Cloning and Expression of a Gene Encoding a Protein Obtained from Earthworm Secretion That Is a Chemoattractant for Garter Snakes*

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The protein ES20, derived from earthworm shock secretion, is a vomeronasally mediated chemoattractant for garter snakes (Jiang, X. C., Inouchi, J., Wang, D., and Halpern, M. (1990) J. Biol. Chem. 265, 8736–8744). Based on its 15-residue N-terminal amino acid sequence, degenerative oligodeoxynucleotide probes were synthesized and used to screen a cDNA library that was constructed in sense orientation using a Uni-ZAP™ XR vector and XL1-Blue MRF™ host. A gene was cloned from a polymerase chain reaction as well as from the cDNA library. A combination of the forward degenerative primer and T7 primer was used to obtain gene-specific DNA fragments, from which probes were synthesized and successfully used in screening the cDNA library. The ES20 gene is about 700 base pairs long and encodes 208 amino residues. The ES20 gene was excised from a recombinant plasmid pSK-ES20, ligated to pQE30 expression vector, and transformed into Escherichia coli strain JM109. The selected recombinant plasmids were transformed into expression host cell, E. coli M15[pREP4]. Three transformants were selected, induced with isopropyl-1-thio-β-D-galactopyranoside for fusion gene expression and an expressed 20-kDa fusion protein purified under denaturing conditions. This protein was refolded and gave a positive reaction against ES20-specific polyclonal antibodies. The fusion protein that had not been denatured remained as an aggregate and was an active chemoattractant for garter snakes.

We have been studying chemosignal transduction in the vomeronasal system using garter snakes (Thamnophis sp.) as model subjects. As is the case for most terrestrial vertebrates, garter snakes possess dual olfactory systems, a main olfactory apparatus and a vomeronasal system. However, unlike most other vertebrates, the vomeronasal systems of most snakes are better developed than their main olfactory systems (2). Snakes appear to be dependent on vomeronasal input for a wide variety of behaviors requiring responses to chemical signals (3). In addition, the two olfactory sensory systems play distinct functional roles in detecting environmental stimuli. The olfactory system detects mainly volatile compounds while the vomeronasal system detects primarily nonvolatile substances. Garter snakes detect prey, such as earthworms (Lumbricus terrestris), with their vomeronasal systems (4–6). Garter snakes respond to earthworm preparations by rapid tongue flicking and attack (5–9). Several chemoattractive proteins to garter snakes have been isolated from earthworms (1, 10, 11). A chemoattractive protein, ES20, derived from electric shock-induced earthworm secretion, was isolated, purified, and characterized to a considerable extent (1). When the vomeronasal organ is irrigated with an aqueous solution of ES20, it induces an increase in the firing rate of mitral cells in the accessory olfactory bulb, the postsynaptic target of receptor cells of the vomeronasal epithelium (1, 12, 13). The chemoattractive protein, ES20, binds to vomeronasal epithelial membrane in a saturable and reversible manner with a $K_d$ value of 0.3 μM (1). The binding of ES20 is tissue-specific, and both the binding and the bioactivity of this chemoattractive protein require the presence of Ca$^{2+}$ (14). When ES20 binds to its G protein-coupled receptors on the vomeronasal receptor cell membranes, the intracellular level of inositol 1,4,5-trisphosphate is increased while that of cAMP is decreased (14, 15). The level of cAMP is regulated mainly by calcium ions rather than by inhibitory G protein or cAMP-phosphodiesterase (15). To facilitate the elucidation of the nature and mechanism of interaction between chemoattractant and its receptors that generate a chemoreceptive signal, it is necessary to have molecular and structural information concerning the chemoattractive protein as well as its receptors. Since a partial amino acid sequence of ES20 protein was previously reported (1), degenerative oligonucleotide primers were synthesized and utilized to clone the gene that encodes this chemoattractive protein. In this communication, we report the cloning of the gene encoding ES20 and its deduced amino acid sequence. The fusion ES20 protein was recognized by polyclonal antibodies specific for the native ES20 protein and was active in a bioassay for chemoattractivity to garter snakes.

MATERIALS AND METHODS

The reagents were obtained from various suppliers: TRIzol and reverse transcriptase from Life Technologies, Inc.; UniZAP™ XR vector and XL1-Blue MRF™ host from Stratagene; poly(A)+ RNA isolation kit and pCR™ II from Invitrogen; [32P]dCTP, DNA sequence kit, and ECL kit (horseradish peroxidase-labeled anti-rabbit antibody) from Amersham Corp.; Taq polymerase from Boehringer Mannheim; pQE30 expression vector and M15[pREP4] cells from Qiagen; and earthworms from Connecticut Valley Biological Supply; garter snakes from various suppliers.

Poly(A)+ mRNA Isolation—Total RNA was prepared from 5 g of earthworm tissues as follows. Freshly dissected earthworm tissues were pulverized by grinding under liquid nitrogen. To the ground tissues, 10 ml of TRIzol were added and allowed to thaw at room temperature. The thawed mixture was incubated at room temperature for 30 min. It was transferred into a Falcon 2059 plastic tube, mixed with 2 ml of chloroform, and then centrifuged. The supernatant was carefully transferred into a new tube, and the RNA was precipitated by mixing
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with 5 ml of isopropl alcohol. Poly(A)$^+$ mRNA was isolated from total RNA according to the procedures described in the manual for poly(A)$^+$ mRNA isolation kit (Invitrogen).

