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Revisiting fifty years of research on pheromone signaling in ciliates

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Abstract

Among protists, pheromones have been identified in a great variety of algal species for their activity in driving gamete-gamete interactions for fertilization. Analogously in ciliates, pheromones have been identified for their activity in inducing the sexual phenomenon of conjugation. Although this identification was pioneered by Kimball more than fifty years ago, an effective isolation and chemical characterization of ciliate pheromones has remained confined to species of *Blepharisma*, *Dileptus* and *Euplotes*. In *Euplotes* species, in which the molecular structures have been determined, pheromones form species-specific families of structurally homologous helical, cysteine-rich, highly-stable proteins. Being structurally homologous, they can bind cells in competition with one another, raising interesting functional analogies with the families of growth factors and cytokines that regulate cell differentiation and development in higher organisms. In addition to inducing conjugation by binding cells in heterologous fashion, *Euplotes* pheromones act also as autocrine growth factors by binding to, and promoting the vegetative reproduction of the same cells from which they originate. This autocrine activity is most likely primary, providing a concrete example of how the original function of a molecule can be obscured during evolution by the acquisition of a new one.

Keywords: Cell-cell communication; Chemical signaling; Conjugation; Pheromones; Protein structure; Self/non-self recognition

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Introduction

The ability of members of a community to communicate via chemical messages has been recognized since ancient times, via popular observations of how strongly the secretions from a bitch in heat attract male dogs, or how promptly bees respond to an injured relative's call to sting. However, modern research on chemical communication practically dates to Karlson and Luscher's (1959) definition of pheromones as "substances that are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behavior or a developmental process", and the concurrent chemical characterization by Butenandt et al. (1959) of the first pheromone represented by 'bombykol' (the sex pheromone of the silk moth). In the recent past, substantial improvements in analytic technologies have greatly contributed to the identification of a remarkable variety of chemical compounds that act as pheromones, usually at very low (picomolar) concentrations, from small molecules that are rapidly dispersed to speed inter-individual communication─signaling alarm, aggression, or aggregation─to high-molecular-weight molecules that tend to function in sexual stimulation and attraction.

For the most, pheromone molecules have been characterized by their structure and activity in bacteria (Dunny and Leonard 1997; Kleerebezem and Quadri 2001) and multi-cellular organisms, animals (Shorey 2013; Wyatt 2014) and fungi (Jones and Bennet 2011; O'Day and Horgen 1981) in particular. Only a minority are known from protists, which require adaptation to grow in stable cultures necessary to arrange appropriate bioassays for pheromone detection. Algal pheromones isolated from Chlorophyta (*Chlamydomonas*, *Volvox*, *Ulva*), Charophyta (*Closterium*), Heterokontophyta (*Ectocarpus*), and Diatomea (*Pseudostaurosira*, *Seminavis*) are extensively reviewed in Frenkel et al. (2014). Here, we revisit and update a fifty-year story of ciliate pheromones.

The pioneering role of Kimball and *E. patella* in the identification of ciliate pheromones

In elevating *Paramecium* to the role of prototypical experimental model in research on the biology of the mating type mechanism that controls conjugation in ciliates, the report by Sonneborn (1937) on the discovery of "Sex, sex inheritance and sex determination in *Paramecium aurelia*" has largely overshadowed a near contemporary study by Kimball (1942) on "The nature and inheritance of mating types in *Euplotes patella*" warranting the conclusion that "A comparison of the mating type determination in *Euplotes* with that found in *Paramecium* reveals no very close similarities."

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On more theoretical grounds, Kimball first noted that the multiplicity of mating types and the behavior of heterozygous mating types as the "simple combination of two homozygous mating types" made the *Euplotes* mating-type determination "quite like those of blood groups in man and self-sterility in flowering plants", rather than like the *Paramecium* sex determination described by Sonneborn. Then, on more experimental grounds, Kimball also observed that *Euplotes* differs from *Paramecium* in "the method of action of the [mating] types in bringing about conjugation", and it is precisely in relation to this difference that his pioneering role in identifying ciliate pheromones was established.

Considering that "in *Paramecium* conjugation is usually found only between animals of different clones, while *Euplotes* conjugation can take place between animals of the same clone" (Fig. 1), Kimball verified whether these intra-clonal mating pairs between genetically identical partners could be induced by suspending clonal cell cultures with cell-free filtrates from other cultures. Not only did these 'homotypic' pairs form, but they also appeared to be as fully viable as the 'heterotypic' ones between genetically diverse partners. With this successful mating-induction assay, Kimball thus provided evidence that, unlike *Paramecium* in which "the [cell-free] fluid from one mating type does not appear to induce conjugation among animals of another mating type", in *Euplotes* "each mating type allele is responsible for the production by the animal of a particular sort of conjugation-inducing substance which gets into the fluid." Kimball could say "little about the nature of these conjugation-inducing substances", that we now usually describe as pheromones. Nevertheless, he insightfully anticipated it to be "highly probable that [their] differences are not simply quantitative but are qualitative", and we now know that also this anticipation is fully correct.

Pheromone-secreting ciliates

Following Kimball's pheromone identification in *E. patella* through successful mating induction assays, diffusible pheromones were then promptly detected in culture filtrates of *Blepharisma* and *Dileptus*, as well as in those other species of *Euplotes.* However, not all ciliates can be induced to form homotypic mating pairs and, hence, are equally ready to reveal their pheromones in solution through a positive response to a mating induction assay. The knowledge of other pheromone-secreting ciliates is also the result of observations unrelated to mating induction, yet directed to detecting context-specific changes in cell morphology and behavior. In *Ephelota gemmipara* (Grell 1953), *Tokophrya infusionum* and *T. lemnarum* (Sonneborn 1978), cell-type specific pheromones in solution are believed to account for the development of pseudopodium-like projections that cells of

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different mating types, grown in proximity of one another, directly orient toward their prospective mate. In *Oxytricha bifaria* (Esposito et al. 1976), they have been inferred from observing that mating pairs form in mixtures between cultures previously incubated in chambers connected via micro-pore filters with a significantly shorter time-lag ('waiting period') than in mixtures between cultures that are not pre-incubated. Pheromone secretion thus appears to be a distinctive trait of the biology of a relatively small number of species. However, the fact that the known pheromone-secreting species belong to genera which radiate into distinct clades of ciliate phylogeny provides a clear indication that pheromone secretion in ciliates is likely a more diffused phenomenon than is commonly assumed.

***Blepharisma* pheromones and their chemical diversity**

The successful isolation and structural characterization of ciliate pheromones was first obtained by Miyake and collaborators in *B. japonicum* (as reviewed in Miyake 1981). As is common among heterotrichous ciliates, this species also systematically manifests spontaneous intra-clonal conjugation (selfing), implying that it lacks a system of genetically determined mating types to control the switching between the growth and mating (sexual) stages of its life cycle (Isquith and Hirshfield 1968). This notwithstanding, non-selfing and cross-mating reactive cell lines of *B. japonicum* were temporarily obtained starting from un-paired cells cloned from selfing cultures (Chunosoff et al. 1965; Isquith and Hirshfield 1968), and two of these lines (one formed by red cells containing the pigment blepharismin and one formed by albino cells devoid of pigment) were elected to represent two "complementary" mating types, I and II, and subsequently used as source of the *B. japonicum* pheromones (Miyake 1968).

