Characterization of a uterine luminal fluid protein ULF-250 using N-terminal microsequencing and RT-PCR identifies a novel estrogen-regulated gene in the rat uterus

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Abstract We had previously identified an estrogen responsive protein ULF-250, synthesized and secreted by the estrous rat uterus, which is immunologically distinct from complement C_3 and α_2-macroglobulin. The N-terminal microsequencing of ULF-250 followed by sequence homology analysis showed that this protein is a new member of a class of estrogen responsive proteins in the uterus. Polymerase chain reaction with a ULF-250 specific primer yielded partial sequence information of its message. The observed pattern of ULF-250 message in the uterus during the various stages of the reproductive cycle in the rat suggested a possible regulation of ULF-250 message by 17β-estradiol. Upstream sequencing of ULF-250 message and its promoter domains would provide insight into the mechanism of its regulation by estradiol.

Key words: ULF-250; Estradiol; Uterine protein; Luminal protein; Polymerase chain reaction; Microsequencing

1. Introduction

The mammalian uterus is an organ which responds profoundly to sex steroids such as estrogen and progesterone. Estrogen and progesterone influence uterine functions in both independent and interdependent fashion. Estrogen is a potent mitogen for the uterus of adult mammals [1]. The mitogenic effect of estrogen involves the regulation of the expression of genes such as c-*fos*, c-*myc*, c-*jun* and *jun*-B, whose products control the cell cycle, and is mediated by an estrogen receptor which can modulate gene expression by binding with estrogen response element (ERE) localized in the proximity of the target genes [2–4]. Induction of c-*fos* gene by 17β-estradiol was observed in the rat uterus [5,6], specially in the luminal and glandular epithelia [7]. Other proteins expressed in the uterus and secreted into the lumen under hormonal influence include the presumptive induced protein [8], alpha uterine protein (AUP) [9,10], the estrogen responsive induced protein (IP) [11], which was later shown to be a creatine kinase [12], a cat uterine protein that is estrogen dependent (CUPED), which was localized in the endometrial epithelial cells [13], and a mouse lactotransferrin [14]. In addition, estradiol regulates the expression of growth arrest specific (*gas*) genes [15,16], growth factors and their receptors [17,18]. Estradiol administered to immature rats stimulated the synthesis and secretion of proteins by the uterus [19–21]. These proteins include complement C_3 and immunoglobulin [22,23].

Uterine luminal fluid (ULF) of the mammal constitutes the immediate environment for early reproductive events and is thus an important factor in successful fertilization and subsequent implantation of the embryo [24]. In addition, ULF proteins are reported to play significant roles in fetal growth and development [25,26] as well as in the facilitation of maternal/fetal interactions [27–29]. ULF contains a complex array of molecules, including secretory proteins of both endometrial and plasma origin [25,30]. Steroid hormones such as estrogen and progesterone are reported to modulate the synthesis of uterine proteins [20,25,31–36], the uptake and transport of certain serum proteins by the uterus [37], and the movement of specific plasma components across the endometrium into the uterine lumen [38].

Our laboratory has recently identified an estrogen responsive protein (ESP) with an estimated molecular weight of 260 kDa (corrected later as 250 kDa), synthesized and secreted by the estrous rat uterus [39], which is immunologically distinct from complement C_3 and α_2-macroglobulin [40], as demonstrated by Western blot analysis. It was also shown that the production of ULF-250 is regulated by estradiol and that this protein is not a component of blood plasma. The biological significance of this protein and the mechanism through which estradiol regulates the synthesis and/or secretion of this protein are not known. In the present study, we attempted to further understand the ULF-250 through protein microsequencing and to partially characterize the message encoding this protein employing a reverse-transcriptase polymerase chain reaction.

2. Materials and methods

2.1. Chemicals

Dulbecco's modified Eagle's medium (D-MEM) was purchased from Life Technologies. 17β-Estradiol and the antibiotics were from Sigma Chemical Company. HiTrap Blue column, QuickPrep mRNA purification kit, Ready-to-Go T-primed first strand synthesis kit and Sephaglass BandPrep kit were from Pharmacia Biotech. BCA protein assay kit (Pierce), Centriprep-10 and Centricon-100 (Amicon) were also procured. All reagents for protein electrophoresis were from BioRad. NuSieve 3:1 Agarose was obtained from FMN Biochemicals.

