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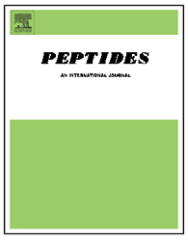

Review

Newly identified water-borne protein pheromones interact with attractin to stimulate mate attraction in *Aplysia*

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ABSTRACT

The water-borne protein attractin is a potent sex pheromone involved in forming and maintaining mating and egg-laying aggregations in the marine mollusk *Aplysia*. Binary blends of attractin and either enticin, temptin, or seductin, three other *Aplysia* protein pheromones, stimulate mate attraction. The four pheromones are thought to act in concert during egg-laying. The new data presented here show that: (1) the water-borne odor of non-laying *Aplysia brasiliana* further increases the attractiveness of attractin and of eggs in T-maze bioassays. This suggests that individual *Aplysia* release additional factors that enhance the effects of attractin, enticin, temptin, and seductin during egg-laying; (2) the N-terminal region of enticin aligns well with the conserved epidermal growth factor (EGF)-like domain of mammalian reproductive proteins known as fertilins, which may mediate intercellular adhesion interactions between eggs and sperm; (3) temptin, according to fold recognition servers, may also have an EGF-like fold. Enticin and temptin also have conserved metal binding sequences that may play a role in their signaling behavior. These results suggest that aspects of mammalian egg–sperm interactions (fertilins) may have evolved from pheromonal signaling mechanisms. We also review the structure, expression, localization, release, and behavioral actions of attractin, enticin, temptin, and seductin.

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1. Introduction

Most, if not all, animals examined use that most ancient form of communication, exchange of chemical signals that are recognized by specific cellular receptors. The examples include animals as diverse as ciliated protozoans [27], yeast [22], insects [17,28,41,43], mollusks [7,8,10–13,15–16,31–33,45, 52,54,55], worms [39,56], fish [25,48], amphibians [20,21,42,53], rodents [26,29,49], and humans [44]. Here, we discuss the protein molecules involved in mate attraction in the marine mollusk, *Aplysia*, which is found in ocean waters in many areas of the world.

Aplysia is a simultaneous hermaphrodite that does not normally fertilize its own eggs. Field studies [23,50,51] have shown that it is a solitary animal that moves into breeding aggregations (''brothels'') during the reproductive season. The aggregations usually contain mating animals, egg-laying animals, and animals doing neither, and are associated with masses of recently laid egg cordons, often deposited one on top of another. Most of the egg-laying animals mate simultaneously as females even though mating does not trigger reflex ovulation [4], suggesting that egg-laying precedes mating in the aggregation and that egg-laying releases pheromones that establish and maintain the aggregation [1,2,18,34]. Attraction requires a bouquet of scents, several of which are derived from the egg cordon [30].

Attractin, the first water-borne protein pheromone to be characterized in invertebrates [32], was purified from eluates of *Aplysia californica* egg cordons. Attractin is a 58-residue N-glycosylated protein with three intramolecular disulfide bonds, and the precursor contains a single copy of attractin (Fig. 1) [15,32,45]. The three-dimensional NMR solution structure of attractin has been determined [16], and a family of attractins has been characterized in six aplysiid species [13,32,33]. Attractin is a highly abundant secretory product of the exocrine albumen gland in the reproductive tract (Fig. 2) [32]. T-maze assays had predicted that attractin acts as part of a blend of water-borne compounds [31,32] since attractin requires the presence of a non-egg-laying animal to be attractive. Isolated egg cordons are also attractive, suggesting that other pheromonal factors work synergistically with attractin to attract *Aplysia* to reproductive aggregates. Consistent with this prediction, three other albumen gland proteins (enticin, temptin, seductin) have recently been identified in *A. californica* egg cordon eluates and characterized (Fig. 1). Binary blends of attractin and any one of these pheromones was sufficient to attract animals, suggesting that they comprise a bouquet that stimulates mate attraction [10,12]. When *Aplysia* makes physical contact with fresh egg cordons, an unidentified contact pheromone is thought to trigger the secretion of egg-laying hormone (ELH) from the neuroendocrine bag cells into the hemocoel and initiate egg-laying [3].

In this paper, we provide new data demonstrating that: (1) animal-derived factors from non-laying *Aplysia brasiliana* act in conjunction with attractin to stimulate higher levels of mate attraction than those seen in the absence of animal-derived factors; (2) sequence and structure prediction results indicate that enticin may be related to the Ca^{2+} binding, epidermal growth factor (EGF)-like domains of mammalian fertilins and other proteins that mediate intercellular surface contacts; (3) temptin may be related to the Ca^{2+} binding, EGF-like domains of the extracellular matrix protein fibrillin. We also review the structure, expression, localization, release, and behavioral actions of attractin, enticin, temptin, and seductin.

2. Materials and methods

2.1. Bioassays

The conduction of T-maze assays using *A. brasiliana* has been described [30–32]. Sexually mature *A. brasiliana* individuals (100–210 g) were used in T-maze attraction assays [30–32,45]. All animals used in assays were sexually mature, as defined by the ability to lay eggs spontaneously or in response to injection of atrial gland extracts [30]. *A. brasiliana* was used as the experimental animal because it is a swimming species and can reach test stimuli in ≤10–15 s, is more reproductively active than *A. californica* [31–33], makes fewer false choices, and can be collected seasonally (May–August) in large numbers from the south Texas coast. Previous T-maze assays demonstrated that individual *A. brasiliana* are attracted to egg cordons alone [12,30], to the pheromone attractin in the presence of a non-laying conspecific, but not to attractin alone [31,45]. The blend of attractin, enticin, temptin, and seductin diffuses from freshly laid egg cordons and comprises a bouquet of scents [10,12].

