prevent the transition into the elongation phase of translation. Insight into the interactions of PrAMPs with their ribosomal target provides the opportunity to further develop these peptides as novel antimicrobial agents.

1 Discovery of PrAMPs

The innate immune system uses a broad range of antimicrobial peptides (AMP) as the first line of defense to kill invading microorganisms. AMPs inhibit the proliferation of bacteria and therefore can prevent the establishment of an infection. They can either be induced through pathogen sensing receptors or are continuously secreted into body fluids.[1] Based on their nature and composition they can be divided into amphiphilic peptides, with two to four β-strands, amphipathic α-helices, loop structures and extended structures.[2] Although most of these five classes inhibit bacterial cells by permeabilizing the membrane, the action of AMPs is not limited to the surface of pathogens.[3] Some AMPs have intracellular targets which affect the metabolism of the invading organism,[4] such as the subclass of Proline-rich Antimicrobial Peptides (PrAMPs).[5–7] PrAMPs belong to the group of cationic peptides that are enriched in proline residues and are often arranged in conserved patterns together with arginine residues (Fig. 1A and B). PrAMPs appear to be irregularly dispersed amongst animals, being so far only identified in some arthropods (insects and crustaceans) and mammals (Fig. 1A). The discovery of the first PrAMP started with apidaecin in the late 1980s.[8] Casteels and coworkers injected a sub-lethal dose of *Escherichia coli* cells into the body cavity of adult bees and subsequently monitored the appearance of AMPs by HPLC.[8] This led to the identification of three active forms of apidaecin which were further characterized with respect to their molecular mass and amino acid sequence.[8] The discovery of apidaecins was quickly followed by the identification of other insect and mammalian PrAMPs. Insect PrAMPs include abaecin from the honey bee *Apis mellifera*,[9] drosocin from the fruit fly *Drosophila melanogaster*,[10] pyrrhocoricin from the firebeetle *Pyrrhocoris apterus*,[11] metalnikowin-1 from the green shield bug *Palomena prasina*[12] and oncocin from the milkweed bug *Oncopeltus fasciatus*[13,14] (Fig. 1A). In crustaceans, the PrAMP Arasin 1 has been isolated from the spider crab *Hyas araneus*[19] as well as a PrAMP with similarity to Bac7 from the shore crab *Carcinus maenas.*[20] Two distinct mammalian PrAMPs have been identified in ruminant species, such as cows (*e.g. Bos taurus*),[15] sheep (*e.g. Ovis aries*)[16,17] and goats (*e.g. Capra hircus*) (Fig. 1A).[16,17] These PrAMPs were named bactenecin 5 and 7 (Bac5 and Bac7) due

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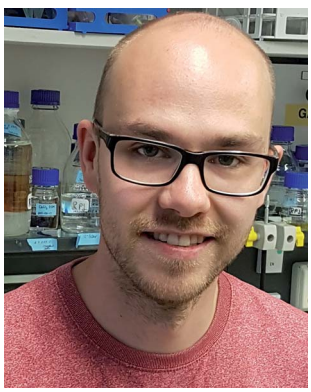
to the molecular weight of the mature peptides being 5 and 7 kDa, respectively.[15] In pigs the corresponding PrAMP homolog to Bac7 has been called PR-39 due to its length of 39 amino acids.[18] Additional bactenecin-like PrAMPs, such as Bac4, Bac6.5 and Bac11, were identified in the sheep genome,[16] but remain to be characterized.

2 Synthesis of PrAMPs

The synthesis of AMPs, including PrAMPs, occurs mainly in response to invading bacteria.[1] While most of the PrAMPs characterized to date are synthesized by the ribosome as inactive precursors, interestingly, their paths of activation differ significantly between species.

Mammalian Bac5/Bac7 peptides are produced, as other non-proline-rich mammalian AMPs, by immature myeloid cells as pre-pro-peptide precursors (Fig. 2A). The Bac5 and Bac7 pre-pro-peptides comprise a 29 aa pre-signal followed by a 101 aa pro-region. Bac5/Bac7 are targeted to large granules, where the targeting signals are cleaved upon import to yield pro-peptides in differentiated neutrophils.[21] When the immune system recognizes invading bacteria, the maturation of pro-Bacs is triggered by secretion and mixing of the contents of large and azurophil granules.[22] The inactive pro-Bacs are then cleaved by elastase, a serine protease that is present in azurophils, either upon (i) fusion with the phagosome, or (ii) exocytosis and release into the extracellular matrix (Fig. 2A).[22,23] The mature Bac5 (43 aa) and Bac7 (60 aa) peptides can then pass through the bacterial cell membrane *via* the SbmA transporter (see next section), where they can subsequently interact with their intracellular target (Fig. 2A).[24,25] Similarly, cDNA analysis showed that the pig PR-39 is synthesized as a 172 aa pre-pro-PR-39 peptide, which is comprised of a 29 aa signal sequence, a 101 aa pro-region and a 42 aa N-terminal PR-39-containing region.



