THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 24, Issue of June 11, pp. 25614–25622, 2004
Printed in U.S.A.

Characterization of *Aplysia* Enticin and Temptin, Two Novel Water-borne Protein Pheromones That Act in Concert with Attractin to Stimulate Mate Attraction*[S]

Received for publication, December 11, 2003, and in revised form, March 29, 2004
Published, JBC Papers in Press, March 30, 2004, DOI 10.1074/jbc.M313585200

Scott F. Cummins‡, Amy E. Nichols‡, Andinet Amare§, Amanda B. Hummon§, Jonathan V. Sweedler§, and Gregg T. Nagle‡¶

From the ‡Department of Neuroscience and Cell Biology, University of Texas Medical Branch, Galveston, Texas 77555, and the §Department of Chemistry and the Beckman Institute, University of Illinois, Urbana, Illinois 61801

Mate attraction in *Aplysia* involves a long-distance water-borne signal (attractin) that is released during egg laying. Other pheromones are predicted to be released during egg laying that act in concert with albumen gland attractin to stimulate attraction, but their identities are unknown. To identify other candidate water-borne pheromones, we employed differential library screening of an albumen gland cDNA library, Northern blot analysis, purification, characterization, cloning, and expression of albumen gland proteins, matrix-assisted laser desorption ionization mass spectrometry, pheromone secretion assays, behavioral bioassays, immunolocalization studies, and comparative genomics. Four genes, *Alb-23*, *Alb-24*, *Alb-69*, and *Alb-172*, were highly expressed in *Aplysia californica* albumen glands and encoded novel proteins. The products of the Alb-24 ("enticin") and Alb-172 ("temptin") precursors were soluble and highly abundant in albumen gland extracts, whereas Alb-23 and Alb-69 were membrane-associated proteins. A comparative analysis showed that the predicted *Aplysia brasiliana* enticin and temptin proteins were 90 and 91% identical, respectively, to their *A. californica* homologs. T-maze attraction bioassay studies have previously demonstrated that egg cordons alone are attractive to *Aplysia* but that attractin alone is not. In the present study, however, the combination of attractin, enticin, and temptin was found to be significantly attractive to potential mates and doubled the number of animals attracted to this stimulus compared with control animals. The combined data strongly suggest that enticin and temptin are novel candidate water-borne protein pheromones that act in concert with attractin to attract *Aplysia* to form and maintain egg-laying and mating aggregations.

* This research was supported by National Science Foundation (NSF) Grant IBN-0314377, John Sealy Memorial Endowment Fund Grant 2579-02R (to G. T. N.), and National Institutes of Health Grant NS31609 (to J. V. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains additional experimental procedures, primer sequences, tables containing cDNAs identified by library screening, predicted amino acid sequences of novel precursors, and MALDI-MS of atrial gland peptides that may correspond to Alb-23 and Alb-24. *The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY161041, AY162288, AY162289, AY236850, AY162290, AY162291, AY236851, AY236852, AY162292, AY236853, AY309079, AY582744, and AY582745.*

¶ To whom correspondence should be addressed: 2.138 Medical Research Building, Galveston, TX 77555-1069; Tel.: 409-772-2834; Fax: 409-772-2789; E-mail: gtnagle@utmb.edu.

Chemical communication is the most ancient form of communication and is used by most, if not all, animals examined, including ciliated protozoans (1), yeast (2), insects (3–5), mollusks (6–10), worms (11, 12), fish (13), amphibians (14–16), rodents (17, 18), and humans (19).

Aplysia are simultaneous hermaphrodites that do not normally fertilize their own eggs. Field studies (20–23) have shown that they are solitary animals that move into breeding aggregations during the reproductive season. The aggregations usually contain both mating and egg-laying animals and are associated with masses of recently deposited 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 (24), suggesting that egg laying precedes mating in the aggregation and that egg laying may release pheromones that establish and maintain the aggregation (25, 27, 28). The pheromonal factors seem to be derived from the egg cordon rather than the egg layer and some are water-borne (29).

One of these water-borne pheromonal attractants (attractin) has been isolated from eluates of *Aplysia californica* egg cordons and characterized. Attractin is a 58-residue *N*-glycosylated protein with three intramolecular disulfide bonds; the precursor contains a single copy of attractin (6, 7, 9). T-maze assays have predicted that attractin acts as part of a bouquet of water-borne odors (7, 8). The three-dimensional NMR solution structure of recombinant attractin has been determined (10), and a family of attractins has recently been characterized in five aplysiid species (7, 30). Attractin is a highly abundant product of the exocrine albumen gland (7), and its cDNAs represent a significant percentage of clones in an albumen gland cDNA library (6).

We hypothesized that other water-borne pheromone cDNAs may also be abundant in this library. In this study, we identified other *A. californica* candidate proteins that might play a role in water-borne pheromonal attraction by characterizing cDNAs isolated by differential library screening. Approximately 42% of the genes had no homology to any sequence, known genes, or expressed sequence tags, in the data base. We selected three highly expressed albumen gland genes, *Alb-23*, *Alb-24*, and *Alb-69*, for further study and purified the abundant product of the Alb-24 precursor ("enticin") from albumen gland extracts. We also purified and characterized an abundant novel protein ("temptin") from albumen gland extracts, cloned the cDNA (*Alb-172*), and showed that *temptin* mRNA levels were highly expressed in the albumen gland. Immunofluorescence localization studies demonstrated that immunoreactive attractin, enticin, and temptin were expressed in albumen gland columnar epithelial secretory cells, consistent with a phero-

monal function. In T-maze assays, the combination of attractin, enticin, and temptin was significantly attractive to *A. brasiliana*, a rapidly swimming species that is genetically closely related to *A. californica* and that can quickly reach a pheromone stimulus in ≤10–15 s. Reverse transcription (RT)[1]-PCR cloning demonstrated that the predicted *A. brasiliana* enticin and temptin homologs were 90 and 91% identical, respectively, to *A. californica* enticin and temptin. We conclude that enticin and temptin are candidate water-borne protein pheromones that act in concert with attractin to attract *Aplysia*, which results in the formation and maintenance of breeding aggregations.

EXPERIMENTAL PROCEDURES

cDNA Library Screening—The albumen gland is a large exocrine organ in the reproductive tract (31, 32) that secretes its products during egg laying. We previously observed that attractin clones represented a significant percentage of cDNAs in an albumen gland *A. californica* oligo-d(T)-primed cDNA library (6). In the present study, we initially selected 200 plaques at random from the same library, and cDNAs were isolated as described previously (6) and sequenced. Of the 171 clones that contained inserts, 35 (20.5%) of them encoded *Aplysia* attractin and 14 (8.2%) encoded a homolog of the *Aplysia kurodai* antibacterial glycoprotein aplysianin-A. To reduce the number of attractin and aplysianin-A clones isolated subsequently by differential library screening, the two plasmids were labeled with [α-^{32}P]dCTP, unincorporated nucleotide was removed, and labeled attractin and aplysianin-A cDNAs were mixed with [α-^{32}P]dCTP-labeled atrial gland-specific cDNA and used as probe in differential library screens.

For differential cDNA library screening, adult *A. californica* (>250 g) were obtained from Marine Research and Educational Products (Escondido, CA), total RNA was isolated from albumen and atrial glands (33) using TRIzol (Invitrogen), and poly(A)$^+$ RNA was selected (Oligotex mRNA Mini Kit; Qiagen). Probes were prepared by RT-PCR (6), and 15,000 plaques were screened from the albumen gland cDNA library. Filters were differentially screened using the two radiolabeled cDNA probes prepared from albumen gland and atrial gland poly(A)$^+$ RNAs, respectively (6), and plaques that preferentially hybridized to the albumen gland probe were rescreened; 33 positive clones were characterized.

Northern Blot Analysis—Total RNA was isolated from *A. californica* tissues using TRIzol, and Northern blot analyses were performed essentially as described previously (6). Aplysianin-A expression is restricted to the albumen gland (34) and was used to confirm that there was no albumen gland RNA contamination of other tissues. As a further control, *Aplysia* actin cDNA was used as a probe to verify the integrity of total RNA.