Before each T-maze assay, 6 l of ASW that had not previously contacted *A. brasiliana* was placed in the maze

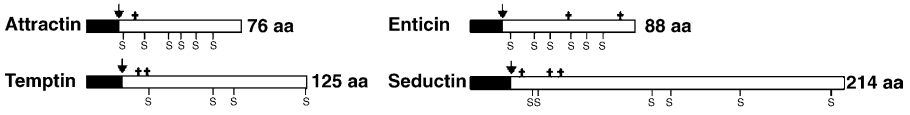

Fig. 1 – Schematic diagrams of attractin, enticin, temptin, and seductin precursors. Black boxes, signal peptides. Experimentally determined (attractin, enticin, temptin) and predicted (seductin) sites of signal sequence cleavage are indicated by arrows. S, cysteine; crosses, predicted N-linked glycosylation sites. Modified from refs. [10,12].

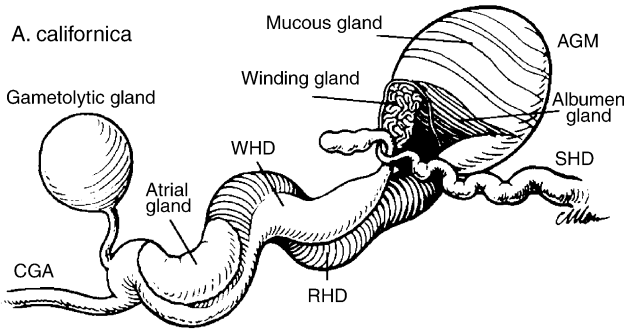

Fig. 2 – Schematic diagram of the reproductive tract of *Aplysia californica*. Following ovulation, eggs are transported from the ovotestis (which would be at the far right) through the small hermaphroditic duct (SHD) to the accessory genital mass (AGM), where they are fertilized and packaged into a cordon. Packaging occurs as the eggs are transported past (or through) the three exocrine components of the AGM: the mucous, winding, and albumen glands. The albumen gland makes up the bulk of the structure. The accessory genital mass is connected to the common genital aperture (CGA; far left) by the large hermaphroditic duct, which is composed of the red hemiduct (RHD; the oviduct) and the white hemiduct (WHD; the copulatory duct). The exocrine atrial gland is associated with the oviduct and secretes into the large hermaphroditic duct. Modified from ref. [35].

[30]; the ASW was stationary during experiments. Cages with or without a non-laying *A. brasiliana* were placed in each upper arm of the T-maze. Known *A. californica* pheromones (attractin, enticin, temptin, seductin) were added to the seawater in one arm of the T-maze, adjacent to a cage (stimulus cage); to avoid directional bias, the addition of potential attractants was alternated from one arm of the T-maze to the other. After 5 min, a non-laying animal was placed in the base of the maze and its behavior observed for up to 20 min. A response was considered to be: (1) positive if the animal traveled to, and remained in contact with, the stimulus cage for 5 min; (2) negative if the animal traveled to, and remained in contact with, the cage in the opposite arm for 5 min; (3) no choice if the test animal did neither. In each case, test animals were choosing between a stimulus in one arm and no stimulus in the other. Statistical significance was assessed using the *G* test. Recombinant attractin and enticin, native temptin, and the fusion protein glutathione S-transferase/seductin (GST/seductin) were prepared as previously described [10,12]. *A. brasiliana* attractin, enticin, temptin, and seductin are 95, 90, 91, and 94% identical to their *A. californica* homologs [10,12,33].

3. Results

3.1. Bioassays

The first series of experiments tested whether a binary blend of attractin and either enticin, temptin, or seductin was as attractive as an egg cordon; these were performed without a stimulus animal in either cage in the upper arms of the T-maze. These results, which have been published [10,12], are summarized in Fig. 3A. Negative control assays (ASW) established chance levels of attraction at 20% (four animals). When freshly laid egg cordons alone were tested, 45% of animals were attracted to the stimulus and remained; egg cordons alone were significantly attractive [$G(2) = 9.42$; $0.005 < p < 0.01$]. When attractin alone (1 nmol) was assayed, 20% of animals were attracted to the stimulus and remained, 5% traveled to the opposite arm and remained, and 75% did neither; the response to attractin alone was significantly different from ASW controls [$G(2) = 8.57$; $0.01 < p < 0.025$], not because it was more attractive but because there were fewer negative choices and more ''no choice'' events. When GST/seductin alone (1 nmol of GST/seductin contains 435 pmol of seductin) was assayed, 20% of animals were attracted to the stimulus and remained. When a combination of attractin and GST/seductin (1 nmol each) were tested, 45% of animals were attracted to the stimulus and remained; this blend was significantly attractive [$G(2) = 15.45$; $p < 0.0005$]. When a blend of attractin and enticin (1 nmol each) was tested, 45% of animals were significantly attracted to the stimulus and remained [$G(2) = 7.67$; $0.01 < p < 0.025$]. Similarly, when a blend of attractin and temptin (1 nmol each) was tested, 45% of animals were attracted to the stimulus and remained; the response pattern to this combination was significantly attractive [$G(2) = 11.98$; $p = 0.0025$].