2.2. Animals

Young adult female rats (Sprague-Dawley) were obtained from Charles River (Wilmington, MA). Vaginal smears were examined to identify the estrus phase of the animals. The estrus phase animals were killed by CO_2 asphyxiation. The abdomen was opened, the uterine horns were exposed and the uterine luminal fluids were collected using a 5 ml syringe fitted with a 23 gauge needle. The fluids from different animals were pooled and were frozen immediately at −20°C. The uterine horns were dissected out, cleaned of fat and connective tissue, slit longitudinally and rinsed three times in D-MEM supplemented with 1 μg/ml streptomycin and 100 μg/ml penicillin. The tissues were incubated in 3 ml of D-MEM for 6 h at 37°C in a 95% air–

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5% CO_2 atmosphere with occasional agitation. After incubation, the tissues were removed and the medium centrifuged. The supernatant was processed as described below.

2.3. Affi-gel blue chromatography

Affi-gel blue chromatography was performed according to the instructions of the manufacturer (Pharmacia), for the removal of albumin. HiTrap Blue 5 ml columns were equilibrated with buffer A (50 mM KH_2PO_4, pH 7.0) at room temperature. The sample was loaded onto the column with a syringe. The eluted albumin-free fraction was concentrated in an Amicon Centriprep-10 concentrator.

2.4. Protein analysis

The protein concentrations of the ULF and the albumin-free supernatant were determined by the bicinchoninic method of Smith et al. [41]. The final concentration of the protein in the sample was adjusted to 5 mg/ml. An aliquot of the sample was denatured in Laemmli sample buffer. Approximately 50 μg protein/well was loaded onto a discontinuous gradient gel (4% stacking, 8–15% linear gradient separating) and electrophoresis was performed at 100 V for 3 h on a MiniProtean minigel format (BioRad). After electrophoresis, the separated proteins were electroblotted onto a 0.2 μm PVDF membrane using CAPS transfer buffer (10 mM CAPS+10% methanol, pH 11.0) at 30 mA for 3 h. After the transfer was completed, the membrane was stained with 0.01% amido black in 40% methanol/10% acetic acid for 5 min and destained 1–3 times with 40% methanol/10% acetic acid (5 min each, followed by several washes with ddH_2O). The 250 kDa bands from 10 lanes were cut out and were processed for N-terminal protein sequence analysis based on Edman chemistry followed by PTH analysis using microbore HPLC (HP G-1000A equipped with a 1090 PTH analyzer) [42]. The protein sequence data were obtained at the Rockefeller University Protein/DNA Technology Center.

2.5. RT-PCR of the 250 kDa protein message

mRNA from diestrous, proestrous and estrous uteri were extracted using QuickPrep mRNA purification kit (Pharmacia) following the instructions of the manufacturer. The mRNA concentration was adjusted to 50 μg/ml and the preparations were immediately processed for first strand synthesis. The first strand synthesis was performed with 2 μg of mRNA, using a Ready-To-Go T-primed first strand synthesis kit (Pharmacia) which utilizes Moloney murine leukemia virus (M-MuLV) reverse transcriptase and NotI-d$(T)_{18}$ primer (5′-d[AACTGGAAGAATTCGCGGCCGCAGGAAT$_{18}$]-3′). PCR of the first strand was performed using a gene-specific sense primer (GSP-250) designed from the reverse-translated N-terminal sequence of ULF-250, NotI-d$(T)_{18}$ reverse primer and other reagents from GeneAmp PCR Reagent kit with AmpliTaq DNA polymerase (Perkin Elmer). PCR was carried out on a Perkin-Elmer Cetus 480 thermocycler. The cycling conditions were: 1 min melting at 94°C, 45 s annealing at 68°C and 1 min primer extension at 72°C. The cycle was repeated 35 times, with 10 s increments in extension time per cycle. After the completion of the cycles, the reaction was held at 72°C for 30 min, after which the samples were recovered.