**cDNA Library Construction**—The construction of a cDNA library was performed according to standard procedures of Sambrook et al. (16) using UniZAP$^TM$ XR vector (Stratagene) and XL1-Blue MR$^B$ host. A cDNA library containing 5 × 10$^6$ recombinants in sense orientation was achieved. One fifth of the recombinants (1 × 10$^5$) were amplified to 1 × 10$^{11}$ plaque-forming units/ml.

**PCR Amplification of ES20 Gene from cDNA Library**—The ES20 cDNA from the recombinants (1 × 10$^5$) was cloned by performing degenerative oligonucleotides-primed amplification. Two oligonucleotides, GCC GTI GTG CCI GGI TTT ACI TAT and GTA ATA CGA CTC ACT ATA GGG C, were used, respectively, as a sense degenerative primer and antisense T7 primer. The sense primer corresponds to the amino acid sequence ALVCPGPFTY of chemotactic protein ES20. PCR was performed in 50 μl of PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl$_2$, 0.001% gelatin) containing 200 μM each of dATP, dCTP, dGTP, and dTTP, 2.5 units of Taq polymerase (Boehringer Mannheim), and 2 μM of each primer. Amplifications were carried out according to the following schedule: 94 °C for 1 min, 36 °C for 3 min, and 72 °C for 3 min (four cycles); 94 °C for 1 min, 50 °C for 2 min and 72 °C for 2 min (30 cycles). PCR products were analyzed with 100-bp ladder DNA markers by agarose (1.4%) gel electrophoresis. A 702-bp PCR product was gel-isolated and cloned into pCR$^TM$ II (Invitrogen). The clones were identified by restriction enzyme digestion and sequencing. The recombinant is referred to as pCR-ES20.

**Determination of the cDNA Sequence of ES20 Clones**—The cDNA of ES20 clones was sequenced by dioxyed-mediated chain termination according to the method of Sanger et al. (17).

**Northern Blot Analysis**—A 200-bp EcoRI fragment of pCR-ES20 was purified, labeled with $^{32}$P[γ-ATP, and used as a probe for Northern blot analysis. Earthworm total RNA (10 μg) and mRNA (50 μg) were size-fractionated on a 1.3% agarose formaldehyde gel, blotted onto a nylon membrane, and cross-linked to the membrane under UV irradiation. Prehybridization was performed in 50% formamide, 5 × SSC, 5 × Denhardt’s solution, 0.4% SDS, and 100 μg/ml denatured salmon sperm DNA at 45 °C for 4 h and hybridization at 45 °C overnight in a similar solution but containing the ES20 probe. Hybridized membrane was washed under high stringency conditions.

**cDNA Library Screening**—The 200-bp EcoRI fragment of pCR-ES20 labeled with $^{32}$P was used as a gene-specific probe for screening the cDNA library. A total of 1 × 10$^{11}$ plaques were screened under high stringency conditions. Four positive clones were isolated following secondary and tertiary screenings. They were excised as pSK-ES20. A 702-bp sequence was determined, and the complete amino acid sequence of 208 residues was deduced from the coding region.

**ES20 Gene Expression in E. coli Cells**—The ES20 gene was excised from pSK-ES20 and amplified by PCR using a gene-specific sense primer containing a BamHI site and an antisense primer containing a HindIII site. Both the pOQE30 expression vector (QIAGEN) and PCR-amplified ES20 DNA were digested by BamHI and HindIII, purified, ligated, and subsequently transformed into JM109 cells for the purpose of selecting the correct insertion of the coding fragment. The selected recombinant plasmids were transformed into expression host cell, M15[pREP4], in a medium containing appropriate antibiotics for selecting the correct insertion of the coding fragment. The selected recombinant clones were excised as pSK(−) from UniZAP$^TM$ XR, and referred to as pSK-ES20. A 702-bp sequence was determined, and the complete amino acid sequence of 208 residues was deduced from the coding region.

**ES20 Gene Expression in E. coli Cells**—The ES20 gene was excised from pSK-ES20 and amplified by PCR using a gene-specific sense primer containing a BamHI site and an antisense primer containing a HindIII site. Both the pOQE30 expression vector (QIAGEN) and PCR-amplified ES20 DNA were digested by BamHI and HindIII, purified, ligated, and subsequently transformed into JM109 cells for the purpose of selecting the correct insertion of the coding fragment. The selected recombinant plasmids were transformed into expression host cell, M15[pREP4], in a medium containing appropriate antibiotics for selection. Three transformants were selected and induced with IPTG for recombinant gene expression. An expressed 20-kDa DNA from earthworm cDNA (linked with 6 residues of His) was purified under denaturing conditions by affinity chromatography with nickel-nitrioltriacetic acid resin. The purified protein was refolded by dialyzing against stepwise dilutions of the denaturant. Such refold recombinant protein was analyzed by Western immunoblot against ES20-specific polyclonal antibodies using an horseradish peroxidase-labeled anti-rabbit antibody (Amersham Corp.) for detection with an ECL kit (Amersham Corp.).