Purification of Albumen Gland Extracts—One *A. californica* albumen gland was extracted at 4 °C in 0.1% heptafluorobutyric acid (HFBA) using a Polytron homogenizer (Brinkmann Instruments) and sonicated. The extract was centrifuged (48,000 × *g*; 20 min; 4 °C) and the supernatant was purified on C18 Sep-Pak Vac cartridges (5 g; Waters Corp.). Peptides were eluted with 50% acetonitrile (CH_3CN)/0.1% HFBA, lyophilized, and the lyophilizate was resuspended in 0.1% HFBA; one third of the sample (33% of one gland) was purified by analytical Vydac C18 reversed phase high performance liquid chromatography (RP-HPLC) using a 2-step linear gradient (0–10% CH_3CN/0.1% HFBA in 5 min; 10–58% CH_3CN/0.1% HFBA in 170 min). Fractions were pooled, lyophilized, and repurified using the same gradient conditions, except that 0.1% trifluoroacetic acid (TFA) was the counter-ion.

Amino Acid Sequence Analysis—Native, recombinant, and tryptic peptides were subjected to microsequence analysis using a Applied Biosystems Procise 494/HT Protein Sequencer.

Trypsin Digestion—An abundant, novel peptide identified by N-terminal microsequence analysis (Alb-172; temptin) was digested with trypsin, the reaction was fractionated by analytical C18 RP-HPLC (data not shown), and four peaks were sequenced.

Oligonucleotide Primers—Sequences of primers (OL1–OL10) used for protein expression and to clone temptin can be found in the supplemental material in the on-line version of this article.

Cloning of Temptin—Based on N-terminal and tryptic fragment sequence information obtained for this novel albumen gland peptide, a 3′-RACE probe was generated to isolate its cDNA. Total RNA was isolated from albumen gland tissue, and first-strand cDNA was generated by RT using antisense adaptor primer OL1 and the Superscript Preamplification System for First Strand Synthesis (Invitrogen). PCR was performed using a degenerate sense primer corresponding to the N terminus of the novel peptide (YPQYQA; OL2) and a semi-nested antisense primer (OL3). Samples were heated at 94 °C for 2 min and amplified for 45 cycles (94 °C, 25 s; 35 °C, 25 s; 72 °C, 1 min), followed by a 7-min extension at 72 °C. Preliminary confirmation of the identity of the specific 3′-RACE product was obtained by PCR using OL2 and a degenerate antisense primer (OL4) corresponding to the tryptic peptide DLSHPGFDEA. 3′-RACE products were cloned into TOPO TA vector (pCR4-TOPO; Invitrogen). Approximately 20,000 plaques from the albumen gland cDNA library were screened using the 3′-RACE insert, and 13 positive clones were rescreened and sequenced.

Behavioral Analyses—*A. brasiliana* (100–500 g) were collected from South Padre Island, TX, and were used in T-maze attraction assays (7–9, 29, 30); all animals used in assays were sexually mature as determined by the ability to lay eggs after injection of egg-laying hormone (ELH)-related peptides (29, 35–37). As in previous studies, *A. brasiliana* was used as the experimental animal in T-maze experiments because it swims rapidly and reaches test stimuli in as little as 10–15 s, is more reproductively active than *A. californica* (7, 8, 30), does not crawl out of T-mazes, 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 (29), to the pheromone attractin in the presence of a non-laying conspecific, but not to attractin alone (7, 9). This suggested that attractin and one or more additional unidentified pheromones diffuse from freshly laid egg cordons and comprise a bouquet of scents that attract potential mates.

Before each T-maze assay, 6 liters of artificial seawater (ASW) that had not previously contacted *A. brasiliana* was placed in the maze (29); the ASW was stationary during experiments. Empty cages were placed in each upper arm of the T-maze. Potential attractants were added to the seawater in one arm of the T-maze, adjacent to a cage (stimulus cage). 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 empty cage in the opposite arm for 5 min; or 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. Stimuli were alternated between arms in consecutive assays. Statistical significance was assessed using the *G* test.

Baculovirus Protein Expression—Using *Alb-23* attB-sense (OL5), *Alb-23* attB-antisense (OL6a, OL6b), *Alb-24* attB-sense (OL7), *Alb-24* attB-antisense (OL8), *Alb-69* AttB-sense (OL9), and *Alb-69* AttB-antisense (OL10) primers, entry clones were generated for use in the Gateway Cloning System (Invitrogen). The *Alb-23* PCR product was used in BP and LR reactions that transfer the gene of interest into pDEST8 for expression of the Alb-23 precursor, and into BaculoDirect Linear DNA for expression of a C-terminal His-tagged fusion protein (His/Alb-23). The *Alb-24* PCR product was cloned into pDEST8 for expression of the Alb-24 precursor. The *Alb-69* PCR product was cloned into BaculoDirect Linear DNA for expression of a His-tagged fusion protein (His/Alb-69). The *Alb-23*/pDEST8, *Alb-23*/BaculoDirect, *Alb*-24/pDEST8, and *Alb-69*/BaculoDirect constructs were used in the Bac-to-Bac System (Invitrogen). Recombinant plasmids containing *Alb-23*, *His/Alb-23*, *Alb-24*, and *His/Alb-69* inserts were transformed into DH10Bac competent cells and insert orientation was confirmed. Sf9 cells transfected with recombinant bacmid DNA were grown in Sf-900 II SFM (Invitrogen) for 72 h, pelleted, and frozen at −70 °C.

Cells transfected with *Alb-24* were resuspended in 0.1% HFBA, sonicated, centrifuged, and the supernatant purified on C18 Sep-Pak Vac cartridges. Peptides were eluted with 70% CH_3CN/0.1% HFBA, lyophilized, resuspended in 0.1% HFBA, and purified on a semi-preparative Vydac C18 RP-HPLC column (10 × 250 mm) using a 2-step gradient (0–10% CH_3CN/0.1% HFBA in 5 min; 10–70% CH_3CN/0.1% HFBA in 212.5 min). Fractions were pooled, lyophilized, and repurified by analytical C18 RP-HPLC using the same gradient conditions, except that 0.1% TFA was the counter-ion. The identity of recombinant Alb-24 was confirmed by N-terminal microsequence analysis.

[1] The abbreviations used are: RT, reverse transcription; HFBA, heptafluorobutyric acid; CH_3CN, acetonitrile; RP-HPLC, reversed phase-high performance liquid chromatography; TFA, trifluoroacetic acid; ELH, egg-laying hormone; ASW, artificial seawater; MALDI-MS, matrix assisted laser desorption ionization-mass spectrometry; RACE, rapid amplification of cDNA ends.

Cells transfected with *Alb-23*/pDEST8 were resuspended in 0.1% HFBA, sonicated, centrifuged, and the supernatant purified on C18 Sep-Pak Vac cartridges; bound sample was eluted with 70% CH_3CN/0.1% HFBA and lyophilized. Attempts to purify recombinant Alb-23 by C18 RP-HPLC proved unsuccessful. Alternatively, cells were transfected with *Alb-23*/Baculodirect plasmid, and His/Alb-23 protein was purified from cell lysates under native and denaturing extraction conditions. For native extraction, cells were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.5, 100 mM KCl, 1% Igepal, and 100 mM phenylmethylsulfonyl fluoride), sonicated, centrifuged, and the supernatant subjected to affinity chromatography (His·Bind Quick 900 cartridges; Novagen). For denaturing extraction, membranes were extracted with 8 M urea, pH 8, sonicated, centrifuged, and the supernatant affinity purified (His·Bind Quick 900 cartridges) and eluted with 8 M urea, pH 4.5. For cells transfected with *Alb-69*/BaculoDirect plasmid, recombinant His/Alb-69 protein was purified from cell lysates under both native and denaturing extraction conditions as described above, and the supernatant affinity was purified.

SDS-PAGE—Sf9 cell lysates, supernatants of centrifuged lysates, and affinity-purified eluates containing recombinant His/Alb-23 and His/Alb-69 protein were fractionated by 12% SDS-PAGE and stained with Coomassie Blue.

Immunolocalization of Attractin, Enticin, and Temptin—Immunofluorescent staining was performed using paraffin sections of albumen glands removed from non-laying and egg-laying *A. californica*. Glands were fixed in fresh 4% paraformaldehyde/0.1 M phosphate buffer, pH 7.4, for 24 h at 4 °C, stored in phosphate buffer at 4 °C, dehydrated in an ascending series of ethanol, passed through xylene, and embedded in paraffin. Serial sections (8 μm) were cut with a microtome (CUT Series Rotary Microtome; Triangle Biomedical), mounted on gelatin-coated slides, deparaffinized in xylene (3×), rehydrated in a descending series of ethanol, and placed in running water for 5 min. Blocking was performed in 4% bovine serum albumin for 30 min. Sections were rinsed in phosphate-buffered saline (3×), incubated overnight at 4 °C in attractin, enticin, or temptin antisera (1:500 dilution), rinsed in phosphate-buffered saline (3×), incubated in fluorescein isothiocyanate-conjugated goat anti-rabbit Ig (Sigma) for 1 h at 22 °C, rinsed in phosphate-buffered saline (3×), and then mounted in fluorescein isothiocyanate mounting solution (90% glycerol, 4% *n*-propylgallate in 50 mM phosphate-buffered saline, pH 8.2). Preparations were examined using an Olympus FluoView confocal microscope (Leeds Instruments), and the image captured on a spot-cooled charge-coupled device camera (Diagnostic Instruments). In preabsorption controls, primary antiserum was replaced with attractin, enticin, and temptin antiserum preincubated with the corresponding antigen (20 μg/ml).