The second series of experiments, which had not previously been performed, were designed to test whether the addition of a non-laying *A. brasiliana* to the stimulus cage increases the attractiveness of attractin, seductin, and a binary blend of attractin and seductin. The data are compared to the attractiveness of an egg-laying *A. brasiliana* with eggs [12], since the latter stimulus more closely mimics a natural egg-laying event. The results are shown in Fig. 3B. Compared to negative control assays (ASW without an animal), when a non-laying *A. brasiliana* alone was tested, 45% of animals were attracted to the stimulus and remained; non-laying animals (NL) were significantly attractive [$G(2) = 6.64$; $p < 0.05$]. When GST/seductin (1 nmol) and an *A. brasiliana* were the test stimulus, 40% of animals were attracted to the stimulus and remained; although seductin did not significantly increase the percentage of animals attracted compared to ASW [$G(2) = 5.03$; $0.05 < p\ 0.10$], there was a non-significant trend in this direction. In contrast, when a combination of attractin (1 nmol) and a non-laying *A. brasiliana* was the test stimulus, 70% of animals were attracted to the stimulus and remained; the combination of attractin and a non-laying animal was significantly attractive compared to ASW [$G(2) = 25.40$; $p < 0.0005$], to a non-layer alone [$G(2) = 6.19$; $0.025 < p < 0.05$], and to attractin alone. Similarly, when a combination of attractin, GST/seductin (1 nmol each) and a non-laying *A. brasiliana* was the test stimulus, 70% of animals were attracted to the stimulus and remained; the combination of attractin, seductin, and a non-laying animal was significantly attractive compared to ASW [$G(2) = 24.56$; $p < 0.0005$] and to a non-layer alone [$G(2) = 7.33$; $0.025 < p < 0.05$]. When an egg-laying *A. brasiliana* with an egg cordon was tested [12], 90% of animals were attracted to the stimulus and remained; egg-laying animals were significantly attractive compared to ASW

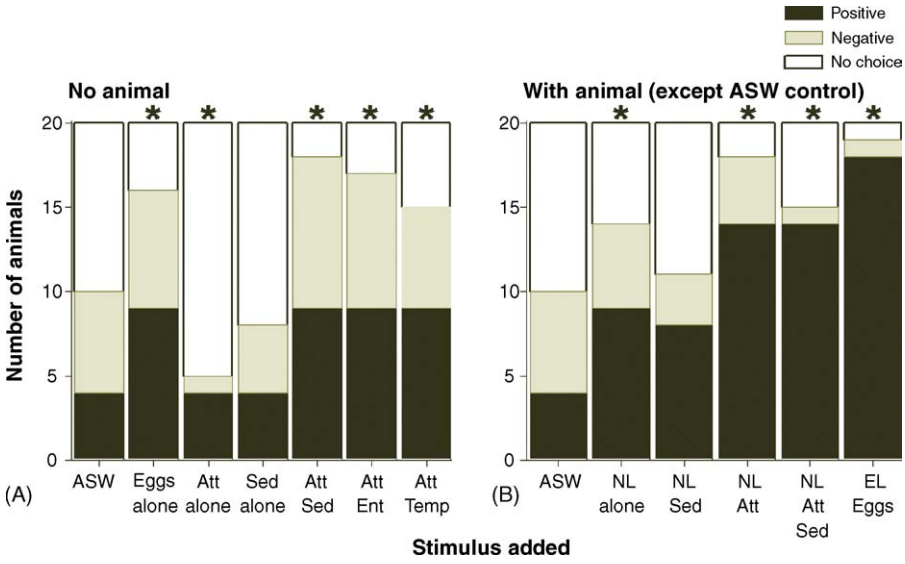

Fig. 3 – T-maze attraction assays. (A) Attractin in combination with either enticin, temptin, or seductin is as attractive as an egg cordon. Compared to negative control assays (ASW) and seductin (Sed) alone, the number of *A. brasiliana* individuals that were attracted increased significantly (*) using the following treatments: eggs alone (eggs; $0.005 < p < 0.01$); attractin alone (Att; 1 nmol; $0.01 < p < 0.025$); attractin and seductin (Att/Sed; $p < 0.0005$); attractin and enticin (Att/Ent; $p = 0.0025$); and attractin and temptin (Att/Temp; $0.01 < p < 0.025$). Attractin alone (1 nmol) was significantly different from ASW controls not because it was more attractive but because there were fewer negative choices and more no choice events. (B) The addition of a non-laying *A. brasiliana* to the stimulus cage increases the attractiveness of attractin and eggs. Compared to negative control assays (ASW), the number of *A. brasiliana* individuals that were attracted increased significantly using the following treatments: non-layer alone (NL; $p < 0.05$); non-layer and attractin (NL/Att; $p < 0.0005$); non-layer plus attractin and seductin (NL/Att/Sed; $p < 0.0005$); and an egg-laying *A. brasiliana* with eggs (EL/eggs; 1.5 ml vol; $p < 0.0005$). The combination of a non-laying *A. brasiliana* and seductin (NL/Sed) was not significantly different from ASW ($0.05 < p < 0.10$), although there was a non-significant trend in this direction. Bar graphs are based on 240 experiments, 20 per stimulus. In each experiment, animals chose between a stimulus in one arm of the T-maze and no stimulus in the other. Panel (A) modified from refs. [10,12].