Two structurally unrelated molecules have been characterized from these two *B. japonicum* mating types and shown to induce behavioral changes, chemo-attraction in particular, in addition to mating (Miyake 1981; Sugiura et al. 2010). The one isolated from the type-I cells and inducing type-II cell mating is a highly-unstable glycoprotein, named Gamone 1 or Blepharmone. It consists of a sequence of 272 amino acids plus six covalently linked sugars (Sugiura and Harumoto 2001), and its synthesis has been reported to be conditioned by environmental and developmental factors (Sugiura et al. 2005). The second molecule, isolated from type-II cells and inducing type-I cell mating, is a very stable tryptophan derivative, namely a calcium-3-(2'-formylamino-5'-hydroxybenzoil) lactate, known as Gamone 2 or Blepharismone, whose fully active racemic form has also been produced via chemical synthesis (Entzeroth and Jaenicke 1981). While Gamone 1

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manifests a close species-specificity with no cross-activity, Gamone 2 has been shown to be structurally identical and active in inducing mating among several distinct morpho-species of *Blepharisma*, such as *B. japonicum*, *B. stoltei* and *B. undulans* (Kobayashi et al. 2015; Miyake and Bleyman 1976). This Gamone 2 cross-activity clearly casts doubt on the genetic separation of these species and complicates any rationalization of the evolution of mating types in *Blepharisma* (Miyake 1996), unless crediting the original Isquith and Hirshfield (1968) proposition that "*B. japonicum* mating types arose in the laboratory and probably would have not survived in nature" and, consistently, that the trigger of *Blepharisma* mating likely resides in binding interactions between the hapten-like non-species-specific tryptophan-derivative Gamone 2 and the species-specific glycoprotein Gamone 1 (Luporini and Miceli 1986).

***Dileptus* pheromones**

Pheromones were identified in culture filtrates of three interbreeding cell types of *D. anser* long time ago (Nikolayeva, 1968). However, a full appreciation of this identification has suffered from descriptions mostly reported in Russian journals that are not readily available abroad. In retrieving 50 years of research on *D. anser* at the Institute of Cytology in St. Petersburg, Uspenskaya and Yudin (2016) have now meritoriously made more accessible their story. Pheromone secretion in *D. anser* has been revealed by observing in addition to mating and chemo-attraction in cells that are suspended with heterologous culture filtrates (Afon'kin and Yudin 1987), also a peculiar mitogenic effect that cells manifest by undertaking supplementary pre-conjugal binary fissions (Tavrovskaya 1974). Although the isolation of the three cell type-specific pheromones of *D. anser* has been only partially successful, evidence has been provided that they are high-thermostable proteins with a presumed mass of 3–4.5 kDa and a strong propensity to associate into unstable oligomers and complexes with a chromophore unit (Parfenova et al. 1989).

***Euplotes* pheromones**

Although *E. patella* pheromones were the first to be identified, their isolation has been sought only partially (Akada 1986). In pushing forward research on the isolation and structural determination of *Euplotes* pheromones, the primary role has been played by other *Euplotes* species, in primis *E. raikovi* and *E. octocarinatus*. The former, originally described from a Caspian Sea sample by Agamaliev (1966), is a widely distributed marine species that has been grown in captivity, starting with a group of interbreeding strains collected from a single location on the Adriatic coasts of Italy (Miceli et al. 1981). The latter, a lacustrine species whose distribution is

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mainly limited to North America, has been cultivated starting with two interbreeding strains collected from an aquarium (Möllenbeck and Heckmann 1999). Analyzed for the mating-induction activity of their cell-free filtrates and their Mendelian patterns of mating-type inheritance, both species revealed a full equivalence with Kimball's *E. patella* system with regard to pheromone secretion and the adoption of co-dominant sets of *mat* alleles to regulate their mating-type inheritance (Luporini et al. 1983; Weischer et al. 1985). However, *E. raikovi* and *E. octocarinatus* show profound differences in the amount of secreted pheromones, the former making it possible to prepare up to 200 μg of homogeneous protein from one liter of cell-free filtrate (Raffioni et al. 1992) and the latter no more than 0.5 μg (Schulze Diekhoff et al. 1987).

Given these quantitative limits in the protein purification from cell-free filtrates, the 85–109 amino acid sequences of nine *E. octocarinatus* pheromones could be determined only through sequencing of the relevant coding genes (Fig. 2) (Brünen-Nieveler et al. 1991, 1998; Meyer et al. 1991, 1992; Möllenbeck and Heckmann 1999). On the other hand, the un-matched secreting capacity of *E. raikovi* has decisively facilitated direct chemical pheromone sequencing and, more important, the determination of the pheromone three-dimensional structures using NMR spectroscopy and X-ray crystallography.

Pheromone structures in *E. raikovi*

In all, nine unique *E. raikovi* pheromone amino acid sequences (designated E*r*-1, E*r*-2, E*r*-7, E*r*-10, E*r*-11, E*r*-20, E*r*-21, E*r*-22 and E*r*-23) have been characterized from distinct cell types manifesting varied degrees of mutual mating compatibility (Table 1), a typical feature of the ciliate high-multiple mating systems containing only genetically partially isolated populations (Valbonesi et al. 1992). These pheromones constitute a homologous family of acidic proteins (isoelectric points in the range 3.3–4) of 37 to 40 amino acids (51 in pheromone E*r*-23) with six cysteines (10 in E*r*-23) paired into intra-chain disulfide bonds whose arrangement is strictly conserved even when all the residues surrounding the cysteines are replaced, as is the case of the pheromones E*r*-1 and E*r*-2 (Fig. 3A) (Luginbühl et al. 1994; Stewart et al. 1992). The percentages of sequence identity are highly variable, with no apparent direct relationship with the affinities/divergences that exist in the mating interactions of cells which are the pheromone source (Raffioni et al. 1992). For example, much stronger mating compatibility exists between the two cell types which secrete E*r*- 1 and E*r*-2 with a sequence identity of only 25%, than between the cell types secreting E*r*-11 and E*r*-20 that have 59% sequence identity. Differently from the sequence variability of the secreted pheromones,

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the sequences of the signal-peptide and, to a lesser extent, of the pro-segment of the pheromone precursors are far better conserved due to their common function in the pheromone secretory pathway (Miceli et al. 1991).

Regardless of the variations in the amino acid sequence, all the *E. raikovi* pheromones take a common structural fold. This implies that, differently from the striking chemical un-relatedness between the glycoprotein and tryptophan-derivative gamones of *Blepharisma*, they can structurally compete with one another for receptor binding, as occurs in higher organisms among growth factors and cytokines members of the same protein family. The adoption of a common fold has been documented by determining the NMR solution structures of a significant number of pheromones, E*r*-1, E*r*-2, E*r*-10, E*r*-11, E*r*-22 and E*r*-23 (Brown et al. 1993; Liu et al. 2001; Luginbühl et al. 1994; Mronga et al. 1994; Ottiger et al. 1994; Zahn et al. 2001). As shown in Fig. 3B, the fold consists of a bundle of three right-handed helices, with the first and third consistently in regular α-conformation and the second varying between a regular and a 3_{10} conformation. The helices run almost parallel with an up-down-up orientation and are closely held together by the disulfide bridges. Although very similar in the fold, each pheromone possesses its own structural uniqueness, apparent even from the simple comparison of the molecular backbone alone, and provides the rationale for different pheromone specificities in the receptor binding reactions. Three main distinctive features have been regarded as functionally significant: (i) the cleft that runs down into the bottom of the molecular surface and contains an asymmetric distribution of charged and non-polar residues; (ii) the bulging loop that leads from the second to the third helix and includes between one and four residues; and (iii) the carboxyl terminal 'tail' that extends from the end of the third helix. This tail varies markedly in length and orientation, providing the most evident hallmark of each pheromone structure. Thus, two pheromones (such as E*r*-11 and E*r*-22) that markedly differ in their amino acid sequence may mimic each other in one or more domains of the molecular structure and, vice versa, two pheromones (such as E*r*-1 and E*r*-10) that have quite similar sequences may diversify markedly in the same domains, providing the rationale for variable specificities of each pheromone-receptor association.