**Protein Determination**—The concentration of protein was estimated using the Micro BCA protein assay reagent and bovine serum albumin as a standard.

**Snake Bioassay**—The snake bioassay was conducted by following the procedures of Jiang et al. (1). Briefly, test samples (200 μl of 53.8 μM fusion ES20 protein or native ES20 protein) or control solution (200 μl of a similar fraction prepared from uninduced Escherichia coli cells) was evenly applied to the surface of a piece of artificial plastic earth-

![FIG. 1. A. PCR amplification of ES20 DNA from earthworm cDNA library using ES20 degenerate sense primer and T7 antisense primer. Lanes: 1, 100-bp ladder DNA marker; 2, β-actin primer; 3, T7 antisense primer; 4, degenerate ES20 primer and T7 antisense primer; 5, ES20 degenerate primer. B, Northern blot analysis of earthworm RNA using a $^{32}$P-labeled 200-bp EcoRI fragment from pCR-ES20. Lanes: 1, total RNA (10 μg); 2, poly(A)$^+$ mRNA (50 ng).](image-url)
yields 200-bp fragments as expected. The results are shown in Fig. 2.

The Complete cDNA Sequence of ES20 Gene and Deduced Amino Acid Sequence—The cDNA sequence of ES20 was determined both manually and by an automated DNA sequencer. Fig. 3 shows the complete DNA sequence of the ES20 gene and its corresponding deduced amino acid sequence.

The Expression and Purification of Fusion ES20 Protein—

The fusion ES20 protein was expressed in pQE30. Fig. 4A shows that the degree of ES20 protein expression was time-dependent following induction with IPTG. A maximum yield of fusion ES20 protein was attained within 3 h following IPTG induction. A relatively pure fusion protein was readily obtained by affinity chromatography using a nickel-nitrilotriacetic acid resin column and showed a similar mobility as the native ES20 protein under SDS-polyacrylamide gel electrophoresis.

Western Immunoblot Analysis of Fusion ES20 Protein—Since the isolation and purification of the expressed fusion protein was carried out under denaturing conditions, the purified fusion ES20 protein was refolded. We prepared previously a polyclonal antibody that is specific against native ES20 and shows no cross-reactivity with several other purified chemoattractive proteins including the EW20 from earthworms (18). A Western immunoblot analysis was performed using this ES20-specific polyclonal antibody. The results are shown in Fig. 4B. Like the native ES20, the refolded fusion ES20 gave a positive immunological reaction, suggesting that the fusion ES20 protein possesses an epitope identical to the native ES20 protein.

The Bioactivity of Fusion ES20 Protein—Despite the fact that the refolded purified fusion ES20 protein gave a positive immunological reaction against ES20-specific antibodies, it exhibited little or no bioactivity when presented to garter snakes in the bioassay. However, when the fusion ES20 protein was recovered in the form of aggregate, without prior denaturation and refolding, it was attractive to garter snakes as shown in Table I. These results suggest that the ES20 gene has been successfully cloned.

**DISCUSSION**

Earthworms, a primary prey of garter snakes, contain a number of proteins attractive to garter snakes. The recognition of and response to these chemoattractive proteins is mediated by the garter snake’s vomeronasal system (5, 6, 9). Several of these chemoattractants have been isolated, purified, and characterized (1, 10, 11, 18, 19). The recognition of the prey is presumably initiated by the binding of one or several chemoattractants to their receptors on vomeronasal sensory neurons. Two of the purified chemoattractive proteins, ES20 and EW3, when delivered as aqueous washes to the vomeronasal sensory epithelium, have been shown to generate action potentials in the mitral cell layer of the accessory olfactory bulb of garter snakes (1, 12, 13). Furthermore, the binding of ES20 and its G protein-coupled receptor(s) results in generation of second messengers (14) and appears to involve cross-talk between two second messenger pathways (15).

Since earthworms contain many proteins attractive to garter snakes, whether these proteins bind to a single type of receptor or to different subtypes of receptors is not yet known. Do these chemoattractive proteins possess a similar type of receptor-binding domain or different types of domains? To answer these
questions, a knowledge of structure-function relationships between ligands and receptors is needed. It is therefore necessary to elucidate the molecular structures of both the ligands and their specific receptors. Toward this end, we have successfully cloned the gene for one chemoattractive protein, the ES20 gene. This clone is the first and only chemoattractive gene that has become available and undoubtedly more chemoattractive genes are needed to map their binding domains.

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### Table I
The attractivity of fusion ES20 protein to garter snakes

| Compounds       | Chemoattractivity positive response/no. snakes tested |
|-----------------|--------------------------------------------------------|
| Control*        | 0/10                                                   |
| Fusion ES20 protein | 23/23                                                 |
| Native ES20     | 10/10                                                  |

* Control is the fraction obtained from equal amount of host cells which had not been induced with IPTG.