[$G(2) = 45.94$; $p < 0.0005$] and compared to a non-layer alone [$G(2) = 18.15$; $p < 0.0005$].

4. Discussion

4.1. *Behavioral aspects of attractin, enticin, temptin, and seductin*

Attractin, enticin, temptin, and seductin are not attractive to *Aplysia* when tested individually, suggesting that these pheromones act as a blend to stimulate mate attraction. In support of this prediction, binary blends of attractin and either enticin, temptin, or seductin more than doubles the number of animals attracted to the stimulus. These minimal protein combinations are as attractive as egg cordons alone [10,12]. In the present study, non-laying *A. brasiliana* were found to be attractive, suggesting that animal-derived pheromonal factors are constitutively emitted that attract other animals during the reproductive season. The addition of attractin to a non-laying animal further increases the attractiveness of the non-laying animal. An actively egg-laying animal is the most attractive stimulus [12], suggesting that animal-derived pheromonal factors emanating from the egg-laying animal act in conjunction with attractin, enticin, temptin, and seductin secreted during egg-laying. Pheromonal attraction results in the formation and maintenance of mating and egg-laying aggregations.

The above conclusions are consistent with observations that known insect sex pheromones are typically mixtures of multiple components [19], that pheromonal specificity is determined by the nature of the components present as well as by their relative concentrations [36,40,41], but that only two components of a complex pheromone blend are generally necessary to serve as an attractant [9,17]. Can a single pheromone compound be behaviorally active? A recent paper by Lin et al. [26] elucidated the chemical identity of a single male-specific pheromone in mouse urine that both activates olfactory bulb mitral cells and elicits behaviors in female mice, demonstrating that even a single pheromone can be behaviorally active. In *Aplysia*, the strongest attractant is an egg-laying animal. In the present study, we show that: (1) non-laying *A. brasiliana* individuals constitutively release an unidentified water-borne attractive factor; (2) a binary blend of attractin and seductin in the presence of a non-laying *Aplysia* individual represents an even stronger attractive stimulus than a non-laying animal alone. Evolutionarily speaking, although egg-laying animals represent the strongest attractive stimulus, it is perhaps not surprising that non-laying *A. brasiliana* individuals are moderately attractive, since

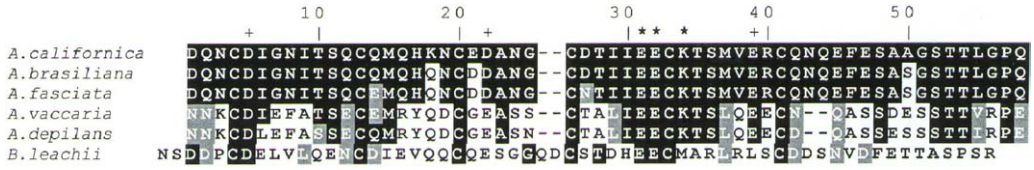

Fig. 4 – Comparison of attractin sequences from *Aplysia californica*, *A. brasiliana*, *A. fasciata*, *A. vaccaria*, *A. depilans*, and *Bursatella leachii*. Identities are shaded black. Similar residues are shaded gray. Asterisks indicate amino acids (Glu-31, Glu-32, and Lys-34) substituted in one *A. californica* attractin triple mutant; plus signs indicate amino acids (Asp-5, Asp-22, and Glu-39) substituted in a second *A. californica* attractin triple mutant. Modified from refs. [13,33].

they still represent a potential partner with which to mate. *Aplysia* mate first as males and then switch roles, subsequently mating as females. This ensures that both animals receive exogenous sperm with which to fertilize their eggs.

The amount of pheromone used in the present behavioral experiments was arbitrarily set at 1 nmol since: (1) conservative estimates indicate that ≥100–1000 pmol of attractin are present in egg cordon eluates during a single bout of egg-laying ([32] and unpublished observations); (2) the relative amounts of each pheromone released during egg-laying are currently unknown. The limited seasonal availability (May–August) and lifespan of *A. brasiliana* precluded further bioassays using other binary blends and amounts of pheromones.

4.2. *Structure of attractin family members, the 3D structural basis of attraction, and interspecific attraction*

Microsequence and 3′-rapid amplification of cDNA ends (3′-RACE) analyses have revealed a single distinct attractin-related protein in albumen gland extracts of all aplysiid species examined to date: *A. californica*, *A. brasiliana*, *Aplysia fasciata*, *Aplysia vaccaria*, *Aplysia depilans*, and *Bursatella leachii* [32,33] (Fig. 4). The five characterized *Aplysia* attractins are representatives of three subgenera that comprise 32 of the 35 known *Aplysia* species and ~15% of all *Aplysia* species. The structural basis for attractin pheromone activity is becoming increasingly clear. The six cysteines, three charged residues (Asp-5, Asp/Glu-22, and Glu-39), and the sequence Ile30-Glu31-Glu32-Cys33-Lys34-Thr35-Ser36 (IEECKTS) are conserved in all five *Aplysia* attractins. The NMR solution structure of *A. californica* attractin demonstrates that it has two helices, and the second helix contains the IEECKTS motif [16]. The IEECKTS sequence is important for biological activity, because a synthetic constrained cyclic peptide that contains the conserved heptapeptide sequence is significantly attractive in T-maze bioassays [11]; altering the three charged amino acids in the IEECKTS sequence (Glu-31, Glu-32, Lys-34; see asterisks in Fig. 4) effectively abolishes attractin activity [33]. In contrast, mutating three conserved charged residues at other areas of the protein (Asp-5, Asp/Glu-22, Glu-39; see plus signs in Fig. 4) slightly reduces but does not destroy attractin activity [33]. The three acidic residues Asp-5, Glu-31, and Glu-32 of *A. californica* attractin are solvent-exposed in the 3D NMR solution structure [16]. Because the triple mutant attractin E31Q, E32Q, K34Q lacks activity in T-maze assays, this suggests that Glu-31, Glu-32, and perhaps Lys-34 may be involved in receptor binding and pheromonal attraction, and may account for the interspecific attraction activity of attractin that has been observed [33].