Among the E*r* pheromones of known structure, the E*r*-23 structure represents a unique case and is probably the result of phenomena of DNA sequence rearrangement of its coding gene (Di Giuseppe et al. 2002). Although secreted from a cell type that is mating compatible with other, but not all *E. raikovi* cell types (see Table 1), its structure deviates considerably from that shared by the other family members. It includes a unique central 11-residue segment and four additional Cys

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residues, which result in the formation of two additional disulfide bonds and two additional short helical structures (Zahn et al. 2001). Despite these multiple modifications, that make the E*r*-23 fold unique in a general classification of small disulfide-rich domains (Cheek et al. 2006), mapping the conservation of functional surface-exposed regions reveals that also this structurally eccentric pheromone maintains a pronounced surface cluster of 8–10 residues for receptor binding in common with all the others (Fig. 4).

Pheromone structures in *E. nobilii*

The second pheromone family that has also been structurally well characterized by NMR spectroscopy belongs to *E. nobilii*, which secretes its pheromones in amounts that are 5–10 folds lower than in *E. raikovi* (Alimenti et al. 2009; Di Giuseppe et al. 2011; Pedrini et al. 2007; Placzek et al. 2007). This species is phylogenetically closely allied to *E. raikovi*, yet distinctly separate from it ecologically, as it lives in the freezing marine waters of the Arctic and Antarctic (Di Giuseppe et al. 2011). It thus provides the twofold opportunity of inspecting the structural diversifications evolved in pheromone families of akin species, and of seeking the structural modifications that adaptive evolution imposes on cold-adapted pheromones and psychrophilic proteins in general.

Compared to the *E. raikovi* pheromones, the *E. nobilii* pheromones (E*n*-1, E*n*-2 and *En*-6 of Antarctic origin, and E*n*-A1, E*n*-A2, En-A3 and E*n*-A4 of Arctic origin) have 52–63 amino acid sequences that include eight instead of six Cys residues (Fig. 5A). However, despite including an additional disulfide bond, they unfold at temperatures in the range of 55–70 °C whereas the *E. raikovi* pheromones maintain their regular secondary structures up to 95 °C (Geralt et al. 2013), which implies that the reason for these inter-specific difference in thermo-stability does not reside in the relative density of the disulfide bridges, but rather in the specific spatial arrangement of one or more of these bridges (Cazzolli et al. 2014).

The structural fold of the *E. nobilii* pheromones (Fig. 5B) overlaps extensively with the up-down-up three-helix fold of the *E. raikovi* pheromones (Fig. 6), and this structural overlapping between pheromones of different species well accounts for why inter-species mating reactions are so frequently observed in *Euplotes* (Alimenti et al. 2011; Kuhlmann and Sato 1993; Nobili et al. 1975). However, although sharing the same molecular fold core, *E. nobilii* pheromones present distinctive structural traits that are clearly functional to their cold-adaptation, in particular improving the flexibility of their molecular backbone by favoring the extension of regions devoid of rigid secondary structures. This extension results from the shortening of the three helices, the

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lengthening of the inter-helix loops, and the differentiation of a largely unstructured 14–17-residue amino-terminal segment which contains only a 3_{10} helical turn as a secondary structure and exposes a large cluster of negatively charged residues that serve to improve the pheromone solubility in the cold by enhancing solvent interactions.

Hints on the evolution of *Euplotes* pheromone structure

Initial information on the evolution of the *Euplotes* pheromone structure has been recently provided by the pheromone isolation and characterization from *E. petzi* and *E. crassus*, species lying in clades of the *Euplotes* phylogenetic tree. These are clearly distinct from the *E. raikovi* and *E. nobilii* clade, as well as from the clade including *E. octocarinatus* along with other freshwater species (Di Giuseppe et al. 2014; Fotedar et al, 2016). Together with *E. sinicus*, *E. petzi*, which lives in sympatry with *E. nobilii* in the polar waters, forms the earliest branch of the tree. On the other hand, *E. crassus* and its sister species, *E. minuta* and *E. vannus*, branch off much later.

Four pheromones have been characterized for their unique sequences of only 32 amino acids with eight cysteines from two inter-breeding Antarctic strains of *E. petzi*, and for one of them, designated E*p*-1 and produced in more abundance, it has finally been possible to determine the NMR solution structure (Pedrini et al., manuscript submitted). This structure is unique due to the presence of only two short α-helices (Fig. 7). However, despite its uniqueness it shows the same up-down-up fold and disulfide bond pattern as the *E. raikovi* and *E. nobilii* pheromones, of which it clearly resembles a smaller and simpler structural precursor. Not only are its two α-helices topologically equivalent to the second and third helices of the *E. raikovi* and *E. nobilii* pheromones, but its amino terminal region also includes a four-residue turn that reminds the first helix in the structure of these pheromones.

With regard to *E. crassus* pheromones, the systematic failure to obtain a mating inducing effect from cell culture filtrates has for long time been taken as evidence that these pheromones are membrane-bound proteins, as such hard to extract and characterize. Based on the common occurrence of inter-specific mating interactions in *Euplotes* (Kuhlmann and Sato 1993; Nobili et al. 1978), it was finally possible to overcome this conviction by assaying the mating induction activity of *E. crassus* culture filtrates not on other *E. crassus* cultures, but on *E. raikovi* cultures which are particularly incline to being induced to mate in homotypic fashion (Alimenti et al. 2011). Two interbreeding *E. crassus* strains, one previously known to be homozygous and the other

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heterozygous at the mating-type locus, were identified as a source of systematically mating-active culture-filtrates on *E. raikovi* cultures. These filtrates were then used for the *E. crassus* pheromone isolation.

Independently of their homo- or heterozygous condition, both strains were similarly expected to secrete a single pheromone. Indeed, a consolidated notion, based exclusively on Mendelian analyses of mating-type inheritance, maintains that the alleles at the mating-type locus of *E. crassus*, as well as its sister species *E. minuta* and *E. vannus*, are regulated by relationships of hierarchical (or serial) dominance (Heckmann 1964; Nobili et al. 1978), not of co-dominance as in the *E. patella* pattern. Differently from expectations, two pheromones were instead identified from the homozygous strain and three from the heterozygous one, thus raising two implications: first, that the *E. crassus* mating-type locus underwent a phenomenon of gene duplication and, second, that the alleles at this locus are expressed with relationships of co-dominance as in *E. patella* and the other *Euplotes* pheromone-secreting species, not of hierarchical dominance (Vallesi et al. 2014).

From the amino acid sequence determination of these five pheromones it turned out that one of them, designated as E*c*-α, in addition to being identical across the two strains, was markedly different from the other three structurally distinct pheromones, designated E*c*-1, E*c*-2 and E*c*-3, primarily because of the inclusion of two presumptive amyloidogenic motifs and of an extended, presumably random coiled inter-cysteine glycine-rich domain of 18 residues (Fig. 8). Altogether these structural specificities would account for a distinctive strong propensity of the E*c*-α pheromone to oligomerize and behave like an adaptor, or a scaffold protein that associates with various degrees of affinity with the other cell type-specific pheromones to mediate their receptor binding reactions (unpublished observations).

Pheromone activity

For many years our knowledge of the ciliate pheromone structures stood anchored to the two chemically unrelated pheromones of *B. japonicum*. Based on this knowledge and the view of the functional equivalence of ciliate mating types with sexes, it was assumed that the only target of pheromone binding are membrane receptors on cells which are not the same as those from which the pheromones are secreted (Miyake 1981). The pheromone interactions between ‘complementary mating types’ would activate a mutual cell-cell stimulation committed to enhancing pheromone production and acquiring competence in uniting in mating pairs. However, a number of

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observations on the physiology of the pheromone secretion in *E. raikovi* (Luporini and Miceli 1986) and *E. octocarinatus* (Kusch and Heckmann 1988) were not in line with this so-called 'gamone-receptor hypothesis'. Varying their pheromone secretion rates in direct relation to the environmental concentrations of the secreted pheromone, *Euplotes* cells revealed to be able to 'sense' these concentrations, and the higher the environmental concentrations of the secreted pheromone the higher the concentrations of the non-self pheromones required to elicit a successful mating induction. In addition, pheromone secretion was observed not to be an exclusive property of the cells which are able to mate, as it initiates from the very beginning of the life cycle when cells do not have this competence (Vallesi et al. 1995).