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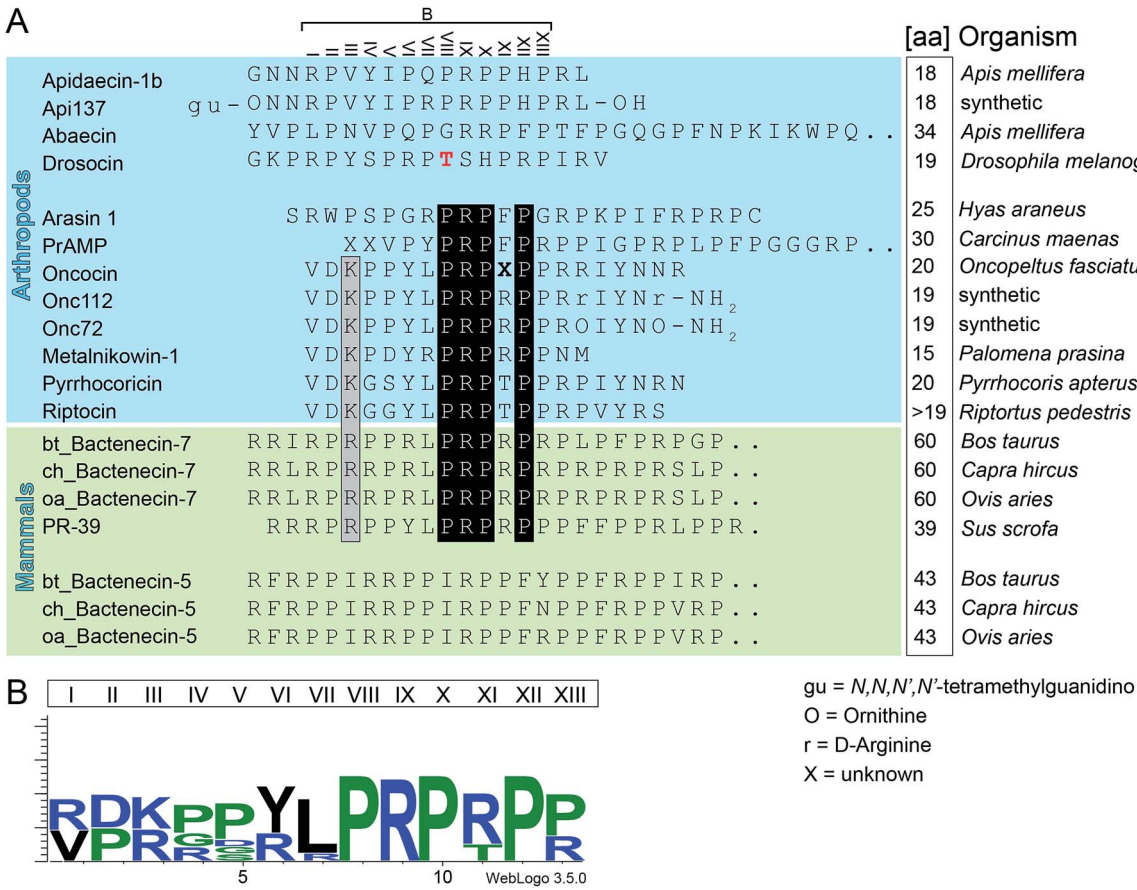

Fig. 1 Sequence alignments of PrAMPs. (A) Sequences of naturally occurring and synthetic PrAMPs derived from arthropods (insects and crustaceans, blue) and mammals (green). The central PrAMPs were aligned first based on ribosome-bound structures of Onc112, Pyr, Met and Bac7 and then on sequence similarity. Similar and identical residues are shown in grey and black, respectively. The *O*-glycosylation (Thr11) of drosocin indicated (red) and position 11 of oncocin is unknown and indicated with an "X". The number of amino acids (aa) comprising the mature peptide is also indicated for each PrAMP. (B) Sequence conservation of the core residues I to XIII of the natural PrAMPs listed in the central region of (A) between oncocin and PR-39 is shown using a WebLogo[62] representation.

The last 3 aa are removed post-translationally to generate the 39 aa active PR-39 peptide.[26]

PrAMPs have been found in many insect species. In *Drosophila melanogaster* one gene encoding the PrAMP drosocin has been identified.[27] The gene encodes a 21 aa N-terminal signal sequence (pre-sequence), the 19 aa PrAMP and a 24 aa inactivating pro-sequence that lies behind the PrAMP.[10] In contrast, some PrAMPs are synthesized as multiple copy peptides encoded within a single ORF, conferring the advantage of a fast signal amplification in response to a bacterial infection (Fig. 2B).[28] Examples include the PrAMPs riptocin [Genebank AB842297.1] and apidaecin.[28] The multiple copy product of apidaecin contains a single pre-signal sequence of 16 or 19 aa followed by a pro-fragment of 13–16 aa in length (Fig. 2B).[28] The mature 18 aa long apidaecin peptide sequence follows in multiple copies containing different isoforms (Fig. 2B).[28,29] In *Apis mellifera* individual apidaecin peptide sequences are separated by an inactivating RR-EAEPEAEP spacer sequence (Fig. 2B).[28] Upon activation amino-, endo- and carboxypeptidases process the linker and liberate the multiple copies of mature apidaecin (Fig. 2A and B). Strikingly, apidaecin is not just encoded as multiple copies within one gene, it is also encoded in several genes containing different isoforms.[28,29] In the Asian honey bee *Apis cerana*, multiple genes encoding four different apidaecin isoforms are evident.[29] Each gene contains a single pre-pro-region that is followed by a variable number of 84 nt repeats containing a linker sequence, a RR or CR dipeptide and a mature isoform of apidaecin.[29]