Pheromone Secretion Assays—*A. californica* were induced to lay eggs by ELH injection. One hour after injection, and at 15-min intervals thereafter, egg cordons were removed, transferred to 100 ml of fresh ASW for elution, and gently shaken for 15 min. Eluates were acidified to a final concentration of 0.1% TFA, filtered (0.45 μm), purified on C18 Sep-Pak Vac cartridges, and the sample eluted with 60% CH_3CN/0.1% TFA and lyophilized. SDS-PAGE and immunoblot analyses of concentrated samples (10–40 μg total protein) were performed using antisera raised against enticin, temptin, and attractin; attractin secretion has been previously demonstrated by RP-HPLC (7, 38). As a control, *A. californica* albumen glands were extracted, sonicated, centrifuged, and purified on C18 Sep-Pak Vac cartridges as described above.

Immunoblot Analysis—Protein was quantified using the BCA Protein Assay Reagent kit (Pierce). Immunoblot analyses were performed essentially as described previously (38, 39) using 12% SDS-polyacrylamide gels; membranes were incubated with enticin (1:1000 dilution), temptin (1:1000 dilution), or attractin antiserum (1:1000 dilution; 1 mg of affinity-purified antibody/ml). As a control, the primary antiserum was replaced with enticin, temptin, or attractin antiserum preincubated with the corresponding antigen (20 μg/ml).

Antiserum Production—Details of enticin, temptin, and attractin antiserum production can be found in the supplemental material in the on-line version of this article.

MALDI-MS—For the Alb-23 and enticin precursors, a list of expected masses based on signal sequence cleavage sites and further processing at basic residues predicted using a basic site cleavage predictor (40) can be found in the on-line supplemental material; a figure showing potential processing products using MALDI-MS can also be found there.

RT-PCR of A. brasiliana Temptin and Enticin Genes—Sequences of primers used to clone *A. brasiliana* enticin and temptin can be found in the on-line supplemental material. Using *A. californica* temptin and enticin cDNA sequences, we performed RT-PCR to determine whether enticin and temptin homologs were expressed in the albumen gland of *A. brasiliana*. Total RNA was isolated from *A. brasiliana* albumen glands using TRIzol, and first-strand cDNA was generated by RT of total RNA using an antisense adaptor primer (OL11) and the Superscript Preamplification System for First Strand Synthesis. PCR was performed using the following primer combinations: sense primer 24s and antisense primer 24r (corresponding to *A. californica* enticin nucleotides 94–307); sense primer 24s2 and antisense primer OL12 (corresponding to *A. californica* enticin nucleotides 273–1038); sense primer 24s3 and antisense primer 24r2 (corresponding to *A. californica* enticin nucleotides 41–225); sense primer 172s and antisense primer 172r (corresponding to *A. californica* temptin nucleotides 82–397); sense primer 172s2 and antisense primer OL12 (corresponding to *A. californica* temptin nucleotides 344–947); sense primer 172s3 and antisense primer 172r2 (corresponding to *A. californica* temptin nucleotides 18–219). Samples were heated for 5 min at 94 °C and amplified using Taq polymerase (Eppendorf) for 36 cycles (94 °C, 30 s; 45–55 °C, 1 min; 72 °C, 2 min), followed by a 7-min extension at 72 °C. PCR products of the expected size were cloned by insertion into pCR 2.1 (Invitrogen), according to the manufacturer's instructions, and nucleotide sequence analyses were performed.

RESULTS

cDNA Library Screening—Because attractin is released at high levels during egg laying (7, 38), we hypothesized that the mRNA and protein levels of other potential *Aplysia* protein pheromones might also be elevated and that the frequency of their cDNAs in the albumen gland cDNA library might also be relatively high. We initially identified abundantly expressed genes by characterizing randomly selected plaques from an albumen gland cDNA library (6).

We obtained high quality sequence (>400 nt) from the 5′ end of cDNAs for 171 clones containing inserts. Thirty-five clones (20.5% of total) encoded attractin, and fourteen (8.2% of total) encoded a protein homologous to the *A. kurodai* antibacterial glycoprotein aplysianin-A, which functions as an antibacterial agent to protect oocytes as they develop within egg cordons (34). *A. californica* aplysianin-A (GenBank accession no. AY161041) shared 85% amino acid sequence identity with the *A. kurodai* protein (data not shown). Sixty-six clones (39.6%) encoded messages related to the cytoskeleton, protein synthesis, cellular metabolism (*e.g.* ribosomal and mitochondrial genes), signal regulatory proteins, and transcription (Fig. 1*A*). The remaining 54 clones (31.6%) were not similar to any other sequences in the databases, including *Alb-24* (*enticin*) that was isolated 14 times (8.2%).

To reduce the number of attractin and aplysianin-A clones isolated in the differential library screen, we added radiolabeled attractin and aplysianin-A cDNAs to radiolabeled first strand atrial gland cDNAs as probes. After obtaining high quality sequence (>400 nt) from the 5′ end of 33 clones that were identified by differential library screening, the percentage of attractin and aplysianin-A clones isolated was reduced to 12.1% (Fig. 1*B*). Fifteen clones (45.5%) encoded messages related to cellular metabolism, transcription, protein synthesis, and the cytoskeleton. Fourteen clones (42.4%) were sequenced completely that had no homology to any known gene or expressed sequence tag. These 14 clones encoded eight proteins, five of which were full-length, including three (Alb-23, Alb-24, and Alb-69) that were predicted to contain hydrophobic signal peptides. Lists of genes identified by random and differential library screening, and the predicted amino acid sequences of novel proteins identified by differential library screening, can be found in the supplemental material in the on-line version of this article.

Alb-24, which was encoded by five of the 14 unknown clones (36%), predicted a full-length 88-residue precursor that was predicted to generate a 69-residue mature protein containing six Cys residues and two consensus sequences for *N*-linked glycosylation (Fig. 2, *A* and *B*). *Alb-23* encoded a full-length 214-residue precursor that was predicted to generate a mature

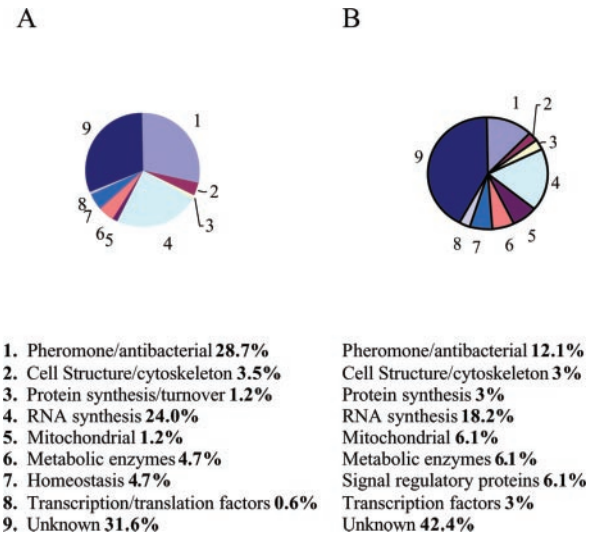

FIG. 1. **Categorization of sequences from random (*A*, *left*) and differential screening (*B*, *right*) of an *Aplysia* albumen gland cDNA library.** The functional categories in *A* and *B*, if known, are indicated. Clones that do not share sequence identity with any known proteins in databases comprised 31.6% and 42.4% of clones isolated from random and differential screens, respectively.

192-residue mature protein containing six Cys residues and three consensus sequences for *N*-linked glycosylation (Fig. 2*B*). *Alb-28* encoded a full-length 57-residue nuclear peptide. *Alb-55* encoded a full-length 122-residue cytoplasmic protein. *Alb-69*, which represented 3 of the 14 unknown clones (21%), predicted a full-length 1790-residue precursor that contained 17 repeat sequences flanked by potential basic residue cleavage sites (RK,RR,KR,KK,KKK; Fig. 2*B*). The N-terminal region of the Alb-69 precursor contained five consensus sequences for *N*-linked glycosylation, and a short peptide bearing a signal for amidation (GKK). *Alb-1*, *-17*, and *-74* were partial clones.