These observations have thus suggested to consider *Euplotes* pheromones as signaling molecules that have evolved to play a dual role (Luporini and Miceli 1986; Vallesi et al. 1995). The first and most likely primary 'self' one is directed to promoting the reproduction, or vegetative growth of the same cells from which pheromones are secreted and to which they continuously bind in autocrine (autologous) fashion. The second one, probably secondarily acquired and of 'non-self' nature, is directed to inducing cell mating through a paracrine (heterologous) binding.

Fundamental support for this 'self/non-self recognition' hypothesis was derived from the finding that the *E. raikovi* pheromone genes, in addition to specifying the soluble pheromones, through a splicing mechanism also specify longer pheromone isoforms which use the un-cleaved signal peptide of the pheromone cytoplasmic precursor to remain anchored to the cell surface (Miceli et al. 1992). As is typical for type-II membrane proteins, the carboxyl-terminal region of these isoforms is directed toward the cell outside, where it forms an extracellular ligand-binding domain that is structurally identical to the secreted pheromone because of the common origin from the same gene. The result is that the mating-type specificity of each *E. raikovi* cell is thus determined not only by the secreted pheromone, but by the secreted pheromone and its membrane-bound isoform that functions as binding site for the soluble form.

Initial evidence that these membrane-bound pheromone isoforms can bind the soluble forms through their extracellular domain and undergo oligomerization, was provided by cross-linking experiments (Ortenzi et al. 1990; 2000) and mass spectrometric analyses of native pheromone preparations (Bradshaw et al. 1990). Taking the case of the E*r*-1 pheromone as an example, the major mass-spectroscopic peak with an isotopically averaged mass of 4410.2, which is close to the molecular weight of 4411.0 calculated from the amino acid sequence, was systematically

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accompanied by a minor peak at m/z 8819 compatible with a homodimeric structure in which the subunits are bound by non-covalent interactions.

More compelling evidence was then derived from the definition of the pattern of pheromone association in crystals (Weiss et al. 1995), recently revisited at a resolution of 0.8 Å (Finke and Marsh, personal communication). These analyses reveal that the pheromone molecules associate cooperatively in the crystal lattice, and that this cooperative association is the result of the formation of two distinct types of dimer which involve intermolecular associations between all three helices and, hence, of all three faces of each molecule (Fig. 9). Dimer 1 is a symmetrical four-bundle structure in which the two molecules are related by a two-fold rotation axis, while dimer 2 forms essentially by stacking the third helix from both monomers in an anti-parallel fashion and involves two units related by a two-fold screw axis.

Both dimers 1 and 2 are predicted to form also in solution, but they are unstable because of their small dimerization energies. Simplifying an analysis of the energetics of dimerization (Weiss et al. 1995), it appears that each molecule involves less than 600 Å^2 of its total accessible area of 3000 Å^2 in the formation of both dimers, whereas 600 Å^2 are the minimal surface area that is required for the formation of a stable oligomer. Oligomerization can however be stabilized by starting with the immobilization of one molecule with respect to another as happens in the crystal, and may happen on the cell surface between partially immobilized membrane-bound pheromone isoforms and soluble pheromone forms. In this case, each molecule contributes with half (1510 Å^2) of its surface to oligomerization and, hence, much more than the required minimum of 600 Å^2.

While the protein-protein complexes that pheromones form with their twin membrane-bound isoforms have been observed to undergo internalization via endocytotic vesicles in growing cells, the complexes between pheromones and not-twin membrane-bound isoforms remain on the surface of cells induced to mate, implying that they may be directly involved in establishing the cell-cell adhesions in mating pairs (Vallesi et al. 2005). Support for this implication derives from considering that the forces for the protein-protein oligomerization would in this case be contributed from both partner cells of a mating pair.

Conclusions

Ciliates rely on the evolution of genetically determined mating types to perceive inter-individual genetic differences through the activity of signaling molecules showing a protein nature, except for

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a tryptophan-derivative signal unique to *Blepharisma*. In the most familiar of the experimental ciliates, *Paramecium* and *Tetrahymena*, these signaling proteins are retained permanently bound to the cell membranes, preventing any attempt of isolation and direct chemical analysis. Only recently, their amino acid sequences could be established from the determination of their DNA coding sequences (Cervantes et al. 2013; Singh et al. 2014). Other ciliates such as *Blepharisma*, *Dileptus* and *Euplotes* release their signaling molecules into the environment as diffusible pheromones, and this has been the key for their isolation and subsequent structural characterization.

In *Euplotes*, the determination of the three-dimensional structure of a variety of pheromones from different species has made it possible to establish that these molecules form species-specific and highly polymorphic families of structurally homologous proteins, that share the same basic molecular architecture and, at the same time, differ from each other at level of local domains and conformations. This structural homology brings strong evidence that pheromones from different cell types can compete with one another to bind to each other's cell receptors, as is the case of cytokine and growth factor signaling networks in multi-cellular organisms, eliciting a cell mating response in the case of paracrine (heterologous) binding, or a cell growth response in case of autocrine (autologous) binding.

Through the activity of the same gene, *Euplotes* cells synthesize their pheromones jointly with membrane-bound pheromone isoforms that function as binding sites of the soluble forms. In addition to fulfilling the basic criterion of any autocrine loop (i.e., that a cell must have a receptor for the specific signal that it releases), this mechanism also suggests a simple explanation for the generation of a new cell type in the open mating systems typical of *Euplotes*. Indeed, a nucleotide mutation in the pheromone gene coding sequence would be sufficient to endow a cell with both the basic units (pheromone and receptor) necessary to effectively interact with other mating types.

The three-helix molecular fold of the *Euplotes* pheromones finds relevant similarities in a variety of other helical and disulfide-rich signaling proteins, such as gastropod attractins, sea anemone peptide toxins, anaphylotoxins and others, produced by multicellular organisms (Cheek et al. 2006). These similarities well account for the intriguing capability of some*Euplotes* pheromones to cross-react with very distantly related cell systems such as mammalian lymphocyte cell lines (Vallesi et al. 1998; Cervia et al., 2013). Although these cross-reactions most likely reflect fortuitous cases of structural convergence, their occurrence implies the early evolution of cell-cell

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signaling mechanisms via water-borne pheromones, and stimulates further structural investigations on ciliate pheromones in view of possible applied purposes.

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Figure legends

Fig. 1. Light microscopy image of homotypic and heterotypic pairs of *E. raikovi*, as they appear in a mixture between cells deprived of food immediately before mixing (looking darker) and cells deprived of food one day before mixing (looking lighter). Scale bar corresponds to 20 μm.

Fig. 2. The *E. octocarinatus* pheromone family. Alignment of the amino acid sequences of eight structurally unique pheromones (designations as in their original descriptions), optimized by deliberate gap insertions. The sequence of pheromone Phr4 is reported separately because of its marked structural difference from all the other sequences. The Cys residues are shadowed and labeled by Roman numerals progressing from the amino-terminus, and sequence positions conserved in all or more than half sequences are marked by filled and empty circles, respectively.