3 Membrane permeability and uptake of PrAMPs

The majority of AMPs act by damaging bacterial membranes and causing thereby metabolite efflux and cell destruction. However, PrAMPs primarily kill bacteria using a non-lytic mechanism *i.e.* without significantly affecting membrane integrity. The first indications for this mode of action came from studies on apidaecin and PR-39, both of which were shown to inhibit bacterial growth without causing cell lysis.[5,30] Moreover, it was shown that apidaecin was internalized by bacteria, indicating that such PrAMPs do not lyse microorganisms but rather kill them from within by inhibiting important metabolic pathways.[6] By contrast, previous investigations indicated that Bac7 permeabilizes the envelope of Gram-negative bacteria

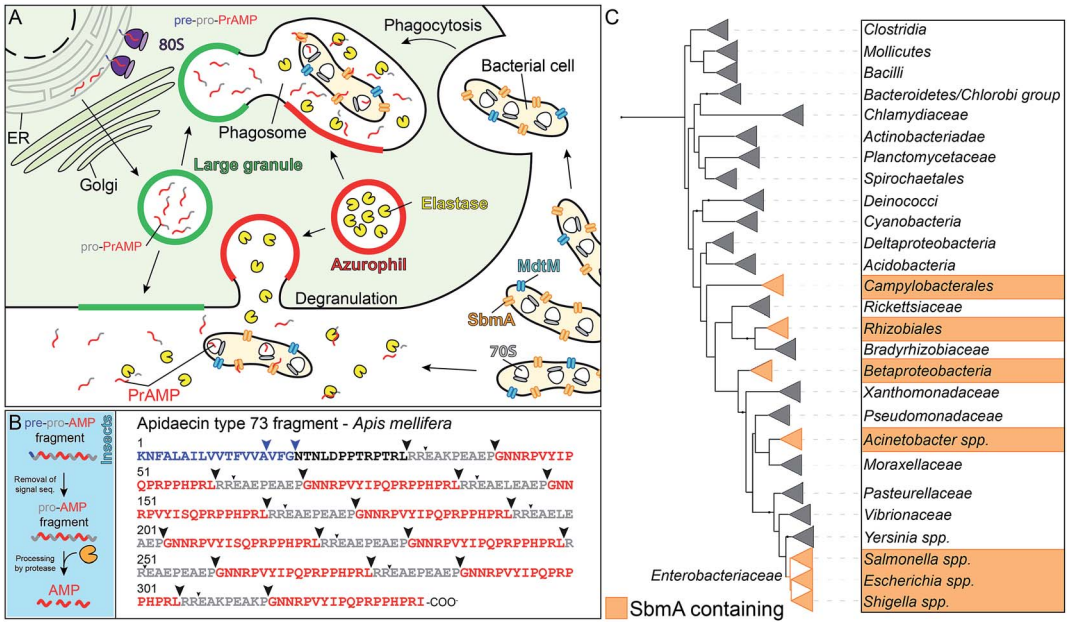

Fig. 2 Synthesis of PrAMPs. (A) Mammalian PrAMPs are synthesized as pre-pro-sequences and targeted to large granules. The PrAMPs are activated upon bacterial infection by fusion of pro-PrAMP containing large granules with the elastase-containing azurophil granules and either the plasmamembrane or the phagosome. Elastase activates the mature PrAMP by removal of the pro-sequence. The activated PrAMP is transported *via* SbmA (or to a lesser extent MdtM) into the bacterial cell. (B) Schematic (left) illustrating the activation of insect PrAMPs synthesized as multiple copies within one open reading frame. Liberation of the mature peptide involves the processing of a pre-pro-AMP by amino-, carboxy- and endoproteases. An example for a pre-pro form (right) of apidaecin type 73 fragment from *Apis mellifera*, which contains several isoforms of mature apidaecin. Putative cleavage sites are highlighted with arrows. (C) Phylogenic tree showing distribution of SbmA (orange) across Eubacteria. Bacterial groups that have some members carrying SbmA have been highlighted in orange. The iTOL software was used to draw the tree.[37]

under restrictive conditions, with a higher permeabilizing activity correlating with longer and more hydrophobic peptides.[31] This contradiction was resolved when a dual mode of action was demonstrated for shorter fragments of Bac7, such as Bac7(1-35), against *Enterobacteriaceae*.[32] The lytic mode of action was relegated to a secondary effect of this molecule, observable only in the presence of high peptide concentrations (starting from 16 μM), much above the bacteriostatic and bactericidal levels (0.5 μM and 1 μM respectively).[32]

The non-lytic mode of action of PrAMPs implies the presence of one or more transporters for cellular uptake into bacteria. The principal "Trojan horse" exploited by insect and mammalian PrAMPs was shown to be the inner membrane protein SbmA.[24] The SbmA transporter is also involved in uptake of the microcins B17,[33] J25,[34] and of the non-ribosomal peptide antibiotic bleomycin.[35] SbmA transports PrAMPs inside the bacterial cytosol exploiting the electrochemical proton gradient across the inner membrane[36] and is the major transporter responsible for their uptake.[24] The physiological role of SbmA still remains unclear, but this protein can be found in phylogenetically distant species of Gram-negative bacteria (Fig. 2C).[37] Evidence for SbmA homologs can be found amongst *Gammaproteobacteria*, in particular, the *Enterobacteriaceae* (*e.g. E. coli*, *S. dysenteriae*, *S. enterica*, and *Klebsiella pneumoniae*) and *Pseudomonadales* (*A. baumannii*), but also amongst *Alpha-proteobacteria* such as *Rhizobiales* (*e.g. S. meliloti*, *A. tumefaciens* and *B. abortus*), *Beta-proteobacteria* (*e.g. Neisseria meningitis*), and *Epsilon-proteobacteria* (*Campylobacter spp.*) (Fig. 2C). The deletion of the *sbmA* gene in bacteria did not fully confer resistance towards PrAMPs, but significantly reduced their sensitivity.[24] This is likely due to a decrease but not abolishment of peptide internalization in bacteria in the absence of SbmA (or presence of non-functional SbmA),[24] indicating that SbmA is not the only transporter for uptake of PrAMPs. Indeed, a second transport mechanism for PrAMP uptake was recently discovered, namely, the inner membrane protein MdtM.[38] MdtM is an efflux pump that extrudes antibiotics from the bacterial cytosol.[39,40] Simultaneous deletions of MdtM and SbmA in *E. coli* further decreased the susceptibility of bacteria to some PrAMPs, but not to all of them.[38] For PrAMPs with a dual mode of action, such as Bac7 or the synthetic A3-APO, the lytic mode of action becomes dominant at high concentrations, thus obliterating the advantage that the deletion mutants have over wild-type bacteria.[38]