In most organisms, sex pheromones attract potential mates (e.g., ref. [21]). If mate attraction were the sole function of attractin, one might expect that the pheromone would attract only conspecifics. However, attractin is a relatively promiscuous signal: *A. brasiliana* is attracted by *A. californica* attractin and *A. vaccaria* attractin, which are 95 and 43% identical to *A. brasiliana* attractin [33]. Furthermore, field observations have reported that multiple *Aplysia* are often found in the same egg-laying and mating aggregations, for example, *A. californica* and *A. vaccaria* individuals from the Pacific Coast [23], which occasionally mate with each other (S. LePage, Marine Research and Educational Products, Carlsbad, CA, personal communication). The 3D structure of attractin [16], which is compact with two helices stabilized by disulfide bonds, may have been conserved during evolution. Other additional functions attributed to attractin, namely the reduced latency of mating and the stimulation of mating [31], may prevent alteration of the attractin structure.

The benefits derived from the aggregation of multiple *Aplysia* species may exceed those derived solely by accessing potential mates. One benefit may be defense from predators. Consistent with this hypothesis, other communal animals that are thought to herd for defense may form aggregates composed of multiple species. Secretion of a pheromone that attracts individuals of a different *Aplysia* species may still be useful in attracting a potential mate, if the individual that is attracted subsequently lays eggs and releases an attractin signal that attracts a conspecific. The concentration of attractin released from the egg cordons of multiple individuals

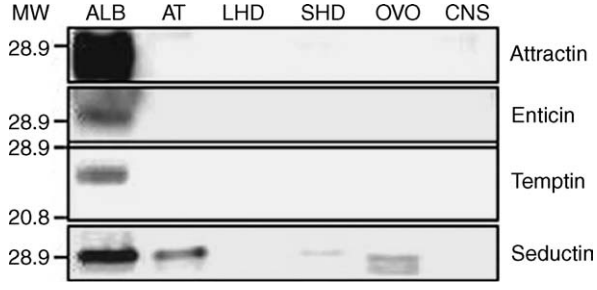

Fig. 5 – Tissue expression of attractin, enticin, temptin, and seductin. Protein (12 μg) was isolated from albumen glands (ALB), atrial glands (AT), large hermaphroditic ducts (LHD), small hermaphroditic ducts (SHD), ovotestes (OVO), and central nervous systems (CNS), and probed by immunoblot analysis using antiserum raised against attractin, enticin, temptin, and seductin. Molecular weight markers (MW) are in kilodaltons. From ref. [12].