Fig. 3. The *E. raikovi* pheromone family. (A) Alignment of the amino acid sequences of eight structurally unique pheromones, optimized by deliberate gap insertions. The sequence of pheromone E*r*-23 is reported separately because of its marked structural difference from all the other sequences. The Cys residues are shadowed and labeled by Roman numerals progressing from the amino-terminus, and the lines above and below the sequences indicate the disulfide bride pairings. In sequences of pheromones with known three-dimensional conformation, the boxes include the segments with helical structure. Sequence positions conserved in all or more than half sequences are marked by filled and empty circles, respectively. (B) Representative conformers of the NMR structures of six pheromones, each showing the common motif of three α-helices, which were identified using the program MOLMOL (Koradi et al. 1996) and are presented as cylinders numbered progressively from the amino (N) to the carboxyl chain end (C). The two additional helical motifs unique to pheromone E*r*-23 are not numbered. Protein Data Bank (PDB) entries: E*r*-1, 1ERC; E*r*-2, 1ERD; E*r*-10, 1ERP; E*r*-11, 1ERY; E*r*-22, 1HD6; E*r*-23, 1HA8.

Fig. 4. Comparison of the molecular structures and surfaces between the pheromone E*r*-22 (top) representing the *E. raikovi* pheromone family and the structurally eccentric pheromone E*r*-23 (bottom). On the left, the two molecules are in ribbon presentation, with sticks and spheres representing bonds and atoms involved in the disulfide bridges. The three topologically equivalent α-helices are numbered progressively from the amino (N) to the carboxyl chain end (C). The two additional helical motifs unique to E*r*-23 are not numbered. On the right, the two molecules are in the same orientation as on the left and show their molecular surfaces, with the shading encoding

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from maximal conservation (dark grey) to maximal variability (light gray), as calculated according to the parameters provided by Glaser et al. (2003) and visualized with the Swiss-Pdb Viewer program, <swissmodel.expasy.org>.

Fig. 5. The *E. nobilii* pheromone family. (A) Alignment of the amino acid sequences of seven pheromones, optimized by deliberate gap insertions. The first three pheromones are of Antarctic origin, while the last four are of Arctic origin. The Cys residues are shadowed and labeled by Roman numerals progressing from the amino-terminus. The lines above the sequences indicate the disulfide bridge pairings. In sequences of pheromones with known three-dimensional conformation, the boxes include the segments with helical structure. Sequence positions conserved in all, or more than half of the sequences are marked by filled and empty circles, respectively. (B) Representative conformers of the NMR structures of four pheromones showing the common motif of four α-helices identified using the program MOLMOL (Koradi et al. 1996) and presented as cylinders. The three core helices topologically equivalent to those of the *E. raikovi* pheromones are numbered progressing from the amino (N) to the carboxyl chain end (C). The fourth helix (located in the N-terminal region and with a 3_{10} conformation) unique to the *E. nobilii* pheromones is not numbered. PDB entries: E*n*-1, 2NSV; E*n*-2, 2NSW; E*n*-6, 2JMS; E*n*-A1, 2KK2.

Fig. 6. Superposition for minimal root-mean-square deviation of the C^{α} atom positions in the three core helices h1, h2 and h3 between the backbones of pheromones E*r*-1 (dark gray) and E*n*-6 (light gray), taken as representatives of the *E. raikovi* and *E. nobilii* pheromone families, respectively. The amino and carboxyl chain ends of the two molecules are identified with N and C, respectively.

Fig. 7. Comparison of the NMR structures of the *E. petzi* pheromone E*p*-1 and a pheromone (E*r*-1) representative of the *E. raikovi* pheromone family. (A) Amino acid sequence of pheromone E*p*-1 (of Antarctic origin). The Cys residues are shadowed and identified by Roman numerals progressing from the amino-terminus. The lines above the sequence indicate the disulfide bridge pairings, and the boxes include the segments with helical structure. (B) Representative conformer of the NMR structure of pheromone E*p*-1 characterized by two α-helices identified using the program MOLMOL (Koradi et al. 1996) and presented as cylinders numbered progressively from the amino (N) to the carboxyl chain end (C). The bracket marks a four-residue segment (Ser_4-Glu-Cys-Ala_7) that looks like a topological counterpart of the first helix (h1) in the E*r*-1 structure, but does not exhibit a regular α-helical conformation. PDB entry: 2N2S.

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Fig. 8. The *E. crassus* pheromone family. In the alignment of the amino acid sequences of three cell-type specific pheromones, dots mark the fully conserved positions. The E*c*-α pheromone sequence, which is identical among different cell types (see text), is reported separately. In all sequences, the Cys residues are shadowed and identified by Roman numerals progressing from the amino-terminus. The boxes highlight potential amyloidogenic domains, and a line below the E*c*-α sequence marks an inter-cysteine 18-residue segment which is unique to this sequence and rich in Gly residues.

Fig. 9. Crystal structure of the *E. raikovi* pheromone E*r*-1. (A) Crystallographic *xy* plane showing the extensive intermolecular helix-helix interactions that cooperatively arrange E*r*-1 molecules (represented in their backbone structure) into two dimer types, 1 and 2. The axes of the two-fold rotation of dimers of type 1 and two-fold screw rotation of dimers of type 2 are represented by full and half arrows, respectively, at the top of the panel. Half of the molecules, shown as thicker wires representing the backbone and sketched as filled triangles in the inset, have their amino chain end oriented towards the back of the figure plane and their carboxyl chain end towards the reader. They are interpreted as mimicking the receptor-binding moieties (membrane-bound pheromone isoforms) on the cell surface. The other half of the molecules, shown as lighter wires and sketched as empty triangles in the inset, have upside-down reverted orientation and are interpreted as mimicking soluble pheromone molecules. (B) On the left, two molecules forming a dimer of type 1 are shown in ribbon presentations as seen in the direction of the black arrow in the *xy* plane of (A), while on the right they are sketched as triangles as seen in top view and the arrow corresponding to the black arrow in (A). (C) On the left, two molecules forming a dimer of type 2 are shown in ribbon presentations as seen in the direction of the grey arrow in the *xy* plane of (A), while on the right they are sketched as triangles as seen in top view and the arrow corresponding to the grey arrow in (A). In both (B) and (C), the three helices are numbered progressing from the amino (N) to the carboxyl chain end (C), and the three molecular faces delimited by adjacent helices are indicated *a* (h1-h2), *b* (h2-h3), and *c* (h3-h1).

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Table 1. Mating compatibility between *E. raikovi* cell types used as sources of pheromones with known amino acid sequences

cell types and pheromones	I/E*r*-1	II/E*r*-2	VII/E*r*-7	X/E*r*-10	XI/E*r*-11	XX/E*r*-20	XXI/E*r*-21	XXII/E*r*-22	XXIII/E*r*-23
I/E*r*-1	—	+	+	+	±	—	—	—	—
II/E*r*-2		—	+	+	±	—	—	—	—
VII/E*r*-7			—	+	±	—	—	—	—
X/E*r*-10				—	±	—	—	—	—
XI/E*r*-11					—	±	±	±	±
XX/E*r*-20						—	+	+	+
XXI/E*r*-21							—	+	+
XXII/E*r*-22								—	+
XXIII/E*r*-23									—

Each cell type carries a homozygous allelic combination at the Mendelian *mat* locus and was obtained from breeding analyses of wild-type strains containing heterozygous *mat* allelic combinations. Only succesful (+), partially successful (±), and unsuccessful (-) mating combinations are indicated, with no weighting for variations in the intensity of mating reactions.