(A)

Met-Pro-Met-Phe-Lys-Phe-Ile-Lys-Pro-Met-Met-Ile-Gln-Gly-Asn-Thr-Gly-

(B)

5	**KFIKPMMIQGNT**	**16**	**ULF-250**
	K+ P +MIQGNT		
219	**KYSAPVMIQGNT**	**230**	**gene45**

Fig. 1. A: The N-terminal amino acid sequence of the uterine luminal fluid protein ULF-250. B: BLASTP alignment of the N-terminal sequence of ULF-250 with hypothetical gene 45 protein (accession number Q00111).

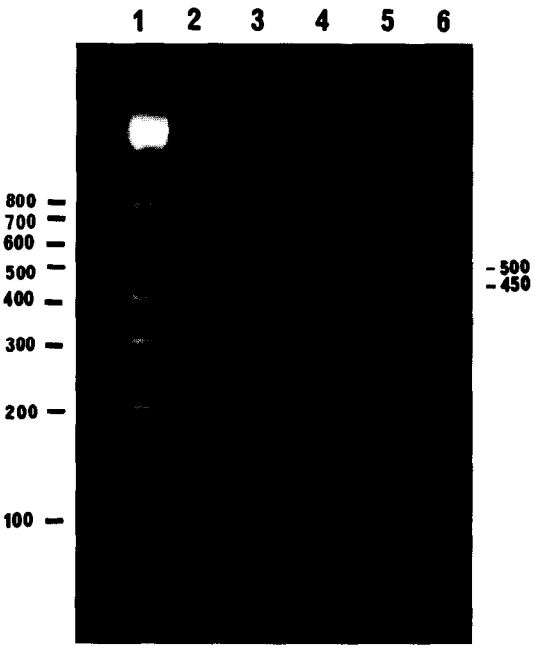

Fig. 2. Agarose gel electrophoresis of the PCR product obtained by amplifying the mRNA preparations from the uteri of rats in various stages of their reproductive cycle. Lanes are as follows: 100 bp ladder (1), and PCR products from diestrous (2), proestrous (3) and estrous (4) rats. Lane 5 shows a 500 bp λDNA. Lane 6 shows a negative control. Arrow points towards a 450 bp band, which appeared in proestrus and estrus stage uteri.

2.6. Agarose electrophoresis

5 μl from each of the reactions was mixed with 1 μl of 6× gel loading buffer (Sigma) and was separated on 4% Nu-Sieve 3:1 agarose (FMN) gel and 1× TAE buffer. Electrophoresis was performed at 5 V/cm^2 for 3 h. The gels were visualized and photographed on a UV transilluminator. The band of interest was sliced off and the DNA was recovered using Sephaglass BP BandPrep kit (Pharmacia). The purified band was subjected to dyedeoxy chain termination sequencing on an ALF sequencer (ABI Prism, Applied Biosystems).

3. Results

3.1. Protein microsequencing

The N-terminal microsequencing of ULF-250 from both uterine luminal fluid and the culture medium produced essentially the same N-terminal sequence. The obtained sequence is shown in Fig. 1A. This sequence was subjected to a BLASTP search [43] using the National Center for Biotechnology Information WWW server, followed by a BEAUTY post-processing [44] provided by the Human Genome Center, Baylor College of Medicine. It was found that our sequence showed 66% identity with a hypothetical gene 45 protein from herpesvirus 1 (strain auburn 1) (Genbank accession number. Q00111). However, this homology is statistically insignificant. A MACAW alignment of these two sequences is shown in Fig. 1B.

3.2. PCR amplification of ULF-250 message

We exploited the available N-terminal protein sequence of ULF-250 to design a gene-specific primer. The N-terminal amino acid sequence was reverse translated using a GCG Backtranslate option and the rat codon usage. This computer-simulated 5′ message was used to design a sense primer

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   1 gacctcctac atatgaacca ttttcattct gtgcagtgca gggattgaac tctgagcctc
  61 atgcatgcta gataagtact ggctgtatct ctagccctga ttttttttctt tttagagaaa
 121 ttgtgaaaat acttataaac atactgaaag tcactttaaa attctggaat tacatgattc
 181 accttgtatg attaataaac aatactattc tgtgaaaaaa aaaaaaaaaa aaaacccggg
 241 ggngggggggg gggcggtttt
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A