Interestingly, there is a link between the presence of transporters for PrAMPs in the membrane of a bacterial species and the mode of action of the PrAMP towards a specific microorganism. For example, *Pseudomonas aeruginosa* does not have SbmA, therefore PrAMPs cannot easily reach the cytosol to inhibit bacterial growth by targeting specific intracellular pathways. The antimicrobial effect of Bac7 fragments is indeed lower on *Pseudomonas sp.* if compared with other bacterial species in which an *sbmA* gene is present.[41] Similarly, insect PrAMPs are also less active toward *P. aeruginosa* strains, and studies optimizing apidaecins with improved antimicrobial activity toward this pathogen, ended up selecting for derivatives

with more membranolytic capabilities.[42] However, if the exogenous *E. coli* SbmA is expressed in *P. aeruginosa* PA01, an increase in internalization and antimicrobial activity was observed.[43] On the other hand, the PrAMP becomes less permeabilizing toward the bacterial cell. SbmA seems therefore to drain Bac7 from the membrane, keeping the local concentration lower and thereby as a consequence reducing membrane damage by the PrAMP.[43]

4 Intracellular targets of PrAMPs

Despite the identification of several PrAMPs, the exact target of these peptides remained elusive for a long time and in the beginning it was even unclear whether PrAMPs were lytic, like most other AMPs, or whether they utilize a completely different mode of action. Five years after the initial discovery of apidaecin, permeabilization assays suggested that the PrAMP apidaecin utilizes a non-lytic mechanism[5] and inhibits bacteria by targeting intracellular components.[6] *In vivo* metabolic labeling assays monitoring the incorporation of radioactive methionine indicated that protein synthesis may be the intracellular target of apidaecin,[6] however, these findings were initially not investigated further. The second target suggested for PrAMP interaction were the chaperones of the Hsp70 family.[44] In co-immunoprecipitation assays the DnaK chaperone was shown to co-purify with PrAMPs, such as drosocin, pyrrhocoricin, apidaecin 1a[44] and Bac7(1-35).[45] Subsequent structural studies visualized the interactions of PrAMPs with DnaK and revealed that PrAMPs bind within the same pocket as natural DnaK substrates.[46–48] The hypothesis that DnaK is the primary target for PrAMP action was challenged when studies reported that DnaK-deficient strains still remained susceptible to Bac7(1-35) treatment.[45] Subsequently, similar results were also obtained for the insect PrAMPs oncocin and apidaecin.[49] Thus, the interaction of PrAMPs with DnaK appeared to be a secondary effect, suggesting the existence of another intracellular target for PrAMP action.

To identify the physiological target of PrAMPs, synthetic derivatives of the insect PrAMP oncocin and apidaecin were biotin-labeled and used to "fish" for interactors within bacterial extracts.[49] This led to the identification of ribosomal proteins, suggesting that ribosomes may be the major target of PrAMPs.[49] Consistently, oncocins and apidaecins derivatives were shown to bind to *E. coli* 70S ribosomes and inhibit *E. coli* protein synthesis using *in vitro* transcription/translation assays.[49] A second independent study reached the same conclusion, demonstrating that the mammalian PrAMP Bac7 co-sedimented with bacterial ribosomes, inhibited *in vitro* transcription/translation reactions using bacterial lysates and blocked protein synthesis in living bacteria.[25] Recently, apidaecin was proposed to have a distinct mechanism of action compared to other insect PrAMPs, such as oncocin, namely, by interfering with the assembly of the large ribosomal subunit.[50] However, it remains to be determined whether this is a direct effect on assembly or an indirect effect resulting from inhibition of translation. Nevertheless, competition assays with other translation inhibitors indicate that the apidaecin binding site on the ribosome may differ somewhat from that of oncocin.[50] A distinct mechanism of action for apidaecins compared to oncocins is also supported by differences in the importance of their C- and N-terminal residues, respectively,[6,41,49,51] as discussed in the following section.

5 Structure activity relationships of PrAMPs

The antimicrobial potency of PrAMPs is most effective against Gram-negative bacteria, especially *Enterobacteriaceae* such as *E. coli*, whereas Gram-positive bacteria are generally less susceptible to PrAMPs, presumably due to the absence of specific transporters, such as SbmA.[7] Given the potential of native PrAMPs for development as antibacterial compounds against Gram-negative bacteria, efforts have been made to identify which residues are crucial or dispensable for their inhibitory activity. The best-characterized PrAMP derivatives are those related to the insect oncocins and apidaecins, as well as the bovine Bac7.