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Figure 1

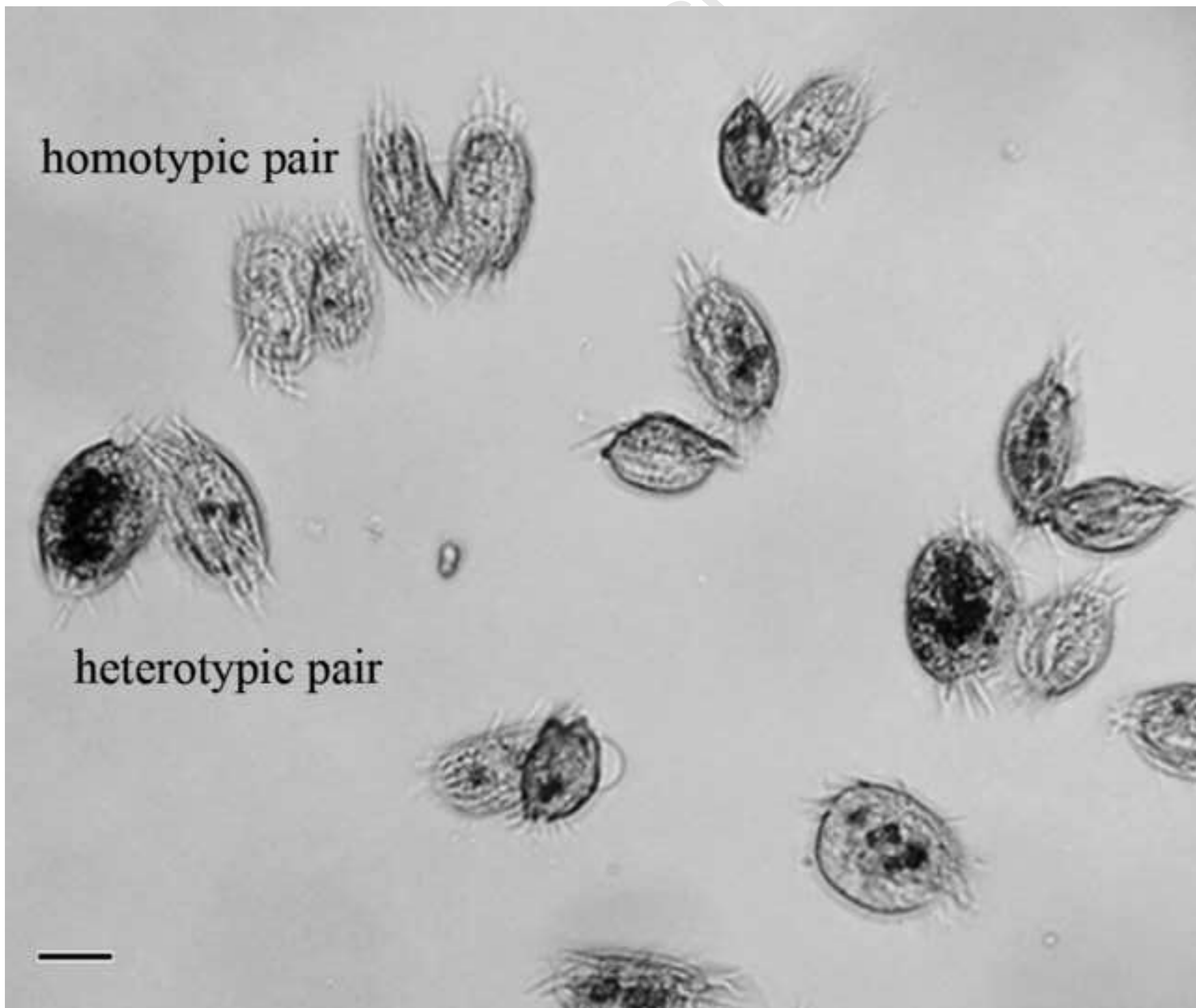

Figure 2

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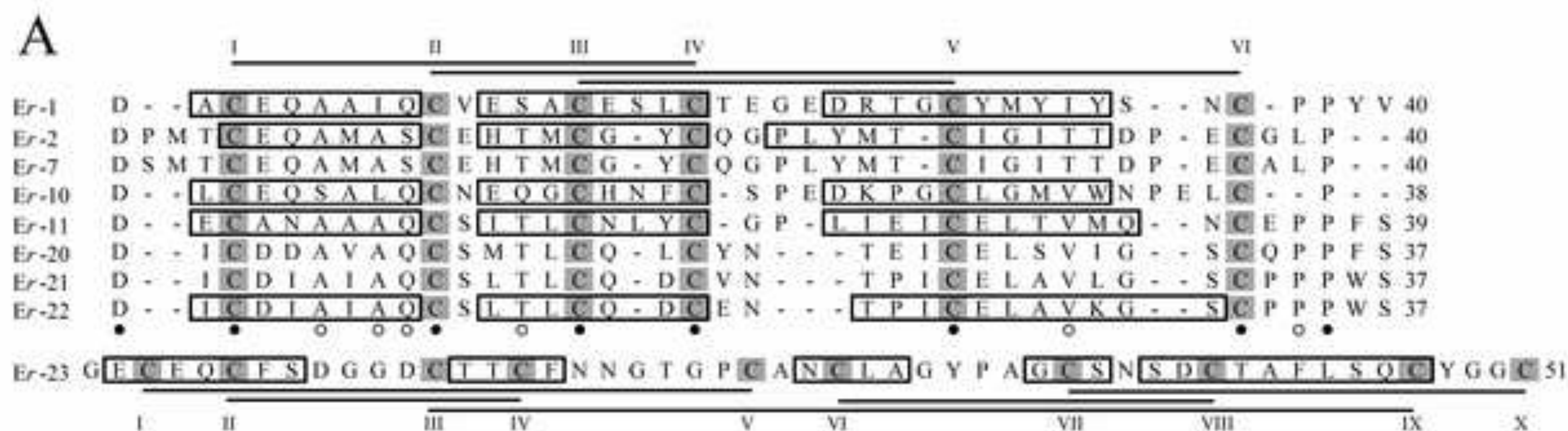
Figure 3