Northern Blot Analysis—Northern blot analyses demonstrated high levels of expression of three of the novel genes in the albumen gland (*Alb-23*, *Alb-24*, and *Alb-69*; Fig. 3); the sizes of the cDNAs were in good agreement with the predicted sizes of the transcripts (both can be found in the supplemental material in the on-line version of this article).

Purification of Native and Recombinant Enticin—We initially investigated the processing product of the enticin precursor because *enticin* represented 36% of the novel clones isolated by differential library screening, and *enticin* mRNA was expressed at high levels in albumen glands. Albumen gland extracts were purified by C18 RP-HPLC, and a representative profile is shown in Fig. 4*A*. The major peak corresponding to fractions 98–109 (Fig. 4*A*) was pooled from several purifications, lyophilized, and repurified by C18 RP-HPLC. Fraction 1 (Fig. 4*B*) was subjected to microsequence analysis, and the N-terminal sequence, TGSQG, was identical to that predicted after signal sequence cleavage of the enticin precursor at Thr20 (Fig. 2*A*).

Two minor peaks in Fig. 4*A* were also identified by microsequence analysis: 1) pedal peptide (41, 42); and 2) acidic peptide, a processing product of the ELH precursor expressed in the neuroendocrine "bag" cells of the abdominal ganglion (43). Both peptides were presumably axonally transported from ganglia to the albumen gland. The presence of acidic peptide is of interest because another processing product of the ELH precursor (delta-bag cell peptide) is hypothesized to be involved in the cellular release of perivitelline fluid from albumen glands (44).

C18 RP-HPLC purification of insect cell lysate supernatants yielded a major peak (enticin) that was repurified using a different counter-ion (data not shown). N-terminal microsequence analysis demonstrated signal sequence cleavage of the enticin precursor at Thr20.

Purification, Cloning, and Expression of Temptin—Repurification of HPLC fractions 98–109 (Fig. 4*A*) also resulted in two additional major peaks (Fig. 4*B*, fractions 2 and 3). Their N-terminal sequences were identical (YPQYQ) but did not match any sequences in GenBank or PIR data bases or any clones isolated by differential library screening. The sequences of four tryptic peptides from fraction 3 were obtained: YPQYQA, QWTTD, SNGVE, and TTDLSHPGFDEATVS. A 3′-RACE probe was generated using primers designed based upon the first and last tryptic peptides, and facilitated the isolation of the full-length cDNA (*temptin*) that encoded a 125-residue precursor (Fig. 5, *A* and *B*). The signal peptide of the precursor was cleaved at Tyr23 based on N-terminal sequence analysis of the native HPLC-purified albumen gland protein (YPQYQ). The 103-residue processing product does not match any other sequence in data bases. Expression of the *temptin* gene was restricted to the albumen gland (Fig. 5*C*), and the size of the cDNA (947 bp) was in agreement with the predicted size of the transcript (1 kb).

Behavioral Assays—Previous studies have demonstrated that the blend of scents that elute from egg cordons contain other unidentified water-borne pheromones that, in combination with attractin, are attractive. The results of T-maze attraction assays of enticin and temptin are shown in Fig. 6. In negative control assays (ASW), five animals (25%) traveled to the right arm and remained, five (25%) traveled to the left arm and remained, and 10 (50%) did neither. These bioassays verify that there is no directional bias in the maze and establish chance levels of attraction at five animals.

In contrast to ASW controls, when native enticin and attractin (1 nmol each) were combined and assayed, nine animals (45%) were attracted to the stimulus and remained, eight (40%) traveled to the opposite arm and remained, and three (15%) did neither; fewer animals failed to make a choice. The combination of enticin and attractin was significantly attractive (G (2) = 10.88; $0.001 < p < 0.005$). When native temptin and attractin (1 nmol each) were combined and assayed, nine animals (45%) were attracted to the stimulus and remained, six (30%) traveled to the opposite arm and remained, and five (25%) did neither; compared with ASW controls, fewer animals failed to make a choice. Although the combination of temptin and attractin was not significantly attractive (G (2) = 5.84; $0.05 < p < 0.10$), there was a non-significant trend in this direction. When enticin, temptin, and attractin (1 nmol each) were combined and assayed, 10 animals (50%) were attracted to the stimulus cage and remained, seven (35%) traveled to the opposite arm and remained, and three (15%) did neither; this combination was significantly attractive (G (2) = 11.35; $0.001 < p < 0.005$). The bar graphs are based on 80 experiments, 20 per stimulus. In each experiment, animals chose between a stimulus in one arm and no stimulus in the other. The limited seasonal availability (May-August) and lifespan of *A. brasiliana* precluded further bioassays of enticin and temptin.

Purification of Recombinant Alb-23 and Alb-69—Alb-23 was not detected by RP-HPLC purification of recombinant Alb-23-containing insect cell lysate supernatants. SDS-PAGE of His/Alb-23-containing insect cell lysates extracted under native and denaturing conditions demonstrated that His/Alb-23 was a membrane-associated protein (Fig. 7*A*); the size of the protein (~37 kDa) was higher than predicted (20.8 kDa). The presence of three potential *N*-linked glycosylation sites and a His tag may account for the higher molecular mass. SDS-PAGE of

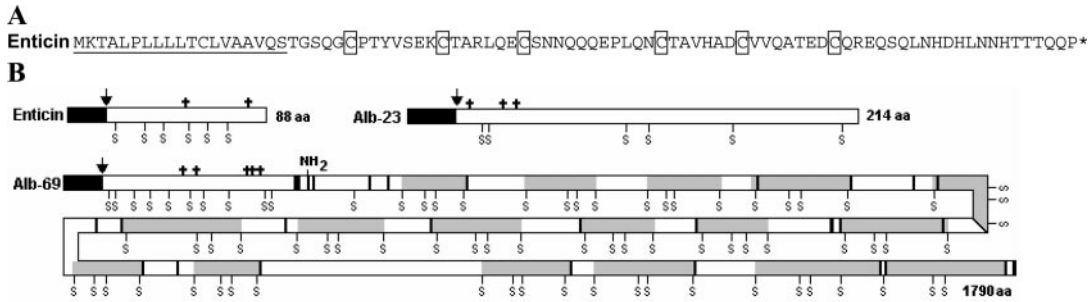

FIG. 2. **Precursors encoded by enticin, Alb-23, and Alb-69 cDNAs isolated by differential library screening.** *A*, predicted amino acid sequence of enticin precursor. Experimentally determined signal sequence in enticin is *underlined*. Cysteine residues are *boxed*. *Asterisk*, stop codon. *B*, schematic diagrams of enticin, Alb-23, and Alb-69 precursors. *Black boxes*, signal peptides. Experimentally determined (enticin) and predicted sites of signal sequence cleavage (Alb-23 and Alb-69) are indicated by *arrows*. *Vertical black lines*, potential basic residue cleavage sites; *crosses*, predicted *N*-linked glycosylation sites; NH_2, predicted amidation site; *shaded regions*, repeat sequences.