The original sequencing analysis of oncocin did not reveal the nature of the residue at position 11 (see Fig. 1A),[13] yet further mutagenesis studies indicated that the antimicrobial efficiency of oncocin derivatives strongly depends on this position.[14] For example, oncocin derivatives containing Pro11 or Thr11 displayed significantly worse MICs (128 μg mL^{-1}) against *E. coli* compared to derivatives with Arg11 (8 μg mL^{-1}). Subsequent removal of Asn18 and addition of an amino group to the C-terminus, coupled with the additional replacement of both Arg15 and Arg19 with either D-arginine or L-ornithine, led to the development of Onc112 and Onc72 derivatives, respectively, both of which displayed increased serum stability without loss in antimicrobial activity against *E. coli* and *Micrococcus luteus* strains.[14,52] Onc72 showed moderate activity against different *E. coli* strains with MICs ranging from 18–44 μg mL^{-1}, whereas Onc112 was more active against *E. coli* in diluted tryptic broth media with MICs of 2.5–6.8 μg mL^{-1}.[53] Alanine-scanning mutagenesis of oncocin revealed that replacement of Tyr6 or Leu7 with Ala led to a 32-fold increase of MIC against *E. coli*,[48] whereas these mutations had little effect on the MIC against *P. aeruginosa*.[54] Onc112 and Onc72 both display potent inhibitory activity in *E. coli in vitro* translation systems.[49] While Onc112 derivatives lacking the last seven C-terminal residues (Onc112Δ7) retained some translation inhibition activity, truncation of an additional two residues (Onc112Δ9) led to complete loss of activity.[55] Both derivatives were unable to inhibit the growth of *E. coli* BL21(DE3) in undiluted LB medium at concentrations up to 383 μg mL^{-1}, while full-length Onc112 inhibited the growth at 60 μg mL^{-1} indicating that the very C-terminus of oncocin is more important for cellular uptake than for ribosome binding and inhibition.[55] An oncocin derivative lacking the first two N-terminal residues (Onc112ΔVD) had reduced capacity to inhibit bacterial growth *in vivo* and protein synthesis *in vitro*,[51] illustrating the significance of N-terminal residues for activity.

Given the increased length (60 aa) of Bac7 compared to insect PrAMPs (<20 aa), structure-activity studies on Bac7 have

so far focussed mainly on analyzing activity of truncated Bac7 derivatives, rather than specific amino acid substitutions.[41] These studies demonstrated that the first 35 N-terminal residues of Bac7 are necessary and sufficient to inhibit bacterial growth with the same efficacy as the full-length native peptide. Bac7(1-35) was shown to display excellent activity (MIC $\leq$ 8.4 μg mL^{-1}) against a range of clinically relevant Gram-negative pathogens, such as *E. coli*, *A. baumannii*, *K. pneumoniae* and *Salmonella enterica*.[41] The Bac7 peptide can be further shortened to encompass only the first 16 N-terminal residues (Bac7(1-16)), sacrificing only partially its antimicrobial potency, whereas further truncation of even one amino acid (Bac7(1-15)) leads to a complete loss of antimicrobial activity.[41] The loss of activity of Bac7(1-15) results from impaired transport into the cytosol, indicating that Bac7(1-16) is the shortest Bac7 PrAMP that is efficiently taken up by bacterial cells.[56] Unlike the C-terminal truncations, removal of the two N-terminal arginine residues of Bac7(1-23) increases the MIC by 8-fold[56] and truncation of the first four N-terminal residues basically inactivated Bac7(1-35).[41] Thus, only the first 16 amino acids of the full 60 of the native Bac7 are crucial for its killing activity. Similarly, N-terminally truncated Bac7(5-35) was shown to have reduced inhibitory activity compared to both Bac7(1-16) and Bac7(1-35) when analyzed using *E. coli in vitro* translation assays.[57] This indicates that the first 16 amino acids of Bac7 are necessary to inhibit bacterial growth, and are also necessary to efficiently block protein synthesis.

The first insights into which apidaecin residues are critical for its inhibitory activity came from a comparative analysis of natural apidaecin-type peptides from a diverse range of insects.[58] Comparison of these peptides revealed a conserved core containing the sequence R/KPxxxPxxPRPPHPRI/L. Deviations from the C-terminal consensus severely reduced the antimicrobial activity of apidaecins, for example, an exchange of penultimate Arg by Ala in hornet apidaecin resulted in an 2500-fold increase in MIC (from 0.01 μg mL^{-1} for the wildtype to 25 μg mL^{-1}).[6] In contrast, substitutions within the middle or N-terminal part of hornet apidaecin produced milder effects.[6] The promising MIC values made apidaecin a potential candidate for the development of new antimicrobial agents, however, apidaecin displayed low stability in mouse serum.[59] In order to improve serum stability, the honey bee apidaecin 1b was modified with an N-terminal tetramethylguanidino-L-ornithine group instead of a glycine, yielding the apidaecin derivative Api137.[59] In addition to increased serum stability, Api137 also exhibited a slightly improved MIC against *E. coli* strains.[59] In accordance with previous studies,[6] the C-terminal of Api137 was shown to be crucial for activity *in vivo*.[49] In the absence of the last C-terminal Leu18 residue, the MIC of Api137 increased by 16-fold (from 4 μg mL^{-1} to 66 μg mL^{-1}),[49] whereas removal of the last two residues (Arg17-Leu18), increased the MIC towards *E. coli* ~140-fold (to 578 μg mL^{-1}). By contrast, mutations within the N-terminal region, for example the Arg4Ala mutation, did not significantly alter the MIC.[49] Thus, unlike oncocin and Bac7 where the N-terminal terminus is critical for antimicrobial activity and the C-terminus is to a large extent dispensable, it is the C-terminus of apidaecins that is important for activity whereas the N-terminus appears to be less critical.