B

C h3 h1 h2 N Er-1

C h3 h1 h2 N Er-2

C h3 h1 h2 N Er-10

C h3 h1 h2 N Er-11

C h3 h1 h2 N Er-22

h3 h2 C h1 N Er-23

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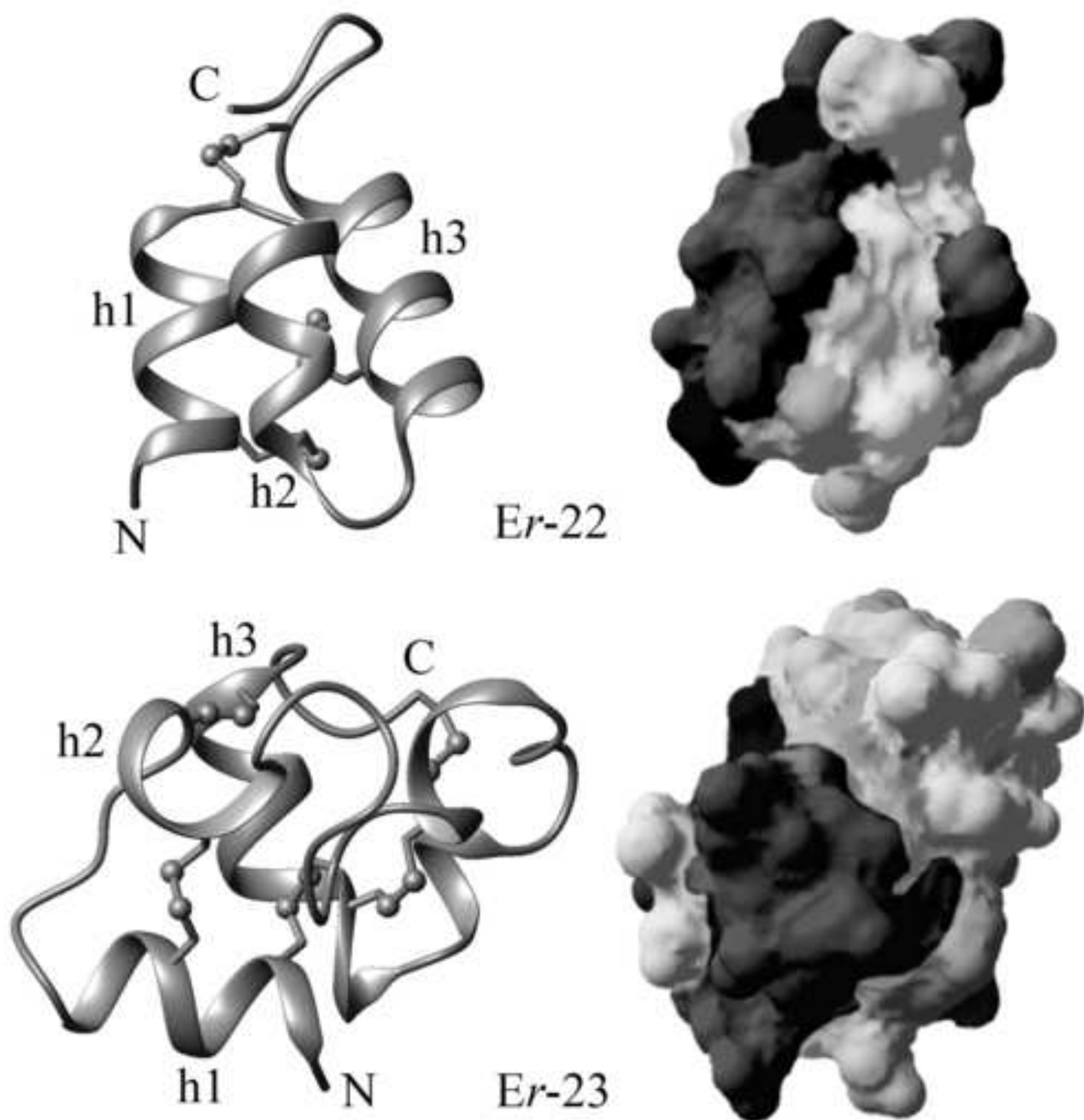
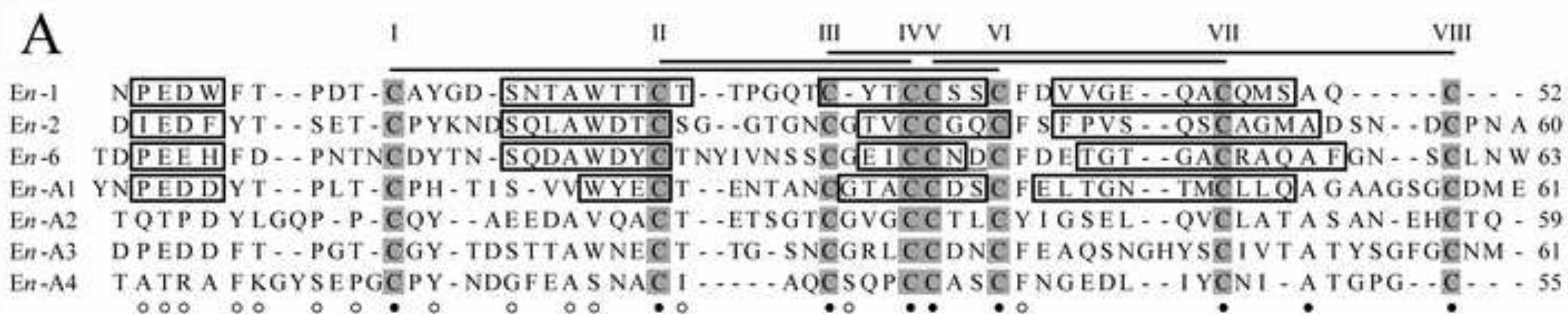