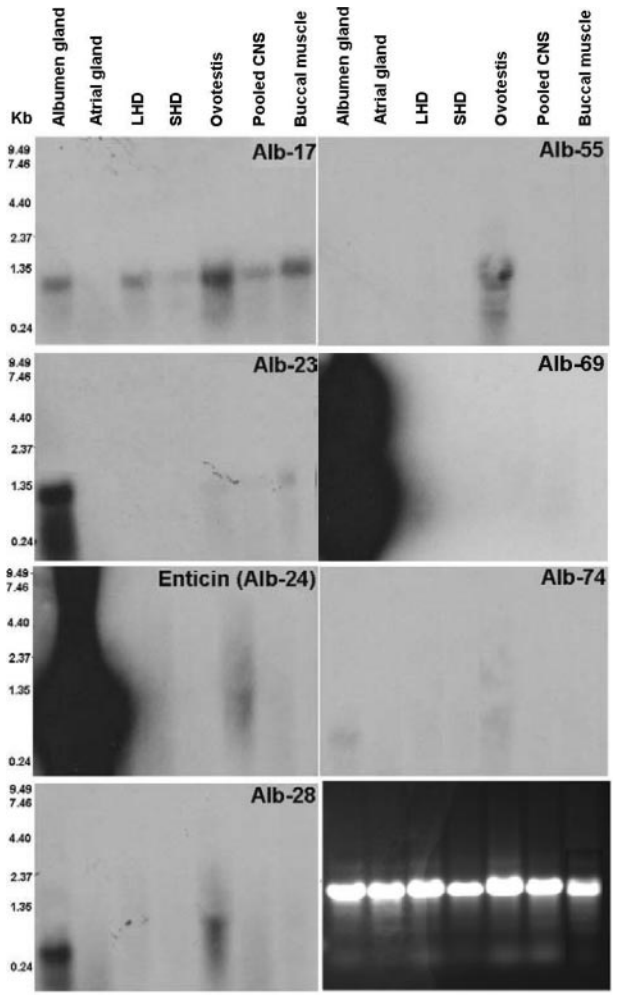

FIG. 3. **Tissue distribution of novel mRNAs isolated by differential library screening.** Total RNA (10 μg) was isolated from the albumen gland, atrial gland, large hermaphroditic duct (*LHD*; combined red and white hemiduct), small hermaphroditic duct (*SHD*), ovotestis, pooled central nervous system (*CNS*; pooled cerebral, pleural, pedal, buccal, and abdominal ganglia), and buccal muscle, fractionated on agarose-1% formaldehyde gels, and the membranes were hybridized with radiolabeled cDNA probes for *Alb-1*, *Alb-17*, *Alb-23*, enticin (*Alb-24*), *Alb-28*, *Alb-55*, *Alb-69*, or *Alb-74*. Autoradiography was performed for 4 h; Alb-1 mRNA was not detected after 18 h of exposure. RNA size markers (Invitrogen) are indicated. Equivalent amounts of RNA were loaded in each lane and confirmed by ethidium bromide staining (*bottom right*). One blot was stripped and reprobed using *Aplysia* actin (26).

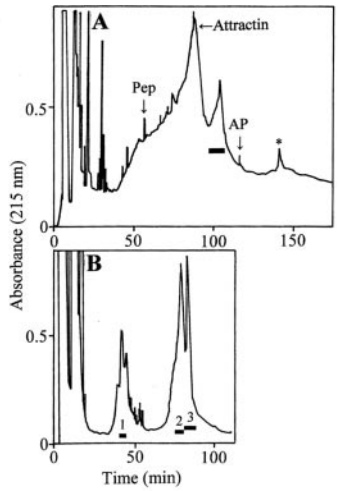

FIG. 4. **RP-HPLC purification and identification of enticin and temptin in albumen gland extracts.** *A*, an extract was eluted with a linear gradient of 0.1% HFBA and CH_3CN/0.1% HFBA. Fractions indicated by the *solid bar* were pooled and lyophilized; the adjacent major peak contains attractin (7). *Pep*, pedal peptide; *AP*, acidic peptide. The peak indicated by an *asterisk* was not a peptide based on microsequence analysis and MALDI-MS. *B*, pooled fractions 98–109 in *A* from several HPLC runs were repurified with a linear gradient of 0.1% TFA and CH_3CN/0.1% TFA. Fractions 1, 2, and 3 were pooled and subjected to N-terminal sequence analysis. Fraction 1 contains enticin. Fractions 2 and 3 share the same N-terminal sequence corresponding to temptin.

His/Alb-69-containing cell lysates extracted under native and denaturing conditions demonstrated that His/Alb-69 was also a membrane-associated protein (Fig. 7*B*); the size of the His-tagged protein (~195 kDa) was in good agreement with the predicted molecular mass (193.1 kDa).

Immunolocalization Studies—Immunofluorescence studies were performed to confirm the expression and localization of attractin, enticin, and temptin in albumen gland secretory cells of *A. californica*. Extensive immunofluorescence was identified in albumen gland secretory cells from non-laying animals using attractin (Fig. 8*A*) and enticin antiserum (Fig. 8*B*), whereas immunofluorescence levels of temptin were less pronounced (Fig. 8*C*). The levels of attractin, enticin, and temptin immunofluorescence were all significantly reduced in albumen gland secretory cells from egg-laying animals (Fig. 8, *E–G*), an indication of their secretion during egg laying. Fig. 8*D* shows a hematoxylin- and eosin-stained section of an albumen gland from a non-laying animal, demonstrating a typical simple columnar epithelium. In control experiments using preabsorbed attractin, enticin, and temptin antisera, no immunofluorescence was observed (data not shown), demonstrating specificity of each antiserum.

Enticin and Temptin Are Released during Egg Laying—To further examine enticin and temptin release during egg laying, immunoblot analyses were performed on eluates of freshly laid

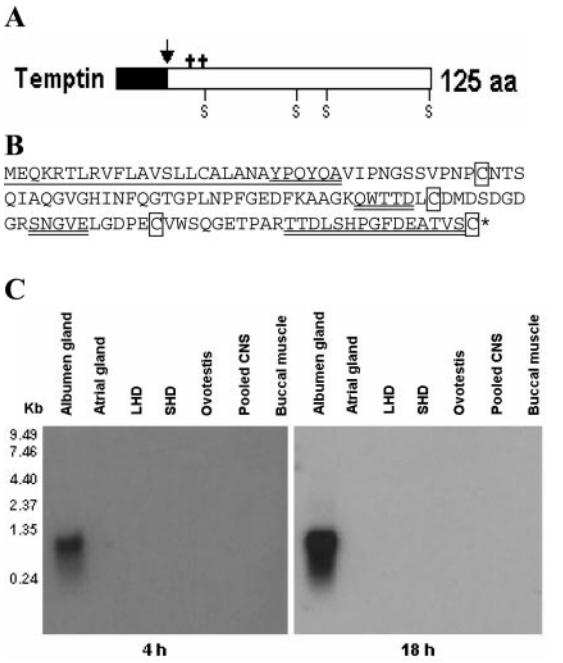

FIG. 5. **Structure and expression of *Aplysia temptin*.** *A*, schematic diagram of temptin precursor. *Black box*, signal peptide; *arrow*, experimentally determined site of signal sequence cleavage; *crosses*, predicted *N*-linked glycosylation sites; *S*, Cys residues. *B*, predicted amino acid sequence of temptin precursor. The signal sequence is *underlined*. Tryptic fragments of temptin that were sequenced are *double-underlined*. *Boxes*, Cys residues; *asterisk*, stop codon. *C*, Northern blot analysis showing albumen gland-specific expression of *temptin*. RNA size markers (Invitrogen) are indicated. Autoradiography was performed for the time indicated.

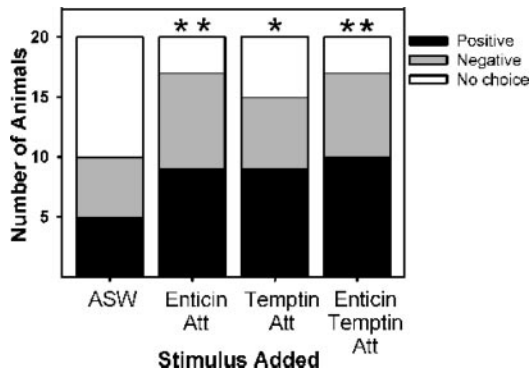

FIG. 6. **The combination of enticin, temptin, and attractin attract *Aplysia* in T-maze attraction assays.** Compared with seawater controls (ASW), the number of *A. brasiliana* that were attracted increased significantly (**, $0.001 < p < 0.005$) when enticin and attractin (1 nmol each) were added to ASW. The number of *A. brasiliana* that were attracted also increased when temptin and attractin (1 nmol each) were added to ASW (*, $0.05 < p < 0.10$); although the attractiveness of this combination was not significant, there was a non-significant trend in this direction. The number of *A. brasiliana* attracted significantly increased (**, $0.001 < p < 0.005$) when a combination of enticin, temptin, and attractin (1 nmol each) was added to ASW.

egg cordons that were purified on C18 Sep-Pak Vac cartridges. Immunoreactive enticin and temptin were both detected in egg cordon eluates, clearly confirming that both are released during egg laying (Fig. 9). The immunoreactive enticin and temptin detected in egg cordon eluates comigrated with endogenous immunoreactive enticin and temptin in albumen gland extracts (Fig. 9). The endogenous and released immunorective enticin and temptin comigrated with recombinant enticin and purified temptin, respectively (Fig. 9). The apparent molecular masses are higher than predicted by their cDNAs (enticin, 7.7 kDa;