6 Interaction of PrAMPs with the 70S ribosome

The reports that PrAMPs bind to ribosomes and inhibit protein synthesis[25,49] prompted two independent studies to determine structures of the oncocin derivative Onc112 in complex with the bacterial 70S ribosome.[55,60] Subsequently, structures were also reported for the insect PrAMPs pyrrhocoricin (Pyr) and metalnikowin-1 (Met) as well as mammalian Bac7 bound to the ribosome.[51,57] These structures revealed that these PrAMPs all interact with the large (50S) subunit of the ribosome, specifically, binding within the ribosomal exit tunnel (Fig. 3A and B). The binding site of the PrAMPs was visualized within the upper region of the exit tunnel, adjacent to the binding site of a peptidyl-tRNA and overlaps with the path of the nascent polypeptide through the tunnel (Fig. 3C).

Within the tunnel, the PrAMPs adopt an elongated conformation, predominantly consisting of random coil interspersed with stretches of *trans*-polyproline helices (type II). The PrAMPs bind with an opposite orientation compared to a nascent polypeptide chain (for example MifM), namely, with the N-terminus located at the tunnel entry and the C-terminus extending deeper into the tunnel (Fig. 3C). For each of the insect PrAMPs, the C-terminal residues (4 aa, 5 aa and 6 aa of Pyr, Met and Onc112, respectively) were not visualized in the structure (Fig. 3B), suggesting that they are not crucial for stabilizing the interaction with the ribosome.[51,55,57,60] Similarly, while all 16 residues of Bac7(1-16) were observed,[57] only 19 residues of Bac7(1-35) were visible with the C-terminal 16 residues being disordered. Consistently, the native Bac7 is 60 aa long however the C-terminus of Bac7 is less crucial for activity and C-terminal truncated derivatives of native Bac7, such as Bac7(1-16), have been shown to retain activity.[41]

PrAMP interaction with the 70S ribosome is facilitated by a multitude of hydrogen bonds and stacking interactions (Fig. 3D–F).[51,55,57,60] The majority of hydrogen bonds are formed between the peptide backbone of PrAMP with the nucleobases of the 23S rRNA. The high content of *trans*-proline residues within PrAMPs seems to be important for maintaining the elongated structure that maximizes the interaction of the peptide backbone with the surrounding rRNA. For insect PrAMPs, such as Onc112, Pyr and Met, additional hydrogen bond interactions are established by amino acid sidechains within the N-terminus of the PrAMP, specifically, Asp2 (D2) interacts with the nucleobase of G2553 and Lys3 (K3) with the phosphate-oxygen rRNA of A2453 (Fig. 3D). Two conserved stacking interactions are observed for the insect PrAMPs Onc112, Pyr and Met, namely, Arg9 (R9) stacks upon 23S rRNA nucleotide C2610 whereas Tyr6 is stacked between C2452 and the neighboring Leu7/Asp7 sidechain of the PrAMP.[51,55,57,60] These stacking interactions are likely to be important, since exchange of Arg9 by Ala leads to a loss of activity when tested in *P. aeruginosa*,[54] and substitutions of Tyr6 or Leu7 with Ala in