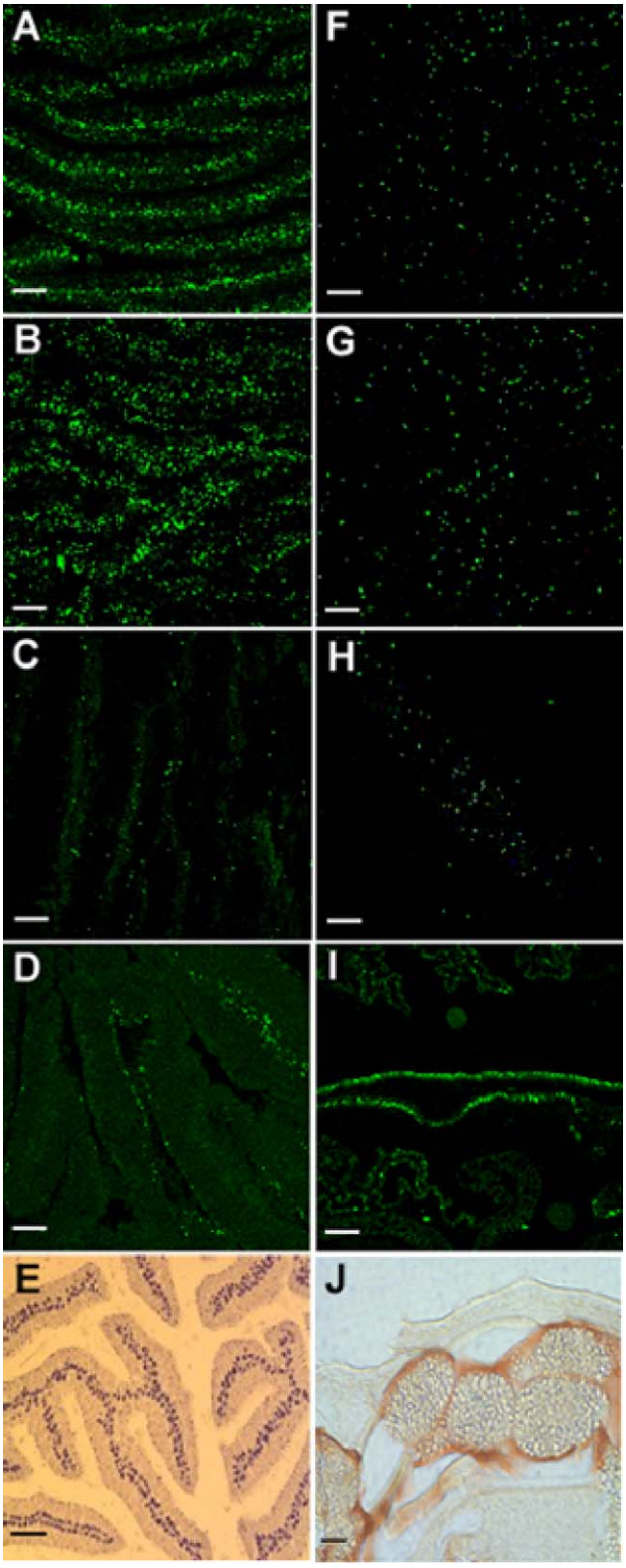

Fig. 6 – Localization of immunoreactive attractin, enticin, temptin, and seductin. Immunofluorescent localization of attractin (A and F), enticin (B and G), temptin (C and H), and seductin (D and I) in the albumen gland of a non-laying *A. californica* (A–D) and an egg-laying *A. californica* (F–I). (E) Section of an albumen gland from a non-laying *A. californica* stained with hematoxylin and eosin. (J) Immunoperoxidase localization of seductin in an egg cordon. Scale bars in A–I = 50 μm; scale bar in J = 25 μm. Modified from refs. [10,12].

should be higher than that from a cordon laid by a single individual. Since attractin is partially degraded within 30 min of eluting from egg cordons [32], a higher concentration of active attractin may be sustained over longer distances, thereby increasing the possibility that additional individuals will be recruited to breeding aggregations. Some of the new recruits may be appropriate partners for mating.

4.3. *Primary structure, expression, localization, and release of enticin, temptin, and seductin*

Earlier studies indicated that water-borne peptide/protein pheromones in the ciliated protozoan *Euplotes* and *Aplysia* are typically small (<110 residues) and *Aplysia* attractin is highly represented in a cDNA library [10,15]. We hypothesized that other water-borne *Aplysia* protein pheromones might also be released in significant amounts during egg-laying, because they may need to travel long distances (tens of meters) before contacting a conspecific. Thus, their corresponding mRNA levels and frequency in cDNA libraries might be relatively high. In differential library screens, we identified cDNAs encoding enticin, temptin, and seductin that are expressed at relatively high levels in the pheromone-secreting albumen gland (Fig. 5) [10,12]. Schematic diagrams of their protein precursors are shown in Fig. 1. Enticin is a 69-residue mature protein containing six Cys residues; temptin is a 103-residue mature protein containing four Cys residues; and seductin is a 192-residue mature protein containing six Cys residues.

Immunolocalization studies have determined the localization of attractin, enticin, temptin, and seductin in *A. californica* albumen glands and eggs [10,12]. In all cases, immunofluorescence has been observed in the secretory cells of albumen glands from non-laying animals (Fig. 6A–D) and egg-laying animals (Fig. 6F–I) using attractin (Fig. 6A and F), enticin (Fig. 6B and G), temptin (Fig. 6C and H), and seductin antiserum (Fig. 6D and I). A section of an albumen gland from a non-layer stained with hematoxylin and eosin shows the typical simple columnar epithelium (Fig. 6E). Immunoperoxidase localization studies indicate that immunoreactive seductin staining is primarily localized on the surface of individual eggs (Fig. 6J).

In contrast to enticin and temptin, which are soluble proteins, native, and recombinant seductin are predominantly membrane-associated proteins [10,12]. Nevertheless, soluble seductin has been detected by RP-HPLC and by immunoblot analyses of albumen gland and egg cordon extracts [12]. Detection of immunoreactive attractin, enticin, temptin, and seductin in egg cordon eluates by immunoblot analyses (Fig. 7) confirm that they are candidate water-borne protein pheromones [10,12].

4.4. *Sequence analysis of enticin and possible relationship to domains of mammalian fertilins*

Enticin, the smallest of the newly identified pheromones, is 90% conserved between *A. brasiliana* and *A. californica* [10], with