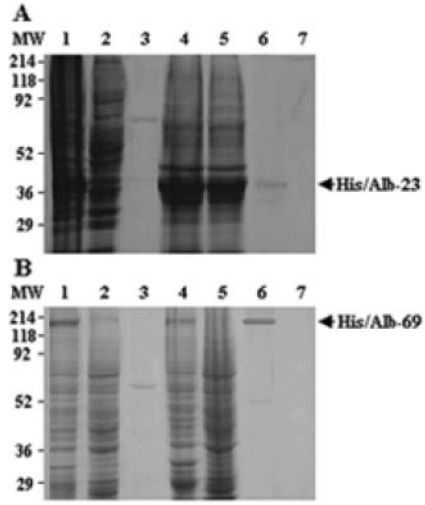

FIG. 7. **SDS-PAGE of Alb-23 and Alb-69 proteins purified from Sf9 insect cell lysates.** *A*, recombinant Alb-23 proteins. *Lanes 1–6*: His/Alb-23-containing cell lysates extracted under nondenaturing (*lanes 1–3*) and denaturing conditions (8 M urea; *lanes 4–6*). *Lane 1*, total cell extract; *lane 2*, wash fraction; *lane 3*, elution; *lane 4*, cell membrane extract; *lane 5*, wash fraction; *lane 6*, elution; *lane 7*, control elution of uninfected cell lysates extracted under denaturing conditions. *B*, recombinant Alb-69 proteins. *Lanes 1–6*: His/Alb-69-containing cell lysates extracted under nondenaturing (*lanes 1–3*) and denaturing conditions (8 M urea; *lanes 4–6*). *Lanes 1–7*, same as in *A*. Molecular mass markers (*MW*) are in kilodaltons.

temptin, 10.9 kDa), perhaps reflecting post-translational modification at the predicted *N*-linked glycosylation sites in each protein pheromone (Figs. 2 and 5). Immunoreactive attractin was also readily detected in egg cordon eluates, as expected based on previous HPLC analyses (7, 38). Native attractin, which is *N*-glycosylated (7), and recombinant attractin, which is not (7, 9), both migrated at a higher molecular mass (Fig. 9) than that previously determined by MALDI-MS (*m/z* 8059) (7). The use of antisera preabsorbed with the corresponding antigen resulted in no immunoreactive bands (data not shown).

A. brasiliana Enticin and Temptin Genes—Because *A. californica* attractin, enticin, and temptin were tested in T-maze assays using a genetically closely related species (*A. brasiliana*), we examined whether enticin and temptin homologs were also expressed in *A. brasiliana*. RT-PCR was performed using *A. brasiliana* total RNA and multiple sets of degenerate *A. californica* enticin and temptin primers. After gel electrophoresis, bands of the size expected for temptin and enticin were cloned and sequenced, and demonstrated that they encoded the *A. brasiliana* homologs of enticin and temptin (Fig. 10*A*). *A. brasiliana* enticin and temptin were 90 and 91% identical to *A. californica*, respectively (Fig. 10*B*).

DISCUSSION

Previous studies showed that water-borne peptide/protein pheromones in the ciliated protozoan *Euplotes* and *Aplysia* are typically small (<110 residues) and that *Aplysia* attractin is highly represented in a cDNA library (6). Consistent with this expectation, attractin cDNAs comprised ~20% of randomly selected clones. We hypothesized that other water-borne *Aplysia* pheromones might also be released in large amounts during egg laying, because they must often travel long distances before contacting a conspecific. In differential library screens, we identified cDNAs encoding eight novel proteins, of which *enticin*, *temptin*, *Alb-23*, and *Alb-69* are novel cDNAs expressed at high levels in the pheromone-secreting albumen gland. Purified enticin and temptin are major constituents of albumen gland extracts. Immunolocalization studies demonstrated that

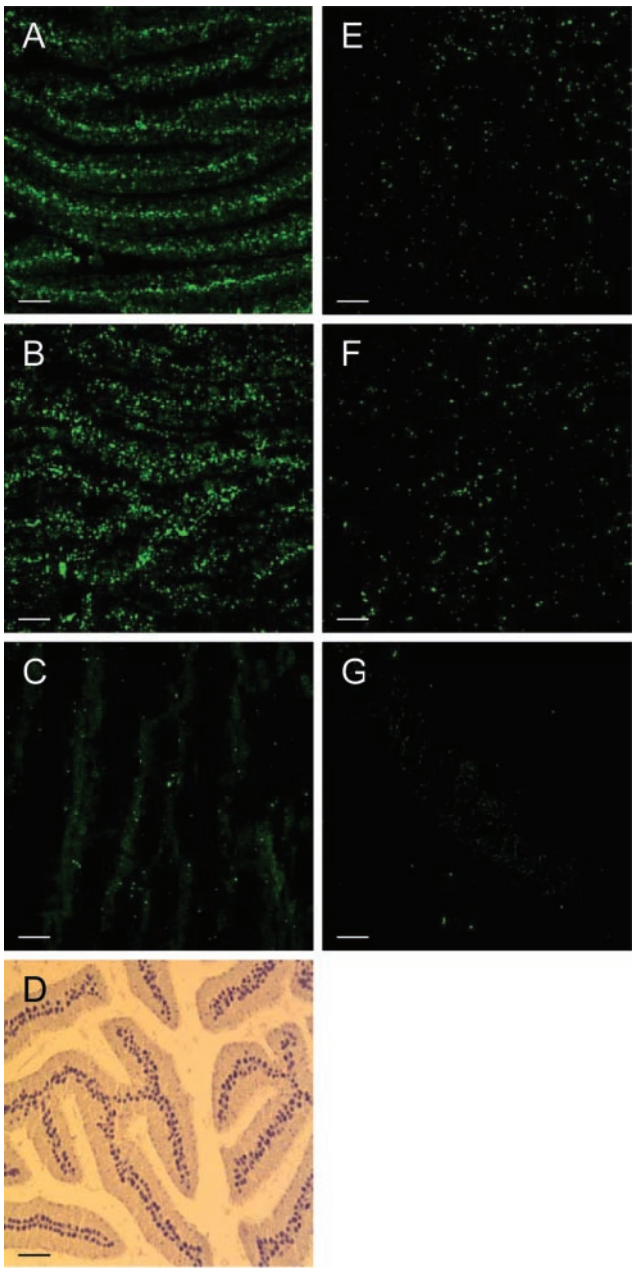

FIG. 8. **Localization of immunoreactive attractin, enticin and temptin in *A. californica* albumen glands.** Immunofluorescent localization of attractin (*A* and *E*), enticin (*B* and *F*), and temptin (*C* and *G*) in the albumen gland of a non-laying *A. californica* (*A–C*) and an egg-laying *A. californica* (*E–G*). *D*, Section of an albumen gland from a non-laying *A. californica* stained with hematoxylin and eosin. *Scale bars*, 50 μm.

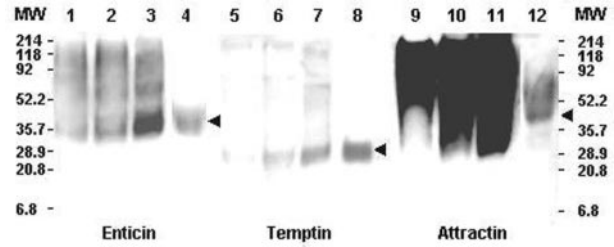

FIG. 9. **Enticin and temptin are secreted during *Aplysia* egg laying.** Egg cordon eluates from individual egg-laying events were purified on C18 Sep-Pak Vac cartridges, lyophilized, fractionated on 12% SDS-polyacrylamide gels, and the concentrated eluates were probed by immunoblot analysis using enticin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

the levels of immunofluorescent attractin, enticin, and temptin in albumen gland secretory cells were significantly reduced in egg-laying animals. Detection of immunoreactive enticin and temptin in egg cordon eluates confirmed that both are candidate water-borne protein pheromones. In contrast, Alb-23 and Alb-69 are membrane-associated proteins. Previous studies clearly demonstrated that attractin alone is not attractive to *Aplysia* (7, 8), suggesting that attractin acts in concert with other unidentified pheromones to stimulate mate attraction. In support of this notion, we demonstrate that *A. californica* attractin, enticin, and temptin seem to act synergistically to attract *A. brasiliana*, because this protein combination mimics the attractiveness of egg cordons (29); attraction results in the formation and maintenance of mating and egg-laying aggregations. These observations prompted us to examine whether *A. brasiliana* also express enticin and temptin homologs, and if so, whether they are similar to *A. californica* enticin and temptin. We found that *A. brasiliana* enticin and temptin mRNA were indeed expressed, and that the predicted proteins were 90 and 91% identical to *A. californica* enticin and temptin, respectively. Overall, the combined data are consistent with the observation that most insect air-borne pheromone attractants are mixtures of several components, and pheromonal specificity is determined by the nature of the components present as well as by their relative concentrations (45, 46).