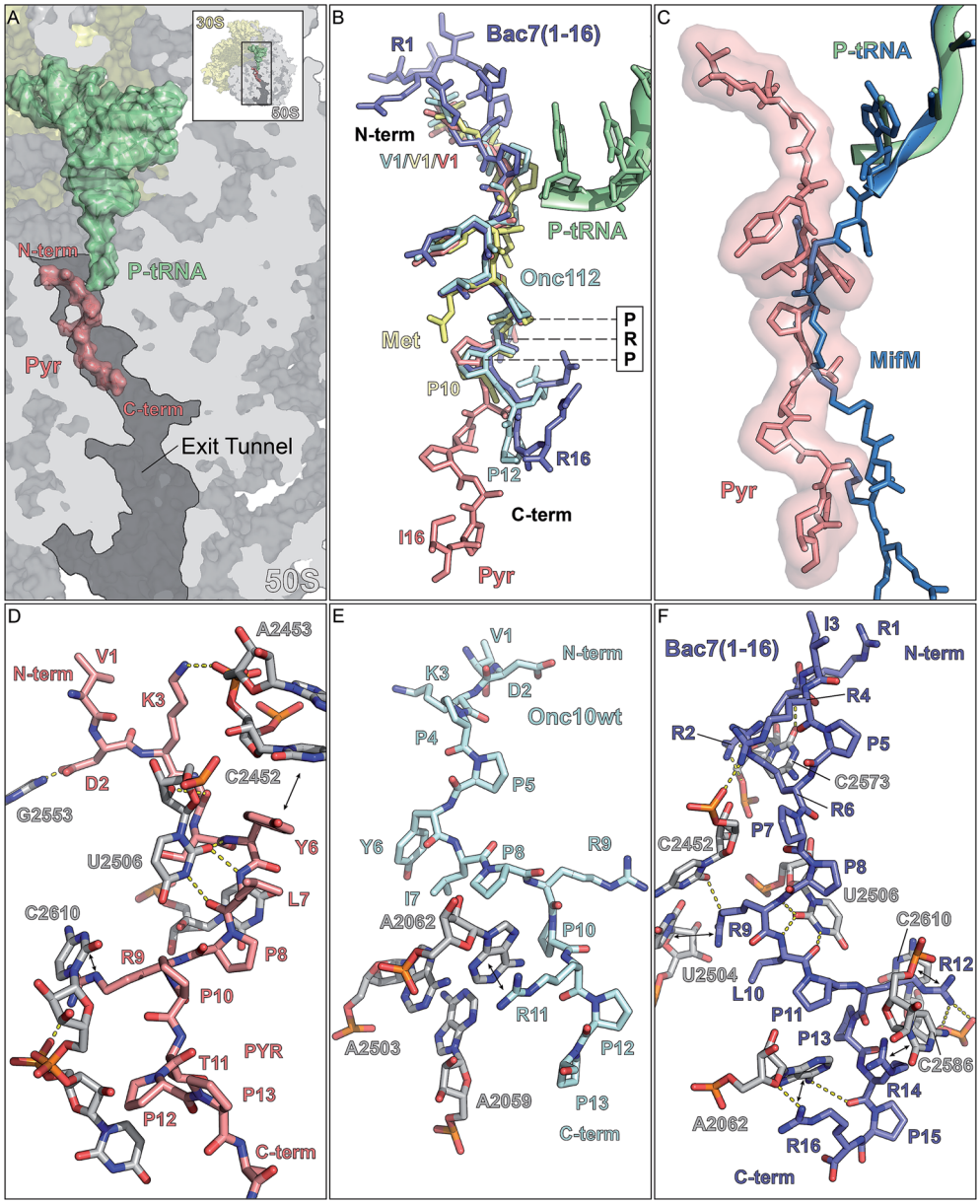

Fig. 3 Binding site of PrAMP within the ribosomal exit tunnel. (A) Overview showing the binding site of pyrrhocoricin (Pyr, salmon) within the exit tunnel of the 50S subunit (grey) with P-site tRNA (green). (B) Superimposition of mammalian Bac7(1-16) (light blue) and insect derived PrAMPs Onc112 (cyan), metalnikowin-1 (Met, yellow) and Pyr (salmon), with the conserved PRP motif highlighted. (C) Binding position of Pyr (salmon) relative to the MifM polypeptide chain (dark blue). (D–F) Interactions of (D) insect Pyr (salmon), (E) Onc112 (cyan) and (F) mammalian Bac7(1-16) with nucleotides situated within the polypeptide exit tunnel. Hydrogen bonds are indicated as dashed yellow lines and stacking interactions with arrows.

oncocin reduce the inhibitory activity in *E. coli* by 32-fold.[48] Mutations within the ribosomal tunnel, namely, A2503C or A2059C, increased resistance against Onc112 by about 4-fold, and the double mutation by more than 15-fold.[51] While neither of these rRNA nucleotides directly contacts Onc112, both residues interact with A2062, which in turn forms stacking interaction with the peptide (Fig. 3E).[51] In addition to revealing the importance of A2062 for Onc112 activity, the mutagenesis data establishes the ribosome as the immediate cellular target of Onc112 and probably other PrAMPs.[51]

Compared to insect PrAMPs, the mammalian Bac7(1-16) contains many more arginine residues. In fact, half (8) of the 16 residues are arginines, which establish multiple hydrogen bonding and stacking interactions with the 23S rRNA (Fig. 3F). The two stacking interactions observed in the insect PrAMPs from Tyr6 and Arg9 have equivalents in Bac7(1-16), namely, Arg9 in Bac7(1-16), which occupies the position of Tyr6, and Arg12, which aligns with Arg9 of insect PrAMPs within the conserved core PRP motif (Fig. 1A and B). This centrally conserved core region is the most structurally conserved region

between PrAMPs, with diverse conformations being observed for the N- and C-terminally flanking regions of the various PrAMPs. Bac7(1-16) establishes another three stacking interactions involving the sidechains of Arg2, Arg14 and Arg16 with 23S rRNA nucleotides C2573, C2586 and A2062, respectively (Fig. 3F).[51,57] The N-terminus of Bac7(1-16) is particularly arginine-rich, comprising Arg1, Arg2, Arg4 and Arg6, which generate a positively charged compacted structure that anchors the N-terminus of the PrAMP to the negatively charged cleft created by the surrounding rRNA. Truncations of four N-terminal residues of Bac7(1-35) inactivated the PrAMP indicating that these interactions are also likely to be critical for Bac7 activity.[41]