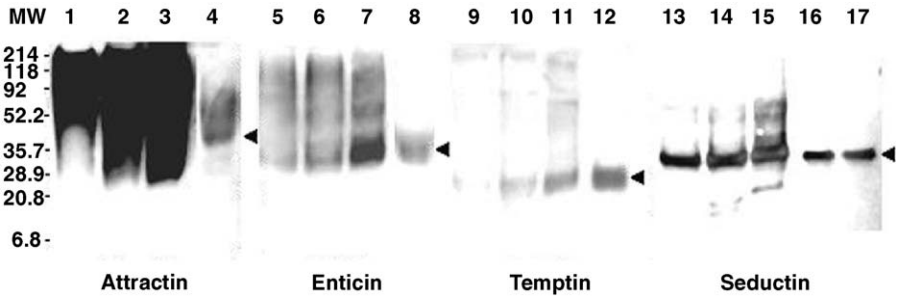

Fig. 7 – Attractin, enticin, temptin, and seductin are secreted during *Aplysia* egg-laying. Egg cordon eluates from individual egg-laying events were purified on C18 Sep-Pak Vac cartridges, lyophilized, fractionated on 12% SDS-polyacrylamide gels, and the concentrated eluates were probed by immunoblot analysis using attractin (lanes 1–4), enticin (lanes 5–8), temptin (lanes 9–12), or seductin antiserum (lanes 13–17). Lanes 1, 5, 9, and 13: 10 μg eluate protein; lanes 2, 6, 10, and 14: 20 μg protein; lanes 3, 7, 11, and 15: 40 μg protein; lanes 4, 8, 12, and 16: 20 μg of C18 Sep-Pak Vac-purified albumen gland acid extract; lane 17: 20 μg of soluble fraction of egg cordon acid extract. In separate experiments, the arrowheads indicate the migration of recombinant attractin, recombinant enticin, purified native temptin, and recombinant seductin, respectively, relative to immunoreactive albumen gland attractin (lane 4), enticin (lane 8), temptin (lane 12), and seductin (lane 16). Molecular weight markers (MW) are in kilodaltons. Modified from refs. [10,12].

the major difference being in the number of –DH– repeats in the C-terminus of the proteins (Fig. 8A). This C-terminal region would appear to be a distinct domain, and is similar to ''acid histidine'' repeat regions found in several metal binding proteins. Since the initial elucidation of enticin's sequence [10], our subsequent BLAST searches with the N-terminal 55 amino acids of enticin reveal similarity to an interior region of mammalian reproductive proteins known as fertilins (Fig. 8A), adhesion molecules that mediate interactions between sperm and egg plasma membranes (Fig. 9). Specifically, the N-terminal region of enticin aligns well with the conserved EGF-like domain in the middle of fertilins (Fig. 8A). Mammalian fertilins and testases are large proteins that are classified as ADAM (an acronym for a disintegrin and a metalloprotease domain) metalloproteases, and are expressed on the surface of eggs and sperm. Human fertilin is the best studied member of the ADAM family of proteins. ADAM proteins have also been found in the invertebrates *Drosophila* and *Caenorhabditis elegans* [38]. There are 15 ADAMs known to be expressed primarily or partially in the testis. Fertilin β, a glycoprotein, was originally discovered to be involved in gamete membrane interactions because it was identified as the antigen of an antibody, PH-30, that blocked fertilization of guinea pig oocytes [37].

The basic fertilin domain structure consists of a signal sequence, prodomain, metalloprotease domain, disintegrin-like domain, cysteine-rich domain, an EGF-like repeat, and a transmembrane segment with a short cytoplasmic tail (Fig. 9B) [5]. Proteolytic processing of fertilin during sperm development separates the disintegrin and metalloprotease domains [6] (Fig. 9B) and is crucial for exposing the disintegrin domain that participates in sperm-egg binding. The cysteine-rich domain and/or the EGF-like repeat of fertilin α, which remain on the surface of mature sperm after processing, are also thought to participate in fertilin α-mediated cell adhesion.

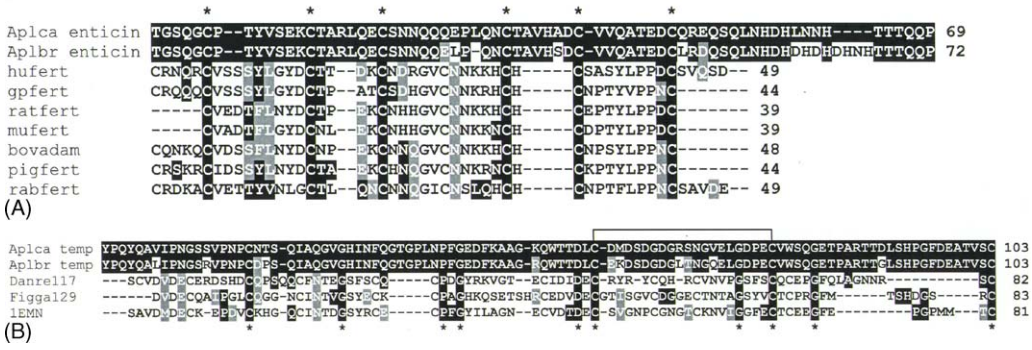

Fig. 8 – Enticin and temptin shares similarities with mammalian proteins. (A) Alignment of *A. californica* (Aplca) and *A. brasiliana* (Aplbr) enticin with the EGF-like domain of human (hu), guinea pig (gp), rat, mouse (mu), bovine (bov), pig, and rabbit (rab) fertilin. Residues that are identical in all the sequences are indicated with an asterisk; similar residues are shaded gray. (B) Alignment of Aplca and Aplbr temptin (temp) sequences with the sequence of the PDB file 1EMN (EGF-like domain of human fibrillin) and two related EGF-like domains from a zebrafish protein (PDB file Danre117) and a fibrillin protein from chicken (Figga129). The solid line indicates a likely disulfide bond, based on the 1EMN structure.