Figure 5



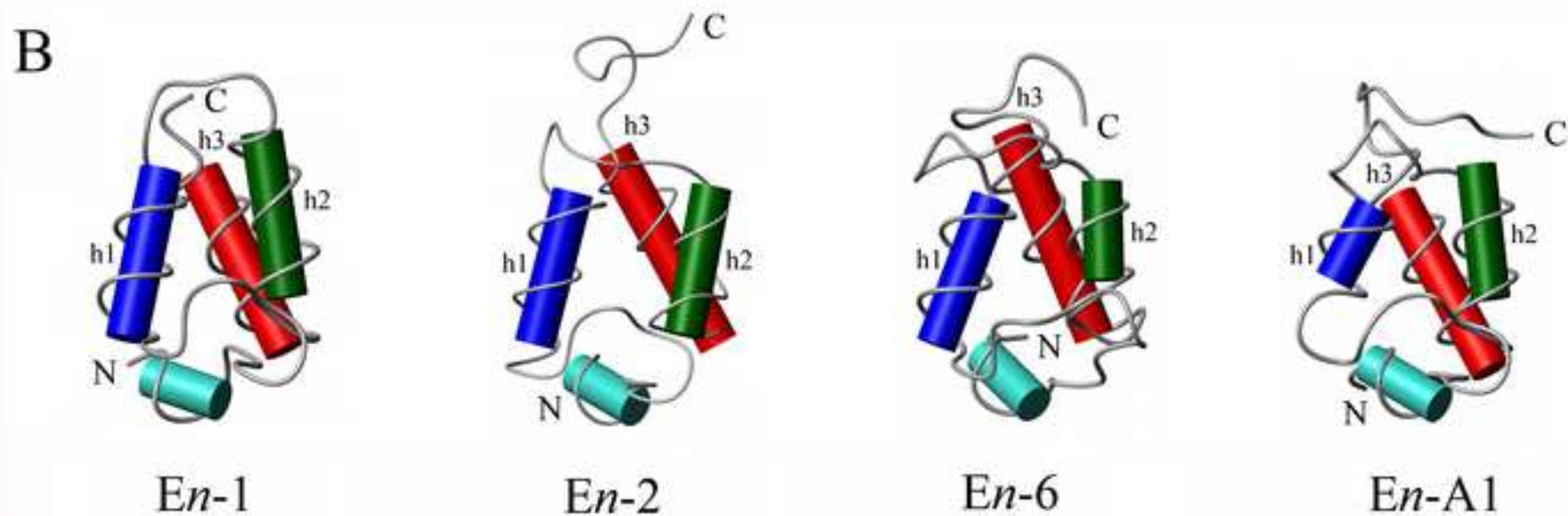


Figure 6

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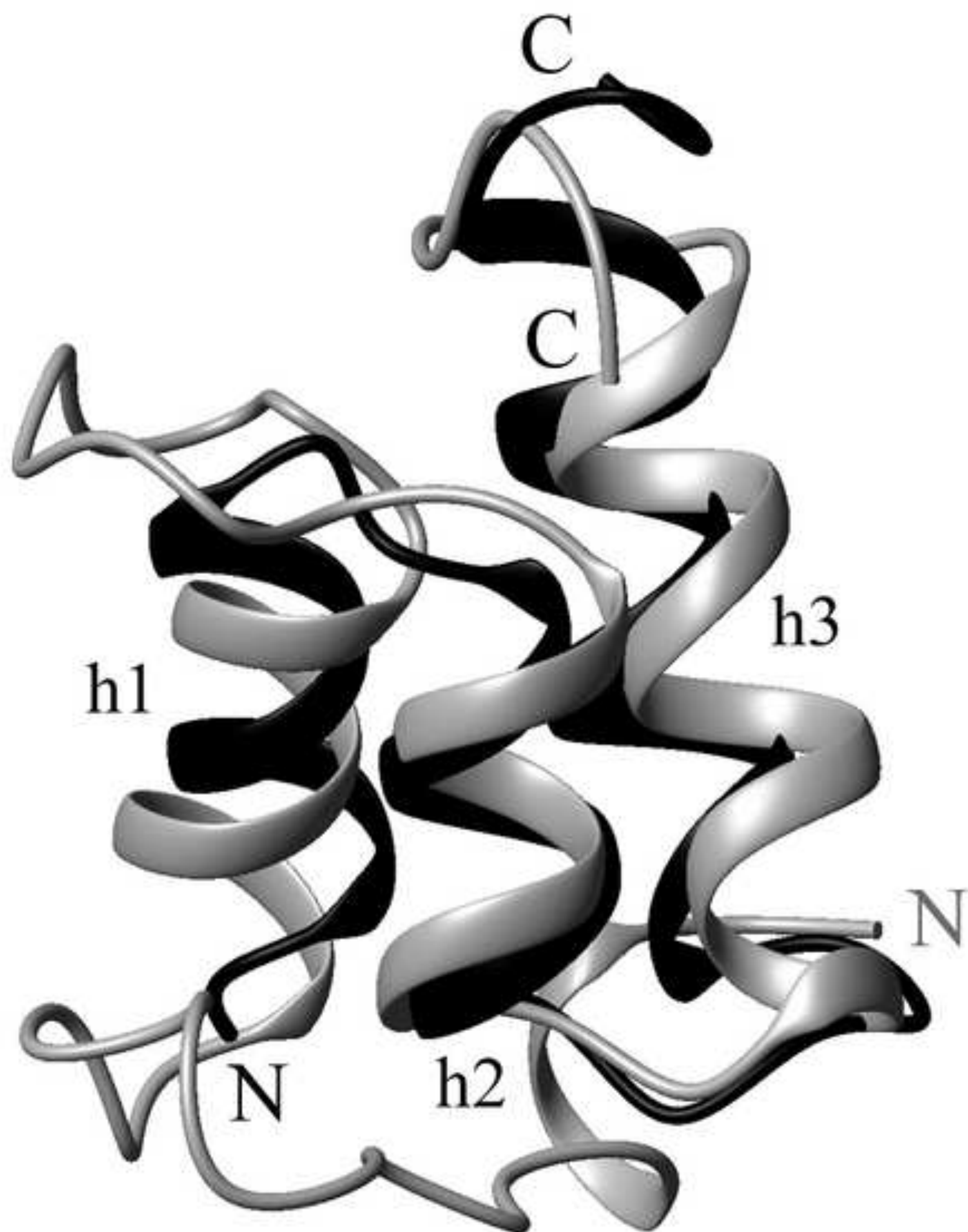
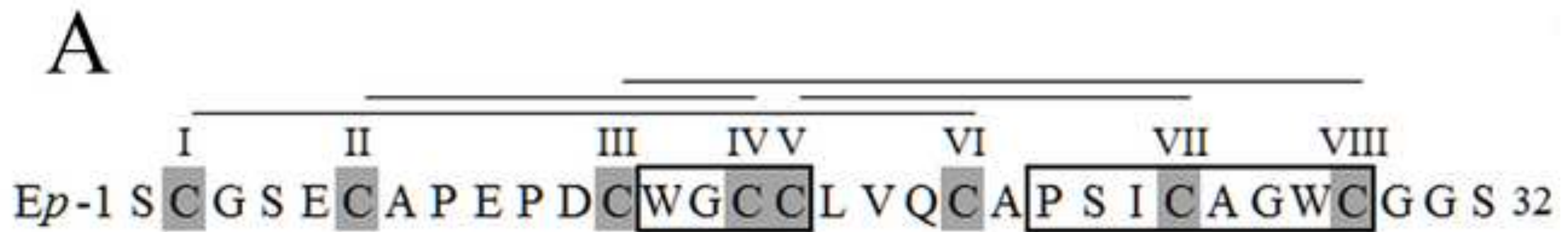
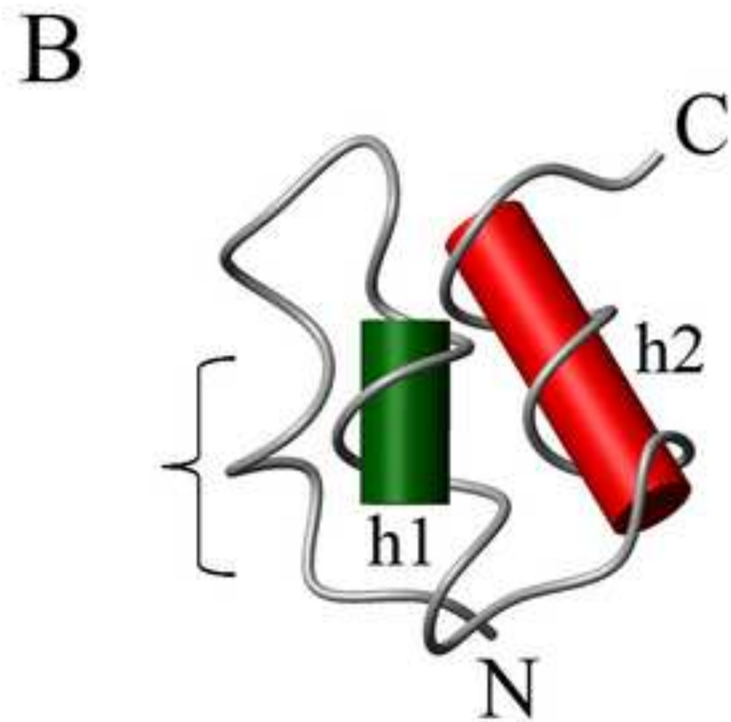
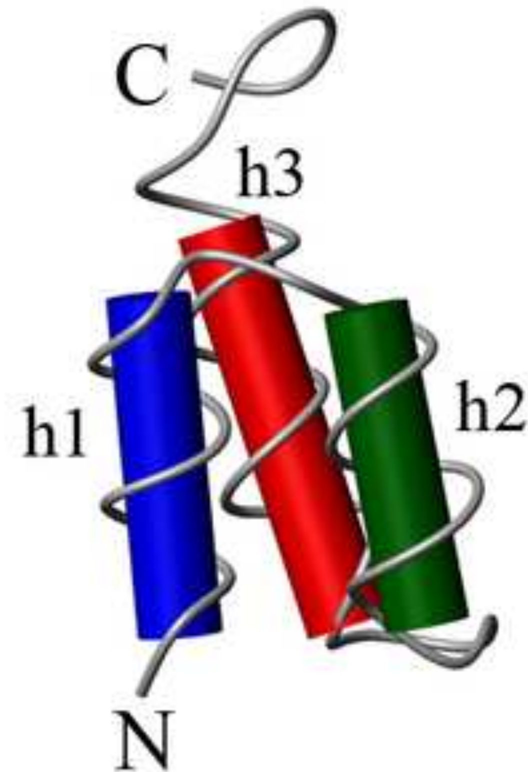


Figure 7







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       I    II        III  IV                       V                VI  VII VIII   IX               X
Ec-1  G C F G C A P T I C Q F C E A I V N P N P D V Y C G D S Q Q Y C H C   C S E C V G H M D C P 45
Ec-2  G C F D C A T N I C Q F C E A I V N P N P D M W C K E A Q E Y C H C   C S E C V G H M D C P 45
Ec-3  L C P G C A P N I C Q L C T Y V V N P N P D V Y C G D S Q E Y C H C   C S G C V G H M D C P 45

         I             II                              III           IV  V VI  VII                 VIII
Ec-α  D D H C P T D V L M T C G Y L Q G R Y N Q G N Y E E V G G L C N M S  A  E F C H C C S A C D E P E V S P Y S N C E 56
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