Enticin accounted for 15% of clones isolated by differential library screening, and the predicted protein was similar (small size; six Cys residues) to the *Euplotes* mating pheromone family and the *Aplysia* attractin pheromone family. In most *Euplotes* pheromones, Cys residues form three intramolecular disulfide bonds, producing stable loops that differ in both size and charge distribution from one pheromone to another within the family. The differences are thought to confer mating-type specificity, whereas the conserved sequences contribute to the conserved structure and preserved function (1, 47–49). Microsequence analyses of purified temptin and its tryptic fragments resulted in the cloning of *temptin*, and Northern blot analysis demonstrated high levels of *temptin* mRNA expression restricted to the albumen gland.

When *Aplysia* 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 (50). Attractin, enticin, and temptin subsequently diffuse from egg cordons, which have a high surface-to-volume ratio. The combination of attractin, enticin, and temptin doubles the number of animals attracted to this stimulus, strongly suggesting that a bouquet of these three water-borne protein pheromones attracts potential mates. The attractiveness of this protein combination is nearly as attractive as egg cordons alone (29). Attractin acts in concert with enticin and temptin to attract and recruit *Aplysia* to freshly laid eggs. Large breeding aggregations then form that may last for several days, and contain animals that alternatively mate and lay eggs. Neither the attractin-, enticin-, and temptin-responsive neurons nor their receptors have yet been identified; therefore the molecular mechanisms of action underlying these pheromones remains to be addressed.

In contrast, the structural basis for attractin pheromone activity is becoming increasingly clear. A single distinct attrac-

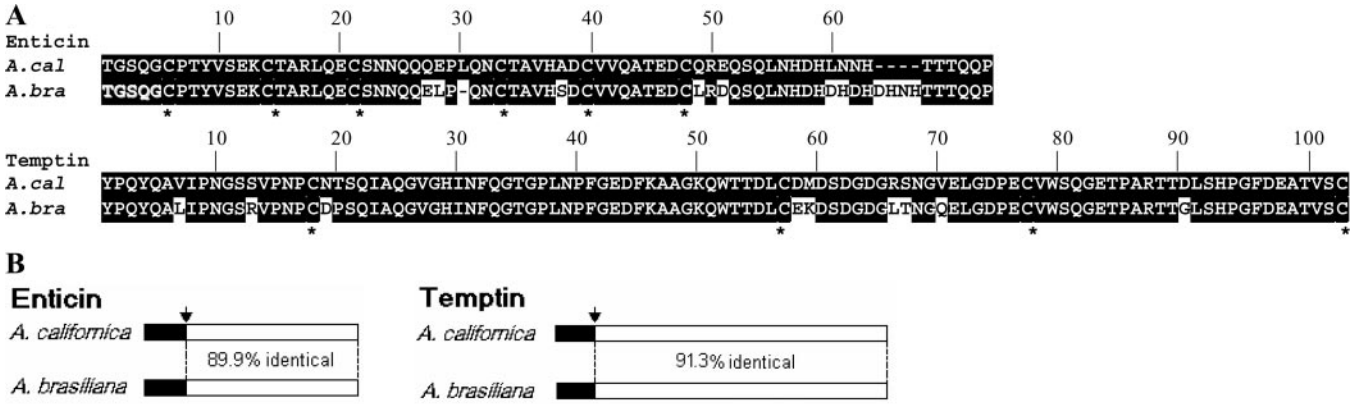

FIG. 10. **Comparison of *A. californica* and *A. brasiliana* enticin and temptin sequences.** *A*, complete amino acid sequences of *A. californica* and *A. brasiliana* enticin and temptin; the signal sequences of each precursor, which were determined, are not shown. Identical residues are indicated in *black*. Conserved Cys residues are indicated by *asterisks*. *B*, schematic diagrams showing the *A. californica* and *A. brasiliana* enticin and temptin precursors, which are 90 and 91% identical, respectively.

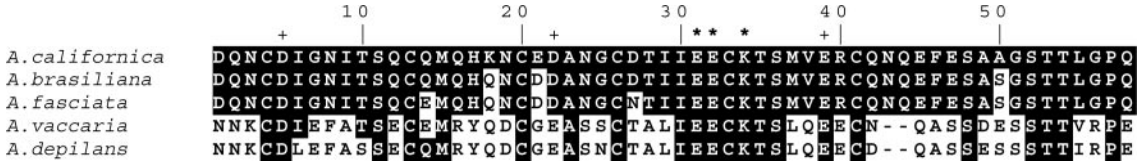

FIG. 11. **Comparison of attractin sequences from *A. californica* (GenBank accession no. A59060), *A. brasiliana* (B59060), *A. fasciata* (A59447), *A. vaccaria* (A59424), and *A. depilans* (A59446).** Identical residues are indicated in *black*. *Asterisks* indicate amino acids (Glu-31, Glu-32, and Lys-34) substituted in one *A. californica* attractin triple mutant; altering these three amino acids abolishes attractin activity. +, amino acids (Asp-5, Asp-22, and Glu-39) substituted in a second *A. californica* attractin triple mutant; mutating these three conserved charged residues slightly reduces but does not destroy attractin activity. See text for details.

tin-related protein is present in five different species of *Aplysia*: *A. californica*, *A. brasiliana*, *Aplysia fasciata*, *Aplysia depilans*, and *Aplysia vaccaria* (7, 30; Fig. 11). The six cysteines, three charged residues (Asp-5, Asp/Glu-22, and Glu-39), and the sequence Ile^{30}-Glu^{31}-Glu^{32}-Cys^{33}-Lys^{34}-Thr^{35}- Ser^{36} (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 (10). The IEECKTS sequence is important for biological activity (30), because a synthetic constrained cyclic peptide that contains the conserved heptapeptide sequence is significantly attractive in T-maze bioassays (51); altering the three charged amino acids in the IEECKTS sequence (Glu-31, Glu-32, Lys-34; Fig. 11) effectively abolishes attractin activity (30). In contrast, mutating three conserved charged residues at other areas of the peptide (Asp-5, Asp/Glu-22, Glu-39; Fig. 11) slightly reduces but does not destroy attractin activity (30). The three acidic residues Asp-5, Glu-31, and Glu-32 of *A. californica* attractin are solvent-exposed in the three-dimensional NMR solution structure (10). Because the triple mutant attractin (E31Q, E32Q, K34Q) lacks activity in T-maze assays, this suggests that Glu-31, Glu-32, and 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 (30).

Although *Alb-23* and *Alb-69* mRNA were expressed at high levels in albumen glands, SDS-PAGE demonstrated that recombinant Alb-23 and Alb-69 were membrane-associated. However, we cannot rule out the possibility that after secretion: 1) Alb-23 or Alb-69 may be proteolytically liberated from membranes and subsequently serve a water-borne pheromonal function; or 2) Alb-23 or Alb-69 may play a contact pheromonal role on the egg cordon surface. A previous study suggested that an unidentified factor diffuses from egg cordons to serve as a water-borne pheromone that stimulates a bag cell discharge and subsequent egg laying, and laboratory observations have shown that: 1) egg laying tends to be synchronized in an aquarium when animals are individually caged; 2) animals downstream from an egg-laying animal tend to lay eggs, whereas animals caged upstream often do not; 3) egg laying is induced among individually caged animals when an egg-laying animal is introduced to the tank (52); and 4) attractin does not induce egg laying, although egg cordons do. Future studies using enticin and temptin and soluble fusion proteins of Alb-23 and of Alb-69 sequences may shed light on these observations.

In conclusion, in most organisms, sex pheromones attract potential mates (*e.g.* Ref. 53). 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* are attracted by *A. californica* attractin and *A. vaccaria* attractin, which are 95 and 43% identical to *A. brasiliana* attractin (30). The three-dimensional structure of attractin (10), which is compact and has two antiparallel helices stabilized by disulfide bonds, may have been conserved during evolution.

Acknowledgments—We acknowledge the assistance of the UTMB Protein Chemistry Lab and Mass Spectrometry Facility, and we thank S. D. Painter for helpful discussions and L. DesGroseillers for the *Aplysia* actin cDNA.