7 Mechanism of action of PrAMPs

The outcome of initiation of translation is the presence of the initiator fMet-tRNA interacting with the AUG start codon of the mRNA located within the P-site of the ribosome (Fig. 4A). Translation elongation ensues with the delivery of an aminoacylated tRNA (aa-tRNA) to the ribosomal A-site of the ribosome by the elongation factor EF-Tu (Fig. 4B). Correct recognition of the codon of the mRNA by the anticodon of the aa-tRNA leads to dissociation of EF-Tu from the ribosome and accommodation of the aa-tRNA on the large subunit (Fig. 4C). The binding position of PrAMPs, such as Onc112 and Bac7(1-35), on the ribosome indicates that they would allow delivery of the aa-tRNA by EF-Tu to the ribosome, but would prevent accommodation of the aa-tRNA on the large subunit (Fig. 4D). Specifically, overlapping the structure of an accommodated aa-tRNA shows a steric clash between the aminoacylated CCA-end of the A-tRNA and the N-terminal residues of these PrAMPs (Fig. 4E and F).[51,55,57,60] Bac7 shows the largest extension into the A-site, surpassing Onc112, Pyr (Fig. 4E) and Met by four amino acids at the N-terminus (Fig. 4F).[51,57] This is consistent with the loss of activity of Onc112 derivatives lacking the first two N-terminal residues and the reduced activity and binding affinity of N-terminal truncated Bac7 derivatives.[51,57] In contrast, the N-terminus of the PrAMPs does not significantly overlap with the binding position of the P-tRNA, which is in agreement with biochemical assays demonstrating that these PrAMPs allow binding of the initiator tRNA at the P-site during translation initiation but prevent the transition from initiation into the elongation cycle to occur.[51,55,57] Presumably, once ribosomes are translating, they are immune to the effects of PrAMPs, such as oncocin, since the binding position of PrAMPs within the ribosomal exit tunnel is likely to be incompatible with the presence of a nascent polypeptide chain (Fig. 3C). Thus, PrAMPs are likely to bind to ribosomes following termination of translation when the polypeptide chain has been released from the ribosome. Additionally, PrAMPs could bind during the late stages of ribosome biogenesis when the binding pocket has formed on the large ribosomal particle.

8 Outlook

A structural understanding of how different PrAMPs interact with components of the ribosome provides further insight into which residues of the PrAMPs as well as which interactions are critical for their inhibitory activity. Importantly, the structures also reveal which regions of the PrAMPs are less important and can be further modified to increase stability and solubility as well as establish additional interactions with the ribosome to increase the binding affinity. This latter point may become important since the binding site of PrAMPs overlaps with many known translation inhibitors, such as chloramphenicol, clindamycin and erythromycin (Fig. 4G).[55,60,61] Therefore, it will be important to assess cross-resistance between such antibiotics and PrAMPs, especially since initial reports reveal some ribosomal mutations that confer erythromycin resistance also

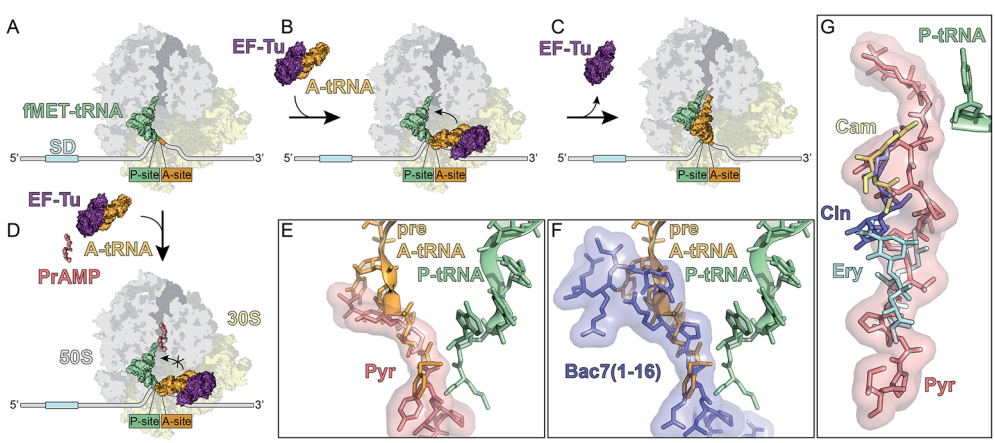

Fig. 4 Inhibition of protein synthesis by PrAMPs. (A–C) Canonical translation in absence of protein synthesis inhibitors, showing (A) translation initiation with initiator P-tRNA (green) bound to the ribosomal P-site. (B) Delivery of aa-tRNA by EF-Tu to the A-site, followed by (C) tRNA accommodation into the A-site on the large subunit and subsequent departure of EF-Tu. (D) In the presence of PrAMPs, such as oncocin, aa-tRNA delivery can occur however the aa-tRNA accommodation is blocked. (E and F) Superimposition of (E) insect Pyr (salmon) and (F) mammalian Bac7(1-16) with accommodated aa-tRNA (bright orange). (G) Superimposition of antibiotics chloramphenicol (Cam; yellow), clindamycin (Cln; slate) and erythromycin (Ery; cyan) with the binding position of the insect PrAMP Pyr (salmon).

reduce PrAMP inhibition.[51] PrAMPs, such as Bac7, also bind to and inhibit translation on eukaryotic ribosomes, albeit less efficiently than on bacterial ribosomes,[57] thus raising issues of toxicity. Fortunately, it seems that most PrAMPs do not penetrate eukaryotic membranes, however, maintaining the non-lytic mechanism of the PrAMPs during optimization will be critical to avoid disrupting the eukaryotic cell membranes. One major concern for development of PrAMPs as an antimicrobial is the ease with which resistance arises in bacteria *via* mutation of the SbmA transporter. Whether these resistant strains can be overcome by the next generation PrAMPs remains to be seen. Lastly, it is unclear as to the full scope of PrAMPs across different species and, in particular, as to the conservation in terms of mechanism of action. Initial indications suggest that PrAMPs such as drosocin and apidaecin may differ from those of well-characterized PrAMPs such as oncocin and Bac7.

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