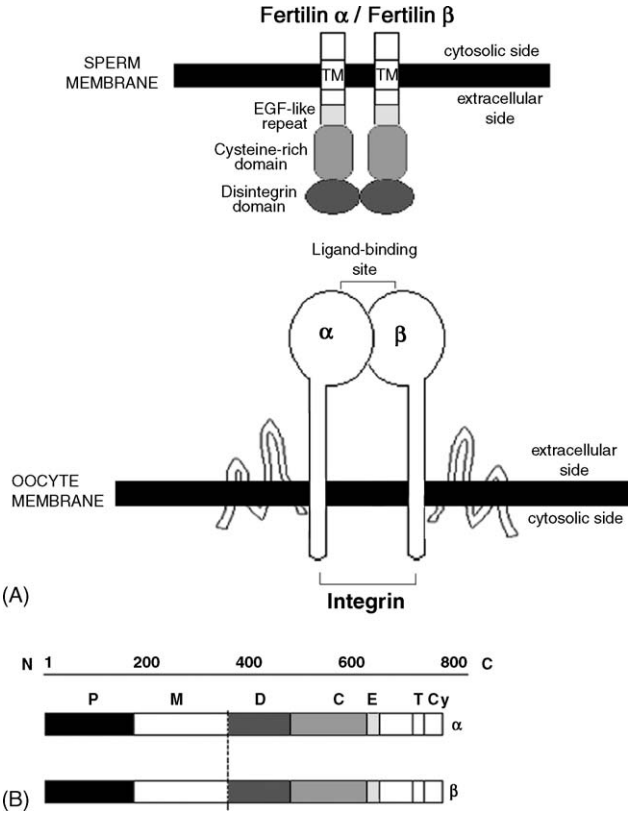

Fig. 9 – Enticin shares similarities with the EGF-like repeat of fertilins. (A) Schematic diagram of sperm and oocyte molecules known to participate in gamete membrane interactions. EGF, epidermal growth factor; TM, transmembrane domain. (B) Domains for fertilin α and fertilin β include: P, prodomain; M, metalloprotease; D, disintegrin; C, cysteine-rich; E, epidermal growth factor-like; T, transmembrane; Cy, cytoplasmic tail. Both α and β subunits are proteolytically cleaved (dashed vertical line) to produce a mature protein containing an N-terminal disintegrin domain. The scale bar indicates relative amino acid position. Modified from refs. [14,38].

4.5. *Sequence analysis, fold prediction, and possible relationship of temptin to domains of fibrillin*

Temptin is longer than enticin, but has only four cysteines. The sequence of temptin, like enticin, differs little between *A. californica* and *A. brasiliana* (91% identity), despite the different appearance and geographical prevalence of these two species. Since the initial elucidation of temptin [10], BLAST searches with the temptin sequences indicate a Ca^{2+} binding sequence in the C terminus. Interestingly, fold recognition servers suggest that temptin might also have an EGF-like fold. Specifically, the servers suggested the NMR structure of the Ca^{2+} binding, EGF-like domains of the human extracellular matrix protein fibrillin (PDB file 1EMN) as a likely template for modeling (these domains would also be similar to the EGF-like domain of human fertilins discussed above for enticin). The temptin sequence aligns reasonably well with the related domains in other fibrillins (Fig. 8B). The four conserved cysteine residues in the temptin sequences align with cysteines in the fibrillin sequences. Fibrillin contains 43 repeats of the EGF-like domains, which are essential for its function in microfibrils. Marfan's syndrome, an autosomal dominant disease affecting connective tissues that are rich in elastic fibers, has been linked to point mutations in the EGF repeats and exon skipping.

The experimental determination of the disulfide bonding pattern of both temptin and enticin is in progress. This information will aid in modeling the structure of these two proteins, as we previously did for attractin [45]. We are also expressing the proteins in bacteria [46,47] to obtain labeled material for NMR studies [16].

5. Conclusions

Mate attraction in the marine mollusk *Aplysia* involves long-distance water-borne signals – the protein pheromones attractin, enticin, temptin, and seductin – which are released during egg-laying. When *Aplysia* individuals make physical contact with freshly laid egg cordons, an unidentified contact pheromone is thought to trigger a synchronous discharge of the neuroendocrine bag cells, resulting in the secretion of ELH into the hemocoel and the initiation of egg-laying [3]. Egg cordons are often deposited on top of one another in aggregations. Attractin, enticin, temptin, and seductin diffuse from egg cordons, which have a high surface-to-volume ratio. The binary blend of attractin and either enticin, temptin, or seductin is attractive, strongly suggesting that a bouquet of these water-borne protein pheromones attracts potential mates and helps to maintain aggregations. The attractiveness of these pheromone blends is as attractive as egg cordons alone. Water-borne animal-derived factors further increase the degree of attraction seen in response to attractin paired with enticin, temptin, or seductin. Attractin is thought to act in concert with enticin, temptin, and seductin to attract and recruit *Aplysia* to the source of freshly laid eggs. Large breeding aggregations then form that may last for several days, and contain animals that alternatively mate, lay eggs, or do neither.

If enticin and temptin are structurally similar to the EGF-like domains of mammalian fertilins and fibrillins, respectively, this would have extraordinary evolutionary significance. In the case of enticin, this would suggest that proteins that regulate egg–sperm interaction (fertilins) were originally part of a pheromone signaling mechanism that perhaps evolved into a gamete recognition mechanism. The eventual 3D structural determination of enticin and temptin should shed light on this evolution.

Neither the attractin-, enticin-, temptin-, and seductin-responsive neurons nor their receptors have yet been identified, although the chemosensory rhinophores of *Aplysia* are known to sense pheromones released during mating and egg-laying [24]. The molecular mechanisms of action underlying these pheromones remain to be addressed. To our knowledge, attractin, enticin, temptin, and seductin are the first bouquet of water-borne protein pheromones to be characterized in invertebrates and vertebrates.

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