REFERENCES

1. Luporini, P., Vallesi, A., Miceli, C., and Bradshaw, R. A. (1995) *J. Eukaryot. Microbiol.* **42,** 208–212
2. Kodama, T., Hisatomi, T., Kanemura, T., Mokubo, K., and Tsuboi, M. (2003) *Yeast* **20,** 109–115
3. Monsma, S. A., and Wolfner, M. F. (1988) *Genes Dev.* **2,** 1063–1073
4. Roelofs, W. L., Liu, W., Hao, G., Jiao, H., Rooney, A. P., and Linn, C. E., Jr. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 13621–13626
5. Saudan, P., Hauck, K., Soller, M., Choffat, Y., Ottiger, M., Sporri, M., Ding, Z., Hess, D., Gehrig, P. M., Klauser, S., Hunziker, P., and Kubli, E. (2002) *Eur. J. Biochem.* **269,** 989–997
6. Fan, X., Wu, B., Nagle, G. T., and Painter, S. D. (1997) *Mol. Brain Res.* **48,** 167–170
7. Painter, S. D., Clough, B., Garden, R. W., Sweedler, J. V., and Nagle, G. T.

(1998) *Biol. Bull.* **194,** 120–131
8. Painter, S. D., Clough, B., Black, S., and Nagle, G. T. (2003) *Biol. Bull.* **205,** 16–25
9. Schein, C. H., Nagle, G. T., Page, J. S., Sweedler, J. V., Xu, Y., Painter, S. D., and Braun, W. (2001) *Biophys. J.* **81,** 463–472
10. Garimella, R., Schein, C. H., Xu, Y., Nagle, G. T., Painter, S. D., and Braun, W. A. (2003) *Biochemistry* **42,** 9970–9979
11. Zeeck, E., Muller, C. T., Beckmann, M., Hardege, J. D., Papke, U., Sinnwell, V., Schroeder, F. C., and Francke, W. (1998) *Chemoecology* **8,** 33–38
12. Ram, J. L., Muller, C. T., Beckmann, M., and Hardege, J. D. (1999) *FASEB J.* **13,** 945–952
13. Li, W., Scott, A. P., Siefkes, M. J., Yan, H., Liu, Q., Yun, S.-S, and Gage, D. A. (2002) *Science* **296,** 138–140
14. Kikuyama, S., Toyoda, F., Ohmiya, Y., Matsuda, K., Tanaka, S., and Hayashi, H. (1995) *Science* **267,** 1643–1645
15. Rollmann, S. M., Houck, L. D., and Feldhoff, R. C. (1999) *Science* **285,** 1907–1909
16. Wabnitz, P. A., Bowie, J. H., Tyler, M. J., Wallace, J. C., and Smith, B. P. (1999) *Nature* **401,** 444–445
17. Stowers, L., Holy, T. E., Meister, M., Dulac, C., and Koentges, G. (2002) *Science* **295,** 1493–1500
18. Novotny, M. V. (2003) *Biochem. Soc. Trans.* **31,** 117–122
19. Savic, I., Berglund, H., Gulyas, B., and Roland, P. (2001) *Neuron* **31,** 661–668
20. Kupfermann, I., and Carew. T. (1974) *Behav. Biol.* **12,** 317–337
21. Audesirk, T. E. (1979) *Biol. Bull.* **157,** 407–421
22. Susswein, A. J., Gev, S., Feldman, E., and Markovitch, S. (1983) *Behav. Neural Biol.* **39,** 203–220
23. Susswein, A. J., Gev, S., Feldman, E., and Markovitch, S. (1984) *Behav. Neural Biol.* **41,** 203–220
24. Blankenship, J. E., Rock, M. K., Robbins, L. C., Livingston, C. A., and Lehman, H. K. (1983) *Fed. Proc.* **42,** 96–100
25. Aspey, W. P., and Blankenship, J. E. (1976) *Behav. Biol.* **17,** 301–312
26. DesGroseillers, L., Auclair, D., Wickham, L., and Maalouf, M. (1994) *Biochim. Biophys. Acta* **1217,** 322–324
27. Jahan-Parwar, B. (1976) *The Physiologist* **19,** 240
28. Painter, S. D., Gustavson, A. R., Kalman, V. K., Nagle, G. T., and Blankenship, J. E. (1989) *Behav. Neural. Biol.* **51,** 222–236
29. Painter, S. D., Chong, M. G., Wong, M. A., Gray, A., Cormier, J., and Nagle, G. T. (1991) *Biol. Bull.* **181,** 81–94
30. Painter, S. D., Cummins, S. F., Nichols, A., Akalal, D.-B. G., Schein, C. H., Braun, W., Smith, J. S., Susswein, A. J., Levy, M., DeBoer, P. A. C. M., ter Maat, A., Miller, M. W., Scanlan, C., Milberg, R. M., Sweedler, J. V., and Nagle, G. T. (2004) *Proc. Natl. Acad. Sci. U. S. A.*, in press
31. Coggeshall, R. E. (1972) *Tissue Cell* **4,** 105–127
32. Painter, S. D., Kalman, V. K., Nagle, G. T., Zuckerman, R. A., and Blankenship, J. E. (1985) *J. Morphol.* **186,** 167–194
33. Chomcyznski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
34. Takamatsu, N., Shiba, T., Muramoto, K., and Kamiya, H. (1995) *FEBS Lett.* **377,** 373–376
35. Nagle, G. T., Painter, S. D., Blankenship, J. E., Dixon, J. D., and Kurosky, A. (1986) *J. Biol. Chem.* **261,** 7853–7859
36. Nagle, G. T., Painter, S. D., Blankenship, J. E., and Kurosky, A. (1988) *J. Biol. Chem.* **263,** 9223–9237
37. Rothman, B. S., Hawke, D. H., Brown, R. O., Lee, T. D., Dehghan, A. A., Shively, J. E., and Mayeri, E. (1986) *J. Biol. Chem.* **261,** 1616–1623
38. Akalal, D.-B. G., Cummins, S. F., Painter, S. D., and Nagle, G. T. (2003) *Peptides* **24,** 1117–1122
39. Akalal, D.-B. G., Bottenstein, J. E., Lee, S.-H., Han, J.-H, Chang, D.-J., Kaang, B.-K., and Nagle, G. T. (2003) *Mol. Brain Res.* **117,** 228–236
40. Hummon, A. B., Hummon, N. P., Corbin, R. W., Li, L., Vilim, F. S., Weiss, K., and Sweedler, J. V. (2003) *J. Proteome Res.* **2,** 650–656
41. Lloyd, P. E., and Connolly, C. M. (1989) *J. Neurosci.* **9,** 312–317
42. Pearson, W. L., and Lloyd, P. E. (1989) *J. Neurosci.* **9,** 318–325
43. Scheller, R. H., Jackson, J. R., McAllister, L. B., Rothman, B. S., Mayeri, E., and Axel, R. (1983) *Cell* **32,** 7–22
44. Nagle, G. T., de Jong-Brink, M., Painter, S. D., Bergamin-Sassen, Blankenship, J. E., and Kurosky, A. (1990) *J. Biol. Chem.* **265,** 22329–22335
45. Roelofs, W. L. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 44–49
46. Plimmer, J. R., Inscoe, M. N., and McGovern, T. P. (1982) *Annu. Rev. Pharmacol. Toxicol.* **22,** 297–320
47. Raffioni, S., Luporini, P., Chait, B. T., Disper, S. S., and Bradshaw, R. A. (1988) *J. Biol. Chem.* **263,** 18152–18159
48. Raffioni, S., Luporini, P., and Bradshaw, R. A. (1989) *Biochemistry* **28,** 5250–5256
49. Raffioni, S., Miceli, C., Vallesi, A., Chowdhury, S. K., Chait. B. T., Luporini, P., and Bradshaw, R. A. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 2071–2075
50. Begnoche, V. L., Moore, S. K., Blum, N., van Gils, C., and Mayeri, E. (1996) *J. Neurophysiol.* **75,** 2162–2166
51. Cummins, S. F., Nichols, A. E., Rajarathnam, K., and Nagle, G. T. (2004) *Peptides* **25,** 185–189
52. Audesirk, T. T. (1977) *Behav. Biol.* **20,** 235–243
53. Kikuyama, S., Yamamoto, K., Iwata, T., and Toyoda, F. (2002) *Comp. Biochem. Physiol. B* **132,** 69–74

Characterization of *Aplysia* Enticin and Temptin, Two Novel Water-borne Protein Pheromones That Act in Concert with Attractin to Stimulate Mate Attraction

Scott F. Cummins, Amy E. Nichols, Andinet Amare, Amanda B. Hummon, Jonathan V. Sweedler and Gregg T. Nagle

J. Biol. Chem. 2004, 279:25614-25622.
doi: 10.1074/jbc.M313585200 originally published online March 30, 2004