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ULF-250 ta ttataaa ata  aaa t a tttaaaatt t  aatta at att a  tt tat 189
ZK-666  taattaaaaa ata  aaa at agctgcaaatg aa atga ct ttt a attatat 5977
CENP-A  aa atttagtatcttctacatagtattgtcaagt ttaaaatttcatgtaaa-------- 1269
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B

Fig. 3. A: Nucleotide sequence obtained from the 450 bp PCR fragment from the estrus stage uteri. B: A MACAW alignment of the obtained sequence with its homologues ZK666 and CENP-A obtained from the database.

using the Xprimer software at ExPaSy. A 27-mer primer (GSP-250) was thus designed (5′-GAATTCTCATCAAGCCCATGATGATTC-3″) and was used as the sense primer. NotI-d(T)$_{18}$ was used as the reverse primer. We could obtain a clear 450 bp band in samples from the estrus stage uterus. This band was absent in diestrous uterine preparations. A weak signal at the 450 bp position was observed in proestrous uterine samples (Fig. 2).

A dye-termination cycle sequencing of this 450 bp band with the GSP-250 primer produced a sequence as shown in Fig. 3A. This sequence showed statistically significant partial homology with *Caenorhabditis elegans* cosmid ZK666 sequence (gi790416) and human centromere protein-A (CENP-A) mRNA (gi602413). A MACAW aligned image of these sequences is provided (Fig. 3B). The nucleotide sequence (accession number RNU 53183) and the predicted amino acid sequence (accession number g1279988) of ULF-250 have been submitted to the Genbank.

4. Discussion

In the present study, we have microsequenced the N-terminus of ULF-250 from estrous rat uterine luminal fluid and the culture medium. The identity in the sequence implies that the two proteins are essentially the same. Earlier reports on ULF-250 (previously called ESP-260) had shown that ULF-250 could be induced by E_2 [39] and that antiestrogens could block the estradiol-mediated ULF-250 induction [39,40]. Because complement C_3 and α_2-macroglobulin are present in the uterine luminal fluid and both of them migrate to a ~200 kDa region, it was necessary to check the possibility that ULF-250 could be one of these proteins. In a recent paper from our lab, we could show on immunoblots that ULF-250 was not recognized by anti-complement C_3 or the anti-α_2-macroglobulin antibodies suggesting that it is not possibly one of these components [40]. Since a BLASTP analysis of the N-terminal amino acid sequence of ULF-250 protein did not show any significantly similar member in the database, it is reasonable to feel that we have identified a novel estrogen-induced uterine luminal protein.

The polymerase chain reaction targeting the ULF-250 message at various stages of the reproductive cycle in the rat showed that this message was absent in the diestrous uteri. A weak ULF-250 message was detectable in proestrous uteri, which became prominent in estrous uteri (Fig. 2). This observation is in perfect agreement with the ULF-250 protein profiles reported earlier [39]. The nucleotide sequence data obtained from this amplified message (Fig. 3) showed that we have captured the 3′ end of a relatively big ULF-250 message. Moreover, the appearance of a 450 bp band in PCR implies that the ULF-250 message contains at least one internal repeat of its N-terminal motif.

The pattern of the appearance of the ULF-250 message in the rat uterus follows the natural 17β-estradiol profiles in the serum of cycling female rats, suggesting a regulation of ULF-250 message by this steroid hormone. 17β-Estradiol has been shown to regulate the expression of a variety of genes, including G6PD [45], TNF-α [46], Raf-1 [47], cystic fibrosis transmembrane regulator (CFTR) [48], lactoferrin [24], c-*jun* [49], srp27 [50], by modulating mRNA synthesis, mRNA translation, protein processing and protein stability. The normal route through which estradiol regulates gene expression in target organs starts with the activation of a transcription factor (ER) which in turn binds a specific hormone response element (ERE) which is a perfect palindrome with the sequence GGTCAnnnTGACC [51].

Thus, we would conclude that ULF-250 is a novel estrous cycle specific gene product. A complete molecular, structural and functional characterization of this novel estrogen inducible gene family member could resolve its possible involvement in contributing to female fertility regulation.

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